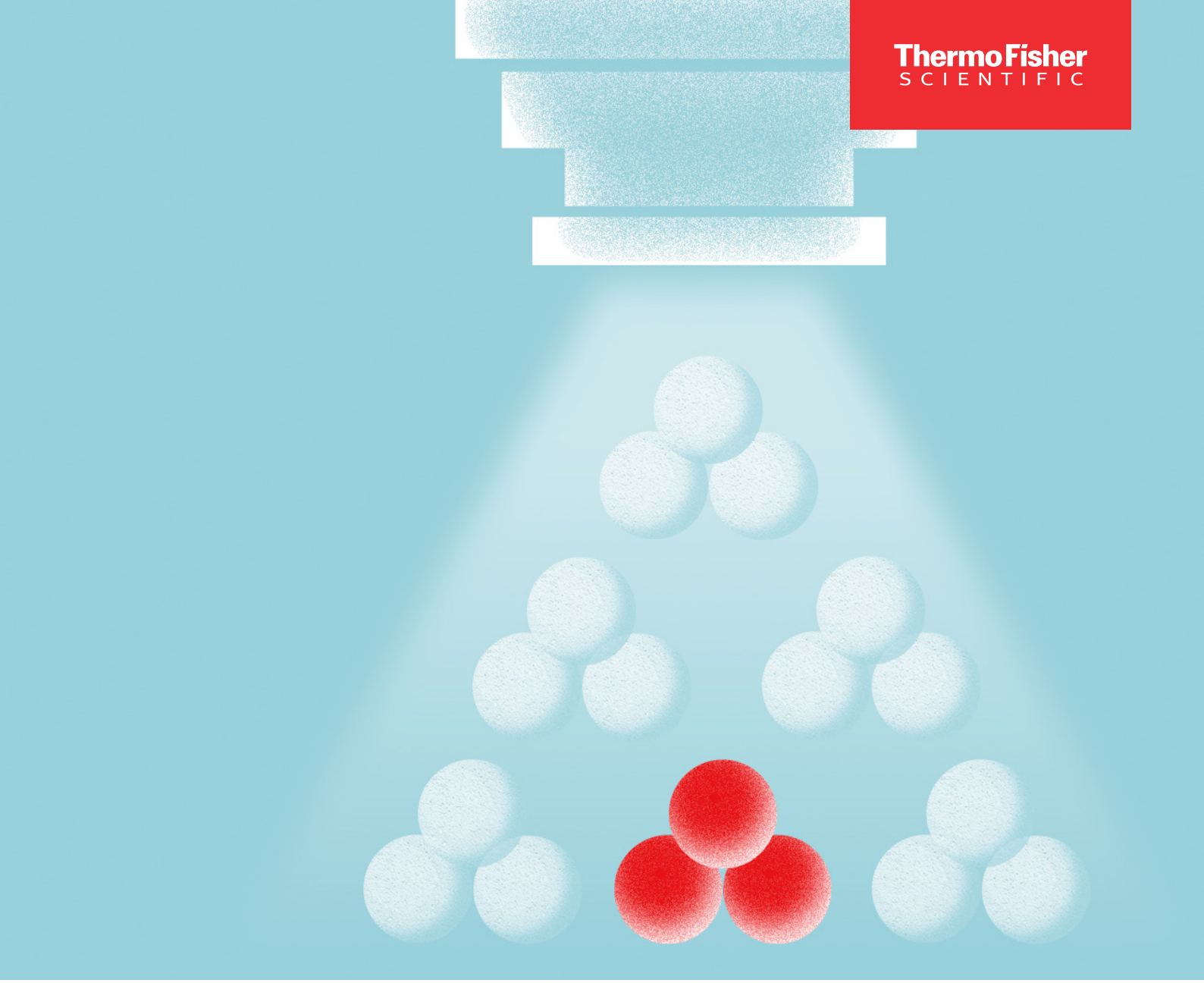

Efficient techniques for antibody therapeutic purification

Foreword

Monoclonal antibodies (mAbs) are fast becoming one of the most sought after therapeutics, with hundreds approved for a wide range of clinical uses, from cancers to infectious disease, in the last few decades.

In order to address more difficult therapeutic targets, novel antibody modalities are being developed, such as bispecific mAbs, antibody fragments and antibody-drug conjugates.

However, these new modalities present purification challenges that traditional methods of purification such as polishing with ion exchange chromatography struggle to overcome. This results in higher costs and lower yields at manufacturing scale.

This eBook explores the recent developments in purification and polishing solutions that can help address the challenges in downstream processing.

Contents

The challenges of therapeutic monoclonal antibodies

Introduction

Since the development of the hybridoma technology in 1975, monoclonal antibodies (mAbs) have become increasingly important in both medical research and disease treatment.[1] The first clinically available mAb, muronomab CD3, was approved in 1986 to block cytotoxic T cell function and prevent rejection in transplanted patients.[2] Since then, hundreds of mAbs have been approved for clinical use for the treatment of a wide range of diseases, from cancers and autoimmune disorders to infectious diseases such as HIV.[3,4]

Despite the success of mAb therapeutics, developing novel antibodies is challenging, as many of the best-understood targets have already been exploited. Remaining targets for potential novel mAb therapeutics pose challenges such as poorly accessible sites of action, low immunogenicity and significant differences in targets between human and the species used for preclinical testing.[5] In order to overcome these roadblocks, novel mAb-based therapies are being developed. These include bispecific mAbs capable of binding two different antigens, antibody–drug conjugates (ADCs), isotype-switched mAbs and antibody fragments.[6]

As biotherapeutics, mAbs must adhere to strict regulatory requirements to help ensure the safety and efficacy of the final product.[7,8] Due to their production in cell-based expression systems, the manufacture of mAbs can result in process- and product-related impurities. These impurities significantly affect the quality and safety of the final product. Therefore, mAbs must go through extensive purification steps before release. However, as the structural complexity of novel mAbs increases, the traditional purification methods have become less effective, resulting in high costs and low yields. Recent developments in affinity purification reagents and polish solutions promise to simplify purification of complex mAbs and improve the efficiency of commercial production of novel mAb therapeutics.

Purifying mAbs for clinical use

In order to meet regulatory guidelines, purified mAbs must have low levels of both process- and product-related impurities, while maintaining a reasonable and cost-effective yield.

Process-related impurities include host cell proteins (HCPs), host cell DNA, leached reagents from other purification steps, process buffers and residual detergents used for viruses. Product-related impurities include high and low molecular weight product aggregates, misformed products, viruses, endotoxins from bacterial expression systems and post-translational modifications.[9]

Following mAbs harvesting from cell culture by centrifugation and filtration, the typical mAb purification process begins with protein A chromatography (Figure 1). Protein A is covalently immobilized onto porous resins. As the samples pass through the column, protein A binds to the Fc region of antibodies at the consensus binding site (CBS), capturing the mAbs and resulting in relatively high purity and recovery in a single step.[9] One of the advantages of protein A chromatography is its sensitivity to low pH. This enables an acidic elution of bound antibodies, and a subsequent low pH hold to inactivate any contaminating viruses (in line with regulatory guidelines for viral safety).[10,11]

The efficiency of protein A chromatography means that downstream polishing steps only need to remove a small amount of the remaining impurities. The most common of these polishing steps are based on ion exchange, namely anion exchange (AEX) chromatography and cation exchange (CEX) chromatography, depending on the impurity to be removed. The net charge of mAbs varies, depending on the relative pH of the antibody's environment. Changing the environment therefore allows the manipulation of mAb charge and enables the removal of a wide range of differently charged impurities.[12] CEX chromatography binds positively charged antibodies and impurities, which are then separated based on charge difference, eluting at different ionic strengths. AEX chromatography binds negatively charged impurities, while positively charged antibodies are allowed to flow through. It is used for the removal of host cell DNA, HCPs, bacterial endotoxins and furhter viral clearance.[13,14] The final polishing steps in purification to help ensure the product meets regulatory specifications, using methods such as ion exchange, or hydrophobic interaction chromatography (HIC).[15]

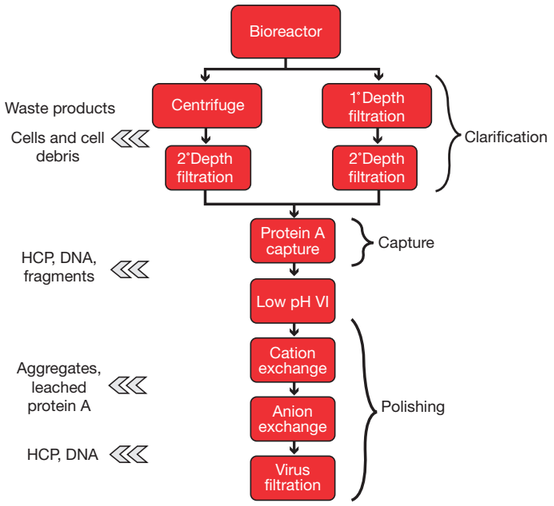

Figure 1. *mAb production process flow diagram. Adapted from Gillespie et al., 2014.*

The Protein A chromatography workflow is highly efficient and selective for traditional mAbs, as it removes the majority of impurities in a single robust step. Both AEX and CEX chromatography have high loading capacities and can accommodate both bind-elute and flow-through modes, for efficient purification at manufacturing scale.

