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The downstream bioprocess toolbox for therapeutic viral vectors

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ABSTRACT

Viral vectors are poised to acquire a prominent position in modern medicine and biotechnology owing to their role as delivery agents for gene therapies, oncolytic agents, vaccine platforms, and a gateway to engineer cell therapies as well as plants and animals for sustainable agriculture. The success of viral vectors will critically depend on the availability of flexible and affordable biomanufacturing strategies that can meet the growing demand by clinics and biotech companies worldwide. In this context, a key role will be played by downstream process technology: while initially adapted from protein purification media, the purification toolbox for viral vectors is currently undergoing a rapid expansion to fit the unique biomolecular characteristics of these products. Innovation efforts are articulated on two fronts, namely (i) the discovery of affinity ligands that target adeno-associated virus, lentivirus, adenovirus, etc.; (ii) the development of adsorbents with innovative morphologies, such as membranes and 3D printed monoliths, that fit the size of viral vectors. Complementing these efforts are the design of novel process layouts that capitalize on novel ligands and adsorbents to ensure high yield and purity of the product while safeguarding its therapeutic efficacy and safety; and a growing panel of analytical methods that monitor the complex array of critical quality attributes of viral vectors and correlate them to the purification strategies. To help explore this complex and evolving environment, this study presents a comprehensive overview of the downstream bioprocess toolbox for viral vectors established in the last decade, and discusses present efforts and future directions contributing to the success of this promising class of biological medicines.

1. Introduction

Viral vectors (VVs) are poised to become fundamental tools in modern medicine and biotechnology owing to their role as delivery agents for gene therapies targeting rare diseases [1], oncolytic agents to fight aggressive forms of cancer [2], vaccine platforms to counter infectious diseases [3], and a gateway to engineer cell therapies [4] as well as plants and animals for a sustainable agriculture [5]. New VV designs are constantly being introduced with improved tissue targeting and gene delivery, as well as low genotoxicity, hepatotoxicity, and immunogenicity [6]. While necessary for their success as next-generation therapeutics, the native complexity and constant upgrading of the viral capsid and transgene design pose arduous challenges to biomanufacturing: *(i)* the biomolecular landscape of VV capsids is inherently diverse – to date, 12 serotypes and over 100 variants of the adeno-associated virus (AAV) and >60 serotypes of adenovirus (AdV) have been isolated in human/nonhuman primate tissues; *(ii)* recombinant capsids selected via library screening for improved therapeutic efficacy and safety can present significant differences – in terms of composition, ratio, and arrangement of virion proteins – compared to the native serotype; and *(iii)* the expression of VVs – whether native or recombinant – by engineered cells is a highly defective process that returns a variety of product-related impurities including partial capsids and capsid fragments, capsid-bound DNA, and Rep-associated capsids with poor or no transduction activity.

The effects of these complexity are well represented in the current landscape of commercial media for VV purification. The first-generation purification toolbox was built by transferring filtration and chromatographic modules originally developed for proteins – with little to no adaptation – to viral vectors [7]. The remarkable differences in biomolecular features between proteins and viruses, however, highlighted the need for a portfolio of purification technologies dedicated to the new

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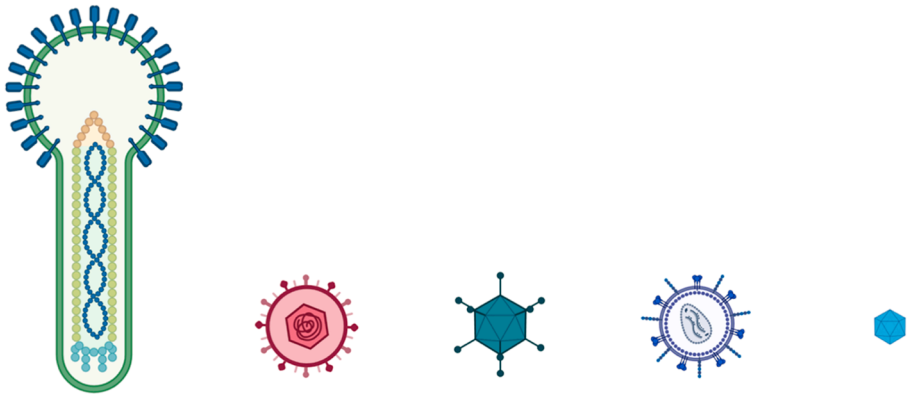
family of products and spurred significant research efforts in both academia and industry. Current innovation efforts in this field are focusing on three main fronts: *(i)* affinity ligands targeting different viral vector families – i.e., AAVs, AdVs, and lentivirus – are being developed for use at the product capture step, the linchpin of any platform downstream process [8,9]; *(ii)* filters and adsorbents whose innovative morphologies – such as nonwoven membranes and 3D-printed monoliths – and flow properties are tailored to the size and biophysical features of viral particles [10]; and *(iii)* bioprocess methodologies and layouts that are flexible and robust, and provide, together with high product yield and purity, the enrichment of intact capsids that carry the gene of interest and possess high transduction activity [11]. Lately, the emergence of new vector families (e.g., herpes virus (HSV), baculovirus, rabies virus), the growth of variants of established families (e.g., the natural and recombinant AAV serotypes), and the introduction of non-mammalian expression host systems have led to formulating the concept of product-agnostic unit operations and processes [12–15]. In this context, particular attention has been devoted to flow-through processes – where impurities are captured and the product flows through – whose smaller footprint, lower complexity, and flexibility enable production across a scale ranging from patient-specific gene therapies to vaccines against pandemics.

