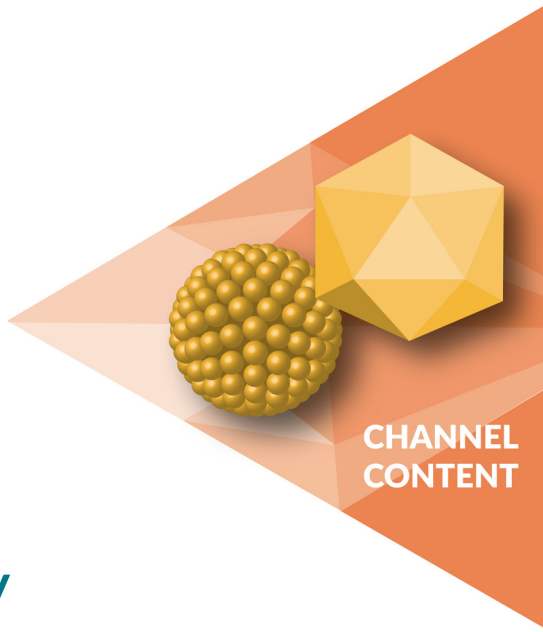
CELL & GENE THERAPY INSIGHTS



SCALABILITY

INNOVATOR INSIGHT

Advancing gene therapy development with a multi-serotype AAV affinity resin

Nicolas Laroudie and Quentin Bazot

AAV purification poses a number of unique challenges to viral vector manufacturers including the need for scalability, a significant impurity burden, and ensuring a good recovery yield. In this article, a multi-serotype AAV affinity resin will be described and its use illustrated by relevant experiences and case studies from a CMDO developing an AAV purification platform for application across various AAV serotypes and client processes.

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AAV DOWNSTREAM PROCESS CHALLENGES

When considering a typical AAV process for viral vector manufacture, the downstream purification process normally includes one or two chromatographic steps. Typically, this consists of an affinity capture step followed by a polishing step. This approach has been developed to address the challenges associated with the purification of complex molecules such as viral vectors and has been made possible by the development of modern tools such as immunoaffinity resins.

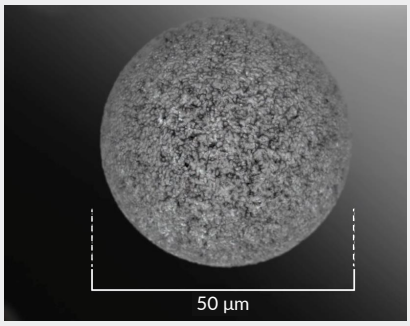
One challenge is the significant impurity burden in the feedstock due to cell lysis. AAVs are not secreted viruses, and it is necessary to break the cells to release the vectors, resulting in a very high amount of impurities. It is also necessary to develop

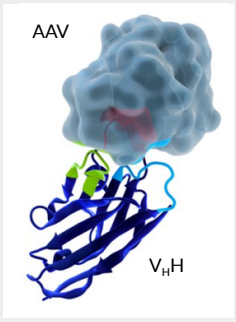
process steps that are robust, reproducible, provide a good recovery yield, and that are easy to scale up.

Most AAV manufacturers will eventually work with various serotypes, and it is therefore extremely convenient to have a platform tool that enables capture of most of the serotypes commonly used in gene therapy without having to redesign the process each time. This is what the POROS™ CaptureSelect™ (CS) AAVX resin has been designed for, and over the last decade it has become the most commonly used tool for AAV purification. However, an immunoaffinity resin such as AAVX is not able to distinguish between full and empty capsids

FIGURE 1

POROS CS affinity solutions to support AAV purification.



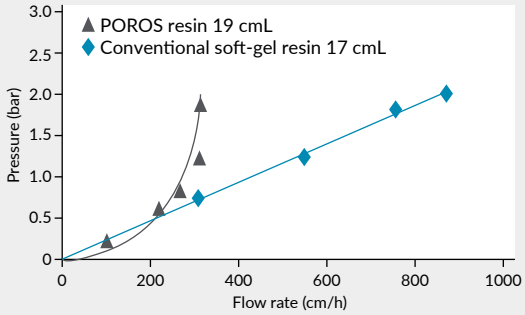


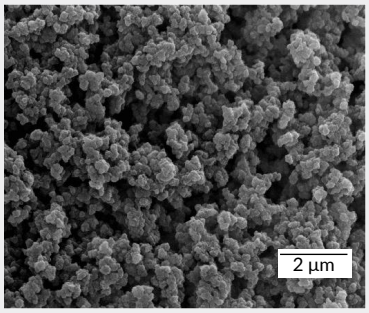
Thermo Scientific™ resin	Serotype affinity
POROS™ Capture Select™ AAV8 resin	AAV8
POROS™ Capture Select™ AAV9 resin	AAV9
POROS™ Capture Select™ AAVX resin	AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, Rh10, recombinant and chimeric vectors

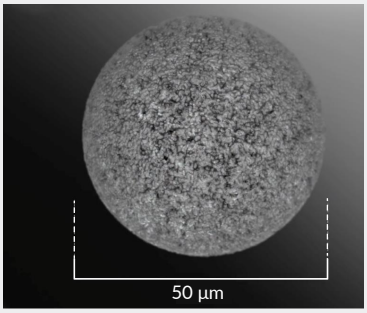
Products come with full regulatory support (RSF) enabling use in commercial manufacturing

FIGURE 2

Features of the POROS bead.







Poly(styrene-divinylbenzene) backbone

- Linear pressure flow curve
- Rigid, linear, and scalable performance
- Easy handling
- Highly robust and chemically stable

Large throughpores

- Reduced mass transfer resistance
- Capacity and resolution well maintained over a wide range of linear velocities
- More efficient purification

50 micron bead size

- Superior resolution
- Improved capacity through novel surface chemistries
- Excellent pressure-flow properties
- Fully scalable

and therefore, a second polishing step is necessary for this purpose. Here, the use of an anion exchanger—the POROS HQ 50, for example—is recommended.

Thermo Fisher has designed and released three immunoaffinity resins for the specific capture of AAV vectors (Figure 1). Along with POROS HQ CS AAV8 and 9, for the capture of serotypes 8 and 9, The POROS CS AAVX has been developed with the capability to target a broader range of serotypes, and for possible use as a platform tool. All three resins utilize CS technology, which involves the use of ligands derived from a camelid single variable domain on a heavy chain (VHH) nanobody which is then grafted onto a POROS base bead. These VHH ligands are synthetically produced in an animal-free system and at large scale.

All three resins are manufactured in a GMP-compliant environment allowing for their use in commercial manufacturing and come with a regulatory support package.