The challenges of traditional polishing

Despite the successes of current purification and polishing processes, these methods still show limitations for both traditional and more complex antibody technologies. Thus, there is a significant demand for better manufacturing and purification strategies. Inherent challenges in mAb production begin even before the purification stages. mAbs are limited to production in a live-cell expression system, which can be difficult to scale to a commercial manufacturing level and can produce variable yields. Historically, mammalian expression systems were used for full-length antibodies, though recent advances have also allowed the use of *Escherichia coli*.[16] Emerging technologies using *in vitro*, cell-free systems could reduce some of the challenges associated with live-cell systems (e.g., reducing the amount of cellular debris and cell-related impurities). However, these methods are not yet ready for industrial-scale manufacture.[17]

Extraction of any high-purity product from a complex mixture such as a cellular expression system is a challenge, but the development of emerging mAb technologies has complicated this process further. The diversity of novel mAb structures has led to increased process- and product-related impurities, for which traditional methods are not suitable. For example, bispecific antibodies may express heavy chains (HCs) and light chains (LCs) from two different parental mAbs. Should these HCs and LCs mispair to form a homodimer during production, they represent an additional impurity that must be removed from the final product, via additional purification steps or specific resins.[18] The complex structure of bispecific mAbs also leads to increased levels of aggregation and the generation of antibody fragments, which can prove difficult to remove by conventional means.[18] Other novel modalities, such as antibody fusion proteins, bring their own unique challenges to purification. In this type of therapeutic, the long half-lives of antibodies are leveraged to enhance the effectiveness of shorter-lived therapeutic proteins such as cytokines. However, these too show increased levels of misformed and mismatched products and aggregates. In addition, instability in acidic conditions can give rise to further aggregation and cleavage during the low pH steps of conventional purification methods.[18]

For both standard and novel mAbs modalities, traditional polishing methods can be further optimized for more efficient purification at almost every stage of the process. In some cases, the type of chromatography itself can inhibit efficient purification. Despite its importance for viral clearance, the low pH hold step results in aggregate formation, exacerbated by the process of protein A chromatography itself.[19] Protein A can leech into the eluted fraction during the chromatography, both as fragments and full-size proteins. This results in additional impurities to be removed from the final product. Furthermore, the protein A fragments can be even more difficult to remove than the whole proteins by ion exchange chromatography.[20] HIC is often used as an orthogonal polishing step during mAb purification due to its high aggregate clearance capability. However, the high concentrations of kosmotropic salts used during the process require extra washing, dilution and ultrafiltration steps to remove the salts from the final product, reducing overall efficiency.[21]

In addition to limitations in the current processes, the impurities to be removed can also cause issues. HCPs are a critical impurity that must be cleared to ensure the safety of the final product. Although the majority of HCPs can be cleared across multiple polishing steps, the remaining HCPs can be highly difficult to remove using traditional means.[22] Residual HCPs can decrease product stability and cause unwanted immune reactions in the recipients.[22] Finally, purification bottlenecks can occur at several steps in the purification process.

Optimizing downstream processing for improved purification

The challenges of traditional purification and polishing methods can be overcome through careful optimization and selection of the most appropriate polishing platforms, ensuring complete removal of impurities. Ion exchange chromatography is a common post-protein A step in most polishing workflows to remove high molecular weight contaminants.[9] Ion exchange chromatography can be employed in both bind-and-elute and flow-through modes, with the optimal method dependent on the isoelectric point of the mAb to be purified. A version of AEX chromatography known as weak partitioning chromatography (WPC) can remove weakly binding impurities, increasing product yield.[9]

CEX chromatography has been shown to effectively remove HCPs, and maximum removal can be assured by selecting an optimal stationary phase with the chemical and physical properties best for the impurities and mAb in question.[23] To reduce purification bottlenecks and increase throughput, high-capacity resins can be selected for CEX which may allow the omission of a rate-limiting protein A capture step.[24] Washing, dilution and filtration steps that increase the time cost of HIC may be reduced through the use of a salt-free method, using an extremely hydrophobic resin in the flow-through step without kosmotropic salts.[21]

As the technology of novel mAb modalities advances, it is only fitting that the technology of mAb purification also advances in step. If optimal yield and efficiency are to be achieved, and regulatory guidelines for purity are to be met, then traditional purification methods and resins are no longer the best, or only, choice. With the wide range of resins now available for all types of chromatography, use of historical and outdated resins is no longer necessary. Resin screening studies can be used to easily select the resin with the highest binding capacity, best selectivity and best resolution for the antibody in question. For example, the ideal resin for bispecific antibody purification and removal of mispaired dimers would consist of a small particle size combined with large pore diameters for perfusive flow and mass transfer.[18]

Further advances in efficiency and purification outcomes have been achieved by the combination of ion exchange chromatography with other methods of purification such as HIC. Known as mixed-mode chromatography, the stationary phases involved consist of both hydrophobic and charged functional groups. High levels of aggregates and high-molecular-weight impurities can be removed in a single step. This eliminates the need for multiple ion exchange chromatography polishing steps, improving cost and time-efficiency, and helping to ensure a highly pure product, even for complex mAb modalities.[25]

Conclusion

Chromatography plays an essential role in therapeutic mAb manufacture and purification. Until recently, lags in the development of updated resins and methods have decreased the efficiency and effectiveness of traditional purification pathways in the face of novel mAb technologies. However, new resins and methods, such as mixed mode chromatography, promise to address the challenges posed by more conventional methods, enabling more thorough removal of impurities in fewer steps. Selection of the best resin and platform for superior mAb purification is crucial, but it doesn't need to be complex or confusing. The following articles will outline the tools available for high-quality mAb purification and polishing, and the novel technologies that can significantly enhance the efficiency and economy of mAb manufacture.

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Purification strategies for monoclonal antibody production

Monoclonal antibodies (mAbs) are becoming an increasingly prominent therapeutic agent which can be engineered to treat a wide range of diseases and disorders, from cancers to autoimmune diseases.[1] Consequently, many developers have multiple mAbs in their pipeline, increasing the demand for rapid, efficient purification processes.

Purification of mAbs is a complex, multistep process. After initial harvesting and filtration steps, mAbs undergo capture, affinity purification and polishing steps to remove impurities.

However, the complexity of engineered mAbs can hinder the purification process, creating mis-formed products, antibody fragments and aggregates. Failure of the process requires modifications to both the capture step and the polish strategy.

This infographic will explore the modifications needed for an optimized platform process, focusing on impurity removal and the Thermo Fisher Scientific solutions available.

Removal of impurities

Typically, an affinity step and two polish steps are needed to remove a range of impurities:

- **Product-related impurities (e.g., aggregates, mis-formed product, dimers, post-translational modifications)**
- **Host cell proteins (HCPs)**
- **DNA**
- **Viruses**
- **Endotoxins (from bacterial expression systems)**

A traditional mAb downstream platform process

A typical purification process consists of three steps: affinity chromatography, often using Protein A, followed by cation exchange (CEX) then anion exchange (AEX).

Affinity chromatography
Captures proteins based on reversible protein/ligand interactions, where the ligand is coupled to a chromatography resin.

Cation exchange
Captures positively charged mAbs and impurities. Captured mAbs are then eluted by increasing conductivity or increasing buffer pH.

Anion exchange
Removes negatively charged product and process-related impurities. It can be used in both flow-through and bind-elute modes.

Typical process steps

Affinity
- Antibody isolation
- HCP reduction

Thermo Scientific™ CaptureSelect™ Affinity Resins
High purity and yield in a single step

Cation Exchange
Removal of:
- HCP
- Leached ligand
- Aggregates and fragments
- Product-related isoforms

Thermo Scientific™ POROS™ CEX Resins:
- POROS XS
- POROS 50 HS

Anion Exchange
For removal of:
- Final HCP
- DNA
- Viruses

Thermo Scientific™ POROS™ AEX Resins:
- POROS XQ
- POROS 50 HQ
- POROS 50 D
- POROS 50 PI

Optimizing polish strategies for complex mAbs

As the complexity and sophistication of mAb therapies increases, structural features such as symmetric, asymmetric and fragment-based bispecifics are becoming more common, resulting in new purification challenges. The traditional polishing process can have limited success for these mAbs, resulting in incomplete impurity removal, and risks non-adherence to regulatory guidelines.

Alternative processes, such as hydrophobic interaction chromatography (HIC) and mixed-mode chromatography (MMC) are emerging as more efficient platforms for more modern, engineered mAbs.

HIC
- Removes proteins based on interactions between hydrophobic ligands on the resin with hydrophobic motifs on proteins.
- Salt buffer conditions promote binding and stabilize mAb structure.
- Removes high levels of aggregates and a wide range of impurities.
- Suitable for bind-elute and flow-through chromatography.

MMC
- Typically combines ion exchange and hydrophobic interaction principles for improved selectivity and resolution of impurities.
- Able to purify diverse populations of proteins.
- Removes high levels of aggregates and a wide range of impurities.
- Suitable for bind-elute and flow-through chromatography.