The complex and rapidly evolving landscape of VV manufacturing can be difficult to navigate – even to subject matter experts – and a summary of the state-of-the-art technology for VV purification is much needed. To this end, this study presents a comprehensive overview of the downstream bioprocess toolbox for VVs, encompassing harvesting and clarification, chromatographic (affinity, ion-exchange, mixed-mode, etc.) and non-chromatographic (centrifugation, two-phase extraction) purification techniques, and final formulation and filling. This review also discusses the current efforts aimed at establishing the layout of platform processes for VV purification, with a special focus on technologies that are conducive to continuous manufacturing, and suggests future directions that biomanufacturing may undertake to ensure the success of this promising class of biological medicines.

2. The landscape of viral vectors

Viral vectors are employed on several fronts, including *(i) in vivo* gene therapy targeting rare acquired or hereditary disorders [16,17], *(ii)* engineering autologous/allogeneic cells for *ex vivo* cell therapies [18], *(iii)* recombinant vector vaccines (platform-based vaccines) [3], and *(iv)* engineering plants and the gut microbiome of animals [5] (Fig. 1). In the field of gene therapy, adeno-associated viruses (AAVs) are the vector of choice owing to their tissue tropism, low immunogenicity and genotoxicity, and efficient transduction with sustained gene expression [6]. To date, ten VV-based gene therapies have been approved for genetic diseases, eight of which utilize AAV (i.e., Glybera, Luxturna, Zolgensma, Upstaza, Roctavian, Hemgenix, Elevidys, and Elaparvovec) and two utilize lentivirus (LV) (i.e., Zynteglo and Skysona), while hundreds more are at different stages in the clinical pipeline [19]. The various AAV serotypes show innate tissue targeting activity: brain cells are targeted by AAV1, AAV2, AAV9, and AAV-rh10; lung cells by AAV5 and AAV6; cardiac cells by AAV4 and AAV8; hepatocytes by AAV2, AAV3, AAV8, and AAV-DJ; skeletal cells by AAV1, AAV6, AAV7, and AAV8; muscle cells by AAV2; spleen cells by AAV11 [20,6]. The small size of the AAV capsid (~ 25 nm diameter), however, limits the size of the gene of interest to ~5 kbases and thus the therapeutic applicability of AAV-based therapies [21]. Conversely, LV and AdV feature higher capsid size (80–120 [22] and 70–100 [23] nm) and allow loading of much larger genetic payloads (~9 kb and ~36 kb) [24]. By providing stable transgene expression, LVs are utilized almost ubiquitously in *ex vivo* cell therapy, chiefly to produce chimeric antigen receptor (Car) T cells for cancer treatment [25], pluripotent stem cells for tissue regeneration



	Baculovirus (BV)	HSV	Adenovirus (AdV)	Lentivirus (LV)	Adeno-Associated virus (AAV)
Size (nm)	length ~200 - 450 diameter ~30 - 100	~80 - 100	~70 - 100	~80 - 120	~20-25
Genome	dsDNA	dsDNA	dsDNA	ssRNA	ssDNA
Genome capacity (kB)	~100	~40	~8-38	~9	~4.8
Biosafety Level	BSL1	BSL1	BSL2	BSL2	BSL1
Advantages and Applications	▸ It can be easily modified to accommodate large insertions of foreign DNA. ▸ BacMan is a technique for the expression of proteins inside mammalian cells. ▸ BV produces high levels of properly folded recombinant proteins.	▸ Strong neural tropism ▸ Large transgene capacity	▸ Efficient transduction in most mammalian cell types and tissues ▸ Infects dividing and non-dividing cells	▸ Continuous transgene expression via host cell genome integration ▸ Infect dividing and non-dividing cells regardless of their proliferation status ▸ Low Inflammatory potential	▸ Low Immunogenicity ▸ Safe transgene delivery ▸ Non-pathogenic ▸ Different serotypes have specific tropism
Limitations	Not fully characterized	High cytotoxicity	Transient transgene expression.	Possible oncogenesis	Low transgene capacity

Fig. 1. Size and genomic capacity of common viral vectors along with their chief applications and limitations.

