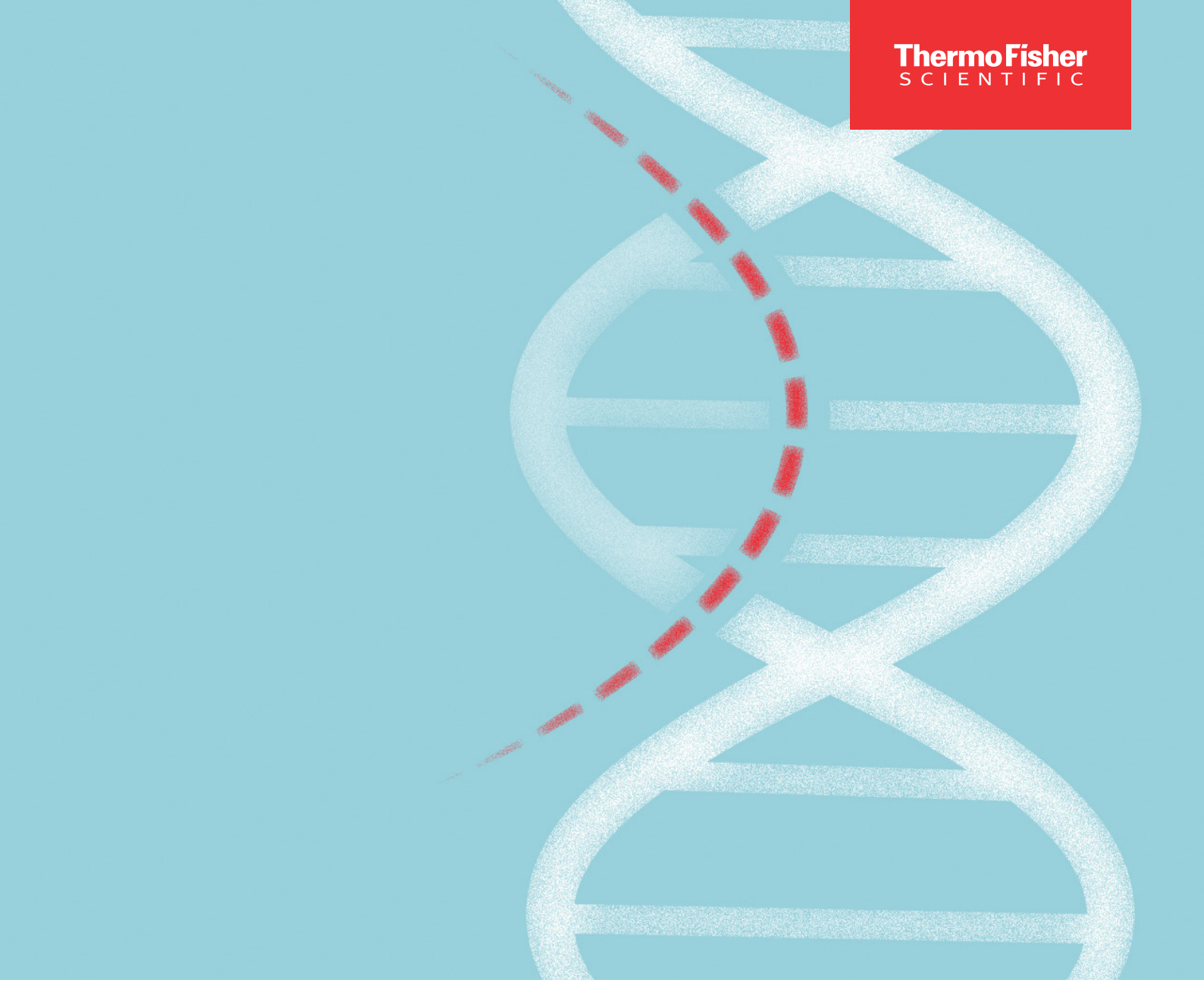

Optimizing gene therapy

A comprehensive guide to AAV purification

thermo scientific

Foreword

We are at the forefront of a gene therapy revolution, with over 30 groundbreaking treatments for a wide range of conditions already approved by the FDA. Central to these advancements are adeno-associated virus (AAV) vectors, which can be highly effective and versatile in therapeutic applications.

However, as the demand for AAV-based therapies grows, so does the need for scalable production methods. Meeting this demand requires a profound optimization of manufacturing processes to help ensure efficiency and scalability, while still maintaining high quality, purity and potency.

Through a comprehensive exploration of current methodologies, technological advancements and industry best practices, this eBook aims to equip readers with a deeper understanding of the obstacles and opportunities in AAV production.

Contents

The importance of AAV purification and analytics

Introduction

The rise of gene therapies marks a radical shift from traditional treatment approaches by addressing the underlying genetic causes of a disease rather than merely treating the symptoms. Initially conceived in the 1970s, gene therapy has rapidly evolved with the advent of more precise and safer delivery methods, leading to several breakthroughs in clinical applications. With the approval of over 30 gene therapies by regulatory agencies worldwide, the field has transitioned from experimental to therapeutic, offering hope for conditions once deemed untreatable.[1]

A key component of gene therapy is the efficient delivery of therapeutic genes into host cells. Viral vectors are often chosen due to their natural capability to efficiently infect human cells. These vectors are derived from viruses like adenovirus, lentivirus and adeno-associated virus (AAV) that have been engineered to be safe and effective. AAVs are one of the most promising vectors in gene therapy due to their ability to provide long-lasting gene expression, target specific cell types and exhibit relatively low immunogenicity.[2]

Large-scale production of AAV-based treatments relies on robust purification and analytical methods to ensure the consistency, potency and safety of the final product. A primary concern is the removal of impurities, such as residual host cell proteins, DNA and empty capsids, which can compromise the efficacy and safety of gene therapies. However, current methods are often difficult to scale, time-consuming and limited to specific AAV serotypes.

As the number of approved AAV-based therapies and clinical trials continues to grow, there is an increasing demand for rigorous quality control measures and improved purification techniques.[3] Continued development and refinement of purification and analytical technologies are therefore pivotal in advancing gene therapy, ensuring that these innovative treatments can safely and effectively reach the patients who need them.

This article explores the challenges associated with large-scale AAV purification and the latest advancements in purification techniques and analytical methods.

Challenges of AAV purification

The production of AAV vectors follows a complex workflow, beginning with vector design and cloning, where the therapeutic gene of interest is inserted into an AAV plasmid vector. This vector is then transfected into host cells, typically HEK293 cells.[4] Following transfection, the cells are cultured under specific conditions to produce the AAV particles. After an incubation period, the cells are harvested and lysed to release the AAV particles. The crude lysate then undergoes several purification steps to isolate and concentrate the AAV particles, followed by formulation into a final product suitable for clinical use (Figure 1).

Purifying AAVs is a critical step in the production process, ensuring that the final product is effective and safe for

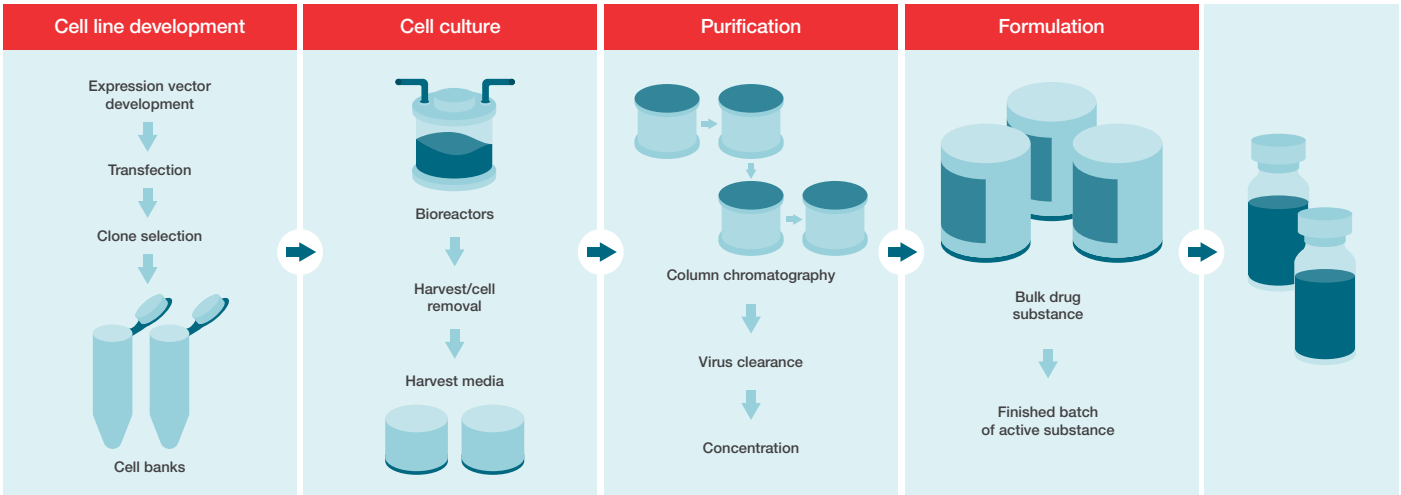

Figure 1. AAV manufacturing process for gene therapy products.

therapeutic use. The primary goal of purification is to remove impurities such as host cell proteins, DNA and other contaminants while maximizing the yield and potency of the AAV vectors. A range of techniques can be used for purification, including centrifugation and chromatography.

Downstream purification of AAV vectors presents several key challenges that significantly impact the yield and efficacy of the final product. One major issue is the increased impurity burden due to cell lysis, which releases a substantial amount of host cell proteins, adventitious viruses and other debris into the crude lysate. However, complex purification processes aimed at removing these impurities often lead to substantial loss of viral particles. Thus, improved purification techniques are key to balance efficient purification with a good recovery yield.

The large variety of AAV serotypes also complicates the purification process. AAV vectors come in many different serotypes, each with distinct capsid proteins and surface properties.[5] This diversity creates challenges in developing a standardized purification process, as different serotypes may require specific conditions or methods for optimal purification. Tailoring purification protocols to accommodate the variety of AAV serotypes can add complexity and cost to the production process.

Perhaps the most difficult challenge is the enrichment of full capsids. The production process often results in a mix of full, empty and partially filled capsids. Full capsids are crucial for delivering the therapeutic gene, while partial and empty capsids can dilute potency and increase the total viral load needed for effective treatment. Moreover, empty capsids can trigger immune responses, compromising transduction efficiency and limiting the potential for repeat dosing due to the development of neutralizing antibodies.[6] Hence, advanced separation techniques are needed to enrich the full capsids effectively.