Complex mAb process steps

Affinity
Antibody isolation
HCP reduction
Thermo Scientific™ CaptureSelect™ Affinity Resins

Complex mAb platform process

HIC
- HCP reduction
- Leached ligand removal
- Aggregate, fragments
- Product related isoforms

Thermo Scientific™ POROS™ HIC Resins:
- POROS ETHYL
- POROS BENZYL
- POROS BENZYL ULTRA

AEX as needed
- Final HCP removal
- DNA removal
- Virus removal

Thermo Scientific™ POROS™ AEX Resins:
- POROS XQ
- POROS 50 HQ
- POROS 50 D
- POROS 50 PI

CEX as needed
- Final HCP removal
- DNA removal
- Virus removal

Thermo Scientific™ Poros™ CEX Resins:
- POROS XS
- POROS HS

Challenging mAb platform process

Mixed Mode-CEX
- HCP reduction
- Leached ligand removal
- Aggregate, fragments
- Product related isoforms

Thermo Scientific™ POROS™ Caprylate Mixed-Mode Cation Exchange Resins

AEX
- Final HCP removal
- DNA removal
- Virus removal

Thermo Scientific™ POROS™ AEX Resins
- POROS XQ
- POROS 50 HQ
- POROS 50 D
- POROS 50 PI

The advantages of Mixed Mode-CEX resins

The POROS Caprylate Mixed-Mode Cation Exchange Resins have a superior pore structure for rapid mass transport and unique selectivity. This can help increase productivity, purity and yield in flow-through modes.

- High aggregate selectivity, effective up to 20% aggregation
- Cost-effective due to reduced chromatography steps and reagents
- Linear pressure-flow curve results in excellent and predictable scalability
- Wide operating range
- 80–90% mAb monomer recovery with minimal optimization

Improve your polish process for engineered mAbs with Thermo Scientific™ POROS™ Caprylate Mixed-Mode Cation Exchange Resin

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Purification of complex mAb modalities: ask the specialists

As the complexity of novel mAb modalities increases, traditional purification and polishing processes may no longer be effective. Outdated resins and archaic polishing methods can decrease the efficiency and effectiveness of mAb purification, while new mAb structures can also bring new, difficult to remove impurities. We asked three in-house specialists at Thermo Fisher Scientific for their advice on how to overcome the challenges of purifiying complex mAbs, and how to optimize your polishing strategies for maximum efficiency.

Jett Appel | *Field Application Scientist, Thermo Fisher Scientific*

Jett Appel has been a Purification Field Applications Scientist at Thermo Fisher Scientific since 2021, supporting purification development and scale-up for monoclonal antibodies, viral vectors, nucleic acids, and recombinant proteins. Prior to joining Thermo Fisher, Jett worked at Avid Bioservices, a contract development manufacturing organization (CDMO) based in Orange County, CA. During his 5 years of working at Avid, Jett first worked in process development and eventually transitioned to a senior engineer role in the Manufacturing Sciences & Technology (MSAT) team, where he supported purification development and technology transfers for monoclonal IgGs, IgMs, bispecific antibodies, scFvs, and recombinant enzymes up to 2000 L bioreactor scale. Jett received a bachelor's degree in Chemical and Biomolecular Engineering from UCLA in 2016. While at UCLA, he was involved in epigenetics research at the Steve Jacobsen Lab, which included studying the pathways involved in RNA-directed DNA methylation in *Arabidopsis thaliana*.

Nicholas Bardol | *Manager, Manufacturing Services, Thermo Fisher Scientific*

Nicholas has been working in the pharmaceutical industry for the past 13 years as a process development scientist focusing on biologics purification and small molecule conjugation. His work at CDMOs has supported over a dozen novel molecules through GMP Manufacturing and FDA filings. He joined the Saint Louis, MO, Thermo Fisher Scientific site supporting Pharma Services location in 2018 as a member of the downstream process development group.

Kelly Flook | *Senior Manager, Product Management, Purification Products, Thermo Fisher Scientific*

Kelly Flook is a dynamic product manager specializing in downstream purification of biotherapeutics and new product development. With over 15 years' experience in product development Kelly has an excellent track record of bringing the right product to the market. She started her career as a scientist specializing in the development of polymer based analytical columns for HPLC before moving into purification R&D and then product management. Kelly went to school in the UK, receiving a bachelor's degree in Analytical Chemistry from University of Northumbria, Newcastle, and a PhD in Polymer Chemistry from University of Durham.

Q: What are the main challenges faced in the purification of complex mAbs compared to traditional monoclonal antibodies?

Jett Appel (JA): It's hard to pinpoint the main challenges because it depends a lot on antibody structure – and antibody structures are diverse, creating diverse challenges. Often, when we talk about complex mAbs, we are talking about bispecific or multispecific antibodies, antibodies where the Fc domain – or even antibody fragments, such as Fabs or scFvs – are modified. So, for example, you may not be able to utilize traditional affinity steps for purification, or it could result in product impurities that are harder to separate. So, good yield or purity tends to be obtained differently from these different complex mAbs.

Nicholas Bardol (NB): An increasing number of drug candidates are bispecific, which are very difficult to separate, because bispecific expression systems produce a lot of parent mAb fragments and paratopes as impurities. Most fragments not only share a lot of the primary characteristics we would usually exploit in traditional purification strategies, but they can also be more biologically active. So if they outcompete the drug candidate for the epitope they're targeting, then the efficacy of the drug candidate really diminishes.

Kelly Flook (KF): Because structures are growing more and more complex, heterogeneity and aggregation become increasingly challenging. However, it's not just complex mAbs that are suffering from increased aggregation levels. As the industry moves towards more cost-efficient mAb production, higher cell densities and processes to increase titer can impose additional challenges downstream with respect to process- and product-related impurities.

Q: How do the structural characteristics of complex mAbs impact the purification process and pose unique challenges?

JA: It depends on the exact structure of the complex mAb. Bispecific or multispecific antibodies often have different heavy chains or light chains and this can result in mispairing. Consequently, these antibodies are very similar in physicochemical properties and can be hard to separate or may get co-purified with traditional Protein A affinity steps.

Other formats of complex mAbs – for example, antibodies that have been multimerized – are much larger than traditional mAbs. This can result in steric hindrance or lower diffusivities. That's a unique challenge because it may lead to lower resin capacities and therefore larger columns, which could ultimately lower productivity as scale increases.

NB: In the case of antibody–drug conjugates, they've been modified specifically to take on a drug linker, and that can pose many challenges – we see issues with free thiol or unbound drug linker. In the case of bispecifics, it's hard to use charge-based separation because the sequence of impurities will overlap a lot with the sequence of the target antibody.

Just as importantly, we may not be able to use our typical polishing strategies of anion and cation exchange. Instead, we have to rely more on things like multimodal or hydrophobic interaction chromatography (HIC) as a different tool to target another characteristic of those impurities and see if we can remove them with a different mechanism than one the industry typically relies on.

Q: What are some of the common impurities or contaminants encountered during the purification of complex mAbs? And how are they typically addressed?

JA: Some common impurities overlap with traditional mAbs – e.g., host cell proteins, residual DNA and aggregates. Often in these complex mAbs, we tend to see higher levels of aggregates because as these structures are engineered the impurities are either produced more in the cell culture itself or can be generated under traditional mAb purification parameters, such as exposure to low pH conditions. The complex mAbs can be less stable and form impurities inherently within the process.

Other common impurities can be mispaired homodimers, for example. They're typically addressed through a traditional ion exchange to separate the target product from these impurities. But, as Nick was alluding to earlier, this can often be a challenge. So, you may need to implement HIC or multimodal chromatography as well.

NB: I already alluded to impurities that we see with unbound drug linker or bispecific isoforms, but the biggest one that we deal with is host cell protein (HCP). In some of the newer mAbs, there is a lot of change in the pI of the target antibody, and it can overlap with the HCP pI. Traditionally, antibodies have high pI relative to the HCPs, making charge-based separation very straightforward: you bind up your antibody, and flow through all the HCP. But, suppose the target is sitting right in the middle. Then you either need multiple steps to capture and resolve everything on either side of the pI or you have to look at another option like multimodal chromatography or HIC again, just to get rid of those impurities that have historically been relatively simple to remove.

KF: Many manufacturers have developed platform purification workflows for their traditional mAbs, but these don't always work for their more complex molecules. This is when you need to turn to HIC or multimodal chromatography. For affinity purification, although Protein A continues to be the workhorse, it's not always the best option, depending on the binding domains of the specific antibody. This is where our CaptureSelect affinity resin product line comes in.

Q: Could you discuss any specific strategies or technologies employed to overcome the challenges associated with complex mAb purification?

JA: We've seen customers implementing unique affinity technologies. Typically, we've utilized traditional protein A affinity chromatography but we can see that, depending on how these complex mAbs are engineered, other affinity ligands that target different domains of the antibody could be advantageous. For bispecifics, we've seen engineered antibodies where one arm doesn't have a CH1 domain. So, for example, you could utilize an affinity resin that targets CH1. That homodimer won't bind to the resin and will just flow through instead of getting co-purified with protein A. Another interesting strategy that customers use is to leverage the avidity of the target molecule or the impurity to the resin and utilize pH gradients. This is used, depending on the antibody format, when there is more than one specific domain, or if the target molecule or the impurity has fewer domains than the other. Where traditional operating conditions for polishing steps work for standard mAbs, they may not work for complex mAbs. These may then need a unique set of conditions; for example, operating cation exchange under a more alkaline condition may help improve resolution. As Nick mentioned earlier, other technologies – such as multimodal chromatography or HIC – can be a useful tool if the standard methods do not work.

