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Scalable Purification of High Yield Adeno-Associated Virus and Lentivirus Gene Therapy Vectors Using Membrane Chromatography

Contents

1 Summary

This paper focuses on the use of scalable membrane chromatography for the purification of adeno-associated viruses (AAV) and lentiviruses (LV). Single-use membrane chromatography provides scalable and robust purification for large scale production of viral vectors in compliance with GMP requirements. Membrane chromatography single-use ion exchange capsules (Mustang® capsules) can be operated alone or for polishing in flow-through mode at high volumetric flow rates while achieving high clearance of serum proteins, host-cell proteins and host-cell DNA. Additionally, Mustang Q can efficiently separate full capsids from empty or partially filled capsids.

2 Introduction

Since the first authorized gene transfer study at the National Institutes of Health (NIH) in 1989, there is a significant increase of gene-therapy programs. In December 2017, the FDA approved Luxturna♦, for a form of hereditary blindness, this can be considered the first directly administered gene therapy for a genetic disorder available in the US. This approval has stimulated investment in gene therapy, with the Alliance for Regenerative Medicine (ARM)'s Quarterly Regenerative Medicine Global Data Report for the first quarter of 2019 identifying 372 gene therapy clinical trials were in progress. Interestingly, a majority (217 or 58%) were studies in Phase II, followed by Phase I (123 or 33%), and Phase III (32 or 9%). Even if a large majority are still in Phase I and Phase I/II, there has been a significant increase of the proportion of trials entering late phases.[1] Moreover, unlike usual biologics which may take somewhere between 6 to 10 years to go from a Phase I status to commercial launch, gene therapy vectors are moving rapidly in timeframes of approximately 3 to 5 years to commercialization, which creates additional pressure on manufacturers.[1] The large-scale production of gene therapy vectors is still exceedingly expensive and puts a high burden on manufacturers to produce adequate quantities, with a strong pressure to reduce cost of goods and treatment cost in the frame of worldwide competition and reimbursing strategies.

3 Vectors for Gene Therapy

No single vector is likely to suit all gene therapy applications. The detailed strategy of vector choice and construction is beyond the scope of this paper. Instead the focus is placed on adeno-associated virus (AAV) and lentivirus (LV) which are the most commonly used vectors for gene therapy (see Table 1).

3.1 Adeno-Associated Virus as Vector of Choice for Gene Therapy

AAVs are single stranded DNA (ssDNA) viruses which can transduce non-dividing as well as dividing cells, resulting in long-term robustly functioning transgenes. Different AAV vector serotypes can be used to specifically target different tissues, organs, and cells, thus expanding the therapeutic applications and commercial potential of AAV-based gene therapies even though their packaging capacity is limited. Their production is achieved typically in adherent cell culture, however suspension cultures are being considered.

3.2 Lentiviral Vectors

Lentiviruses (LV) are retroviruses and have single-stranded RNA (ssRNA) genomes of 7-11 kilobases, two of which homodimerize and package in lipid-enveloped viral particle. The spherical particles have a genome size about 9 kb and measure ~80-120 nm in diameter. LV vectors have been used successfully in clinical trials, in a first instance for the treatment of rare diseases of primary immunodeficiencies and in neurodegenerative storage diseases.[2,3] LVs are frequently used in gene-modified cell therapies, such as CAR-T therapies, since they can infect many cell types, dividing or non-dividing, easy-to-transfect or hard-to-transfect cells, and have a high affinity for blood and bone marrow cells. There has been a more

recent trend to use LV for the treatment of more frequent genetic and acquired diseases, including treatment of β-thalassemia, Parkinson's disease, and chimeric antigen receptor-based immunotherapy of cancer. LVs can stably integrate into the target cell genome, thus allowing persistent expression of the gene of interest.[4,5] LV particles have traditionally been generated by the co-transfection of three plasmids in human embryonic kidney 293T cells (HEK 293T). Use of optimized lentiviral vectors that incorporate biosafety features is essential to prevent the reacquisition of replication competence.