The CS ligands are supported by POROS base beads (Figure 2), with a unique pore structure for the efficient purification of large molecules such as viral vectors.

CONSIDERATIONS FOR PLATFORM AAV AFFINITY CAPTURE

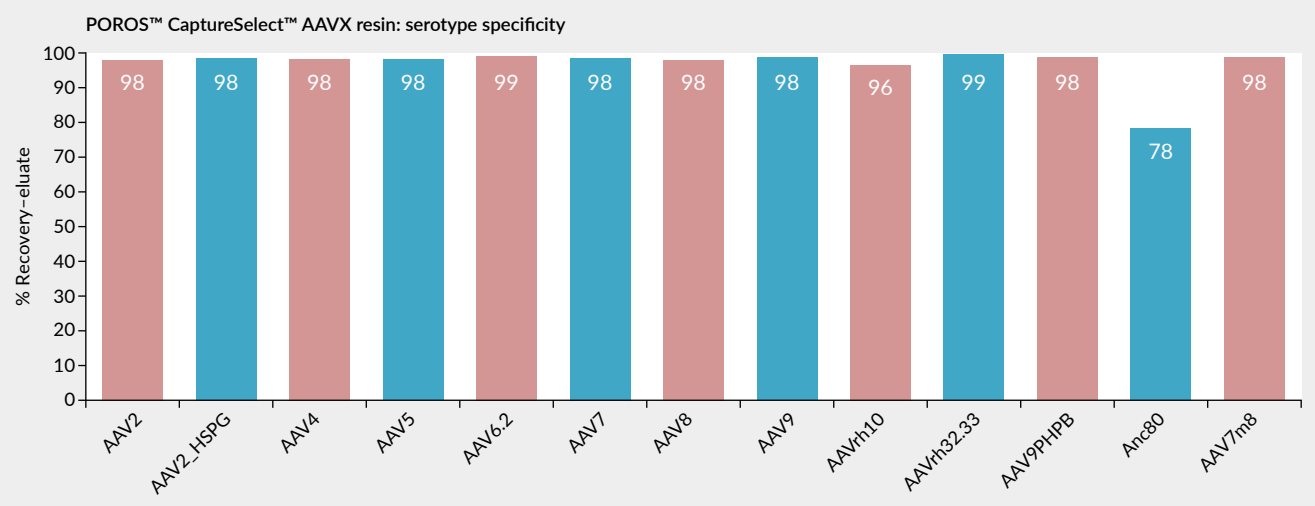
In order to develop a platform for AAV capture there are a number of requirements to be considered, including:

- Broad specificity to different AAV serotypes;
- High dynamic binding capacity;
- High purity and recovery;
- Scalability; and
- Reusability

The system must allow for the recognition of any serotype, whether naturally occurring or engineered. There is a need for high binding capacity to reduce column size requirements and maximize productivity. High purity and high recovery are two characteristics one can expect from immunoaffinity technology, and they can both be achieved via finetuning of intermediate washes and elution buffer optimization. Scalability, with consistent performance upon scale-up, is also key. Reusability

FIGURE 3

Broad specificity of POROS CS AAVX. Batch binding experiments performed and data provided by Massachusetts Eye and Ear.



AAV quantified by qPCR [1].

can reduce the COG in particular at the small- and pilot-scale or in routine manufacturing for the same serotype and same transgene. Reusability is therefore crucial for reducing cost and maximizing productivity.

Specificity

The POROS CS AAVX medium has the capability to capture a very broad range of serotypes, which is a unique feature in the current market. In a set of experiments designed and conducted by the Massachusetts Eye and Ear Institute in the US, researchers took different AAV serotypes and placed them in tubes with AAVX resin. The supernatant was incubated and then removed and the resin was washed. The bound material was eluted by adding acidic solution. The amount of AAV recovered was then measured and compared to what was initially added.

As shown in Figure 3, all of the serotypes evaluated were recognized and captured on the resin. There is currently no identified AAV serotype that is not recognized by the AAVX ligand. This makes the POROS CS AAVX resin an ideal candidate for the establishment of a platform purification tool.

Binding capacity

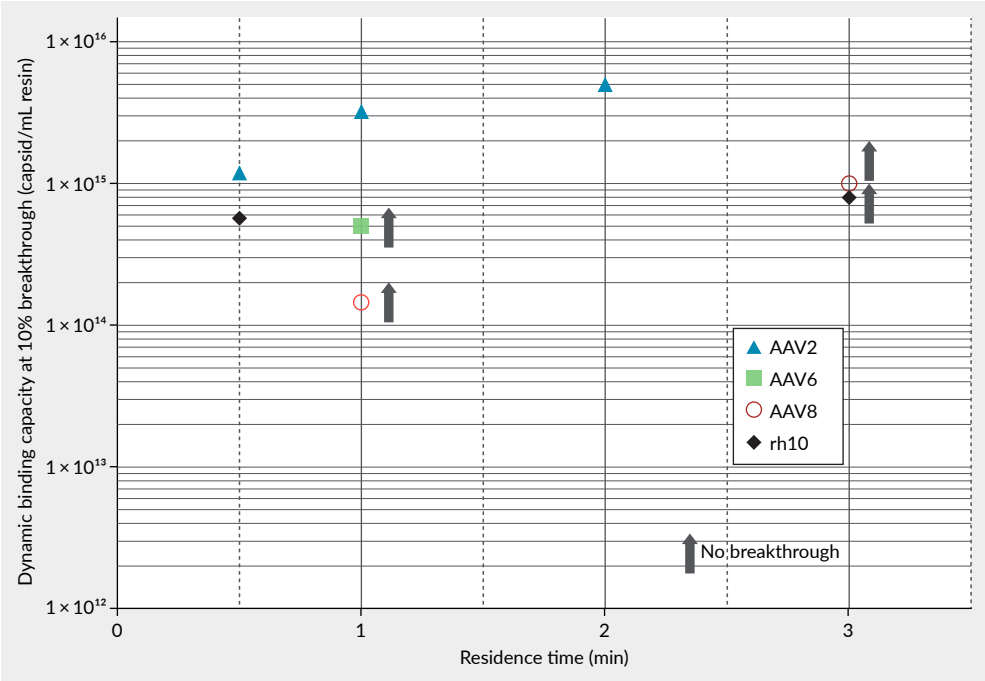
The POROS CS AAVX resin exhibits a high dynamic binding capacity for many serotypes as shown in Figure 4. For AAV2, the capacity at 10% breakthrough was measured as higher than 1×10^{15} capsids/mL for a residence time as short as 30 seconds, and at least 1×10^{14} capsids/mL for serotype rH10 for the same residence time. For AAV8, it was not possible to saturate the resin due to the shortage of material, and this is indicated by the grey arrows that suggest a higher dynamic binding capacity than measured is expected.