KF: Our ion exchange resins have high capacity and robust salt tolerance to be able to handle higher titers in bind-and-elute mode. Using a flowthrough process, your capacity becomes capacity for the impurity, which is typically <5% at this point, meaning you can significantly improve productivity and reduce resin consumption.

NB: I want to highlight a couple of things both upstream and downstream of purification. The industry has been chasing high titers in their clone selection for as long as biologics have been around, but choosing the clone with the highest titer may not be the best strategy. Instead, choosing the cell line with the lowest levels of a complex impurity might be the best mechanism. So be very forward when thinking about clone selection.

Another thing is analytical strategies. A lot of these new impurities come with bispecifics and isoforms that share the same charge; using all of the traditional analytical techniques, you might not get total resolution. They may co-elute and you won't know the contaminant is present until the end stages, or you may chase something that isn't there. So, looking at orthogonal assays for size or isoform pattern and charge heterogeneity can be hugely beneficial. Some less commonly used techniques can be really powerful here; using 2D-LC and native mass spec to see those hidden isoforms can save a lot of time when you're trying to develop your purification strategy.

Q: Are there any particular purification techniques or methods that have shown promising results in effectively purifying complex monoclonal antibodies?

JA: Utilizing innovative affinity technologies that target different domains can be useful to leverage the unique structures of these complex mAbs. That's one technique that has shown promise. Innovations looking at different optimization conditions for polishing steps and unique polishing resins can be a useful strategy as well. A lot of these complex mAbs may not be as stable and can form higher-level aggregates, therefore the ability to operate a wider set of conditions and separate species using resins that might be more selective for different product species can be useful.

NB: Affinity capture is the big space where we're seeing major changes and alternative mechanisms for purifying complex mAbs. For bispecifics, they have both a lambda and kappa light chain, which is something that you can target – you can capture one side, then capture the other side. That's a perfect mechanism to remove all of the fragments of the parent antibodies that would otherwise need convoluted polishing strategies to remove.

KF: We recently launched the new POROS Caprylate, our first mixed-mode cation exchange resin. As many may know, caprylic acid is used to pull down high levels of aggregates upstream. Attaching this to the bead allows aggregation to be effectively removed downstream, reducing aggregation from 20% to less than 2% without the need to then remove the caprylic acid. An added benefit is that this resin was designed specifically to be used in flow-through mode to allow high recovery over a wider range of loads compared to other resins positioned for aggregate removal in flow-through mode.

Q: How do the scalability and throughput of complex mAb purification processes compare to traditional mAbs? And what considerations need to be taken into account?

JA: It depends on the antibody and the specific challenges present. Some of these complex mAbs can have lower capacities, so you may need to utilize resins at lower loading densities or, in order to achieve appropriate resolution, operate at lower flow rates. Ultimately, this can lead to larger columns as you scale up, increased buffer consumption and longer processing times, which can reduce efficiency.

Therefore, it's important to optimize early on and utilize different strategies – e.g., using conditions that allow you to operate at faster processing times and higher capacities to reduce that burden as you scale up more complex mAbs.

NB: We've seen a need to prioritize resolution, and we were seeing lower loading on columns through the entire downstream process as we tried to get higher resolution. This can mean more unit operations in manufacturing and taller polishing columns that are more difficult to work with, as well as big increases of buffer volumes and longer residence times. So, the variations may become a little more complicated to transfer and scale up. To alleviate these issues, many programs are using very specific pH ranges or non-traditional buffers (e.g., Good's buffers such as MES or bicine) that we haven't seen used on a manufacturing scale historically. Pressure-tolerant resins can also be very beneficial – you can get the tall bed heights and the high resolution that you need. New inline dilution strategies to make the buffer volumes more manageable are also hugely beneficial when you're scaling up.

Q: What role does process optimization play in improving the efficiency and yield of complex mAb purification? And what are some key factors to consider during optimization?

JA: Process optimization plays a critical role because your goal is to maximize purity and recovery and still maintain the quality of the product. But you also want to ensure that it's scalable, robust and overall low-cost once you have a fully scaled-up process. Monitoring the process is important to better understand how it operates – ensuring that you're not operating at the edge of failure and identifying the parameters that could impact your process

NB: Complex mAbs tend to have lower bioreactor titers overall because they are more difficult to grow, so even a couple of percent gain can be meaningful. Relying more heavily on DOEs and quality by design frameworks can help labs to get the most information possible with the least material, so that those optimization strategies can be executed with just one batch.

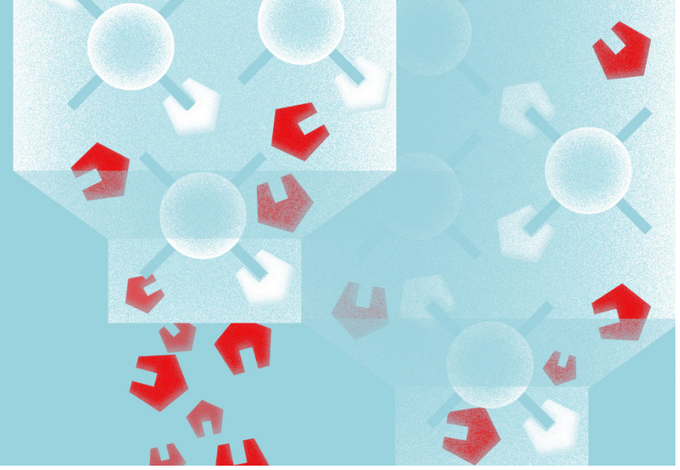
Q: What are the potential future advancements or innovations in complex mAb purification that could address existing challenges and further improve the process?

JA: Firstly, the continuing advancements of different affinity technologies. Different affinity ligands target different domains – so designing new ligands that are specific for different domains or have different characteristics that can be leveraged for separating difficult-to-resolve impurities in these complex mAbs is important.

Another interesting advancement is the use of chromatography modeling software. Mathematical models can reduce the number of DOE experiments required and help us have a better understanding of the process. We're even starting to see customers utilize AI or machine learning techniques for their large datasets of different antibody sequences and structures. AI can look at these datasets and use pattern identification to predict the behavior of different chromatographic resins.

NB: The other thing that we're seeing customers move towards is process analytical technologies. Not all of these mAbs can be tightly controlled just by looking at a UV signal. Having inline or online detection or mass-based detection – instead of just looking for chromophores that may not exist or may completely overlap with your antibody – has real potential to solve challenging separation issues that we may not be able to resolve at scale.

KF: Achieving high purity is all about having the right resin for your molecule. Chromatographic efficiency can be achieved by reducing the particle size, but that comes with the expense of flow rate or bead height due to the pressure limitations of hardware. Modulating or designing the right chemistry or ligand on the surface to be selective between target and impurities is a more effective way to increase purity and yield. But as molecules become more complex, this means more tools are needed across different variations from the traditional mAb. mAb developers can engineer in things like a C-tag to enable selective removal or a specific sequence that interacts with specific ion exchange or HIC groups.



Antibody therapeutics

Advances in flow-through technology to enhance mab polishing

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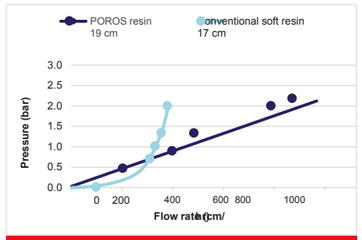
Summary

The rapidly evolving landscape of monoclonal antibody (mAb) development has led to increasing demand for innovative purification approaches, particularly during the polishing stage. Advances in flow-through technology have enhanced mAb polishing, with a growing emphasis on hydrophobic interaction chromatography (HIC) and mixed-mode chromatography. These approaches can remove challenging species not easily removed by other methods, enhancing product quality and manufacturability. This makes flow-through technology an essential tool to address the increasing complexity and variety of mAbs in clinical development.

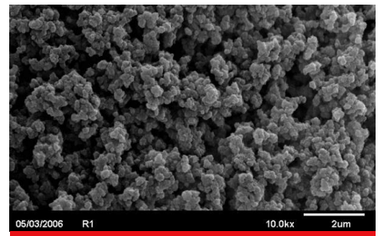
Increasingly complex antibody molecules

In recent years, the variety of mAbs has expanded beyond traditional IgG formats. The emergence of alternative antibody derivatives such as antibody-drug conjugates (ADCs), bispecific mAbs, Fab fragments and Fc-fusion proteins presents unique challenges (Figure 1). For example, some of these formats display absent or altered protein A binding sites, overexpression of free light chains or increased propensity for aggregation. These challenges place considerable pressure on downstream development teams to continuously evolve and maintain a robust purification approach.