[26], or hematopoietic stem cells for hematopoietic reconstitution [27]. Their application *in vivo* has focused on cell types within the central nervous system (i.e., neurons, astrocytes, adult neuronal stem cells, oligodendrocytes, and glial cells), but has been limited by concerns of genotoxicity [28]. Adenoviruses have risen to prominence as oncolytic agents and platform vaccines owing to their innate immunostimulatory behavior and adjustable replication activity [29]. Oncolytic adenoviruses (OAds), engineered via capsid modifications, insertion of tumor-specific promoters, and addition of immunostimulatory transgenes, display excellent tumor cell targeting and cytolytic activity [30]; when loaded with tumor-associated antigen transgenes, OAds can act as cancer vaccines, providing tumor-specific immunity. AdVs are known to trigger strong antiviral immune responses: while this has curbed their application in anti-cancer therapy [31], it has also highlighted their potential as a vaccine delivery platform, with recent applications targeting Ebola and COVID-19 having received significant press coverage [32]. Non-replicating vectors can be easily engineered with features that ensure compatibility with clinical manufacturing and thermal stability, and elicit robust transgene antigen-specific T cells and humoral immune responses [33]. The fourth VV family of clinical and industrial relevance is baculovirus (BV). Unlike AAVs, LVs, and AdVs, which have been identified in mammalian cells, baculovirus is an insect-derived vector [34]. As such, it has been applied as an environmentally friendly insecticides in agriculture, horticulture, and forestry [35]. Being also capable of *in vitro* and *ex vivo* gene delivery into a wide variety of vertebrate cells, BV has been broadly applied in stem cell manipulation and engineering of human tissue models [36], engineering of transgenic plants and animals [37], vaccination and oncolytic applications [38,39], and – likely the main application to date – large scale manufacturing of AAVs [40]. At the same time, the transient gene expression and complement activation limit gene expression *in vivo*; while the fragility of the capsid is a concern in large scale applications [41]. Additional vectors of growing interest in cell and gene therapy as well as vaccination include alphaviruses, flaviviruses, herpes simplex viruses, measles viruses, Newcastle disease virus, poxviruses, pseudorabies virus, rabies virus, retroviruses, rhabdoviruses, and vesicular stomatitis virus. While promising, these VVs are not yet the focus of biomanufacturing-aimed technologies and their purification is still carried out empirically using a combination of orthogonal filtration and chromatographic devices. Accordingly, this review focuses on the process technologies and operation modalities dedicated to the AAVs, LVs, AdVs, and BVs, owing to their immediate relevance in the current clinical and biotechnology scenario.

3. Harvest and clarification of cell culture fluids containing viral vectors

The downstream processing of viral vectors starts with the harvest and clarification of the cell culture fluid in order to collect the viral particles and remove large impurities like cells and cellular debris [42]. The harvest method depends on whether the viral particles are expressed intracellularly or secreted and whether the system is operated in batch or continuous mode (e.g., perfusion systems [43]). Fluids containing secreted viruses – such as the Human Embryonic Kidney 293 (HEK293) cell culture supernatants containing AAV9 and LV – can be sent directly to the clarification unit, since no cell lysis is required. For viruses that require the collection of intracellular material, like most AAVs and AdVs, the harvesting step involves cell lysis [44]. Cell lysis can be conducted using chemical lysing agents (e.g., Tween 20 and Triton X-100 [45]; *note:* Triton X-100 has been placed on the European REACH banned substances list), freeze-thaw cycles [46], sonication [47], and mechanical homogenization [42]. Currently, chemical lysis is the most common method for large-scale operations [48]. A DNase treatment using commercial products Benzonase® (nuclease), Denarase® (endonuclease), or Turbonuclease™ typically ensues to digest host cell and plasmid DNAs (hcDNA and pDNA), whether free or capsid-associated, that can affect the performance of subsequent chromatographic steps as well as product efficacy and safety [49,50]. DNases, however, are typically rather expensive and add significant costs to manufacturing. Therefore, significant in this context is the recent introduction of producer or helper cells that secrete the nuclease during VV expression, such as the SecNuc™ system [51]: this approach, applicable across different vector platforms, bypasses the DNase addition, thereby lowering manufacturing costs and streamlining operations.

Following harvest, clarification is accomplished primarily by membrane filtration and centrifugation in order to reduce the bioburden presented to subsequent downstream unit operations [52,53]. Besnard et al. (2016) presented a comprehensive review on the clarification of vaccines, which represents a good resource for those preparing a viral vector clarification strategy [54]. Bench-scale methods typically rely on centrifugation followed by 0.45 μm microfiltration. For larger scales (> 1 L), the current filtration method involves either normal flow filtration (NFF), articulated in depth filtration (DF) followed by surface filtration (SF), where the liquid flow direction is perpendicular to the filtration membrane, or tangential filtration flow (TFF), where the flow is parallel to the membrane [55]. A recent advancement combines the two into tangential flow depth filtration (TFDF) [56]. A summary of selected filtration units covering a wide range of targets, filtration modes, and filter materials may be found in Table 1.

These methods are vector-agnostic, allowing for adaptability to new targets such as Borna disease virus [65]. Nikolay et al. presented a systematic method for selecting TFF membrane modules and operating conditions, utilizing a perfusion system producing yellow fever virus (~50 nm) as a case study [66]; by providing multiple criteria beyond the mere pore size for selecting TFF membranes, their approach bears general applicability and can be implemented to guide the adoption of clarification systems for other VVs.

Generally, these methods also allow for early concentration of the capsids and adjusting buffer composition to promote the efficiency of the subsequent chromatographic steps. Ma et al. demonstrated that modulating buffer conductivity via TFF greatly enabled virus purification via a single step of anion exchange chromatography [67]. Key concerns for both methods include product loss due to filter clogging and adsorption of the viral particles, along with insufficient consideration of membrane size [61,68,69], which may fail to clear particulate impurities and reduce yield. To circumvent these issues, Labisch et al. employed diatomaceous earth as a novel filter aid to improve LV clarification, achieving faster processing, improved impurity removal, and reduced filter consumption in an easily scalable format [70]. However, as the diatomaceous earth concentration in the filter increased, the LV titer decreased, indicating that process economics would dictate the appropriate trade-off.