Table 1. Main types of viral vectors used for gene therapy

	Adeno-Associated Virus (AAV)	Lentivirus
Genome	ssDNA	ssRNA (+)
Coat	Naked	Enveloped
Genome size	5 kb	9 kb
Infection/tropism	Dividing and non-dividing cells	Dividing and non-dividing cells
Host genome interaction	Non-integrating	Integrating
Transgene expression	Potential long lasting	Long lasting
Packaging capacity	4.5 kb	6 kb
Immune response	Very low	Low

4 Challenges Associated with Downstream Processing During Large Scale Gene Therapy Viral Vector Manufacturing

Current viral vector manufacturing techniques are often characterized by low yields and inefficient processes. Downstream processing (DSP) challenges are such that losses of greater than 60% and sometimes up to 90% of the product can be observed. This leads to an increased cost of goods per dose, and more importantly, an inability to produce enough virus to treat the patients that need it. In contrast to mAb production methods, platform purification processes that suit different viral vectors are not yet available. Chromatography is key to the purification of most biologics, however current chromatographic techniques for gene therapy vectors, specifically those based on affinity, are very expensive at large scale and often require harsh conditions that reduce the infectivity of viruses. Often, poor virus recovery is achieved because the sequential unit operations used for DSP are suboptimal, and this is in part because equipment was originally designed for monoclonal antibody purification. All of these factors culminate in inefficient processes that can significantly elevate costs.

In parallel with chromatography, the development of rapid analytics that can measure viral vector quality during the downstream processing steps are critical to support process decision making and optimization, as well as for final product quality control. Traditional cell-based assays are not rapid enough to perform in process testing so standard protein and DNA-based methods are employed while novel methods such as next generation sequencing (NGS), digital drop polymerase chain reaction (ddPCR), nested PCR and others are currently in being explored. However, longstanding protocols that have been widely used continue to be the preferred methodology to monitor viral critical quality attributes.

Empty, broken or partially filled capsids, a result of incomplete viral assembly during viral synthesis, may need to be removed as they contribute to the immunogenicity of the product without providing any therapeutic effect. Their removal can be particularly challenging because the surfaces of these particles are structurally similar to full capsids. At laboratory scale, this is typically done using cesium chloride gradient ultra-centrifugation, but this technique is not practically scalable. Ion exchange chromatography has been successfully used to separate empty and full capsids by taking advantage of their charge differences (*see below Section 5*).

Note: The upstream process, including the engineered vectors, can have a strong impact on the overall percentage of empty capsids generated. Depending on the AAV serotype, it might lead to as little as 1% encapsulated with the right material of interest, compared to 99% empty or partially full capsids or containing other materials. Optimizing the upstream process may help to get a better load of full capsids material which will 'reduce waste' and improve DSP yield.[6]

5 Chromatography Approaches for Viral Vector Purification

5.1 Conventional Packed Bed Chromatography

There is a large choice of packed bed chromatography methods that can potentially be used for viral vectors purification, as summarized in Table 2. However, not all are cost-effective and/or and amenable to large-scale purification.

Table 2. Examples of packed bed chromatography methods used for viral vector purification

Method	Principle	Technology
Ion exchange	Net charge of proteins outside the viral capsid	Anion and cation exchange can be used
Affinity	Specific ligands	Variable domains of camelid heavy-chain-only antibodies (VHH)
Heparin affinity	Heparin ligand	Binds heparan sulfate receptors
Size exclusion	Size	Not scalable
Mixed mode	Charge and hydrophobicity	Dual-mode separation mechanism
Hydrophobic	Hydrophobic interaction	Needs high lyotropic salt concentrations
IMAC	Metal chelate	Not easily scalable

5.2 Membrane Chromatography

Membrane adsorbers like Pall's Mustang Q® membrane capsules offer significant benefits for large-scale purification of viral vectors: the Mustang capsules consist of disposable, ready-to-use, ion exchange chromatography units thus dispensing with the need to pack, clean and validate conventional chromatography columns, and are ideally suited for GMP protocols. In the biopharmaceutical industry, Mustang Q capsules have also been routinely used to remove contaminants in large-scale cGMP mAb purification processes using the 5 L Mustang Q capsule.

Figure 1. Chromatography membrane adsorbers

Pall Mustang Q membrane has become a standard in purification of gene therapy vectors, since they offer a simple scalable purification method and are the ideal complement of single-use bioreactors. With the advances made in instrumentation, single-use systems are highly amenable to automation, which can further reduce timelines and cost and ensure consistent product quality.