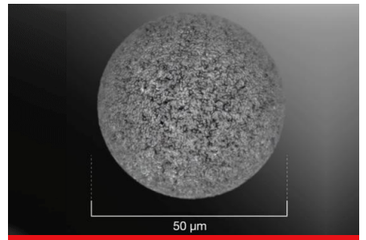
Figure 1. Different mAb modalities that demand additional tools for efficient purification.



Linear pressure flow curve



Efficient purification



Superior resolution

Poly(styrene-divinylbenzene) backbone **Large through-pores** **50-micron bead size**

Figure 2. Key features of the POROS bead.

Key considerations in downstream processing

Developing effective downstream antibody processing involves balancing multiple factors. First and foremost, product quality is of paramount concern. Chromatography resins must provide high resolution to effectively separate the product of interest from impurities, such as aggregates and host cell proteins (HCPs). Ideally, resins should offer high capacity and throughput, allowing researchers to minimize costs, reduce processing times and manage intermediate pool volumes. POROS chromatography resins are designed with these factors in mind and allow for simple downstream processing.

The POROS base beads are comprised of polystyrene-divinylbenzene, a rigid polymer that results in a stable packed bed and a linear relationship between flow rate and pressure (Figure 2). This makes scale-up, as well as optimization of flow rate and process efficiency, more straightforward with respect to column pressure-drop. The POROS base bead also has large through-pores, which reduces resistance to mass transfer. This translates to more robust binding capacity and resolution with respect to flow rate. Moreover, the bead itself has an average diameter of 50 µm. This size allows for a proper balance between resolving power and the ability to maintain scalability and sufficient pressure flow characteristics.

Chromatography can be operated in either bind-and-elute mode or flow-through mode. In bind-and-elute mode, the resin binds both the product of interest and impurities (such as aggregates), and then the product of interest is selectively eluted from the column. This mode is advantageous for separating closely related species,

Figure 3. Comparison of key characteristics of POROS beads compared to select competitor products, highlighting higher capacity and resolution, independent of flow rate.

such as charge variants. However, for aggregate and host cell protein removal, comparable product quality can be achieved using flow-through chromatography. In flow-through mode, only impurities bind to the stationary phase, allowing for higher mass loading, which results in reduced resin usage, fewer processing steps and lower buffer consumption. All these benefits contribute to shorter processing times and a smaller equipment footprint.

POROS hydrophobic interaction chromatography resins

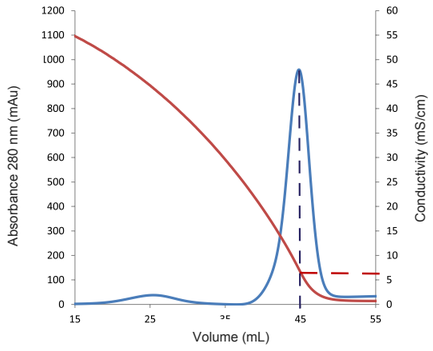
The POROS HIC family of resins includes POROS Ethyl, POROS Benzyl and POROS Benzyl Ultra resins. POROS Ethyl is the least hydrophobic while POROS Benzyl and POROS Benzyl Ultra offer higher hydrophobicity. POROS Benzyl Ultra is designed specifically for flow-through mode under low-salt conditions. These POROS HIC resins display higher capacity and resolution independent of flow rate compared to competitors' products (Figure 3). Moreover, they display consistent lot-to-lot performance as well as the linear pressure-flow drop, which is characteristic of the POROS base bead, making them ideal for large-scale bioprocessing.

HIC can be used in a range of applications across different therapeutic areas. For example, it can also be used for enzyme, recombinant protein and virus purification. It is also useful for reducing aggregates and other product and process-related impurities during mAb purification, including ADC purification for the resolution of individual drug–antibody ratio (DAR) species and Fc fusion type molecules. The following case studies highlight the effective use of flow-through applications for HIC.

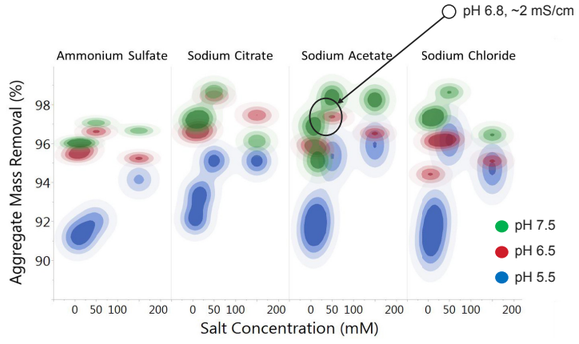
Case Study 1: Optimizing a mAb purification polishing step in flow-through mode using POROS HIC chromatography in flow-through mode

The first case study focuses on mAb A, a clinical-stage antibody with an existing process involving affinity capture, depth filtration, low pH hold, anion exchange in flow-through mode and a mixed-mode bind-and-elute step to reduce high aggregate levels (>12%) (Figure 4). Despite achieving 99% monomer purity and 90% recovery with the mixed-mode step, the throughput was limited to 25 g/L of resin at a 6-minute residence time.

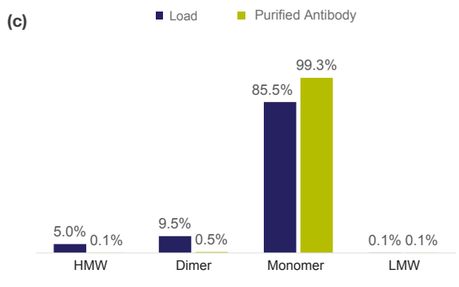
(a) mAB A: Bind-Elute POROS Benzyl Ultra



(b) Conditions: 0–150 mM Salt, pH 5.5–7.5



(c)



(d)

mAb-A Process	Mixed-Mode BE	POROS Benzyl Ultra-FT
Load density (g/L resin)	25	**80**
Monomer purity Pool (%)	99	**>99**
Monomer recovery (%)	90	**98**
Residence time (min)	6	**1.2**
HCP (ppm)	<LLOQ	**<LLOQ**

Figure 4. Bind and elute experiment (a) performed with POROS Benzyl Ultra to determine the starting point for flow through conductivity, with peak elution at ~7 mS/cm. (b) Heat map highlighting aggregate mass removal from high throughput screening performed using POROS Benzyl Ultra and four salts (ammonium sulfate, sodium citrate, sodium acetate, sodium chloride) from 0–150 mM, pH 5.5–7.5. (c) Verification run to show effective reduction of aggregates in no-salt FT process with high recovery, carried out at flow rate 500 cm/hr, 1.2 min residence time, load density 80 g/L. (d) Comparison of product quality, with FT showing an 8% increase in monomer recovery and ~threefold increase in load density.

To optimize this process, the mixed-mode step was replaced with a flow-through POROS HIC step. The process development work involved three stages:

1. Determining optimal flow-through conductivity: A low-loading bind-and-elute experiment was conducted with a decreasing conductivity gradient to establish a starting point for flow-through conductivity optimization.
2. High throughput screening (HTS): During this step, various salt types, concentrations and pH levels were evaluated to optimize conditions, focusing on POROS Benzyl Ultra.
3. Scale down model: Column loading studies were performed to assess residence time effects.

Results showed that the POROS Benzyl Ultra resin operated in flow-through mode provided comparable aggregate removal to the mixed-mode separation operated in bind-and-elute mode. Although product quality in terms of aggregate removal was equivalent in both modes, the flow-through HIC step noticeably improved recovery, with a boost of 8% (Figure 4). Similarly, column loading capacity was almost three-fold higher with the flow-through HIC step, with column loading increased to 80 g/L. Furthermore, the residence time, or flow rate, was five times faster with the flow-through HIC step. Thus, the flow-through HIC step matches the product quality of the mixed-mode bind-and-elute step and is more efficient with respect to binding capacity and flow rate, resulting in productivity gains.

Case Study 2: POROS Benzyl Ultra viral clearance and impurity removal in an ADC manufacturing process

The next case study involved the evaluation of the POROS Benzyl Ultra resin for viral clearance and impurity removal during an ADC manufacturing process. The company producing this ADC utilizes synthetic amino acids that allow for site-specific conjugation of the drug linker, creating a highly homogenous DAR. However, this process can result in high levels of aggregates (7–11%).

The POROS Benzyl Ultra resin was used to reduce high molecular weight (HMW) species as well as host cell proteins for pre-conjugated mAbs in three different processes. The results showed good reduction of host cell proteins and HMW impurities using high loading densities (Figure 5). In a viral clearance study for mAb A, yield was comparable across qualification, XMuLV-spiked and MVM-spiked runs, averaging 85%. A log reduction value (LRV) of >5.97 was achieved for XMuLV and a LRV of 4.56 was achieved for MVM, demonstrating effective viral clearance of a model parvovirus and retrovirus for this process.

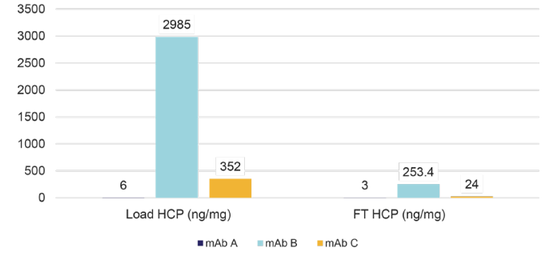
Flow-through high aggregate mAb polishing using POROS Caprylate Mixed-Mode Cation Exchange Chromatography Resin

Thermo Scientific POROS Caprylate Mixed-Mode Cation Exchange resin is a unique mixed-mode chromatography tool designed for high aggregate selectivity in flow-through mode that became commercially available in 2024. The ligand, caprylic acid, imparts both hydrophobic and weak cation exchange characteristics.