Performance overview

A rigid chromatography medium means that the pressure evolves linearly with the flow

FIGURE 4

The POROS CS AAVX resin dynamic binding capacity or multiple serotypes, including at short residence times.



Data sources: AAV2, AAV6: internal work; AAV8: [2]; rh10: [3].

increase, as compared to a semi-soft material such as agarose, for which the pressure increases exponentially with the flow rate when it exceeds a certain value. Figure 5 illustrates an example with POROS CS AAV9; the behavior is identical with AAV8 or AAVX.

With rigid material, the backpressure relies on the flow and on the bed height but not on the column diameter. This makes it simple to anticipate the expected backpressure when scaling up a process. In addition, backpressure remains limited even at high velocities, allowing processes to be run at high speed, resulting in increased productivity with linear and predictable scalability.

Reusability

To address the question of reusability, an internal cycling study with an AAV2 feedstock was performed (Figure 6). No significant decrease in recovery yield was observed over 35 cycles, and the performance of the resin in terms of purity was consistent from run 1 to 35.

Polishing step: separation of full and empty AAV capsids with POROS HQ

As mentioned previously, one capability the immunoaffinity resin lacks is the ability to distinguish between full and empty capsids, and therefore the utilization of an affinity column requires a polishing step after capture to separate empty capsids from full ones. Ion exchange chromatography has shown to be most suited for this application, making use of the difference in pI of full and empty AAV particles. Since the difference in pI is only 0.4 units, a high resolution ion exchange resin with superior selectivity is required.

Thermo Fisher's portfolio includes two strong anion exchangers—POROS HQ50 and POROS XQ—that have been successfully used for the separation of full and empty capsids, and which share the same POROS backbone.

Data from Lavoie *et al.* demonstrates capsid enrichment using POROS AEX resins. A

FIGURE 5

POROS CS AAV9 pressure flow curve. Low back pressure: <3.0 bar at 700 cm/h in 22 cm length column.

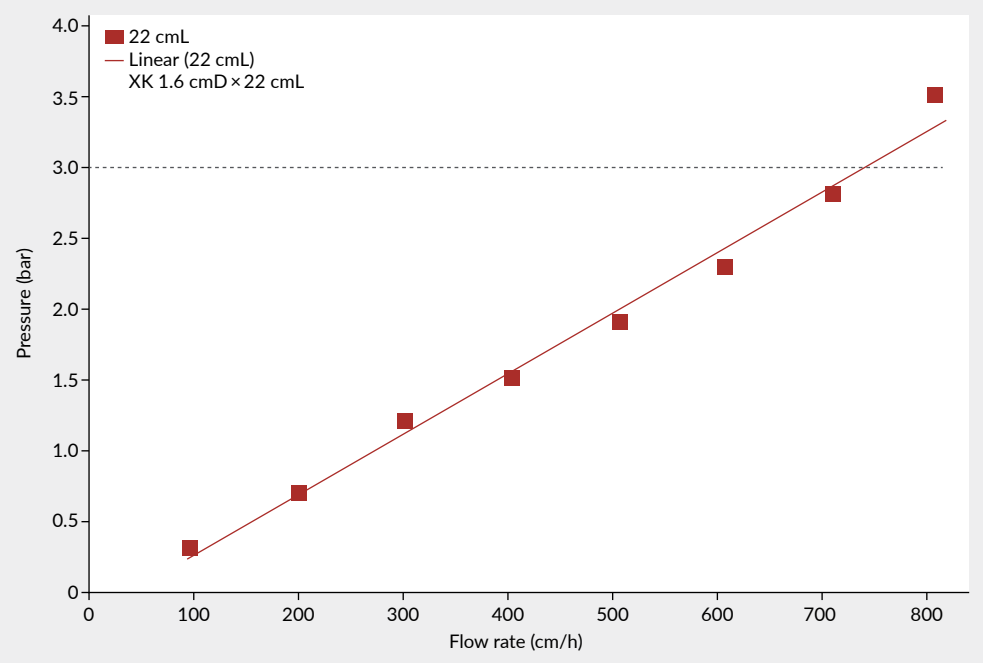
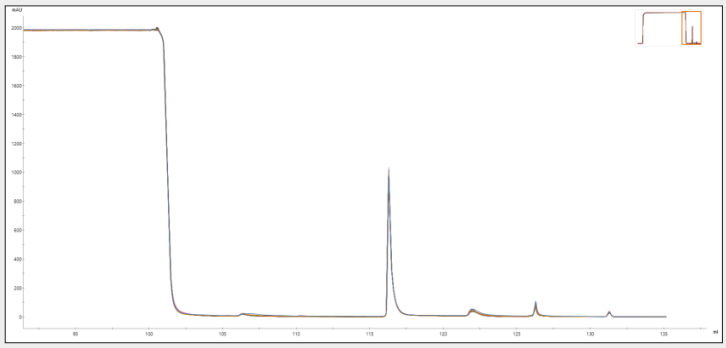
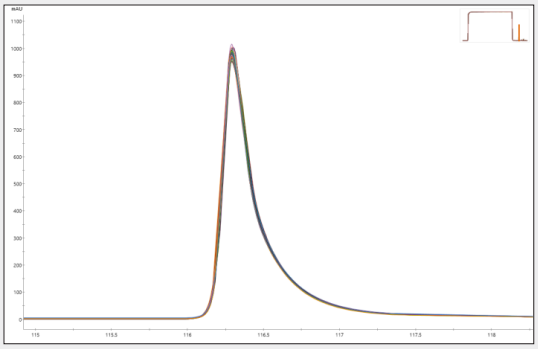
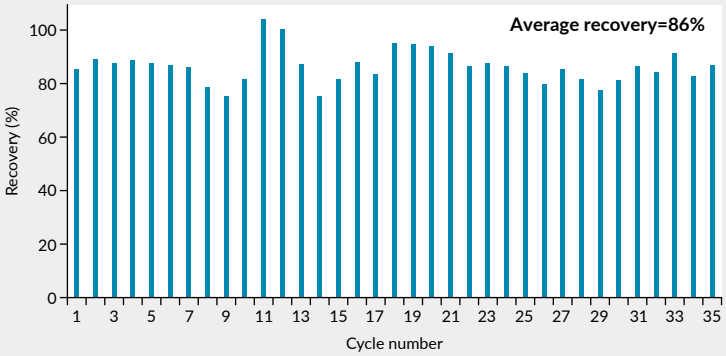


FIGURE 6

POROS CS AAVX reuse study with AAV2.