Analytical techniques in AAV purification

Analytical techniques are vital for accurately assessing critical quality attributes (CQAs), such as the purity, potency and safety of AAV products. Several CQAs are essential for characterizing AAV products, including (i) virus titer, (ii) capsid aggregation and (iii) the ratio of full to empty viral particles (Figure 2).[7] Several analytical methods are employed to assess these attributes, each offering unique insights into the quality of AAV products.

For example, analytical ultracentrifugation (AUC) is widely regarded as one of the main methods for determining the ratios of full, partial and empty capsids, in addition to providing detailed aggregation profiles. Viral titer and content ratios can be measured using a combination of quantitative PCR (qPCR) and enzyme-linked immunosorbent assay (ELISA), where qPCR measures the viral genome and ELISA quantifies capsid protein content. Additionally, transmission electron microscopy (TEM) can provide detailed visualization of AAV particles, allowing for the evaluation of capsid integrity, aggregation and morphology.

To date, manufacturing purification methods for AAV vectors typically rely on a sequence of chromatography techniques due to their ease of scalability. The process generally begins with affinity chromatography which purifies the AAV particles from crude mixtures. In this stage, specific ligands within the affinity column are engineered to selectively bind to AAV capsid proteins. As the mixture flows through the column, AAV particles are captured by these ligands, while impurities are allowed to pass through, resulting in a significantly purified AAV product. This product can then be further refined through additional chromatography techniques, such as anion-exchange high-performance liquid chromatography (HPLC). Anion-exchange chromatography (AEC) is particularly effective in enriching full capsids based on their distinct charge properties, thus enhancing the overall quality and consistency of the final AAV preparation.

However, these techniques come with various challenges.[7,8] Many of them face issues related to scalability and throughput, which are crucial for large-scale production. For instance, TEM and AUC are labor-intensive and have long turnaround times, making them less suitable for high-throughput analysis. Techniques like qPCR and ELISA, while highly sensitive, can

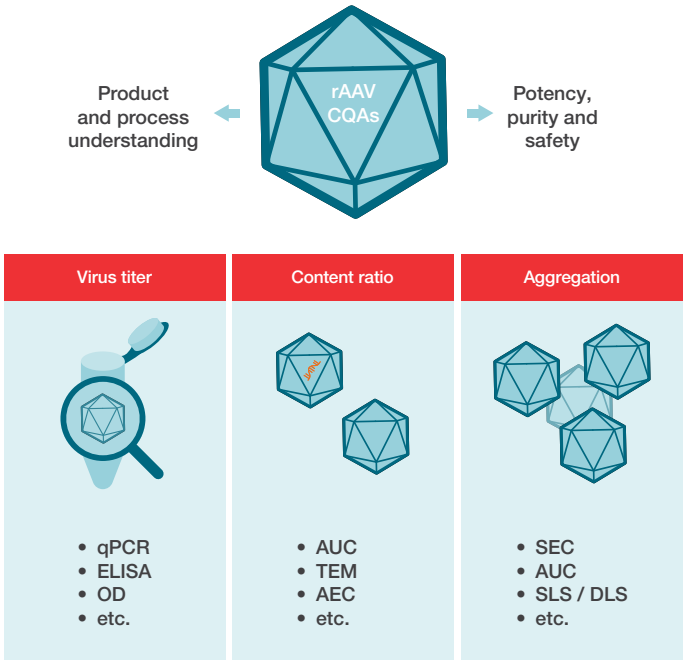

Figure 2. Several techniques can be used to measure the potency, purity and safety of AAV therapies including optical density (OD), size exclusion chromatography (SEC) and static/dynamic light scattering (SLS/DLS).

only quantify limited serotypes and often require multiple steps, increasing the complexity and time required for analysis. Anion-exchange HPLC, although effective in separating AAV particles, can be hampered by resins with small pores, limited binding capacities and long turnaround times, leading to suboptimal performance in large-scale operations.

Hence, developing more robust and streamlined analytical workflows is essential to support the growing demand for AAV-based therapies. By addressing these limitations, the gene therapy field can achieve more efficient production and quality control, ultimately leading to safer and more effective treatments for patients.

Trends and innovations

As the field of AAV-mediated gene therapy progresses, there is an increasing need for highly scalable methods for AAV purification. As a result, one-step affinity chromatography protocols have become attractive for accelerating the purification of viral vectors while meeting good manufacturing practice (GMP) requirements.[9]

HPLC-based affinity chromatography is quick and efficient, enabling the determination of both capsid titer and content ratio. With a quick run time and no need for manual sample handling or pretreatment steps, the process is ideal for rapid, high-throughput analysis in both research and production environments.[10] However, the specificity that makes affinity chromatography effective also presents a notable drawback. Most ligands are designed to capture only one or a few AAV serotypes, meaning that any change in the virus particle often necessitates a new capture ligand. This can be time-consuming and costly, hindering the flexibility needed for platform production processes.

As a result, affinity columns based on AAV-specific camelid antibodies have started to dominate the field. These resins can target a broad range of natural and synthetic AAV serotypes, significantly simplifying the purification process across various vector types.[11,12] Additionally, they have very high binding capacities, demonstrate robust viral clearance and are stable against harsh clean-in-place and regeneration methods, making them suitable for repeated use.[12,13] Similar advances in AEC are also contributing to more effective polishing steps, resulting in the enrichment of full capsids by 90% in some cases.[14]

These trends and innovations are pivotal in meeting the growing demand for AAV-based therapies, facilitating the production of safe and effective treatments on a larger scale. As the field continues to evolve, these advancements will likely play an integral role in the future of gene therapy, driving further improvements in both efficiency and product quality.

Future directions

As the number of gene therapy programs advancing to the clinical phase and commercialization continues to rise, the optimization of large-scale AAV production is essential for the future of the field. Achieving this requires analytical tools and methods capable of providing rapid and accurate assessments of sample purity at increasing scales.

Innovations in purification techniques, particularly in chromatography, are helping to pave the way for more efficient and effective viral vector purification. Future developments will likely focus on optimizing these methods, integrating advanced analytical tools and leveraging automation to enhance consistency and scalability. Overall, continued advancements in viral vector purification will be essential to meet the growing needs of the gene therapy field and bring innovative treatments to patients worldwide.

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Viral vectors in gene therapy: innovations and simplified solutions

Gene therapy involves the introduction of specific genetic material into a patient to alter and improve cell function. Recent advancements have resulted in over 30 approved cell and gene therapies worldwide, addressing a variety of conditions ranging from congenital disorders to solid cancers.[1,2]

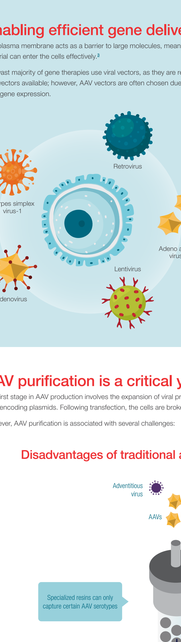
These breakthroughs have been possible thanks to the development of sophisticated delivery systems and the refinement of gene-editing technologies. Innovations such as viral vectors, including adeno-associated viruses (AAVs), have enabled precise delivery of genetic material to the target cells, enhancing the efficacy and safety of treatments.

This infographic explores current trends, delivery mechanisms and manufacturing challenges in gene therapy.

The two paths to gene therapy

There are two main ways to deliver gene therapy: *ex vivo* and *in vivo*. Each method offers its own set of benefits and considerations.

In vivo methods are often used when treating a singular gene or when targeting an internal organ, like the heart, brain or lungs.

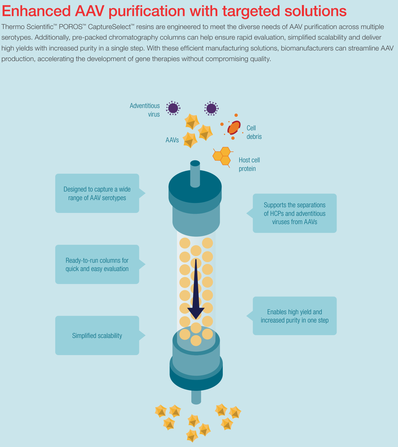


Ex vivo gene therapies are most commonly used to treat blood disorders.

Enabling efficient gene delivery

The plasma membrane acts as a barrier to large molecules, meaning that specialized methods are needed to ensure the genetic material can enter the cells effectively.[3]

The vast majority of gene therapies use viral vectors, as they are remarkably efficient at gene delivery.[4] There are different types of viral vectors available; however, AAV vectors are often chosen due to their efficiency, low risk of insertional mutagenesis and long-term gene expression.

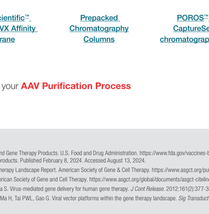


AAV purification is a critical yet challenging step

The first stage in AAV production involves the expansion of viral producer cells in culture, prior to their transfection with one or more AAV-encoding plasmids. Following transfection, the cells are broken down and the lysate is harvested for AAV purification.

However, AAV purification is associated with several challenges:

Disadvantages of traditional affinity chromatography resins



Enhanced AAV purification with targeted solutions

Thermo Scientific™ POROS™ CaptureSelect™ resins are engineered to meet the diverse needs of AAV purification across multiple serotypes. Additionally, pre-packed chromatography columns can help ensure rapid evaluation, simplified scalability and deliver high yields with increased purity in a single step. With these efficient manufacturing solutions, biomanufacturers can streamline AAV production, accelerating the development of gene therapies without compromising quality.

The future is in our genes

Gene therapies are transforming the future of medicine. As we advance our understanding and technology, these therapies are expected to become more precise, effective and accessible. Thermo Fisher Scientific is at the forefront of this evolution, providing innovative tools and solutions that help streamline the development and production of gene therapies. By supporting purification techniques, Thermo Fisher is working to pave the way for safer, more effective treatments that can help to improve patient outcomes.

Explore AAV purification solutions for gene therapy development

Thermo Scientific™ GoPure™ AAVX Affinity Membrane

Prepacked Chromatography Columns

POROS™ and CaptureSelect™ chromatography resins

Thermo Scientific™ POROS™ GoPure™ AAVX 96-well screening plate

Simplify your **AAV Purification Process**

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AAV purification trends and techniques: ask the specialists

Specialists

Alejandro Becerra

Principal Scientist and Global Technical Lead for Purification Products, Thermo Fisher Scientific

Dr. Alejandro Becerra is a Principal Applications Scientist and Global Purification Technical Lead. Alejandro has over 15 years of experience in downstream processing and customer support having worked as Purification Team Manager and other bioprocess engineering roles prior to joining Thermo Fisher Scientific in 2018. Dr. Becerra is a subject matter specialist in preparative chromatography with expertise in the development, optimization and scale-up of antibodies, recombinant proteins, viral vectors, and nucleic acid purification processes. Alejandro holds a Ph.D. in Chemical Engineering from Cornell University.