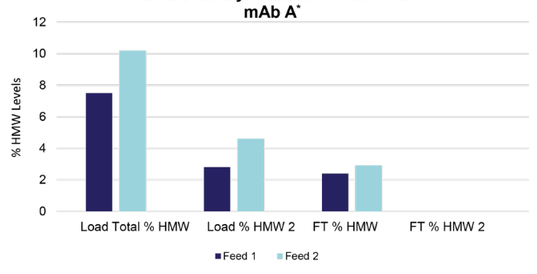
POROS Caprylate Mixed-Mode Cation Exchange resin is suitable for moderate to high aggregate levels (up to 20%) and operates over a broad pH (4.5–7.0) and conductivity range (10–30 mS/cm). To demonstrate the aggregate removal capability of the resin an IgG1 mAb was purified via Protein A capture and subjected

Loading densities — mAb A – 100 g/L | mAb B – 150 g/L | mAb C – 150 g/L

(a) POROS Benzyl Ultra HCP Reduction



(b) POROS Benzyl Ultra HMW Removal mAb A*



Reduction of host cell proteins and HMW impurities achieved at high loading densities

Figure 5. (a) Host cell protein reduction for three different mAbs using POROS Benzyl Ultra and (b) a summary of aggregate removal for mAb A using two feed streams. *Post POROS Benzyl Ultra HMW levels for mAb B and C were <1%.

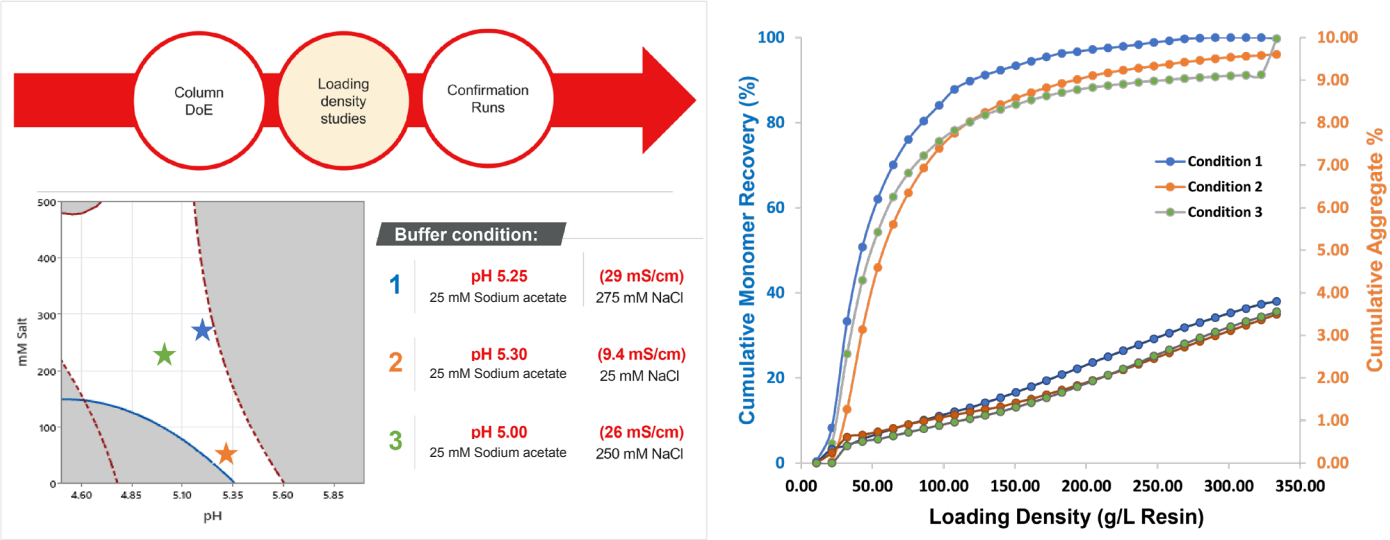


Figure 6. Loading density studies, performed across three buffer conditions, to confirm that POROS caprylate can facilitate effective polishing with high monomer yield and purity.

to high and low pH adjustments to generate up to 10% aggregate in the feed stream. A bench-scale design-of-experiment (DOE) was performed to evaluate the impact of pH (4.5–6.0) and sodium chloride concentration (0–500 mM) on the responses of yield and high molecular weight (HMW) reduction using POROS Caprylate Mixed-Mode Cation Exchange resin in flow-through mode. Column loading was kept constant at 100 g/L resin in the DOE. The results showed >75% monomer recovery and robust aggregate removal (<2% aggregate) across a wide range of conditions, with monomer recovery expected to increase with higher column loading.

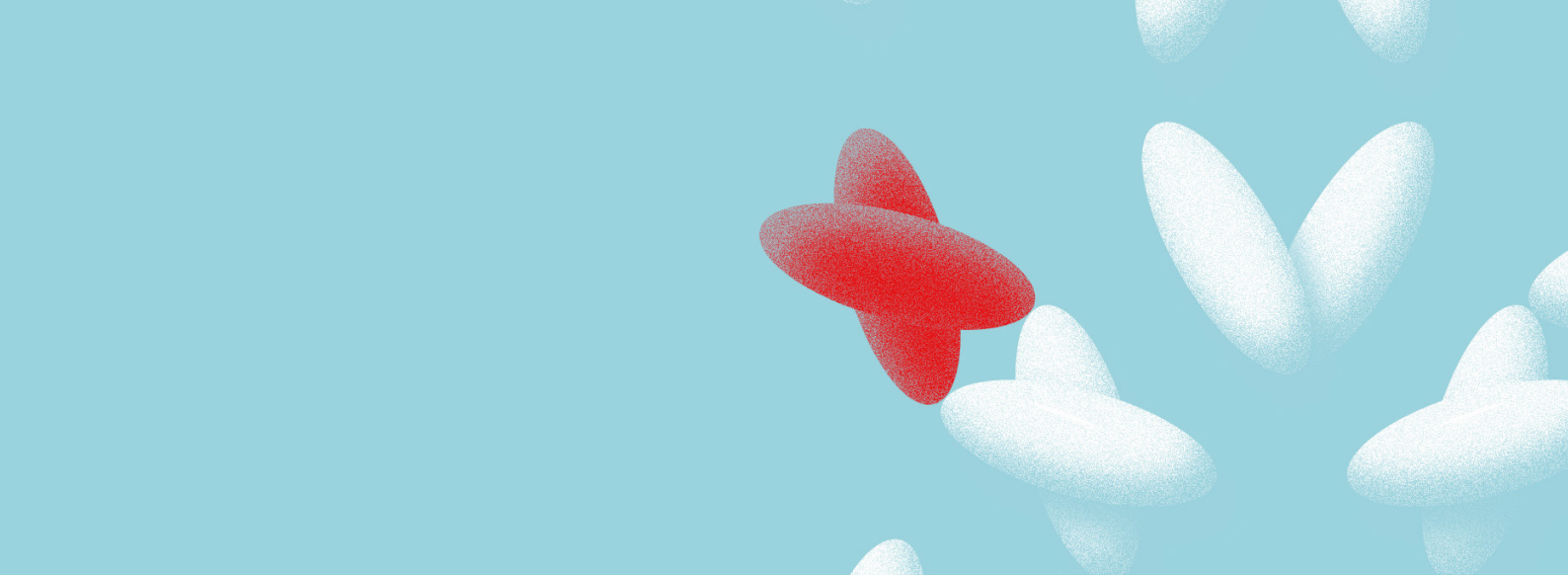
Next, loading density studies were performed at three different conditions within the DOE space (Figure 6). For all three conditions, <2.0% aggregate in the product pool was achievable with ≥90% monomer recovery at 160–180 g/L loading density. Additionally, HCP and leached Protein A were reduced by approximately 95% for all three operating conditions. Further characterization of HCPs by HPLC-MS/MS showed that POROS Caprylate Mixed-Mode Cation Exchange resin was able to reduce the number of individual HCP species from 380 to 79, with complete removal of many HCPs considered to be high risk or challenging to remove in mAb processes.

Conclusion

Advancements in flow-through chromatography technology, particularly with POROS chromatography resins, offers significant enhancements for polishing in mAb manufacturing processes. The case studies highlighted here demonstrate the applications and benefits of these advanced resins, paving the way for more efficient and effective bioprocessing strategies.

Watch the complete webinar with Robert Stairs **here**.

Learn more at **thermofisher.com/mixed-mode-chromatography**



5 key considerations for optimizing resin selection for efficient mab purification

Introduction

Monoclonal antibodies (mAbs) have become a major class of therapeutics and are now approved for clinical use in various fields, including cancer, autoimmune disorders and infectious diseases such as Ebola and COVID-19.[1] In order to adhere to regulatory guidelines, mAbs must meet certain purity standards, making purification a critical step in the bioprocessing workflow.