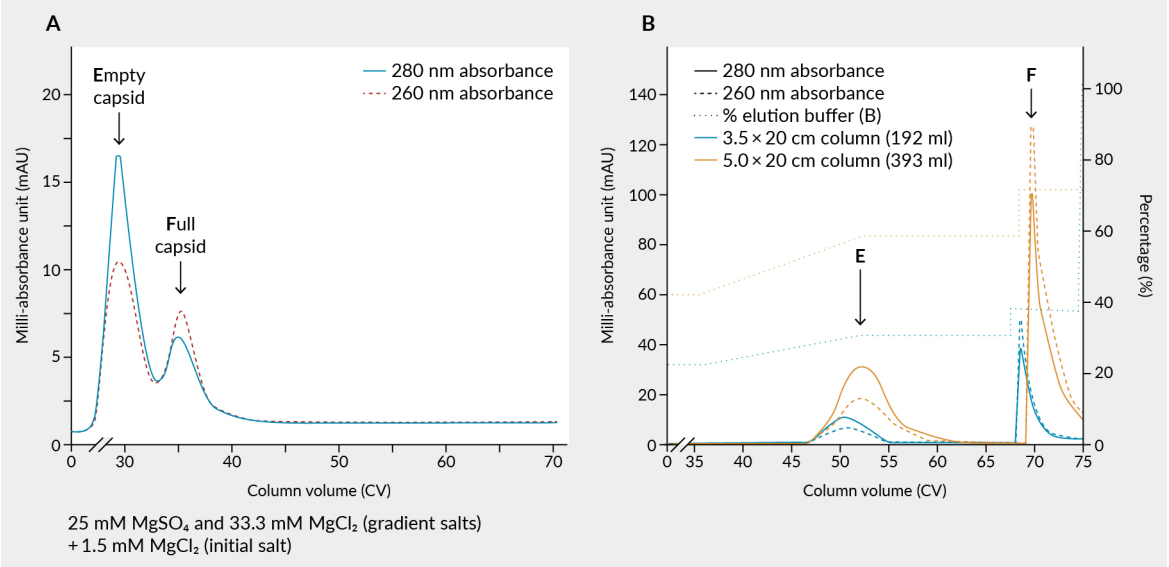




Study format	
Sample	Clarified lysate AAV2
Load preparation	Concentration and buffer exchanged
Resin	POROS CaptureSelect AAVX
Column details	1 mL (0.5 cmD × 5 cmL)
Loading/cycle	6.6×10^{14} cp/mL resin
Regeneration	0.2 M phosphoric acid (15 min)
Cleaning	6 M guanidine–HCL (15 min)

FIGURE 7

Optimized elution profiles of AAV5 on POROS HQ using 'dual' salts.



Percentage full was determined by AUC, measuring a 75% and 79% full respectively. Data from Lavoie et al. [4].

dual salt gradient was utilized to separate full and empty capsids in AAV5, as shown in the chromatogram in Figure 7A. A gradient was used, then converted into a more elaborate purification process to achieve baseline separation of full and empty capsids (Figure 7B).

TABLE 1

Performance of elution of AAV5 on POROS HQ at various scales.

Scale	vg yield(%)	cp yield (%)	Eluate % full (vg/cp)	Enrichment factor
(0.66 cm × 20 cm) 6.8 mL	69	22	80	4.0
(3.5 cm × 20 cm) 192 mL	80	22	80*	4.0
(5.0 cm × 20 cm) 393 mL	63	24	63*	3.2

cp: capsid; vg: vector genome.

Using POROS HQ, the process was then scaled up to different column sizes and geometries, as shown in Table 1. Notably, this approach enriched percentage of full capsids in purified feedstock by a factor of three to four, reaching up to 80% full capsids.

CASE STUDY SERIES: INTEGRATION OF THE POROS AAVX AND POROS HQ50 INTO AN AAV PURIFICATION PLATFORM

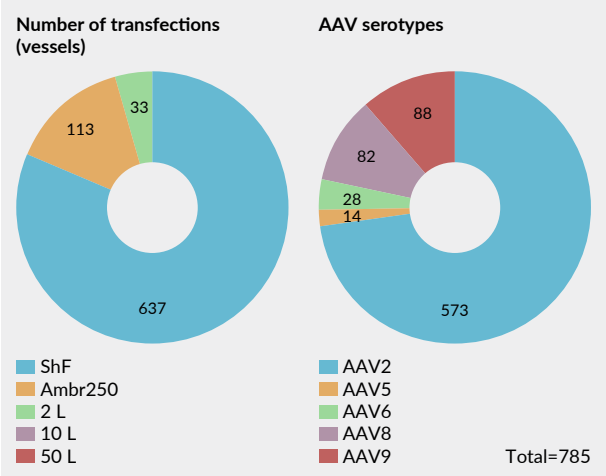
ABL Europe, now Oxford Biomedica, is a pure-play CDMO specialized in viral vector production that has manufactured a broad range of viral vectors for various applications, including vaccines, oncolytic virotherapies, and gene therapies.

These case studies will focus on AAV vectors and the development of an AAV platform based on HEK293 suspension cells, using transient transfection. The goal of this work was to create a scalable platform that could easily be implemented for different AAV serotypes and at various stages of a project in order to meet client needs.

The transfection step is key to producing AAV, and ABL Europe's process development and innovation lab screened a number of different HEK293 suspension cell lines and media, along with various transfection reagents and production systems, including one-, two- and classical three-plasmid systems. In terms of production scale, all projects start with transfection in shake flasks, and can go up to 50 L in the process development lab. Optimization work was done using serotypes AAV2, 5, 6, 8, and 9. A summary of the various vessels and AAV serotypes utilized is outlined in Figure 8.

FIGURE 8

Summary of AAV upstream platform development work undertaken by ABL Europe's development and innovation laboratory.



For optimizing the downstream process, various detergents were screened for the cell lysis step, along with filters and tangential flow filtration (TFF) cassettes, and chromatography media for the capture and polishing step. An additional goal was to retain an optional first TFF step before capture and after clarification, for client processes that required it.

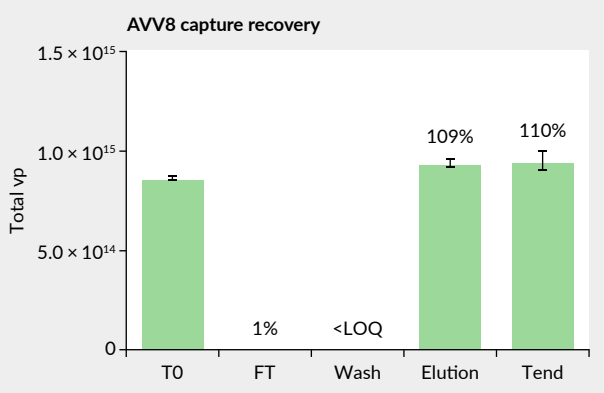
AAV PLATFORM DEVELOPMENT: ASSESSING THE AAV CAPTURE STEP

For the AAV capture step, the AAVX resin was an obvious candidate for evaluation when developing a pan-serotype platform.