Chantelle Gaskin

Field Applications Scientist, Purification Business Unit, Thermo Fisher Scientific

Chantelle is a Field Applications Scientist, specializing in protein and viral vector purification and downstream process development. With over 10 years of experience in gene therapy, Chantelle has accumulated comprehensive knowledge of standard industry practices and regulatory standards, applying this knowledge to advance the development of therapies for a variety of indications including ocular, CNS and systemic disease. Chantelle holds a Master's degree in Chemistry from the University of Florida and a Bachelor's in Chemistry from Smith College.

Adeno-associated viral (AAV) vectors are an increasingly popular choice for gene therapies; however, a major bottleneck in the production of AAVs is their efficient purification. A broad range of serotypes, residual contaminants and a disparity in genetic filling increases the complexity of purification processes. These factors can affect the consistency and quality of the final product, making it challenging to achieve the high purity levels required for safe and effective therapies.

We asked two in-house specialists at Thermo Fisher Scientific for their advice on overcoming AAV purification challenges and implementing innovative strategies to enhance the efficiency and reliability of AAV purification.

Q: What are the current trends in AAV purification? What challenges do process development scientists typically face in this process?

Alejandro Becerra (AB): Today, most of the industry is adopting a similar approach to purification with two chromatography steps after cell lysis and clarification. The primary challenge now lies in further optimizing this standardized process. Unlike other purification processes, AAV purification can be limited by difficulties in obtaining sufficient material for proper process development.

Another challenge closely related to purification is the quality of analytics. The effectiveness of any purification process depends heavily on the robustness of the associated analytical techniques, in addition to the sample type and the stage of the process. These limitations impact the ability to detect and quantify various elements accurately and develop robust unit operations.

Current efforts in clinical and commercial manufacturing are focused on eliminating product-related impurities, as these are more difficult to address. In contrast, process-related impurities are largely removed during the pre-capture and capture stages. These product-related impurities include empty particles, partially filled capsids and, occasionally, over-packaged and aggregated AAVs. The primary focus is on achieving high product purity, particularly a high percentage of full particles. As previously mentioned, the field has adopted a common downstream approach for AAV particle purification, with affinity and anion exchange chromatography serving as key unit operations.

Chantelle Gaskin (CG): There are three main trends in AAV purification that I'd like to highlight. Firstly, there's an increasing focus on engineered capsids. Many companies generate novel capsids, driven by their R&D teams, for various reasons. This includes developing viral vectors with intellectual property protection and capsids with specific tropism to target particular tissues. Additionally, there's a strong emphasis on safety and reducing immunogenic responses.

This trend towards novel and engineered capsids has escalated, and many customers are using the Thermo Scientific™ POROS™ CaptureSelect™ AAVX Affinity Resin for this purpose. However, purifying engineered capsids requires downstream purification steps to be further optimized. Our role as field application scientists (FAS) is to support customers in optimizing their entire downstream process for these novel capsids.

Another significant trend is the enrichment of full capsids, which is generally achieved using non-affinity polish resins combined with different buffer compositions. Moreover, there is a growing focus on characterizing partially filled and overfilled capsids. Historically overlooked, this aspect is now gaining attention as companies are exploring purification processes and upstream strategies to address these issues.

Lastly, the importance of analytics in the purification process cannot be overstated; effective purification is contingent upon robust analytical methods. We're seeing ongoing trends in developing analytical assays for titer determination of various capsid species and methods for quicker titer readouts. These advancements are critical for accurately characterizing the purification process and are increasingly prevalent in the field.

Q: How does affinity chromatography contribute to achieving high purity and yield in AAV purification? Can you explain the underlying principles and mechanisms involved?

CG: Affinity chromatography has become a staple in biologics purification due to its efficiency and specificity. The technique relies on highly specific binding sites on the chromatography media, allowing only the target molecule to bind to the column while other impurities in the starting material flow through. This results in highly purified material in just one step, unlike the two or three steps often required with non-affinity chromatography.

Our Thermo Scientific™ CaptureSelect™ portfolio, for example, uses camelid antibody fragments to achieve this level of purification. These antibody fragments have high specificity for the target molecule, helping to ensure that only the desired AAV particles are retained on the column. This approach not only shortens the purification process but also increases its efficiency, delivering high purity and yield with fewer steps.

AB: Affinity chromatography offers numerous advantages for AAV purification. For example, it eliminates the need to adjust the sample before loading it onto the chromatography column. Thus, high purity can be achieved in a single affinity chromatography step. For instance, the initial load sample may contain less than 1% of the target product, with the rest being process-related impurities. After affinity chromatography, purity levels can exceed 90–95%, effectively removing these impurities. Additionally, this step concentrates the sample by several hundred-fold, depending on the specific process.

Affinity chromatography is highly specific, eliminating the need for additional unit operations, and thereby reducing the number of steps in the process. Each additional step can lead to product losses, so minimizing these steps enhances the overall AAV recovery.

Q: What are the recent advancements in affinity chromatography for AAV purification?

AB: One recent development in AAV purification using affinity chromatography involves optimization of the overall downstream process to reduce product losses. Traditionally, many processes include a concentration step using tangential flow filtration (TFF) before the affinity chromatography step. This concentration step helps to increase the amount of AAV in the sample, thereby reducing the time needed for affinity chromatography. However, it also introduces an additional unit operation, which may lead to potential product losses.

However, an innovative approach to eliminate the TFF step can be achieved by using chromatography resins with high capacity and high permeability, such as the POROS™ CaptureSelect™ AAVX Affinity Resin or the Thermo Scientific™ POROS™ CaptureSelect™ AAV9 Affinity Resin. By operating at higher flow rates and using shorter bed heights, these resins can process larger volumes quickly without the need for prior concentration.

This method not only simplifies the process but also helps to minimize product losses associated with additional steps.

CG: Recent advancements in affinity chromatography for AAV purification have primarily focused on optimizing the surrounding downstream processes to enhance the efficiency of the affinity purification step. One approach involves implementing DNA removal protocols prior to affinity purification. This step helps increase the purification efficiency of the affinity chromatography process.

Additionally, there has been a focus on optimizing the cleaning and reuse of AAV resins to extend their lifecycle. This is particularly important in process development and GMP manufacturing, where the ability to reuse columns can significantly help to reduce costs. Historically, single-use affinity columns were preferred in GMP settings due to concerns around handling viral vector product. However, to support the development of larger-scale AAV processes, we have demonstrated that POROS CaptureSelect AAVX affinity resin can be used for multiple cycles, enabling more sustainable and cost-effective manufacturing.

These advancements, while not the most glamorous aspects of AAV purification, are crucial for improving the efficiency and cost-effectiveness of both process development and large-scale production. We have been actively supporting customers in implementing these strategies to achieve better results in their purification workflows.

Q: The POROS CaptureSelect AAVX affinity resin can capture a wide range of AAV serotypes. How does it do that?

CG: The AAVX ligand was developed through an extensive screening process of multiple ligand candidates to find the one with the highest specificity across a broad range of AAV serotypes. The key to its success lies in its ability to bind to specific sequences on the AAV capsid. These sequences are conserved across different viral vector serotypes, allowing AAVX to effectively capture a wide variety of AAV serotypes.

This binding mechanism is documented in a white paper that highlights that these conserved sequences are crucial for the ligand's broad specificity.[1] However, when customers engineer capsids and alter these proteins, the binding efficiency of AAVX can decrease. To assist with this, we provide access to the published epitopes so that customers can avoid modifying these critical binding sites during their engineering processes.

Q: What are the key factors to consider when selecting an affinity chromatography method for AAV purification? Are there any specific ligands or matrices that have demonstrated superior performance?

AB: When discussing AAV purification, it's crucial to recognize the wide range of serotypes used and the field's efforts to engineer these particles for various applications. Therefore, the first factor to consider is the specificity of the affinity chromatography resin, i.e., ensuring the affinity resin can target the specific serotypes used by an organization. The POROS CaptureSelect AAVX affinity resin has demonstrated broad specificity, effectively binding to all natural serotypes as well as engineered capsids.

The second consideration is scalability. Chromatography has a long history and is easily scalable. However, given the typically low concentration of AAV and the relatively long processing times, it's essential to consider the binding capacity of the affinity resin, particularly at shorter residence times and higher flow rates. In this regard, chromatography resins like POROS CaptureSelect AAVX affinity resin are particularly advantageous as they offer high binding capacity at high flow rates. This capability reduces overall processing time and allows smaller columns to be used, thereby lowering the overall costs.

CG: There are several key factors to consider when selecting an affinity chromatography method for AAV purification. First, you need to look at binding capacity, as this will impact the efficiency and yield of your purification process. Next, consider the material of construction, which affects flow pressure and flow characteristics. These factors can be critical when scaling up your process.

Another important aspect is the ability to clean your resins. Efficient cleaning protocols are essential for maintaining resin performance and longevity, especially in large-scale operations. Additionally, the ability to pack resins effectively is crucial. While many AAV purification processes use pre-packed columns, those who pack their own columns need a resin that is easy to pack consistently.

Q: Can you provide examples where affinity chromatography has successfully enabled one-step capture of AAV with high purity and yield?

AB: It's important to note the difference between producing recombinant AAVs for research or preclinical purposes versus clinical studies in humans. Several research groups and industry specialists have used one-step purification with affinity resins and demonstrated their effectiveness in *in vitro* or early-stage *in vivo* models.

CG: One example comes from a study, in which they used AAVX to purify 15 divergent AAV serotypes, including AAV2, AAV9 and even the ancestral AAV serotype Anc80, known for its excellent tropism but difficult purification.[2] The results demonstrated high levels of purification in a single step. They compared this approach to ultracentrifugation with an iodixanol gradient, a common method in early-stage research that, while effective, is difficult to scale. This comparison highlighted the advantages of AAVX, particularly in scalability, making it suitable for larger-scale applications like preparing materials for extensive animal studies.

A published case study utilized an AAVX affinity column for analytical purposes.[3] This method is particularly beneficial for titer determination of crude samples.