As the complexity of novel antibody modalities has increased (e.g., antibody–drug conjugates, bispecific antibodies, etc.), traditional purification techniques have struggled to meet required purity levels while also maintaining high yields and cost-effective processes. Complex engineered antibodies can be prone to aggregation and fragmentation and may have increased sensitivity to pH. In addition, they may also have altered or absent protein A binding sites, complicating purification further.

Recent advances in purification methods have resulted in the emergence of novel affinity and polish purification tools that can help to overcome many of these challenges. Something as simple as selecting the optimal resin for purification can help achieve high purity and efficient yields. This guide will discuss some of the key considerations for mAb purification, helping you to optimize your purification and polishing processes while still balancing cost efficiency.

1. Addressing challenges in complex purification

Although leaps are being made in polishing and purification processes, inherent challenges in the purification of complex mAbs still arise at every stage of the mAb manufacturing process. Cellular expression systems lead to high levels of process-related impurities, such as residual host cell proteins (HCPs) and host cell DNA. In addition, the inherent heterogeneity of mAbs leads to product-related impurities such as aggregates, misformed products and post-translational modifications.

These impurities must be removed before product release to meet stringent regulatory and safety guidelines and prevent adverse effects in recipients. This is a complex process, especially for novel antibody modalities. Instead of relying on traditional purification and polishing methods, look to recent developments and emerging tools in mAb processing for solutions to complex challenges.

Hydrophobic interaction chromatography (HIC) (which removes proteins based on hydrophobic interactions between resins and target proteins), and **mixed-mode chromatography (MMC)**, which typically combines ion exchange and HIC properties for improved selectivity, are two such alternative processes, already addressing a range of challenges.[2,3] For example, Chinese hamster ovary (CHO) cells are one of the most common cell lines for mAb production.

However, these cells produce several notoriously difficult-to-remove HCPs, including clusterin, which can co-elute with the target mAb.[4] MMC has been shown to be effective in removing or significantly reducing the most challenging and high-risk HCPs to acceptable levels.[5]

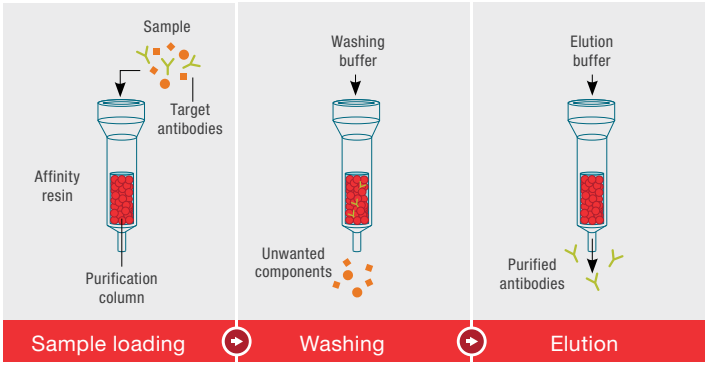


Figure 1. Protein A chromatography.

2. Evaluating affinity chromatography options for mAb capture

Affinity chromatography is widely accepted as one of the most efficient and effective techniques for the initial stages of mAb purification, due to its specificity, ease of operation, and high yields, purity, and throughput.[6] While other chromatography methods rely on separation by differences in size or ionic charge, affinity chromatography relies on specific binding interactions between an immobilized ligand and the mAb to be purified (Figure 1).

Due to the specificity of affinity chromatography, it is important to select the right resin for the mAb in question. The most common resin for affinity purification is **protein A**, which binds primarily to the Fc region between the CH2 and CH3 domains of most IgG subtypes.[7,8]

Although protein A is considered a trusted resin of choice for the purification of traditionally structured mAbs, it can underperform with novel modalities such as bispecific antibodies (bsAb), due to formats that produce Fc-containing mispaired product variants, formats that have modified Fc regions that limit or eliminate binding to protein A, or fragment-based antibody formats. In addition, the acidic elution used during protein A chromatography can lead to aggregation of some modalities, making impurity removal more difficult. In these cases, alternative resins such as **protein L** are available. Though still requiring a low pH, protein L binding is specific for the variable light chains and can bind most antibody classes and fragment fragment antigen-binding region (Fab) fragments.[6,9] Other resins based on **camelid-derived single-domain antibody fragments** have been developed specifically for bsAbs and antibody fragments. These come in a range of different binding sites and have high affinity, specificity and capacity, allowing for a streamlined workflow and high-purity products.[10] They can also enable mild elution, preventing pH-induced aggregation.

In addition to careful resin selection, recovery rates can also be maximized by optimizing the purification process and its steps. If bind/elute steps are providing suboptimal monomer recovery, changing the workflow to a flow-through (FT) operation using a HIC resin can improve recovery. HIC resins are particularly well suited for manufacturing-scale workflows, due to their high linear binding capacity over a wider range of flow rates.

3. Strategies for effective polishing steps

Following mAb affinity capture, product purity of non-complex antibodies may be as high as 95%. For more complex, engineered mabs, purity levels of 80% or below are not uncommon. To achieve higher purity, the next steps in the downstream mAb purification process are intermediate and final polishing. This typically consists of a cation exchange chromatography step (CEX), followed by anion exchange chromatography (AEX) and sometimes HIC. For relatively clean feeds, a single polishing step may be sufficient, provided the product purity and safety requirements are met.

Selecting an appropriate resin combination along with optimized process conditions enables orthogonal separations that maximize yields, reduce process and product-related impurities, and improve process robustness. Resins should align with the purification step and the charge properties of the target molecule or impurities to be removed. In AEX, the chromatography matrix is positively charged, so negatively charged molecules can be captured. For example, impurities such as viruses, HCPs, DNA, endotoxins and aggregates can be captured and removed from the final product. In comparison, CEX captures positively charged mAbs, which can then be eluted by increasing the conductivity or buffer pH.

For complex mAbs, different polishing strategies may be needed. IEX using standard conditions may result in inadequate impurity removal, risking non-adherence to regulatory guidelines. HIC and MMC offer unique selectivity and can often be more

effective platforms for novel mAb modalities. HIC resins can be utilized in either bind and elute or flow-through mode, depending on the hydrophobicity of the resin, the salt concentration and the salt type used (e.g., kosmotropic salts like ammonium sulfate). This allows for highly customizable options for targeting specific impurities, such as product variants in bind and elute mode or removal of aggregates and HCPs in flow-through mode. HIC resins can support high resolution, even under low conductivity conditions often encountered post protein A, making HIC an excellent option for manufacturing scalability. HIC resins are often utilized for the purification of engineered mAbs, including antibody fragments and antibody–drug conjugates. MMC combines the benefits of IEX and HIC for even greater selectivity and purification due to a unique stationary phase that contains both hydrophobic and charged functional groups (Figure 2). MMC is ideal for a diverse range of proteins and particularly challenging mAbs with high aggregate levels and complex impurity profiles, as it can reduce 10% aggregation to around 1% in a single polish step.[3]

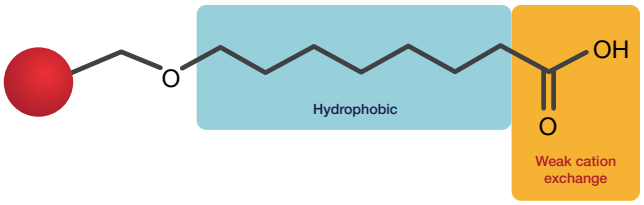


Figure 2. The structure of an MMC resin backbone.

4. Enhancing recovery rates through optimal resin selection

A generic purification workflow may be able to meet regulatory guidelines, but if it isn't achieving maximal recovery rates, it could be costing you product and efficiency. Additionally, a process that works well at a pilot or development scale may not run efficiently at a manufacturing scale. It's important to optimize your workflow in a manner that maximizes recovery rates while still maintaining high purity and efficiency. To achieve this, resin screening studies can be used to select the resins with the highest binding capacity, best selectivity and best resolution for the antibody in question.

Recovery can be affected by resin characteristics such as binding capacity, elution efficiency and stability, therefore these are important aspects to consider when selecting the best resin. Some engineered antibodies can have altered, blocked or absent protein A binding sites, causing issues for traditional protein A-based purification. In these cases, specialized matrices can be used, designed to target different binding sites while still maintaining high dynamic binding capacity. For example, for Fab purification, ligands that bind the constant heavy (CH1) domain of IgG subclasses, or the kappa and lambda Fab regions, can be used. For Fc-fusion proteins and chimeric antibodies, ligands that bind only the constant heavy domain (CH3) are available.

Resin selection is also key in polishing steps. While IEX is often considered as a first choice for mAb polishing, HIC resins are an effective alternative to address high levels of aggregation or in antibody–drug conjugates (ADCs), where further purification is needed to refine the drug–antibody ratio (DAR). HIC resins can operate using various salt types, over a range of salt concentrations, and come in a range of hydrophobicities. They offer improved resolution and can help achieve enhanced clearance of aggregates with higher productivity. You can also consider mixed-mode (MMC) resins. As previously mentioned, these resins combine properties of IEX and HIC, and they can remove even high levels of aggregates in a single flow-through step, thereby reducing the number of polishing steps and improving efficiency.[11]

5. Balancing cost and performance in resin selection

The core goal of purification is to isolate a high-purity product in a reliable and cost-efficient manner. In addition to ensuring optimal performance, economic aspects – such as initial costs, lifespan and regeneration capabilities – must also be considered when selecting a resin.