4. Chromatographic purification

Following treatment with DNase and filtration, the clarified cell culture harvest contains, together with the target VV product, a wide variety of process- and product-related impurities [71]. The former include oligonucleotides and host cell proteins (HCPs), several of which possess enzymatic activity that can damage the viral vector capsid or coat (e.g., proteases and lipases), or immunogenic potential that poses a threat to the patient's safety (e.g., Vero cells and non-mammalian expression systems such as Sf9 insect cells) [72]. The latter include capsid fragments, full capsids that do not contain the gene of interest (GOI), or full capsids that contain the gene of interest but whose transduction activity is compromised (e.g., by denaturation or incorrect post-translational modification of the capsid/coat proteins) [71]. Successful clearance of these impurities – necessary to ensure the efficacy and safety of the VVs administered to patients – relies on the chromatographic train. This consists of series of adsorbents, each featuring an orthogonal biorecognition method and thus providing a unique contribution to clearing a subset of impurities, ultimately affording a

Table 1
Filtration techniques and products for the clarification of viral vectors.

Filtration Mode	Filter Manufacturer	Filter Model	Filter Material	Host Cell Line	Purification Target	Recovery	Virus Size (nm)	Refs.
NFF	EDM Millipore	MilliStack D0HC®	Cellulose	HEK-293SF	Influenza	~35–79%	80 - 120	[57]
NFF and TFDF	1) Cytiva followed by Sartorius Stedium Biotech 2) Repligen followed by Merck Millipore	1) 5.0 μm ULTA GF filter followed by 0.8/0.2 μm Sartopore 2 XLG 2) 5.0 μm KrosFlo TFDF followed by Millistak X0SP	1) Glass microfiber for the Cytiva product and polyethersulfone for the Sartorius product 2) Polypropylene with polyethylene terephalate for the Repligen product and silica filter aid with polyacrylic fiber for the Merck Millipore product	HEK-293	AAV	1) 72% global yield 2) 90% global yield	20 - 25	[58]
TFDF	Repligen	Krosflo TFDF	Polypropylene with polyethylene terephalate	Mammalian	Lentivirus and HIV-1 vector	95%	80 - 100	[56]
TFF	Repligen	TangenX	mPES	HEK293	AAV2 & AAV8	~108.2 × 10^3 total yields per cell compared to legacy, $CsCl_2$ gradient purification of 42.2 × 10^3 total yields per cell.	20–25	[59]
TFF	1) Sartorius 2) GE Healthcare	1) 100-kDa MWCO Hydrosart flat sheet membrane held by Sartocon Slice 1000 holder or 2) 300-kDa MWCO GE Healthcare hollow fiber filter (both followed 1.2, 0.8, and 0.45 μm depth and dead-end filtration)	Cellulose	sBHK	AAV	1) Flat sheet: 96±7% 2) Hollow fiber: 59 ±10%	20 - 25	[60]
TFF and HPTFF	Sartorius	Sartocon® Slice 200 cassettes of 30, 50, 100, and 300 kDa MWCO.	Cellulose	Aedes Albopictus	AeDNV	1) TFF: 3 30 kDa: 0% 50 kDa: 0% 100 kDa: 0% ~5 × 10^5 virus e/mL 2) HPTFF 30 kDa: 0% 50 kDa: 0% 100 kDa: ~1 × 10^6 virus eq./mL 300 kDa: ~5 × 10^7 virus eq./mL	20	[61]
TFF	Pall	Omega™ Membrane Cassette	PES	HEK293T	Lentivirus	100% with both 100 kDa and 300 kDa membranes	80 - 100	[55]
NFF and TFF	EMD Millipore	1) Millistak+® HC Pro-depth filters with C0SP media filters followed by 2) Pellicon 2 Mini with 300 kDa BioMax PES membrane	1) Polyacrylic fiber and silica 2) PES/mPES	HEK293T adapted to suspension	Adenovirus	1) > 90% 2) ~80%	90 - 100	[62]
NFF	EDM Millipore	CE25 followed by DE45	Cellulose/Cellulose	PER.C6	Adenovirus	70%	90 - 100	[63]
NFF and TFF	Sartorius	1) 3 μm and 0.65 μm Sartopure PP2 filter capsules followed by 2) 100 kDa MWCO Hydrosart membranes	1) Polypropylene 2) Cellulose	Sf9	Baculovirus	1) 95 ± 5% 2) 70 ± 5%	150 - 200	[64]

pure product. Fig. 2 presents the configuration of chromatographic trains that has become prevalent in platform processes for VV purification: the various modalities and substrates utilized in the listed unit operations are presented in the following sections. Finally, viral vectors, with their extraordinary costs and fragility, may benefit from renewed efforts into continuous chromatography for process intensification and negative mode, flow-through purification.

4.1. *Affinity chromatography*

Chromatography represents the key segment of every scalable viral vector purification process [10]. Within the chromatographic train, a critical role is played by the first step – namely, product capture – which relies on either affinity or ion exchange modalities to concentrate the product and remove the bulk of process-related impurities from the clarified harvest. Affinity adsorbents leverage the biorecognition