Q: Are there some serotypes that prove to be more challenging when developing an AAV affinity capture step? Have these challenges been addressed?

AB: While many AAV serotypes are quite similar, which is why we can use a single affinity resin to capture all of them, they also have some key differences. One notable difference is the stability of the AAV particle itself. For example, serotypes like AAV2 are more prone to aggregation, especially under low conductivity conditions where there's not enough salt.

In affinity chromatography, we bind the particles at neutral pH and elute them at low pH. Generally, low conductivity is beneficial for good recovery. However, for serotypes prone to aggregation under these conditions, we need to address the challenge of balancing recovery and stability. We do this by including excipients like arginine to prevent aggregation while still achieving good recoveries. Additionally, after elution, we can add different salts to the neutralization buffer to prevent aggregation.

Another example is AAV5, which binds very strongly to the AAVX ligand. This means we need slightly more stringent conditions for elution, such as a lower pH – maybe half to one pH unit below what we'd use for other serotypes. We can also use excipients or modifiers to facilitate elution and maintain good recoveries.

For engineered capsids, the situation can be different. Sometimes, the binding to the resin isn't sufficient. In such cases, we can adjust the binding conditions or explore alternative custom ligands or resins to achieve the desired capture efficiency.

CG: AAV9 and AAV9-like serotypes tend to resist binding, making purification difficult. This serotype crosses the blood-brain barrier, making it particularly useful for neurological applications and diseases involving the central nervous system (CNS).

Some companies are making small modifications to the AAV9 capsid to improve its suitability as a viral vector. Despite these challenges, the AAVX resin is capable of purifying AAV9 capsids. We recommend certain considerations to optimize the purification process for AAV9, but overall, AAVX shows great binding capacity for this serotype. Additionally, we have also developed the POROS CaptureSelect AAV9 affinity resin, which is made specifically to bind this species.

Q: In your experience, what are the main benefits of affinity chromatography compared to alternative AAV purification methods?

AB: One of the main benefits is the ability to take the sample from the previous step without needing to adjust pH or conductivity. For example, if you use cation exchange chromatography for capture, you need to lower the pH and adjust the conductivity. Some impurities may precipitate after these adjustments. This adds extra steps that require further optimization and can lead to product losses.

The second benefit is achieving very high purity levels in a single step. Affinity chromatography is scalable, and in that same step, it also concentrates the load sample. Depending on the initial concentration and specific conditions, you can achieve a concentration increase of 100- to several hundred-fold.

Compared to ultracentrifugation, the scalability of chromatography resins is also clear. Ultracentrifugation faces scalability issues, especially as the field moves toward larger doses for larger patient populations, making it challenging to produce the required amount of vector.

CG: Affinity chromatography offers significant benefits by effectively reducing the number of purification steps needed. With just one affinity chromatography step, you can achieve the same level of purification that might otherwise require two or three ion exchange steps. This translates to greater process efficiency, as you're eliminating additional chromatography steps, along with their associated costs for resins, buffers and manpower.

Q: How does affinity chromatography fit into the overall process of AAV production? Are there any considerations regarding scalability and cost-effectiveness?

AB: Affinity chromatography plays a crucial role in both research and larger-scale AAV production. Typically, it fits into the process after several initial steps and before any final processing steps. Chromatography resins have been used for decades, so the underlying physical principles remain the same, with a range of column diameters and bed heights providing flexibility compared to other adsorptive methods.

In terms of cost-effectiveness, affinity chromatography offers significant benefits by potentially eliminating the need for additional steps. While the cost of affinity chromatography resins is a factor, it should be compared to other expensive raw materials, such as nucleases and plasmids. Importantly, these resins can be reused in both research and GMP settings. They can be cleaned and utilized multiple times, which helps reduce the overall cost of the process. We have demonstrated that these resins can maintain comparable performance over 35 cycles. Similarly, research by Florea *et al.* has shown good reproducibility over six cycles.[2]

The ability to reuse chromatography resins significantly lowers the cost per cycle, and this exponential reduction in cost with reuse makes affinity chromatography a cost-effective choice.

However, it's crucial to validate the resin reuse using a qualified scale-down model and ensure the necessary analytics are in place to support this approach.

CG: Affinity chromatography streamlines the purification process. For instance, compared to ultracentrifugation – which is labor-intensive and has significant scalability issues, such as the need for precise manual band extraction from gradient tubes – affinity chromatography offers a more efficient and consistent approach.

The manual aspect of ultracentrifugation, often described as tedious or even an art form, can vary greatly between operators, further complicating scalability. In contrast, affinity chromatography using POROS CaptureSelect AAVX affinity resin provides excellent scalability. The resin's robust material construction supports large-scale applications and enables multiple reuse cycles. This allows affinity chromatography to be not only more cost-effective but also more scalable compared to traditional methods. Overall, the efficiency, consistency and reusability of affinity chromatography contribute to its advantages in AAV production.

Q: Are there any limitations associated with affinity chromatography in AAV purification? How can these be addressed or optimized?

AB: One key limitation is specificity. While the AAVX ligand has been effective for many engineered capsids, there have been instances where the resin, or even the AAV9 resin, hasn't bound to certain capsids, particularly with AAV particles similar to AAV9. Future engineered serotypes might also face similar issues.

When these challenges arise, there are a couple of options. One is to explore non-affinity approaches, such as cation exchange chromatography. However, this method involves an additional step before chromatography and requires optimization for each specific case.

The second option is to develop a custom ligand. At Thermo Fisher, we offer the capability to create tailored affinity ligands and resins for various biomolecules, including AAVs. We've successfully developed custom solutions in the past, and this could be a viable route when dealing with new engineered capsids that don't bind well with standard resins.

CG: There are some limitations and challenges with affinity chromatography in AAV purification, particularly when dealing with novel capsids. Novel capsids can present unique issues, as they may not bind as effectively or predictably to the affinity resin. This challenge extends to upstream processes, where suboptimal production conditions for the novel capsid can lead to lower viral titers, complicating downstream purification.

The Gibco™ AAV-MAX Helper-Free AAV Production System is designed to help enhance upstream AAV production by optimizing culture media, additives and cell lines. Despite these advancements, issues with novel capsids can still arise, and overcoming them often requires careful troubleshooting and optimization.

FAS and purification specialists work closely with customers to navigate these difficulties, developing workarounds and refining processes to support effective purification even with novel capsids.

Q: What are the current methods to remove any additional impurities that remain after an optimized AAV capture chromatography step?

AB: The main methods used are anion exchange chromatography and ultracentrifugation. Anion exchange chromatography, especially with specific resins, is commonly employed. Each has its advantages and disadvantages depending on whether you're working in research or scaling up for GMP production.

These are the primary approaches because removing product-related impurities – similar in size and charge to the target product – is quite challenging. Fine separation is required, which is something that ion exchange chromatography and ultracentrifugation currently handle most effectively.

CG: After capturing AAV through affinity chromatography, the next step typically involves using ion exchange chromatography, with anion exchange being the most common choice. Anion exchange chromatography effectively addresses the remaining impurities, such as empty, partially filled and overfilled AAV capsids, as well as trace amounts of host cell DNA and proteins. These impurities usually account for about 3–5% of the purified material.

The focus of the anion exchange step is often on enriching the full capsid population. This step is crucial for removing empty capsids, which could potentially trigger an immunogenic response in patients. Since most of the AAVs produced upstream are empty, it is essential to effectively separate these from the full capsids.

Thermo Scientific™ POROS™ 50 HQ Strong Anion Exchange Resin and Thermo Scientific™ POROS™ XQ Strong Anion Exchange Resin are strong anion exchangers that are particularly effective in this process. Recent publications have explored advanced techniques, such as using dual salt buffer systems to create step gradients rather than linear gradients. Step gradients are more suitable for large-scale purification, enabling better separation of different capsid species and improving scalability.

Q: What particular resins are used and how does a process scientist evaluate and choose the best candidate for the process?

AB: To select the most optimal resin, a process scientist needs to start by defining the goals of the step. This involves understanding the target enrichment of the full particles required for the process and determining the acceptable levels of other impurities, like residual ligands or DNA.

Once those targets are set, it's crucial to leverage existing knowledge and resources. For instance, since the approval of AAV therapies in the U.S. about six or seven years ago, the field has accumulated significant insights, particularly in anion exchange chromatography. Scientists should use this knowledge to guide their initial conditions and step development.

Scalability is another key factor. Anion exchange resins offer more size options compared to other adsorbents, like membranes or convective materials. Typically, this polishing step is conducted at an alkaline pH (between 8 and 9.5) because AAV particles exhibit poor binding at lower pH levels. The separation is also performed at low conductivity. Additionally, different counter ions or salts, like magnesium, have been found to positively impact the separation. While the exact mechanism might not be fully understood, it's generally considered as an additive during the process evaluation.

CG: In downstream purification, POROS™ 50 HQ Strong Anion Exchange Resin and POROS™ XQ Strong Anion Exchange Resin are commonly used strong anion exchange resins. These are preferred because anion exchange chromatography effectively handles the diverse characteristics of AAV and its impurities, such as isoelectric points and binding strengths. While anion exchange is the predominant choice, there are instances where cation exchange might be used, for which Thermo Scientific™ POROS™ 50 HS Strong Cation Exchange Resin and Thermo Scientific™ POROS™ XS Strong Cation Exchange Resin are available.

When selecting the most optimal resin, process scientists evaluate several factors, including the specific binding properties and the nature of the impurities. Downstream scientists have many options here, but POROS XQ strong anion exchange resin and POROS HQ strong anion exchange resin are highly recommended due to their robust performance and extensive published data supporting their efficacy.

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Harnessing CDMOs for optimized AAV production: ask the specialist

Specialist

Pouria Motevalian
Director, Viral Vector Process and Analytical Development, Thermo Fisher Scientific

As Director of Process Development, Pouria Motevalian oversees the development, scale-up and analytical characterization of novel and compliant manufacturing processes for gene therapy. He also holds a key position as a member of the Senior Leadership Team for Thermo Fisher Scientific's Plainville, MA site, one of the largest viral vector development and manufacturing site in North America. In this role, Pouria provides strategic guidance, shaping scientific and operational plans for implementing bioprocess technologies to meet the needs of viral vector clients. Pouria received his PhD in chemical engineering with a minor in computational science from Pennsylvania State University.

Adeno-associated viruses (AAVs) are a versatile tool in gene therapy, promising to treat a range of previously incurable genetic disorders by delivering therapeutic genes directly into patients' cells. Despite their potential, the large-scale production and purification of AAVs still faces significant challenges. Contract development and manufacturing organizations (CDMOs) play a crucial role in addressing these complexities, using their expertise to streamline and enhance production.