5.2.1 Chromatography Strategies Applicable for Production-Scale AAV Purification: Packed Bed Affinity Followed by Ion Exchange Polishing on Membrane Chromatography

Affinity chromatography has become the method of choice for AAV selective capture, and different affinity ligands are available for specific capture of various AAV serotypes. The method is based on the utilization of the variable domains of camelid heavy-chain-only antibodies (VHH) which offer full functionality in antigen-specific recognition and also high-affinity binding. These sorbents offer capacities in the range of $1x10^{13}$ to $1x10^{14}$ genome copies/mL (GC/mL) of resin according to manufacturers. However, even after clarification the affinity purified feed still contain various impurities (AAV, HCPs, genomic DNA, serum protein, helper DNA/virus). These impurities are cytotoxic, reduce transmission efficiency, may elicit inflammatory responses in patients and must be removed. But, the main challenge in purification of such viral vectors is separating AAV from closely related impurities in structure such as empty capsids (Isoelectric point (pI) of empty capsid typically ~ 6.3 vs. pI of full capsid pI_F 5.3). The affinity step alone is not sufficient to accomplish this task, and a polishing step using ion exchange membrane adsorbers has proven to be very effective (i.e. using Mustang Q membrane in flow-through mode). Ion exchange chromatography also has the advantage that it can remove other impurities such as serum, host cell proteins and host cell DNA. The simple elution methods also separate efficiently filled capsids from empty or partially filled ones.

5.2.2 Membrane Chromatography for AAV Purification

The purification conditions for AAV and the binding capacity of the membranes are serotype-specific and depend on their pI. When the solution pH value is above the pI of the AAV, AIEX (Mustang Q membrane) is the good choice. Average AAV capacities of Mustang Q membrane are in the range of $1x10^{12}$ to $1x10^{13}$ GC/mL. On the contrary, when pH value is below the pI_F, rAAV is positively charged, favoring the selection of CIEX (Mustang S). Figure 2 shows the calculated pI values for different AAV serotypes, that could serve as guideline to design the best strategy. In the vast majority of cases Mustang Q anion exchange membranes are used, however the option exists to use Mustang S membrane (for contaminant removal prior to load on a Q membrane). Table 3 gives examples of purification conditions and buffers used for various AAV serotypes, using either anion exchange or cation exchange membrane adsorbers. The optimization of the elution conditions is important: using salt washes of different concentrations, it is possible to separate HCPs, host-cell DNA and other impurities from viral vectors, which initially bind to the Mustang Q membrane. Simple inexpensive buffers like 20 mM Tris HCl, pH 8.5-9.0 supplemented with 300 mM ammonium acetate or 1 M NaCl are used for this elution.

Figure 2. Calculated isoelectric points of various AAV serotypes[7]