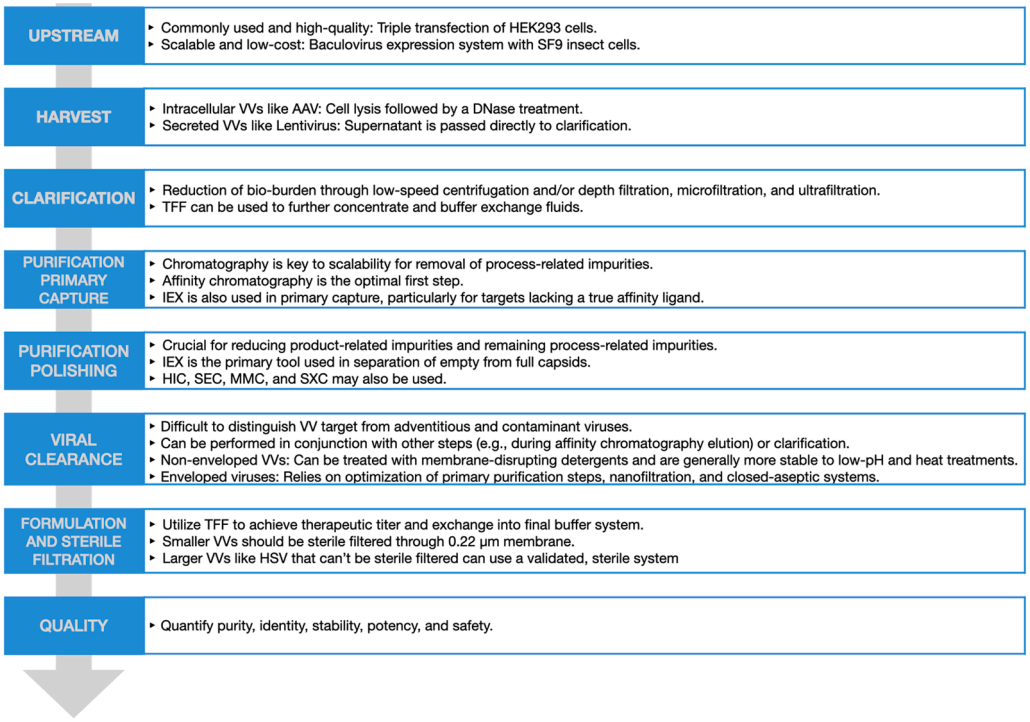


Fig. 2. Process flow diagram highlighting the main unit operations in the downstream purification or viral vectors.

activity of biomolecular ligands towards the epitopes displayed on the virion capsid or coat to achieve selective capture of the target VV, while letting impurities flow-through. Following adsorption and wash, the bound VVs are eluted in a highly concentrated and purified form, ready for the subsequent polishing steps. Accordingly, affinity chromatography operated in bind-and-elute mode is key to achieve high product yield and purity, reduced batch-to-batch variability, and process scalability, and is therefore the method of choice for product capture [73].

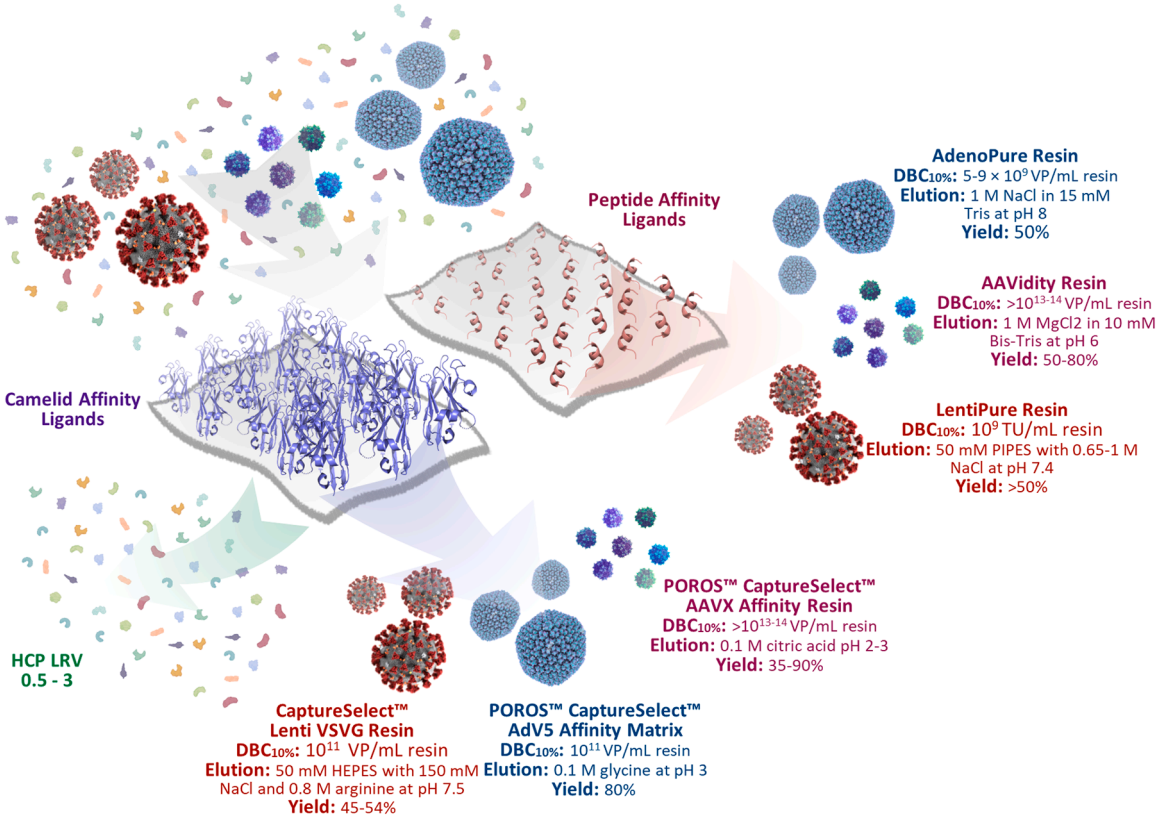


Fig. 3. A depiction of two different classes of affinity ligands, camelid-derived and peptide-based, for the purification of viral vectors and their associated performance in terms of $DBC_{10\%}$, elution conditions, and yield.