To gain deeper insights into how CDMOs can tackle these purification challenges and optimize AAV production, we spoke with Pouria Motevalian, Director of Viral Vector Process Development at Thermo Fisher Scientific.

Q: What have been the key advances in AAV manufacturing technology over the last few years?

A: One of the most notable advancements has been the development of scalable production platforms, particularly in the areas of triple transfection-based systems, baculovirus expression systems, and producer cell lines. These platforms have evolved significantly, enabling the establishment of scalable production processes for each approach.

Another major advancement is in capsid design and engineering, which enables the development of vectors with improved specificity, stability, and a reduced immune response. Equally notable are improvements in downstream processing, particularly in chromatography and filtration. Enhanced affinity chromatography, with improved resins for the affinity capture step, has resulted in more robust and efficient processes. Depth filtration technologies have also been significantly improved, increasing throughput and reducing process and product impurities. These advancements in recent years have propelled the field forward.

Q: What is the main bottleneck for manufacturing of viral vectors?

A: Scaling up the production process while maintaining purity, consistency and potency is a major challenge. Despite all the advancements we've seen in recent years, inconsistencies can still arise during scale-up, particularly with titer levels, impurity removal and overall product quality. These inconsistencies can

ultimately affect the final product's potency, making this a key bottleneck in viral vector manufacturing today.

Q: Are there any unique considerations or challenges that arise when purifying viral vectors compared to other types of biologics?

A: The goal is always to reduce process- and product-related impurities. Removing upstream impurities, such as host cell DNA or proteins, is standard for any biologic. However, for viral vectors, particularly AAVs, the downstream process must also remove empty and partial capsids. This is especially challenging because empty, full, and partial capsids are similar in size and have minimal differences in isoelectric points—often just 0.2 to 0.6 units—making their separation extremely difficult.

To address this challenge, we rely on high-throughput resin and mobile phase screening for optimization of anion exchange and affinity chromatography steps. Anion exchange is particularly crucial for empty/full capsid separation, and utilizing high-throughput technologies for screening is essential for developing a well-optimized process. This approach allows for substantial removal of empty and partial capsids, significantly enhancing the effectiveness of the purification process.

Q: How do CDMOs ensure the scalability and reproducibility of viral vector purification processes, particularly when dealing with large-scale production for gene therapies?

A: There are two key aspects to consider. First, it's crucial to rely on a scalable, well-developed scale-down model for each unit operation. Ensuring that the scale-down model used during process development accurately reflects larger-scale operations is essential. If the scale-down model doesn't faithfully represent large-scale production, the development process loses value as the results won't be transferable.

Second, it's important to keep the end goal of large-scale production in mind throughout the course of process development. The focus should always be on ensuring that the process designed in the lab can be successfully scaled for clinical and commercial manufacturing. This means that when developing processes and determining normal operating ranges, we must work to ensure that these parameters are feasible for large-scale production.

Q: How do gene therapy developers ensure compliance with regulatory guidelines and standards when purifying viral vectors?

A: The answer is straightforward: embrace Quality by Design (QbD) throughout the entire development process and ensure strict compliance with GMP best practices and guidelines during clinical and commercial manufacturing. By adhering to these principles—integrating QbD from the outset and following GMP guidelines—developers can ensure they meet regulatory requirements and align with industry standards.

Q: What further innovations would you like to see in viral vector manufacturing in the future?

A: First, plasmid design and optimization should be prioritized early on, because the optimized design of the plasmid (especially ITR regions) has been shown to significantly boost productivity, especially for AAV viral vectors. Second, we would like to see advancements in resins that allow for enhanced separation – not just when it comes to separating empty and full capsids, but also in removing impurities. Lastly, implementing process analytical technology (PAT) tools such as Raman, FTIR and NIR for real-time measurement of critical quality attributes is highly desired. This would help reduce bottlenecks in QC testing without compromising product quality, enabling real-time measurement and release.

Q: What are the advantages of working with a CDMO to solve purification challenges early on in the process?

A: The first major advantage is the expertise and experience that CDMOs bring. Specializing in specific areas, CDMOs possess the technical knowledge and deep understanding necessary to develop and scale processes efficiently for clinical and commercial manufacturing. Their experience working with a variety of vectors and clients, each with unique requirements, gives them a broad perspective on industry challenges. This accumulated expertise allows them to provide tailored solutions to the specific challenges each client faces.

Another key advantage is the ability to accelerate development timelines. CDMOs, equipped with advanced tools and technologies, can offer accelerated development. This is partly due to economies of scale—they handle multiple projects and pipelines simultaneously, enabling a more standardized and efficient approach. As a result, they can speed up the development process, which is particularly beneficial when time is critical.

Q: Are there any particular purification challenges for which your company has interesting and valuable solutions for AAV manufacturers?

A: One of the major challenges in AAV purification is the separation of empty and full capsids, as well as the removal of residual host cell impurities like DNA and proteins. Now,

the tricky part is that each client's process is unique, so the challenges they face are unique too. Because of that, the solutions we offer need to be tailored to each client's specific needs.

Instead of providing a one-size-fits-all purification solution, we take a more versatile approach to downstream processing, development and optimization. We rely on high-throughput technologies and techniques, which have consistently proven effective in tackling these major downstream challenges across multiple clients.

Q: Are there any specific recommendations for AAV therapeutic downstream scientists when developing a process planned to be transferred to a CDMO?

A: First, ensuring process robustness is essential, though it's a broad concept. To clarify, when transferring process parameters for a specific unit operation, it's important to provide a range of acceptable parameters rather than just a single target. This flexibility allows for a better facility fit, particularly in a GMP setting for clinical and commercial manufacturing.

Another key aspect of process robustness is the manufacturability of mobile phases used in critical chromatography steps. At times, the passing criteria for parameters like pH and conductivity are so stringent that it becomes challenging to prepare and release these buffers in a GMP environment. When developing mobile phase formulations, it's important to consider manufacturability, ensuring that the release criteria are broad enough to be practical without compromising quality.

The second point is establishing appropriate hold times for intermediates. Undefined hold times can force critical operations to be performed during less optimal shifts, increasing operational risk. Planning for these operations to occur during shifts with full manpower and expertise reduces this risk.

Lastly, it's crucial to ensure that unit operations are scalable throughout the process. For example, ultracentrifugation is often used for viral vector purification but becomes difficult to scale beyond a certain point, leading to the need to scale out rather than up, which poses operational challenges and increases capital and space requirements. Designing scalable unit operations from the start is critical for a smooth and successful tech transfer to a CDMO.

Benefits of affinity chromatography for AAV purification

Explore techniques and strategies to improve your downstream purification of viral vectors

Introduction

Affinity chromatography is a powerful technique used in downstream purification processes, particularly for the purification of adeno-associated viruses (AAVs). With its ability to selectively bind and isolate target molecules, affinity chromatography offers several key benefits that can significantly improve the efficiency and yield of AAV purification. Here, we will explore the five main benefits of affinity chromatography for AAV purification and discuss various techniques and strategies to enhance your downstream purification of viral vectors.

1. High specificity and selectivity

Affinity chromatography relies on the specific interaction between a target molecule, such as a specific protein of the AAV capsid, and an immobilized ligand on the chromatography resin. This interaction allows for the selective binding and isolation of AAV particles from complex mixtures, resulting in a highly pure sample. The high specificity and selectivity of affinity chromatography helps ensure minimal contamination and maximize the recovery of AAV particles.

2. Increased purity and yield

By specifically targeting AAV from a complex feedstock, affinity chromatography effectively recovers AAV particles and allows unrelated impurities to flow through. With optimization, this results in high yield and purity in a single step.

3. Versatility and adaptability

Affinity chromatography can employ a wide range of ligands that can be tailored to specific AAV purification needs. Leveraging a robust ligand generation platform, VhH antibodies can be generated to target specific properties of AAV, resulting in both serotype-specific targeting or pantropic AAV binding.

4. Gentle purification conditions

One of the advantages of affinity chromatography is its ability to operate under gentle purification conditions. This is especially important for the purification of delicate viral vectors like AAV, as harsh purification methods may compromise their structural integrity and functionality. Affinity chromatography allows for purification under mild conditions, preserving the quality and functionality of the AAV particles.

5. Scalability and automation

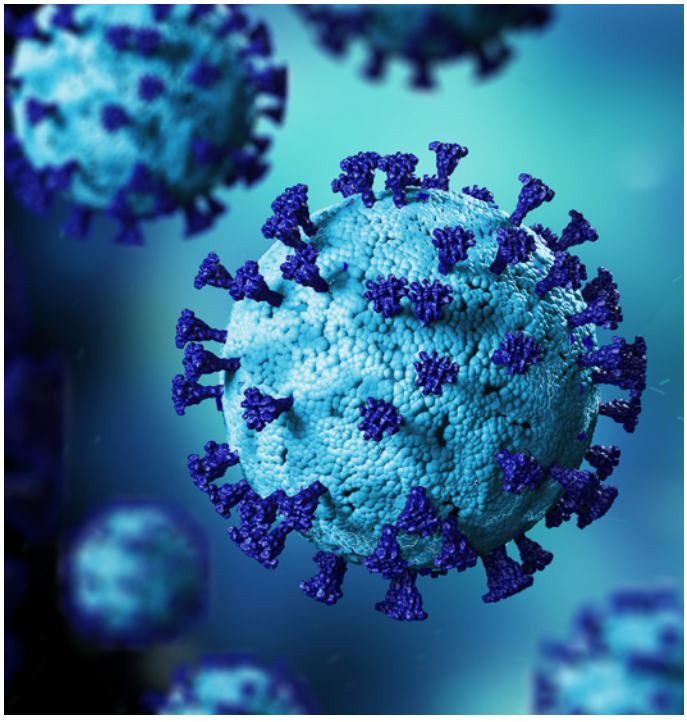
Affinity chromatography techniques can be easily scaled up to accommodate large-scale AAV production. The purification process can be automated, facilitating high-throughput purification and reducing the time and effort required for purification. This scalability and automation make affinity chromatography a practical option for industrial-scale AAV production.

Conclusion

Affinity chromatography offers significant benefits for the downstream purification of AAV vectors. Its high specificity, high purity and yield, versatility, gentle purification conditions, and scalability make it an essential technique for downstream process development scientists involved in AAV purification. By exploring various techniques and strategies to optimize affinity chromatography, scientists can enhance the efficiency and yield of AAV purification, ultimately advancing the field of viral vector-based therapies.