Evaluation began with AAV8, and the material was taken directly from clarification, with no TFF step. Capture recovery was assessed as shown in Figure 9, showing very high recovery with AAV8 using the AAVX resin.

FIGURE 9

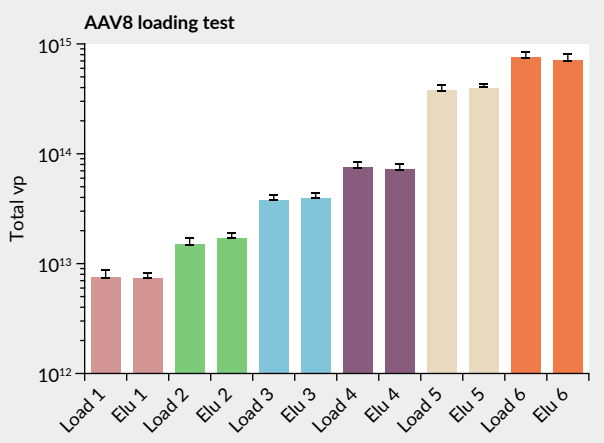
Evaluation of AAV8 vp recovery using the POROS AAX resin.



vp: viral particle.

FIGURE 10

Evaluation of loading capacity and recovery with the POROS AAVX resin using AAV8.



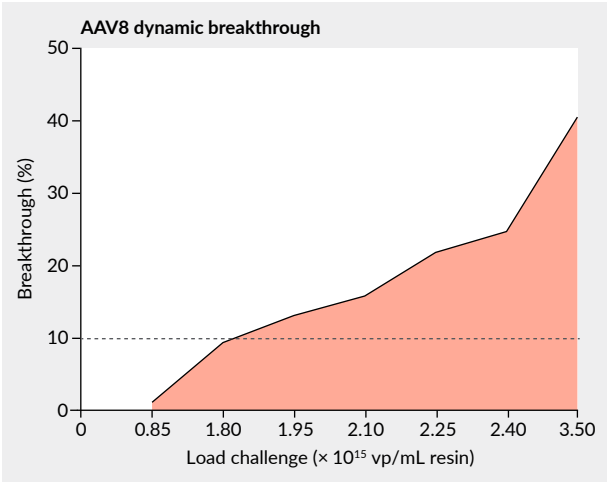
Run	Load total vp/ml resin	Recovery (%)
1	8.0×10^{12}	98
2	1.6×10^{13}	112
3	4.0×10^{13}	105
4	8.0×10^{13}	95
5	4.0×10^{14}	104
6	8.0×10^{14}	94

Next, the loading capacity of the resin with AAV8 was evaluated, as shown in Figure 10. Experiments were carried out with different amounts of AAV8 per mL of resin. Recovery of above 90% was achieved in all experiments, with high recovery of >2 log difference.

Next, resin dynamic binding capacity was determined (Figure 11). Loading of 1.7×10^{15} viral particles (vp) of AAV8 per mL

FIGURE 11

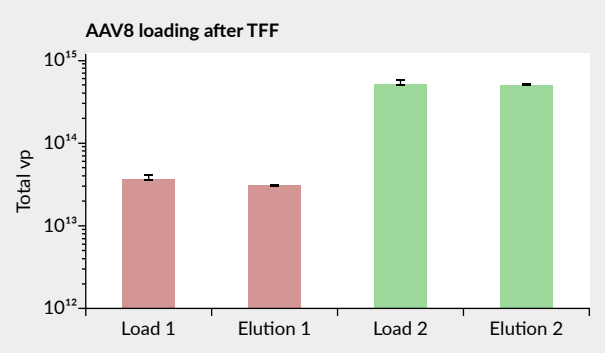
Determination of AAVX resin dynamic binding capacity for AAV8.



Loading of 1.7×10^{15} vp of AAV8 on AAVX resin leads to 10% breakthrough. Breakthrough curve realized with 1 min residence time. vp: viral particle.

FIGURE 12

Impact of concentration/diafiltration step on AAVX resin performance.



Run	Load total (vp/mL resin)	Recovery (%)
1	4.0×10^{13}	83
2	5.5×10^{14}	98

of resin led to a 10% breakthrough, indicating that no more than 1×10^{15} vp per mL of resin should be loaded.

As noted above, retaining TFF as an optional process step was one of the goals of the platform development process. Various TFF cassettes were screened before one with good recovery was selected. The impact of the TFF step on resin performance was then assessed. TFF was performed after clarification, then a capture step was carried out with two different amounts of AAV8 loaded

FIGURE 13

Characterization of protein impurities during the capture step using the POROS AAVX resin.