Fig. 3 offers an overview of affinity purification, showcasing both camelid-derived and peptide-derived ligands, complemented by relevant details such as elution conditions, $DCB_{10\%}$ values, and yields. While capable of clearing most process-related impurities, however, current affinity ligands do not afford any enrichment of GOI-loaded vectors by excluding product-related impurities. Furthermore, as complex biomolecules, they feature significantly higher cost and lower stability compared to synthetic ligands (e.g., ion-exchange or mixed-mode), which introduces the risk of immunogenic fragments being released in the product stream and limits their lifetime to 15–20 cycles. Two options for addressing some of these challenges could be continuous or semi-continuous manufacturing and/or flow-through operations. Mendes et al. (2022) utilized periodic-counter-current (PCC) affinity chromatography to achieve a 3-fold productivity improvement and >82% recovery with Capto AVB [74]. Sripada et al. (2022) demonstrated flow-through affinity chromatography with peptides for HCP capture that allow mAb targets to flow-through [75]. This method, if applied to viral vectors, could facilitate continuous manufacturing and has the added benefit of not subjecting the target to potentially denaturing conditions.

The initial attempts at the affinity purification of AAVs relied on immobilized metal affinity chromatography (IMAC). While providing yields from HEK293 above 90% for AAV2and AAV8 qualitatively high purities based on SDS-PAGE, IMAC requires engineering an oligo-histidine tag (His-tag) on the viral capsid [76], possibly interfering with protein expression, folding, and activity. Furthermore, IMAC purification of recombinant his-tagged products has the additional disadvantage of co-eluting impurities with natural histidine hot-spots [77–80]. Capitalizing on the display of heparin-binding motifs by different viral genera, other studies reported the use of resins functionalized with heparin sulfate to purify AAV [81] and LV [82] from HEK293 lysates and supernatants: while affording 53-to-96.7% vector yield, heparin-based affinity chromatography affords modest purities due to the presence of numerous heparin-binding HCPs [83]. Additional efforts on heparin-based chromatography have accomplished the purification of foamy virus from HEK293 supernatant with one group achieving a step-yield of ~69% with no discussion on host cell protein clearance [84], and another group reporting a 50% infectious yield and 99.9% HCP clearance when used in series with Capto Core 700 [85]; and baculovirus from SF9 supernatant with 54–85% recovery of intact, infectious particles (based on transmission electron microscopy (TEM) and transduction assay) with some reduction in HCPs qualitatively determined by silver stained gels [86,87]. Since AAV5-based vectors do not bind heparin, an alternative mucin-functionalized Sepharose was developed. This method provided high yields of 4×10^7 TU/ml (the feed concentration was undefined) and almost completely removed host cell proteins, as visualized by SDS-PAGE [88].

Affinity tag chromatography is a less common purification scheme. A streptavidin/biotin strategy afforded LV purification from HEK293 supernatants with yields of >60% with LRVs of 2 and 3+ for HCPs and DNA, respectively [89]. While useful in non-clinical studies, affinity tag chromatography presents challenges when considered for clinical or large scale manufacturing, as the regulatory agencies worldwide likely require post-purification cleavage of the tag. Pseudo-affinity ligands, like dextran sulfate (e.g., Capto DeVirS (Cytiva)) [90] and cellulose sulfate (e.g., Cellufine Sulfate (JNC Corporation)), have primarily been used in vaccine production processes or purification of non-therapeutic vectors like West Nile and Dengue viruses [91], though some processes use it as an additional step in a larger chromatographic train as shown in an rAAV purification study which achieved >90% purity and 30% infectious rAAV recovery [92].

Immunoaffinity chromatography (IAC) is currently the prevalent method for VV capture, as the result of several academic and industrial efforts conducted over the past two decades to deliver a portfolio of VV-targeting antibodies and antibody fragments for use as affinity ligands. The first application of IAC for VV purification was demonstrated by Grimm et al., who utilized NHS-activated HiTrap-Sepharose conjugated with anti-AAV2 monoclonal antibody A20 to purify AAV2 from HEK293 lysate. They achieved 65–70% yield measured from replication assays and western blots and 80% purity based on silver staining of polyacrylamide gels [93]. A key breakthrough in IAC technology was achieved with Cytiva's AVB ligand, a single-domain camelid antibody (V_HHs or Nanobodies®) that binds AAV serotypes 1, 2, 3, and 5 [94] by targeting the SPAFKA epitope [95]. Notably, the ligand can target serotypes other than the listed ones, provided that the epitope's display is engineered on the capsid [11].