Learn more about optimizing your purification process today. Let's simplify purification together.

Visit our webpage to find out more.



Gene therapy purification

High recovery and purity of AAV2 via capture and anion exchange polish chromatography

Introduction

Gene and cell therapies have been active areas of research for at least the past two decades. Adeno-associated virus (AAV) became the delivery vector most used for gene therapy due to its unique safety and efficacy. As of today, seven products have been commercialized that rely on recombinant AAVs to deliver functional genes to patients with genetic disorders, including Duchenne muscular dystrophy and hemophilia B.

Over the years, the number of clinical trials involving recombinant AAV has dramatically increased, as has the demand for larger quantities of highly purified material. For contract development and manufacturing organizations (CDMOs), the manufacturing effort is mainly focused on robustness and productivity. Effective downstream purification to generate a clinical product of high titer, potency, and purity while maintaining high AAV recovery is essential.

One of the challenging aspects of AAV manufacturing is inefficient packaging of the gene of interest (GOI), which results in large quantities of empty or partially filled capsids. Clinical studies support the role of the total capsid dose as a determining factor of AAV vector immunogenicity [1], so it is crucial to develop purification processes to minimize these species in the final product. However, the development of a robust and scalable purification process to separate empty and full AAV particles at large scale remains a challenge due to the similarity between the physicochemical characteristics of these two AAV populations. Separation of the two species has been achieved by iodixanol gradient and cesium chloride (CsCl) gradient ultracentrifugation based on slight density differences between full and empty capsids. However, this method is not easily scalable, and it is time- and labor-intensive. Due to the small difference between the isoelectric points of full and empty capsids, anion exchange chromatography–based separation was found to be a scalable and robust alternative to ultracentrifugation.

Here, we describe how affinity chromatography using Thermo Scientific™ POROS™ CaptureSelect™ AAVX Affinity Resin followed by anion exchange (AEX) chromatography using Thermo Scientific™ POROS™ XQ Strong Anion Exchange Resin results in successful purification and full capsid enrichment of AAV2-GFP particles. VVector Bio's AAV downstream processing (Figure 1) starts with lysis of the cells. As the majority of AAV remains intracellular, the cell lysis step allows the release of AAV into the supernatant. This step also releases cell debris and other impurities such as host cell proteins and DNA, which is mostly digested with a nuclease treatment. The removal of cell debris is performed by either centrifugation for small-scale production or depth filtration for larger-scale production. This step is followed by a second clarification using membrane filtration before the capture of AAV by affinity chromatography using POROS CaptureSelect AAVX resin.

In this study, the affinity-purified AAV was used to develop a method to separate full and empty capsids. A linear gradient elution approach was used as the baseline to develop a hybrid elution method to further separate full and empty capsids.

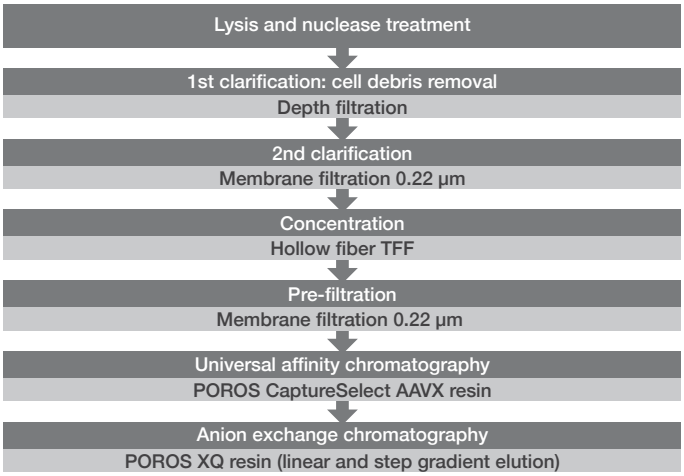


Figure 1. Downstream process workflow.

Materials and methods

Purification of AAV2 using POROS CaptureSelect AAVX Affinity Resin

The affinity chromatography capture step utilizes POROS CaptureSelect AAVX Affinity Resin. The resin consists of rigid poly(styrene-divinylbenzene) Thermo Scientific™ POROS™ base beads (50 µm mean particle size) functionalized with the Thermo Scientific™ CaptureSelect™ AAVX affinity ligand. The details of this affinity capture step, including the buffers used, are listed in Table 1. Immediately after elution, the eluted sample was diluted with 10% (v/v) neutralization buffer to prevent further aggregation and stabilize the AAV.

Separation of full and empty AAV2 with POROS XQ resin—linear gradient development

Prior to the separation of empty and full capsids, the purified AAV sample was buffer exchanged to anion exchange chromatography equilibration buffer using a Cytiva™ PD-10 Desalting Column. The material was then loaded onto a 1 mL Thermo Scientific™ POROS™ GoPure™ XQ pre-packed column containing fully quaternized amine functional groups. The details of this chromatography step are presented in Table 2. In brief, after column equilibration and loading, elution was performed using a 40 CV (column volume) linear gradient from 50 mM to 500 mM sodium acetate in 20 mM Bis-Tris propane (BTP), 10 mM $MgCl_2$, and 0.01% Pluronic™ (pH 9.0) on the POROS XQ column.

Table 1. Steps for the purification of AAV2 with POROS CaptureSelect AAVX resin (CV: 5 mL).

Step	Buffer	Column volume	Flow rate (mL/min)
Equilibration	50 mM Tris, 0.5 M NaCl, pH 7.5	8	2.5
Loading	1.6 x 10^{12} vector genomes per mL of resin	–	2.5
Wash	50 mM Tris, 0.5 M NaCl, pH 7.5	15	2.5
Elution	0.1 M glycine, pH 2.5	3	2.5

Table 2. Steps for the separation of full and empty AAV2 on a POROS XQ anion exchange resin (CV: 1 mL).

Step	Buffer	Column volume	Flow rate (mL/min)
Equilibration	10 mM sodium acetate, 20 mM BTP, 10 mM $MgCl_2$, 0.01% Pluronic, pH 9.0	5	1.0
Loading	Purified AAV2, approx. 1.2 x 10^{12} vector genomes per mL of resin	7	0.5
Elution	A: 20 mM BTP, 10 mM $MgCl_2$, 0.01% Pluronic, pH 9.0 B: 500 mM sodium acetate in Buffer A	40 Linear gradient 10%–100% B	0.5

Hybrid elution optimization—linear gradient and isocratic hold

To improve the separation between empty and full capsid peaks, we adopted a hybrid elution strategy that combined step elution and a linear gradient with a manual hold of the system [2,3]. The linear gradient and the buffer compositions used were the same as described in Table 2.

Analytical methods

As part of the dosing and potency analysis of AAV vectors, the vector genome (vg) titer needs to be accurately quantified. This titer refers to AAVs that package the GOI in full length or truncated form. Droplet digital PCR (ddPCR) can provide an absolute count of viral nucleic acids, enabling the precise quantification of AAV vectors containing the GOI. However, not all full AAV capsids are functional particles if there is insertion of host cell DNA or truncated GOI into the capsids. The estimated viral genome titer (vg/mL) does not always directly translate into functional titer or transduction units (TU). Hence, the titration of functional particles is critical to determine vector efficacy. A gene transfer approach was developed in-house to detect expression of the transgene. To determine the transduction efficiency, or the potency of the AAV2-GFP vector, a cytometry-based assay was developed to quantify the percentage of Green Fluorescent Protein (GFP)-expressing cells.

ddPCR

The concentrations of all relevant samples in vg/mL were determined using ddPCR as previously described [4]. Briefly, the Roche™ High Pure™ Viral Nucleic Acid Extraction Kit was used to extract the viral DNA corresponding to the AAV following the manufacturer's instructions. Then, with forward primer 5'-CTGCTGCCCGACAACCAC-3' and reverse primer 5'-TCACGAACTCCAGCAGGAC-3' obtained from Integrated DNA Technologies, PCR was carried out with a pre-incubation step at 95°C for 15 min followed by 40 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min, and a 5 min final extension at 72°C. The plates were scanned on a Bio-Rad™ QX100™ Droplet Reader, and analysis was carried out with BioRad™ QuantaSoft™ Software.

Potency assay

A transduction assay was performed in which samples containing AAV were added to HEK293 cells that had been transduced with adenovirus 5 as previously reported [4]. At 24 hr post-transduction, cells were harvested and fixed in 2% paraformaldehyde at 4°C for 30 min, followed by two analyses: first in a BD™ Biosciences BD Accuri™ C6 Flow Cytometer to account for all cells, then with BD Accuri™ C6 Plus Analysis Software to quantify enhanced green fluorescent protein (eGFP) expression. The linear range of quantification was established between 2% and 20% of GFP-positive cells. The functional titer was calculated in TU/mL.

Mass photometry

The ratio of full to empty capsids was determined by mass photometry as reported elsewhere [5]. Briefly, a Refeyn™ SamuxMP Auto Mass Photometer was used according to the manufacturer's instructions, with Refeyn™ MassFerence™ P2 as the calibrant. The samples and calibrant were automatically processed, and the resulting data were analyzed using the system software.

Results

Purification of AAV2 using POROS CaptureSelect AAVX Affinity Resin

Figure 2 shows the affinity capture chromatogram demonstrating successful AAV2-GFP purification with POROS CaptureSelect AAVX resin (CV: 5 mL).

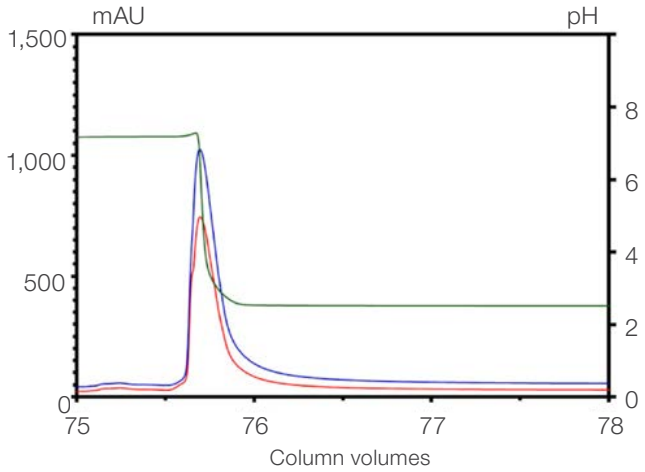


Figure 2. Successful purification of AAV2-GFP with POROS CaptureSelect AAVX resin. The red and blue lines represent the mAU at A_{260} and A_{280}, respectively, during the elution step. pH is represented in green.