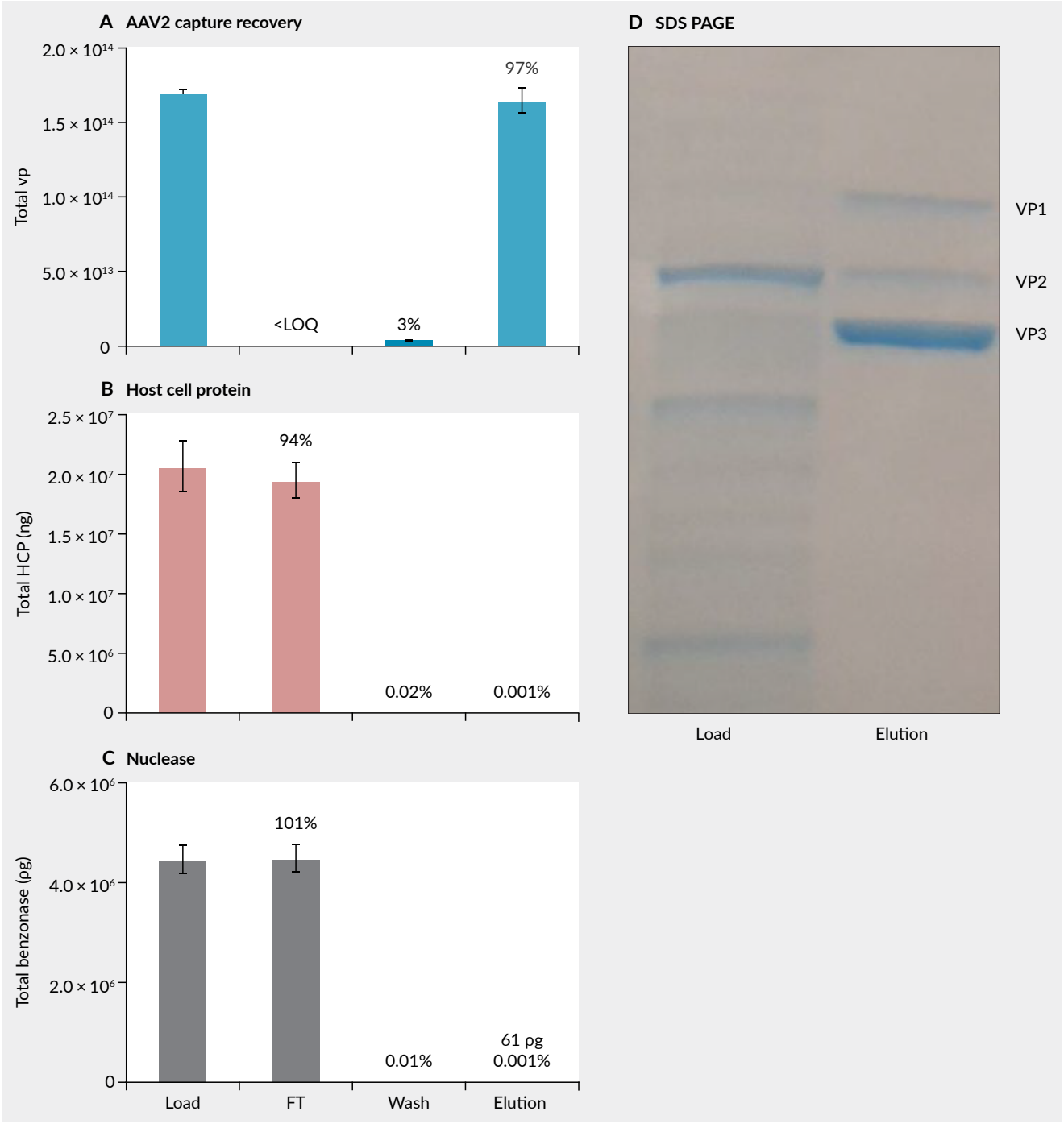
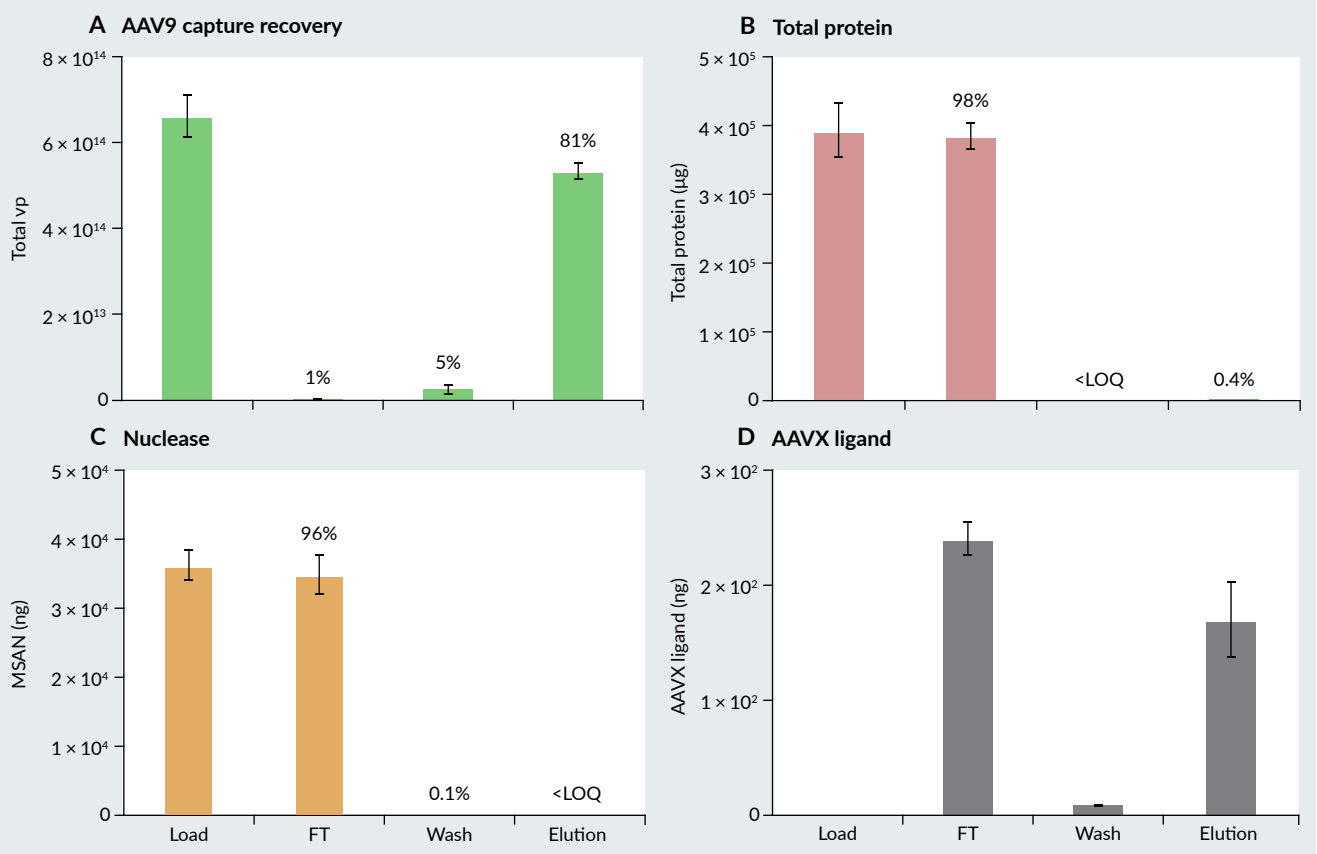


FIGURE 14

Characterization of protein impurities and of AAVX ligand during the capture step using the POROS AAVX resin.



(Figure 12). It was observed that the concentration step appears to slightly reduce recovery at low load, such as 1×10^{13} vp. However, 83% would still be considered a highly satisfactory recovery.

Another question to be addressed was whether the AAVX resin would perform similarly for different AAV serotypes. After working on elution parameters using AAV8, the AAVX resin was tested and evaluated on a number of additional serotypes (AAV2, 9, and 6), and good overall recovery of total vp was achieved for all serotypes tested.