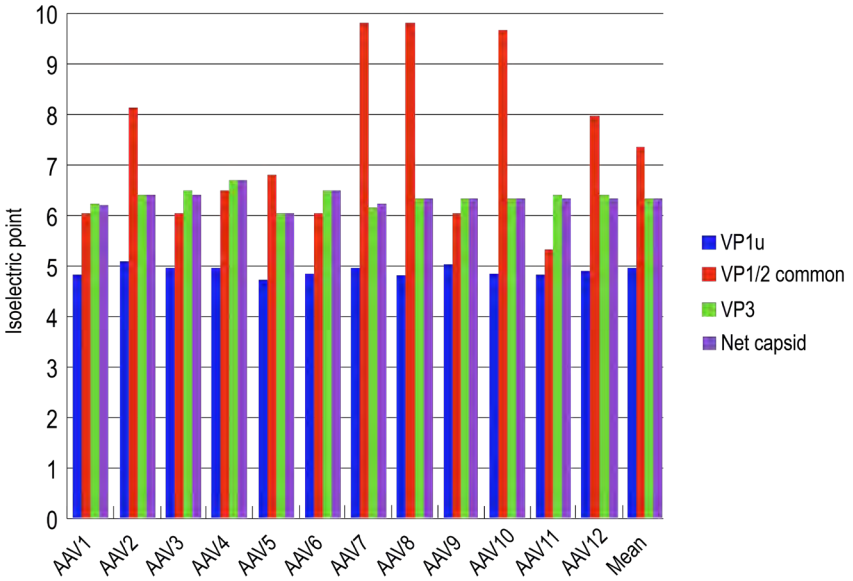

Table 3. Membrane chromatography conditions used for AAV purification

Strategy	Serotype	Purification	Equilibration & Wash Buffer	Elution Buffers
CEX	All	Mustang S	25 mM Citrate, pH 3 - 3.9	50 mM Citrate + 0.5 M NaCl, pH 6.2
CEX in FT mode	1-2-5-6-8	Mustang S	3.3 mM MES, 3.3 mM HEPES, 3.3 mM Na Acetate, pH 6.5	Non applicable since Mustang S is operated in FT for contaminant removal before Mustang Q
AEX	1-2-5-6-8	Mustang Q	3.3 mM MES, 3.3 mM HEPES, 3.3 mM Na Acetate, pH 6.5	3.3 mM MES, 3.3 mM HEPES, 3.3 mM Na Acetate + 1 M NaCl, pH 8.0
AEX	1-2-5-6-8-10	Mustang Q	20 mM Tris-HCl, 100 mM Ammonium Acetate, pH 8.5	20 mM Tris-HCl + 300 mM Ammonium Acetate, pH 8.5
AEX	All	Mustang Q	20 mM Tris-HCl, pH 8.5 - 9.0	20 mM Tris-HCl + 1 M NaCl, pH 9.0

5.3 Membrane Chromatography for Lentivirus Purification

The most significant challenge for upstream processing of LV is low product titer, whereas the main challenge for downstream processing is maintaining infectivity. LVs are more fragile than AAVs and are correspondingly more sensitive to process conditions (pH, ionic strength, temperature and pressure) that may lead to conformational changes and loss of infectivity. Therefore, conditions used for AAV vectors are usually not applicable to LVs. Moreover, LVs also have specific electrostatic charge surface properties that make them bind non-specifically to surfaces, resulting in yield losses of up to 80-90% during final filtration steps. Besides their purification, understanding filtration challenges is also important from the manufacturing standpoint and the final productivity.

Note: A particular advantage that lentivirus may have over AAV is that LV is a secreted virus as opposed to most serotypes of AAV which are intracellular and therefore require a lysis step. Therefore, LVs can then actually lend themselves quite nicely to continuous processing.

As shown in Table 4, membrane chromatography (Mustang Q membrane) has been successfully used to purify LV. Binding capacity for Mustang Q membrane was $5x10^9$ to $5x10^{10}$ TU/mL. The virus capture is achieved at pH 8.0, while elution is achieved by a linear salt gradient (elution occurs at around 1 M NaCl in most cases); however, LV are sensitive to salt, and a quick dilution/diafiltration should be performed immediately after elution. Also, great care should be taken during tangential flow filtration (TFF) since LVs are sensitive to shear stress.

Note: Expressing LV titers as transduction units per mL (TU/mL), one TU producing one integration event in target cells, represent a better measure of virus infectivity (compared to conventional physical titers or PFU [particle forming units] which only measure viral particles formed in a specific volume, not functional particles).

Table 4. Purification of lentivirus by membrane chromatography on Mustang Q membrane[8]

Technology	Mode	Equilibration	Wash	Elute
Mustang Q membrane	Step	25 mM Tris, 0.2 M NaCl, pH 8.0	25 mM Tris, 0.3 M NaCl, pH 8.0	25 mM Tris, 0.3 M-1.5 M NaCl, pH 8.0

6 Discussion and Conclusions

Gene therapies have the potential to revolutionize medicine because of their ability to cure diseases rather than just treat symptoms. However, significant challenges still remain in manufacturing these drugs at an affordable cost so that they can be accessible to all patients that need them.

Downstream processing for purification is a key unit operation of the manufacturing process. Membrane ion-exchange chromatography offers significant benefits compared to packed bed: the method uses single-use devices that are scalable and easy to use, achieving high viral vector purity and yields. They can be used for various vector types capture but also as efficient polishing devices, for example after affinity chromatography to remove trace contaminants as required by regulatory authorities. This easy scalable approach using single-use chromatography membranes allows an optimal manufacturing process that enables more efficient and cost-effective production and ultimately will ensure that patients have access to the therapy.

7 References

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