The viral genome titers (VGs) and the TUs of the affinity chromatography load and elution samples are presented in Table 3. A recovery of 95% functional AAV particles was achieved, demonstrating that the process maintained the functionality of the AAV particles. The data also showed a difference of approximately 1 order of magnitude between VG and TU. This variation was expected, and the purification process did not change the ratio of VG to TU. The difference between TU and VG seems highly dependent on the serotype and gene of interest (data not shown).

Table 3. Process recovery using POROS CaptureSelect AAVX resin (CV: 5 mL).

Step	Volume (mL)	vg/mL	TU/mL	Total VG	Total TU	VG recovery (%)	TU recovery (%)	log (VG/TU) ratio
Load	294.0	2.73×10^{10}	1.75×10^{9}	8.03×10^{12}	5.15×10^{11}	–	–	1.2
Elution	3.9	1.67×10^{12}	1.27×10^{11}	6.43×10^{12}	4.89×10^{11}	80	95	1.1

Separation of full and empty AAV2 with POROS XQ resin

The linear gradient elution performed to separate full and empty particles is presented in Figure 3. The chromatogram displayed two overlapping peaks. A qualitative analysis based on UV absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) suggested that the first peak consisted primarily of empty particles, while the second peak consisted mainly of full particles. This is because an increase in the A_{260}:A_{280} ratio indicates enrichment of full capsids containing DNA. Preliminary quantitation of the TUs in the fractions collected from the two peaks showed that the fraction from the second peak consisted of around 25 times more functional particles than the fraction from the first peak (data not shown). These data suggest that empty capsids elute first, followed by full capsids.

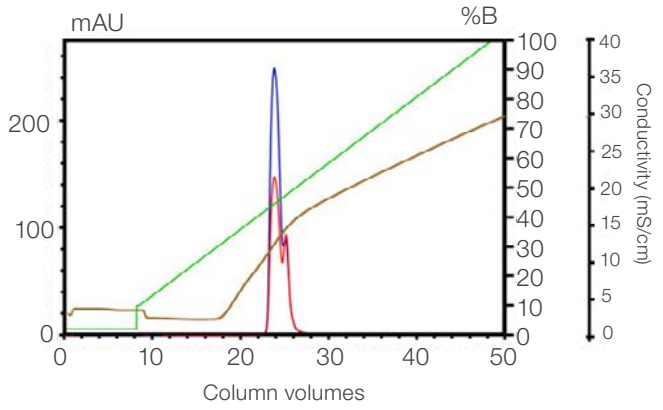


Figure 3. Linear gradient elution for the separation of full and empty AAV2-GFP with POROS XQ resin. The red and blue lines represent A_{260} and A_{280}, respectively, in mAU. Conductivity is presented in brown and %B (500 mM NaOAc in Buffer A) in green.

In addition, these results confirmed that the buffer formulation used during the process was suitable for the separation of full and empty capsids. The chromatogram showed that the empty capsids started to elute at a concentration of 216.5 mM sodium acetate (NaOAc) and 11.57 mS/cm conductivity. The peak maximum was found at 12.57 mS/cm and 222.5 mM NaOAc and the full capsids started to elute at 232.5 mM NaOAc and 13.84 mS/cm. It is worth noting that there was only a difference of 2.27 mS/cm between the start of the elution of the full and empty capsid peaks.

A closer look at the chromatogram showed a delay between the increase in the NaOAc concentration (%B) and an increase in conductivity. This phenomenon is expected based on the equipment configuration. There is a delay volume between the percentage of B and an increase in conductivity, because the percentage of B is determined at the mixing pump upstream of the column while the conductivity is measured downstream of the column.

Considering the high sensitivity of the AAV elution peaks to conductivity, the inherent challenge of maintaining consistent conductivity during buffer preparation, and the difference of ~2 mS/cm between elution of full and empty capsids, it was decided to develop a separation based on conductivity rather than molarity to increase the resolution between the empty and full peaks.

Hybrid elution optimization—linear gradient and isocratic hold

The timing of the isocratic step hold accounts for the equipment configuration when the conductivity sensor is downstream of the column. Once the isocratic step hold is started, conductivity will continue to increase before stabilization due to the holdup volume between the mixing chamber and the conductivity sensor. It is critical that conductivity stabilizes before reaching 13.58 mS/cm, as this will trigger the elution of the full capsids. Previous experimental data (data not shown) indicated that initiating the isocratic hold step at 12.5 mS/cm, which was the conductivity at the maxima of the empty peak, led to poor separation, because the conductivity continued to rise and stabilized at 13.8 mS/cm, resulting in full capsid elution. Based on these data, the isocratic step hold should be initiated at the beginning of the elution of the empty peak, when the conductivity is around 11.5 mS/cm, to stabilize at the target conductivity of 12.5 mS/cm. For the experiment presented in Figure 4, the linear gradient was held when the conductivity reached 11.57 mS/cm and until UV absorbance returned to baseline. The linear gradient was then resumed to allow the elution of the second peak corresponding to the full capsids.

The resulting chromatogram (Figure 4) showed that the isocratic step hold initiated at 11.57 mS/cm resulted in conductivity stabilization around the target conductivity of 12.5 mS/cm. The UV traces also showed that this elution strategy resulted in two fully resolved peaks. As expected, the second peak displayed a higher A_{260}:A_{280} ratio, which reflected an enrichment of AAV capsids containing DNA. The elution of the first peak started at 11.57 mS/cm as expected. However, the concentration of NaOAc was determined to be 158 mM (31.6% of B), while the previous separation showed elution at 217 mM NaOAc for the same conductivity of 11.57 mS/cm. These data indicate that conductivity is a more robust criterion for the elution of AAV2-GFP than the salt concentration.

The characterization of the fractions collected in terms of viral genome and functional titer is shown in Table 4. The column and method used for the separation of full and empty capsids recovered approximately 60% of viral genomes, while the recovery of functional particles was approximately 46% (Table 4). The recovery of the full capsid fraction yield could be further improved by optimizing the compositions of buffers.

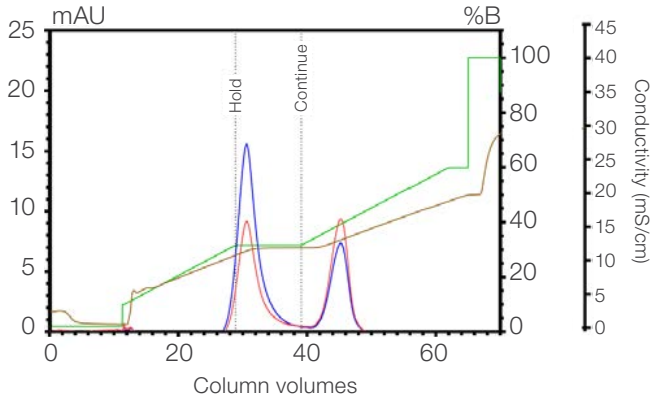


Figure 4. Combination of linear and isocratic elution for the separation of full and empty AAV2 using POROS XQ resin. The red and blue lines represent A_{260} and A_{280}, respectively, in mAU. Conductivity is presented in brown and %B (500 mM NaOAc in Buffer A) in green.

Table 4. Step recovery and AAV particle characterization of load and elution fractions using POROS XQ anion exchange resin (CV: 1 mL).

Step	Volume (mL)	Total VG	Total TU	VG recovery (%)	TU recovery (%)	log (VG/TU)	Full capsid (%)
Loading	3.5	2.15×10^{12}	7.18×10^{10}	–	–	1.5	14
Empty capsid (1st peak)	6.0	3.20×10^{10}	8.94×10^{8}	1.5	1.2	1.6	~1
Full capsid (2nd peak)	5.5	1.30×10^{12}	3.30×10^{10}	60.7	46	1.6	75

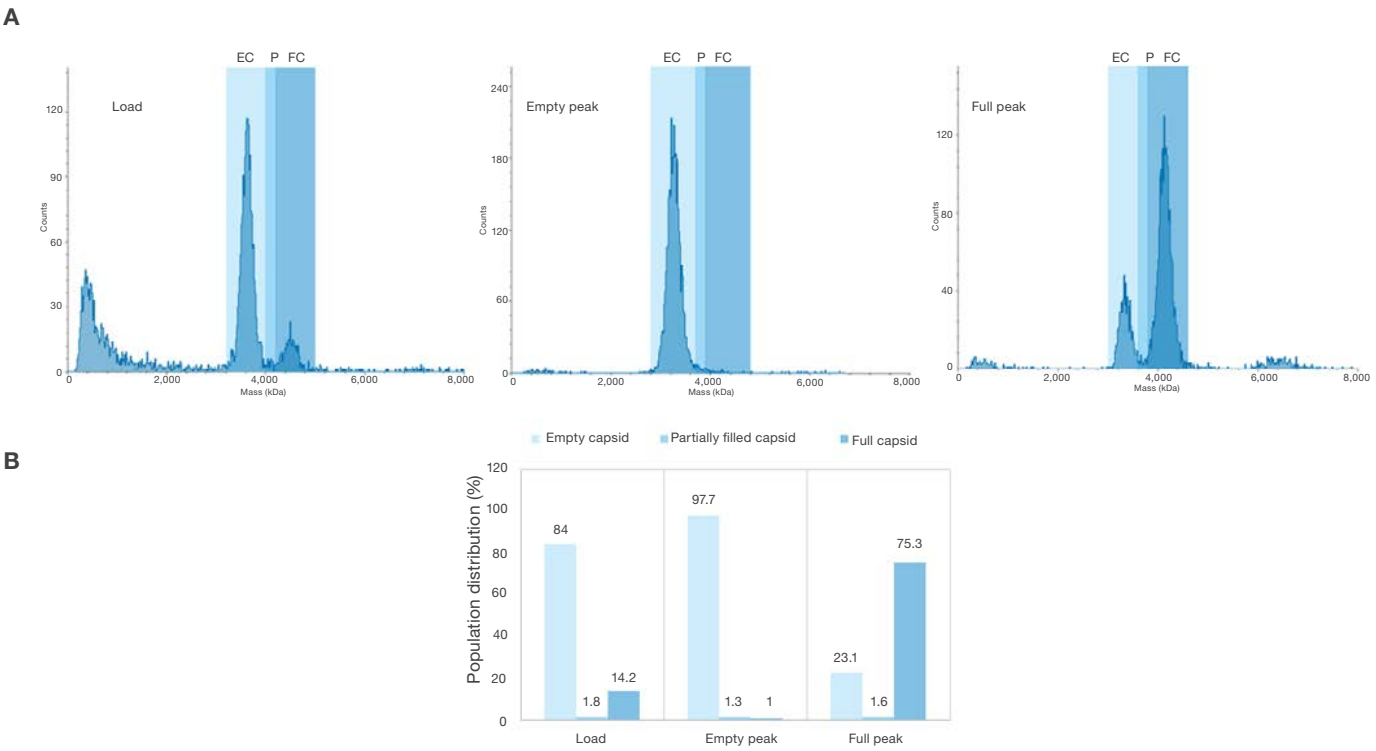


Figure 5. (A) The mass distributions of three fractions (load, empty peak, and full peak) comprising different proportions of AAV subpopulations. EC, P, and FC represent empty capsid, partially filled capsid, and full capsid, respectively. **(B)** Proportion of AAV2 subpopulation (EC, P, FC) from load (initial material), first peak (or empty peak), and second peak (or full peak).