IMPURITY REDUCTION CASE STUDIES

Characterization and reduction of protein impurities during the capture step was evaluated. Two case studies were performed to evaluate purity reduction. The first was an AAV2 run where a capture step was performed. As shown in Figure 13A, nothing was detected in the flowthrough, 3% of AAV2 was detected in the wash, and 97% in the elution. Host cell protein was then evaluated using analyzer kits specialized for HEK293. Almost all host cell protein was detected in the flowthrough, with small traces in the wash and the elution (Figure 13B).

Finally, this case study looked at another impurity introduced at the beginning of downstream processing: nuclease (in this case, Benzonase). ELISA kits were used for the Benzonase and as for the host cell protein, Benzonase was found in the flowthrough, with traces in the wash and in the elution (Figure 13C).

Additionally, SDS-PAGE was performed during these experiments (Figure 13D). In the load a number of impurities can be seen, but for the elution, mainly VP1, VP2, and VP3 are observed, i.e. the capsid protein.

A second case study was performed with AAV9. In this case, 81% recovery was achieved with the capture step (Figure 14A). Total protein was assessed using a Micro BCA Protein Assay kit, and 98% was found in the flowthrough, an amount below the limit of quantification in the wash, and 0.4% in the elution (Figure 14B). In this case, salt active nuclease was used and traces were found in the wash (Figure 14C). In this set of experiments, the AAVX ligand—an impurity introduced during the capture step—was assessed using an analyzer kit. As shown in Figure 14D, nothing is detected in the load, which is to be anticipated as there is no resin before this step. AAVX was detected in the flowthrough, wash, and elution. One important point to note is that this experiment was continued onto the polishing step. AAVX ligands were tested for at the end of the polishing step and were below the limit of quantification.

FIGURE 15

Effect of glycine- and citrate-based elution on polishing step with AAV9.

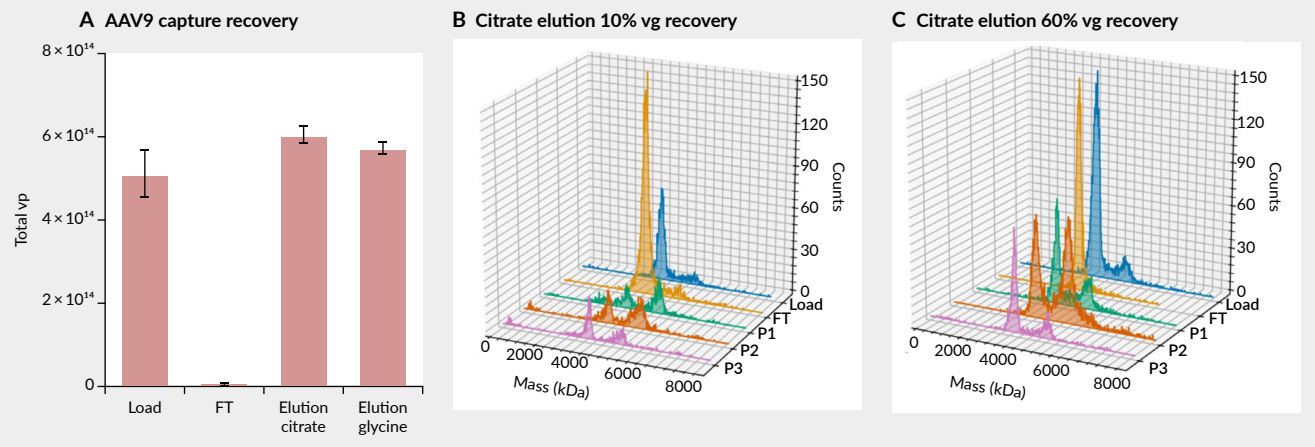
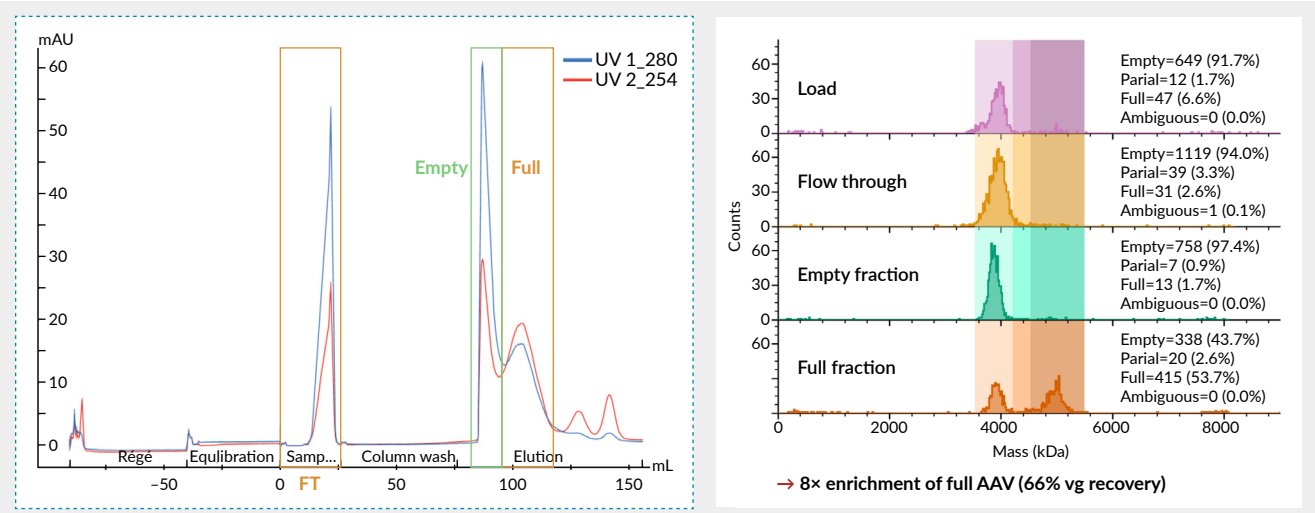


FIGURE 16

Poros HQ resin used for AAV full enrichment.



AAV POLISHING STEP

The polishing step is arguably the most technically challenging step in an AAV manufacturing process, and one that is quite specific to AAV vectors. The relationship between the capture and polishing step, and more specifically the choice of the elution buffer for the capture step, may have an important impact. In this set of experiments, the effect of glycine and citrate-based elution on the polishing step was studied. Utilizing AAV9 capture runs, both citrate-based and glycine-based elution buffers were tested. As shown in Figure 15A, both buffers worked well with up to around 100% recovery. Via chromatographic analysis, it was observed that around 10% vector genome (vg) recovery was achieved with citrate, as compared to glycine where 60% recovery was achieved (Figure 15B and C). Glycine-based elution was therefore found to have a positive relative impact on the polishing step.