The AVB technology spurred the development of ligands that were engineered by selecting libraries of single-chain camelid antibody fragments against the various viral genera, resulting in the recent commercialization of a number of affinity adsorbents for VV purification. Together with AVB Sepharose HP and Capto AVB by Cytiva [96], there are now nine adsorbents available for AAV purification, including ThermoFisher's pan-selective POROS™ CaptureSelect™ AAVX [97] as well as the serotype-specific CaptureSelect™ AAV8 (CSAL8) and AAV9 (CSAL9) [98]; the AVIPure-AAV2, -AAV8 and -AAV9 by Repligen [99]; and the ViraBind™ AAV Purification Kit by Cell Biolabs for the purification of AAV2 and AAV-DJ [108]. Together with AVB Sepharose, the POROS™ CaptureSelect™ AAVX affinity resin is regarded as the state-of-the-art adsorbent for AAV capture, featuring a dynamic binding capacity ranging between 10^{13} and 10^{14} viral particles per mL of resin (vp/mL), allowing up to 10 liters of cell culture lysates to be processed with as little as 1 mL of resin; product yield of 35-to-90%; and logarithmic removal value of HCPs (HCP LRV) of 0.5-to-3 [47,74,100–108]. Most importantly, these resins demonstrate that – as with monoclonal antibodies – a platform purification process is achievable for AAV purification from a variety of recombinant and chimeric serotypes [73,106,107,109,110]. While – to our knowledge – every natural AAV serotype tested has demonstrated affinity towards AAVX, recombinant vectors (rAAVs) of more recent design may only demonstrate weak affinity [103]. The comprehensive mapping of ligands' binding sites on AAV, as completed by Mietzsch et al. offers substantial guidance on selecting commercial resins for the purification of known AAVs and engineering AAV vectors suitable for affinity purification [8,111].

While providing the high binding strength and selectivity needed to isolate AAVs from feedstocks with low product titer and a wide abundance of impurities, all IAC resins mandate the use of strong acid buffers ($pH \leq 3$) to release the bound capsids. Such harsh elution conditions often cause capsid aggregation or denaturation, resulting in a loss of transduction activity [112,113], and may also degrade the antibody-derived ligands, thus reducing the resin's lifetime. These issues prompted a search for ligands with comparable biorecognition and binding capacity, but stronger chemical stability. Pulicherla and Asokan identified the heptapeptide GYVSRHP and evaluated it by purifying AAV8 from HEK293 cell culture supernatant [108,114]: while showing modest binding capacity (~10^{12} vector genomes per mL), their GYVSRHP-agarose resin afforded a yield of 71%, higher than those obtained via IAC, and a purity comparable to that provided by iodixanol or cesium chloride density ultracentrifugation. While harsh elution conditions were still required (0.2 M Glycine-HCl, pH 2.2), this work provided the first demonstration of the use of small peptide ligands for AAV purification.

Recently, our team sought to develop peptide affinity ligands (AAVidity resin) that combine high binding capacity and selectivity with mild elution conditions (1 M $MgCl_2$ in 10 mM Bis-Tris buffer at pH 6.0) [108,115]. The first group of peptides were identified by screening a focused solid-phase library using a selection device that returns ligands with bespoke binding strength [116–120]. The lead sequences were evaluated *in silico* to select new candidate ligands that target homologous sites at the interface of the VP1-VP2 and VP2-VP3 virion proteins with mild binding strength ($K_D \sim 10^{-5}$–10^{-6} M). When conjugated to Toyopearl resin, the ligands demonstrated the ability to target AAV2 and AAV9 with values of dynamic binding capacity > 10^{13} vp per mL of resin

and product yields > 50%. The peptide-based adsorbents were then utilized to purify AAV2 from a HEK293 cell lysate, affording product recovery up to 80%, 80-to-400-fold reduction of HCPs, and high transduction activity (up to 80%) of the purified viruses [108]. In a subsequent study, our team introduced a cohort of cyclic peptide mimetics of the AAV receptor (AAVR) and anti-AAV2 antibody A20: the peptides were designed *in silico* to be pan-selective, enable product elution at pH 6.5, and grant extended reusability [115]. Peptide CVIDGSQSTDDDKIC demonstrated excellent capture of serotypes belonging to distinct clades – AAV2, AAV5, AAV6, and AAV9; when conjugated on Toyopearl resin, it features binding capacity ~ 10^{14} vp per mL and product yields ~60–80%. This peptide successfully purified AAV2 from a HEK293 cell lysate affording 80% yield, a remarkable 980-fold reduction of HCPs, and transduction activity up to 80%.

In the field of LV purification, most of the affinity chromatography studies published to date leveraged the biotin-streptavidin interaction, IMAC, or heparin and only recently have groups begun to develop LV-specific ligands [121]. The biotin-streptavidin mechanism requires the expression of a cyclic biotin-mimicking peptide cTag8 on the virion coat to capture LVs on a streptavidin-functionalized resin [89]. Accordingly, biotin is introduced to elute the bound LVs from magnetic beads, affording step yields ~60% active virus and 2- and 3-log reductions of HCPs and dsDNA, respectively [122]. However, recovery drops to 20% when the same strategy is tested on a streptavidin based monolith column. Similarly, IMAC requires engineering the LV coat to display His-tags, and when applied on monoliths functionalized with iminodiacetic acid and nickel affords a binding capacity of 6.7 × 10^8 TU/ml of adsorbent, and 69% recovery [123]. The authors do not report values of HCP or DNA removals nor reusability studies of the ligands. These methods may not be translatable to clinical production, since both the integration of the cTag8 tag and the use of imidazole for LV elution from the IMAC column reduce the infectivity of the LV particles. Heparin-functionalized resins overcame these issues as a cost-effective tool that provides good yield (51–61%), purity (HCP LRV ~ 0.5–1.5), and binding capacity ~1.3 * 10^{10} TP/mL while safeguarding the infectivity of purified LV particles [78,124–126]. Recent studies by Rayat and Peixoto offer a comprehensive review of upstream, downstream, and analytical processes for LV production [22,127]. Immunoaffinity resins have been recently introduced by Peixoto's team for LV purification. The team developed a V_HH-based ligand targeting the Vesicular stomatitis virus G (VSV-G) protein displayed on the LV envelope and conjugated them to agarose beads [9,128,129]. Dynamic binding capacity at 10% breakthrough ($DBC_{10\%}$) studies were conducted using LV clarified harvest at 4.0 × 10^9 TP/mL and a 2 min residence time resulting in the $DBC_{10\%}$ of 1.0 × 10^{11} TP/mL of resin. Binding and elution experiments were conducted with buffers composed of 50 mM HEPES, 150 mM NaCl, pH 7.5 with addition of 800 mM arginine for elution. Arginine has been studied as an alternative for low pH buffers for elution of antibodies, however, there are studies that show reduction of viral titer at arginine concentrations as low as 350 mM [130,131]. For example, McCue et al. reported a 2 log reduction of retrovirus xenotropic murine leukemia titer when exposed to 500 mM arginine for 15 min [132]. Regarding stability of Capture Select Lenti VSVG Affinity Matrix, the authors do not report reusability of resin and resistance to NaOH. The stripping condition was described as 50 mM sodium phosphate at pH 12, which is not comparable to 0.5–1 M NaOH with a 30 min contact time which is the CIP method recommended by GMPs. A lack of stability under NaOH CIP conditions may lead to low reusability of resin and bio-fouling of beads which would decrease binding capacity.