Mass photometry was used to further characterize each collected fraction. Figure 5 shows that the AEX load material was primarily composed of empty capsids (84%) while full capsids represented only a small portion, about 14% of the total amount of particles. The empty peak comprised mostly empty capsids (98%), while the proportion of full capsids remained low at around 1%. These data demonstrate efficient empty capsid removal while minimizing full capsid loss. Finally, the characterization of the full peak showed a composition of 75% full capsids and only 23% empty capsids. The separation method developed here achieved a full capsid enrichment factor of 5-fold and a 4-fold reduction in empty capsids compared to the AEX load material. Achieving a high concentration of AAV can be challenging due to potential aggregation. Thus, removal of empty capsids offers the possibility to further concentrate full capsids carrying the therapeutic gene while minimizing the risk of aggregation. As the total capsid dose appears to be responsible for immune-mediated toxicities, removing empty capsids with no therapeutic benefit is crucial [1].

Conclusion

The work described here is the result of a collaboration between VVector Bio and Thermo Fisher Scientific to develop a robust method for separating full and empty AAV particles. The experiments were conducted at VVector Bio under conditions jointly optimized by both teams. The results demonstrate that the combination of affinity chromatography using POROS CaptureSelect AAVX Affinity Resin and subsequent anion exchange chromatography using POROS XQ Strong Anion Exchange Resin as outlined here enables efficient and reliable separation of full and empty AAV2 particles.

We hypothesize this method can be successfully applied to other AAV serotypes, and further optimization of the buffer compositions will result in higher recovery yield and consistent full capsid enrichment. The enrichment of full particles is crucial to achieve highly concentrated AAV preparations, which is a critical factor to obtain an effective response *in vivo*. Reducing the quantity of empty capsids in final preparations can also reduce the risk of aggregation if the sample needs to be further concentrated. This work offers detailed insights in the operation protocol for separation of empty and full capsids and emphasizes conductivity as the primary factor to consider when developing the separation method.

Acknowledgments

We thank Nils Williston and Alejandro Becerra (Thermo Fisher Scientific) for performing the mass photometry analysis and reviewing this manuscript, respectively.

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Ordering information

Description	Cat. No.
POROS CaptureSelect AAVX Affinity Resin, 10 mL	A36739
POROS CaptureSelect AAVX Affinity Resin, 25 mL	A36740
POROS CaptureSelect AAVX Affinity Resin, 50 mL	A36741
POROS CaptureSelect AAVX Affinity Resin, 250 mL **Note:** Larger volumes are available.	A36742
POROS XQ Strong Anion Exchange Resin, 250 mL	4467820
POROS XQ Strong Anion Exchange Resin, 1 L	4467818
POROS XQ Strong Anion Exchange Resin, 5 L **Note:** Larger volumes are available.	4467817
POROS GoPure XQ Pre-packed Column, 1 mL	A25812
POROS GoPure AAVX Pre-packed Column, 5 mL	A36651

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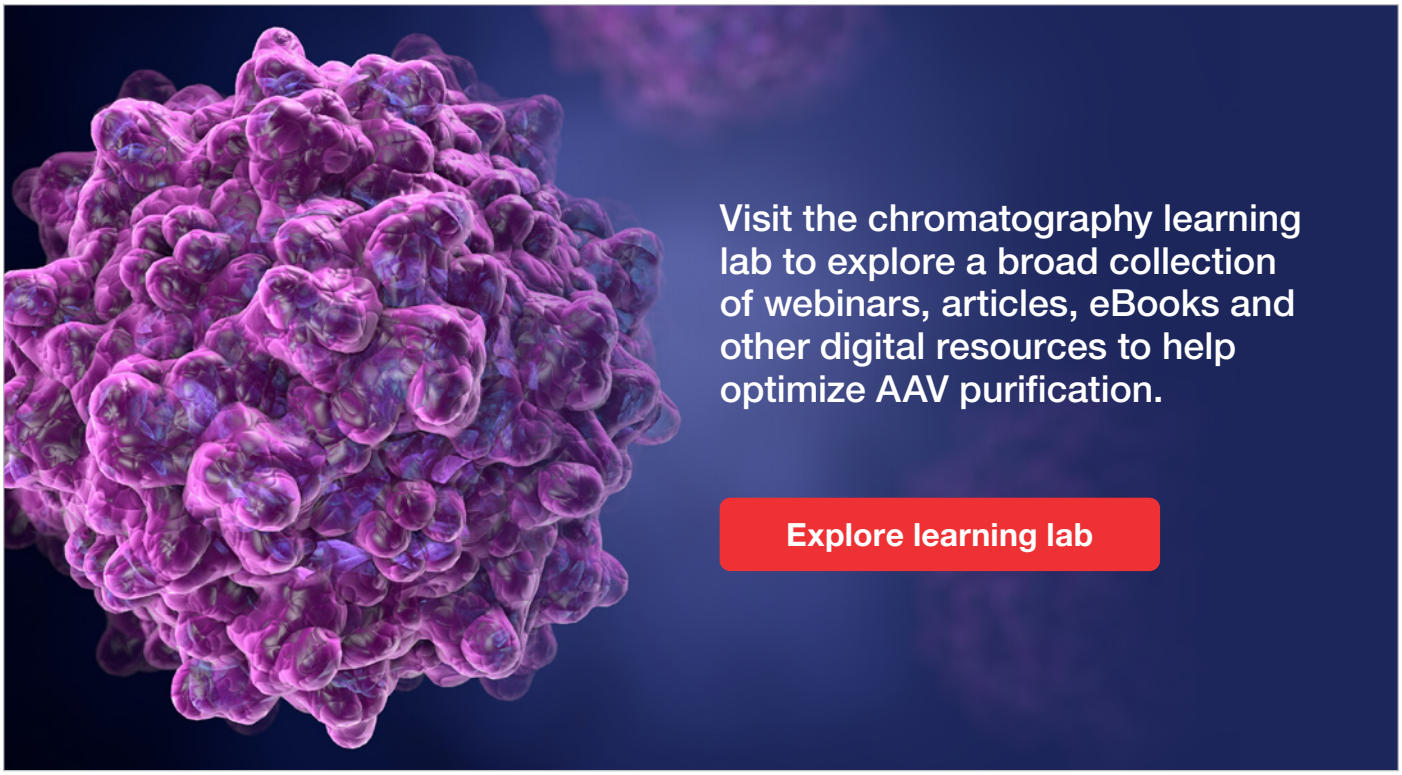

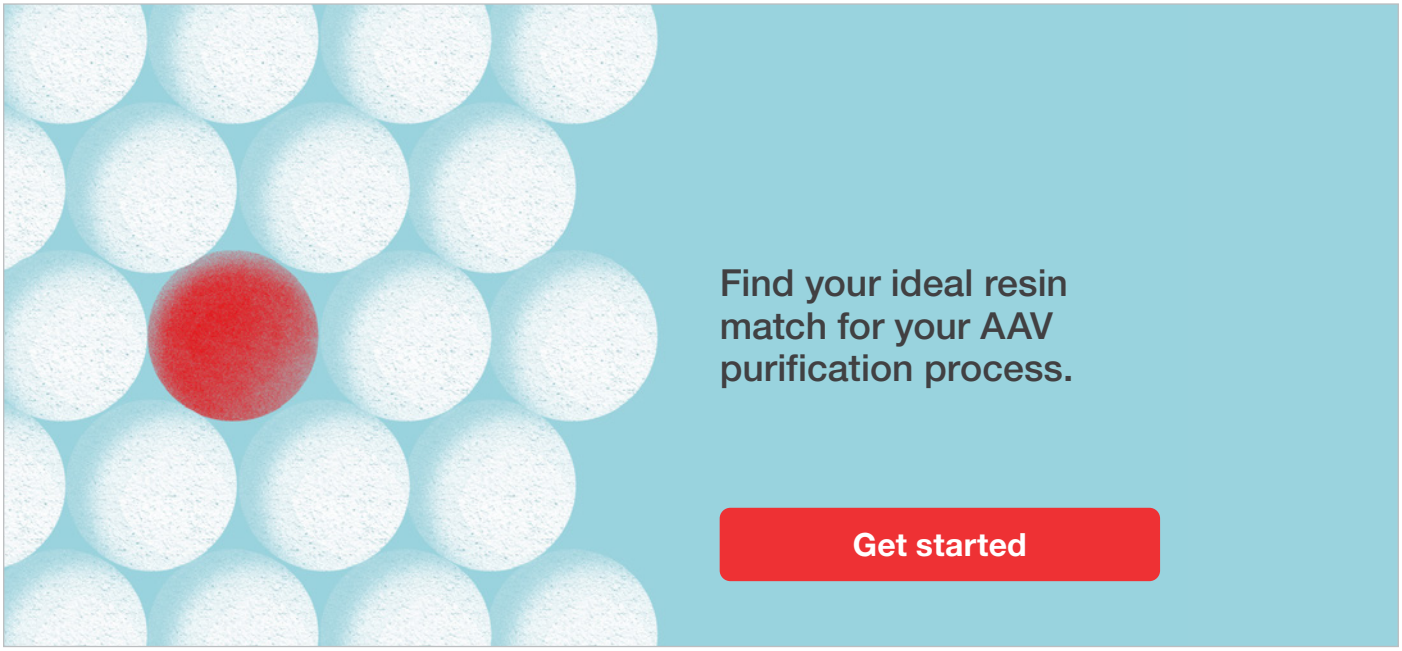

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