USE OF POROS HQ RESIN FOR AAV POLISHING STEP

In collaboration with a client, ABL Europe developed an AAV2 polishing step using the POROS HQ 50 resin. An example chromatogram of the optimized process is shown in Figure 16. A challenge of this project was starting with a low amount of full AAV capsids (4–8%).

Looking at the flowthrough fraction, this was comprised mainly of empty AAVs, with around 97% being empty particles. The full fraction shows 53% full AAV (i.e. 8 × enrichment of full AAV) with 66% vg recovery.

CONCLUSION

To summarize, the pan-affinity ligand POROS resin AAVX offers high capacity, high yield, and high purity for different AAV serotypes. It is the AAV affinity resin of choice for ABL Europe, as it can be used for a variety of projects and performs well across different serotypes. The POROS HQ resin is efficient at enriching full AAV capsids. While data on AAV2 has been outlined in this article, internal work has also been performed on AAV9 and AAV8, which has also performed well and enriched for full fractions.

Q&A

Nicolas Laroudie (left) and **Quentin Bazot** (right)

Q Did you compare the performance of the AAV9- or AAV8-specific resin against the AAVX?

QB: I don't think we have done AAV8, but we have compared AAV9. The AAV9-specific resin works well. However, for us, the binding capacity was better with the AAVX resin for AAV9 compared to the AAV9-specific resin.

Q What analytical assay did you use to assess the total vector particles?

QB: We used ELISA methods. For AAV2, AAV6, and AAV8 we used an automated ELISA, and for AAV9, we used a classical ELISA kit.

Q Are you producing AAV on microcarriers or free suspension cultures?

QB: In the work I presented, we are only using suspension cells. I've worked with micro-carriers and AAV previously, before my time at ABL Europe, and it works well. Obviously, there are different challenges, and it requires different optimization because you are working with adherent cells. You need to find the right microcarrier, and look at the confluency and the cell growth. In my opinion, the process is easier with suspension cells, especially when you scale up and when considering GMP processes.

Q Should the process parameters be adapted to each serotype or is there a universal protocol used with AAVX?

NL: That is the beauty of the AAVX resin—it can be used as a platform as demonstrated by Quentin. The parameters that you have developed for one serotype can be used for other serotypes with very minimal adaptation. For exotic serotypes, you may have to play a little bit with the elution buffer to improve the efficiency of elution. You also have to be careful with some serotypes like AAV2, for instance, because it tends to aggregate somewhat. To prevent this, some salt must be added to the elution buffer. Therefore, some very small changes must be applied. Otherwise, yes, it is a universal protocol.

Q How should the CS with AAVX resin be scaled up?

NL: Resins based on POROS material are rigid and not compressible, so they are easy to scale up because the pressure versus flow curve is linear. It is really easy to predict the back pressure generated for a specific flow rate, whatever the scale of the column. Because it is rigid, back pressure does not depend on the column diameter but only on the bed height.

Therefore, when you want to scale up such a process, you maintain the bed height constant and simply increase the diameter of your column. The idea is to keep the velocity or the residence time constant when you increase your scale, maintain the number of CVs for each step and if possible, maintain the ratio between the volume of loading material to the volume of resin that is used.

Q Were you able to define a platform buffer to wash and elute all the AAV serotypes on AAVX?

QB: We worked on different process parameters and elution buffers. As mentioned above, for some serotypes it is better to add or to reduce the concentration of some buffer components. We have buffers that we prefer for different serotypes, especially AAV2, where we alter some things in order to avoid any problems such as aggregation.

NL: A recommendation and a good practice would be to have a little bit of surfactant and especially Pluronic F-68 in all the different solutions and buffers used for AAV purification. AAV tends to stick to plastics and to the walls of tubes. We sometimes see bad recoveries due to this, but the AAV is not still on the resin or on the column itself. I recommend paying special attention to this and to the analytics as well. Always take care to close the mass balance.

Q Was impurity or vector carryover quantified during resin reuse?

NL: On reusability, we have run some experiments where we use the resin up to 35 times. We have data regarding the potential carryover over 14 cycles, with the resin cleaned for each cycle with phosphoric acid and guanidinium HCl. We have not seen carryover of capsids in the eluate. Even after 14 cycles, it is below the detection limit.

QB: It is the same for us—we did some work on that, and we don't see any specific carryover of AAV between the runs.

Q Regarding POROS resin stability over time, there is no stated expiration date. Is there an effect of resin storage on its binding performance?

NL: We do not provide an expiry date with our POROS resins, but we support this with stability data that we provide to our customers when available. Typically, we provide stability data for three years with POROS CS AAVX, but that does not mean that the resin is not stable for a longer time. It is up to the customers—with our support, of course—to generate more stability data to fit with their specifications.

Q What is the capacity of POROS HQ resin for AAV?

NL: We don't have numbers for every serotype, but typically, we use the same column size for POROS HQ and AAVX when we have a full purification process. Nevertheless, for development purposes, we recommend loading much less on the POROS HQ: in the range of 3×10^{12} vg/mL for development, and then once your parameters are well established, you can increase the loading up to 2×10^{14} vg/mL, the same as on the AAVX.

Q What are your recommendations for cleaning the resin?

NL: AAVX or CS resins are not alkaline-resistant, so please don't use caustic to clean them—use acids, and especially, phosphoric acid or citric acid (0.1 M for cleaning after each cycle). This works pretty well. Guanidinium HCL up to 6 M can be used as well to improve the cleaning. POROS HQ is resistant to caustic so it can be cleaned with sodium hydroxide.

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BIOGRAPHIES

NICOLAS LAROUDIE worked at Généthon, France between 2001 and 2011 as Head of Downstream Development. He led a team developing and scaling-up purification processes for AAV, retroviral, and lentiviral vectors used for gene therapy treatments. He then joined Merck Millipore as a BioManufacturing Engineer and supported European customers for all DSP technologies with a strong focus on chromatography. In particular, he took an active role in the establishment of a fully continuous, large-scale disposable DSP process for the purification of a monoclonal antibody, within the framework of a large multi-company European consortium. Next he joined ThermoFisher Scientific in 2019 as Field Application Specialist for purification, supporting the technical implementation of POROS and CS chromatography products in south-western Europe.

QUENTIN BAZOT is a molecular virologist. He received his PhD from the École Normale Supérieure (ENS) de Lyon in 2012. After a postdoctoral training at the Imperial College London, he joined the CGT Catapult where he led viral vector process and manufacturing development activities. In 2020 Quentin joined ABL where he is currently leading AAV process development and innovation programs working towards the optimization of AAV production and purification.

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