Reducing the number of steps in the downstream purification process can improve cost-efficiency, as it reduces processing time and is faster to optimize. A streamlined process can also reduce the amounts of reagents needed and the labor and utilities costs. For example, high-performing techniques such as affinity chromatography for purification and MMC for polishing can achieve higher yield and purity than other methods in a single step, increasing efficiency without compromising recovery. To streamline IEX steps, resins that have large through-pore structures can allow higher capacity, while resins optimized for low to high flow rates can help ease the scale-up process from small-scale to process-scale production.

Ensuring protocol validation and compliance can be complex, costly and time-consuming. Therefore, consider resins that can help address these issues; for example, resins with animal-free origins can help ease adherence to regulatory guidelines. Good availability and quality of technical support from the resin vendor can also help with validation and optimization, while choosing a vendor with a reliable supply chain and a proven history of delivering high-quality product can help mitigate the risk of unforeseen circumstances and facilitate optimal product manufacture.

Resin reuse is another effective method of improving cost-efficiency, though this should be approached carefully so as not to affect performance. Consider the chemical stability of the resin, dynamic binding capacity over several cycles, monitoring of product quality attributes, and other parameters when assessing a column lifecycle. For example, single-domain V_HH antibody fragment affinity resins are inherently stable, making them suitable for large-scale processing.

Conclusion

The ultimate goal of every mAb purification process is to produce a high-quality therapeutic that meets stringent efficacy and patient safety guidelines. However, it is also important to consider workflow productivity, in order to keep therapeutics affordable and manufacturing efficient. Significant leaps in efficiency and performance can be achieved by simple changes, such as informed resin selection. Selecting the right resin for your process can address aggregation challenges, improve cost-efficiency and maximize recovery rates, while still adhering to regulatory guidelines. Optimize your purification workflow today and see the real-world benefits to your mAbs.

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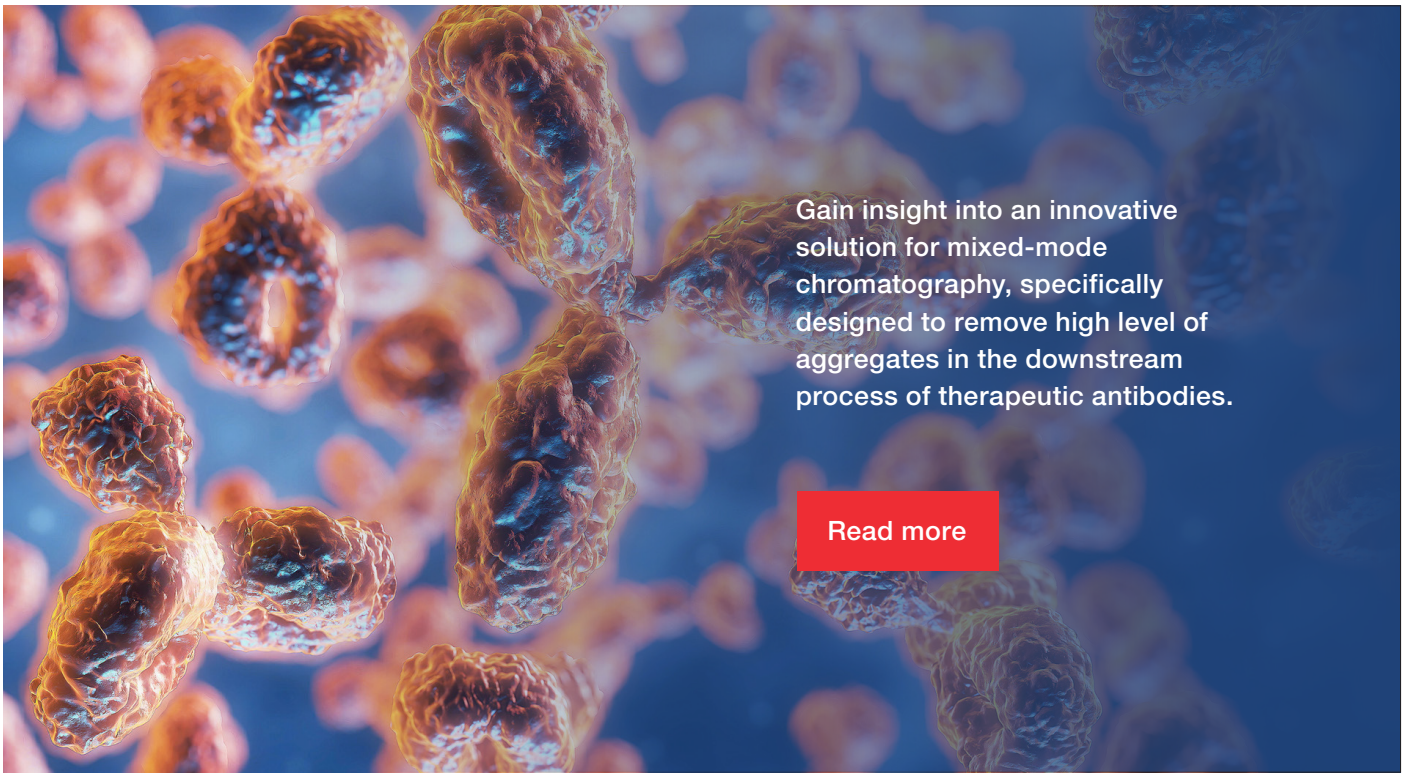

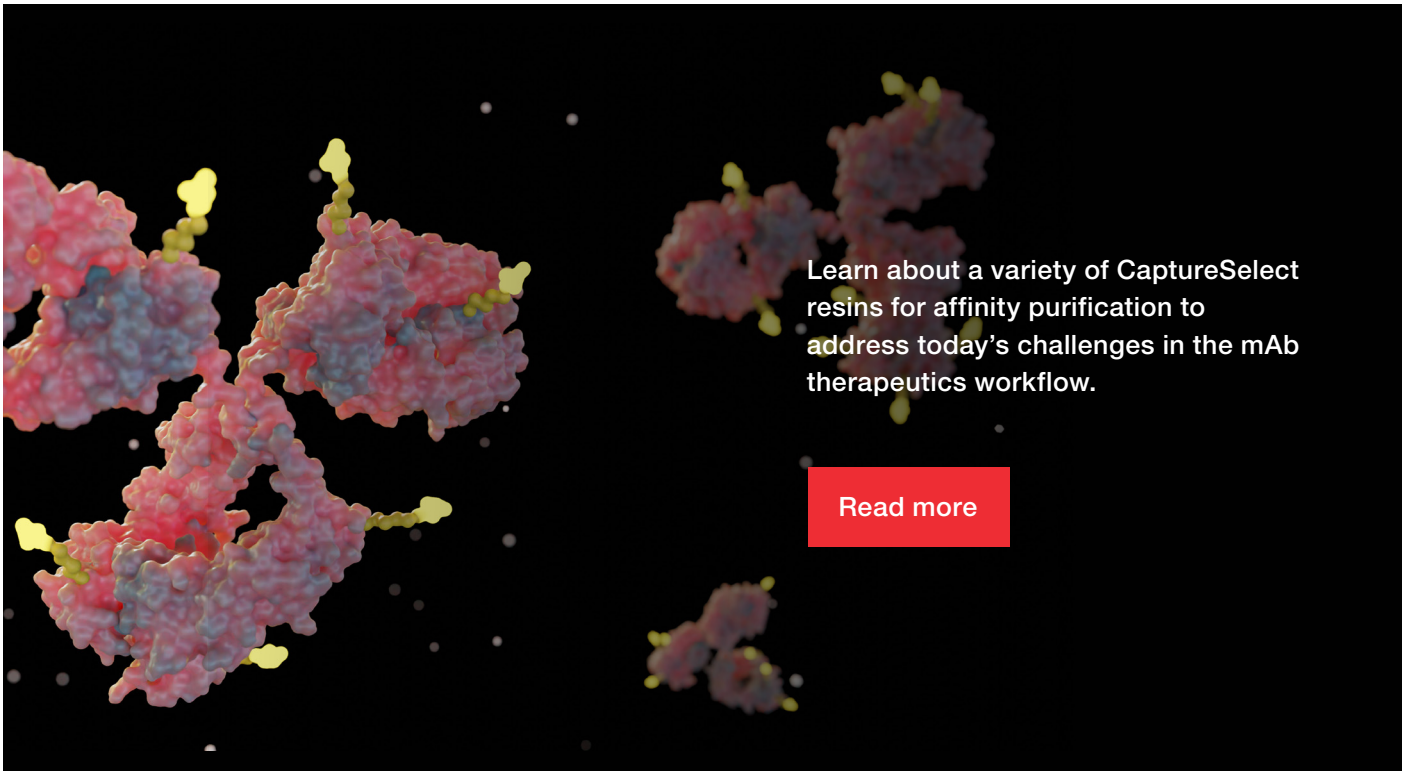

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