Inspired by this work, our team sought to develop VSV-G-targeting peptide ligands (LentiPure resin) that provide comparable binding capacity and selectivity, while at the same time featuring milder elution conditions as well as stronger chemical stability and lifetime. Peptides were selected by screening a focused solid-phase library against the ectodomain of VSV-G to possess high binding strength in 50 mM PIPES 100 mM NaCl pH 7.4 and release the bound LVs upon exposure to 50 mM PIPES 0.65–1.0 M NaCl pH 7.4. Selected peptides were conjugated to various chromatographic resins (POROS™ 50 OH Hydroxyl Activated Resin, Toyopearl AF-Amino-650 M, and Eshmuno™ epoxy activated resin). These peptides achieved high binding capacities (0.5–3 × 10^9 transducing units per mL of resin) and purified LVs from a HEK293 cell culture supernatant with a yield of infectious particles > 50% and an HCP LRV of 1.8–2.4 [326]. The eluate from the peptide-functionalized Poros resin was polished using a Capto Core 700 resin, affording a global 56% yield and a 2.9-to-4.0-log reduction of HCPs. Notably, the peptide-Poros resin features strong caustic stability, providing above 50 cycles of use with a CIP consisting of 0.5 M NaOH at a 30 min contact time.

Similarly to LV, baculovirus (BV) accesses cells by targeting surface heparan sulfate proteoglycans (HSPG). Specifically, baculovirus binds CD138 - also known as syndecan-1 (SDC1), which comprises a protein core functionalized with HS chains – whose N- and 6-O-sulfation is critical for the virus to dock onto and transduce mammalian cells [133]. Accordingly, heparin affinity chromatography is commonly used for BV purification from Sf9 cell lysates. Because buffers with high conductivity (1.5 M NaCl) are required for BV elution from HS-functionalized resin – which may cause viral inactivation – an immediate 10-fold dilution of the eluate is required immediately upon collection [86,87]. Concanavalin A (ConA) can also be used as a ligand for BV as it targets the baculoviral envelope glycoprotein gp64, affording yields of ~30% using α-D-methylmannoside as elution agent [134]. Because neither HS nor ConA are selective to BV and can co-elute other glycoproteins present in the cell lysate, Moleirinho et al. sought to develop affinity ligands dedicated to baculovirus by screening a phage display library of V_HH fragments. The identified ligands afforded binding capacities between 4.44 × 10^9 and 1.15 × 10^{11} vg per mL of resin and BV yields of 60–70%. At the same time, the identified ligands showed cross-binding of other viral particles, namely AAV2 and Hepatitis C VLPs, and were not able to clear Sf9 host cell proteins and DNA (40–60% residue) [135]. Accordingly, the commercial format of the resin, POROS CaptureSelect BacuClear Affinity Matrix, is utilized for the removal of baculovirus in flow-through mode from baculovirus-based systems for the expression of recombinant proteins [136].

Finally, the purification of AdV has not benefited from comparable efforts. To date, one commercial adsorbent, POROS™ CaptureSelect™ AdV5 Affinity Matrix [137–139], functionalized with adenovirus-binding camelid V_HH ligand is available, which provides binding capacity ~ 10^{11} viral particles per mL of resin and high product purity. Despite the harsh conditions required for product elution (0.1 M glycine, pH 3.0), the resin undergoes rapid buildup of viral particles and its yield decreases from 80% to 50% in just 3 cycles [140]. Having demonstrated the potential of peptides as alternative to antibody-based ligands, our team developed Adenovirus-binding peptides (AdenoPure resin) and utilized them to purify AdV5 from HEK293 and Vero cell lysates. Adsorbents functionalized with these peptides display binding capacities of 5.0–9.0 × 10^9 vp per mL of resin and afford high yield (up to 50.1%) and purity (1.7-to-2.1-log reduction of HEK293 HCPs and 1.5-to-1.7-log reduction of Vero HCPs) under mild elution conditions (0.15 M Tris–HCl buffer, 1 M sodium chloride pH 8) (unpublished).

4.2. Ion-exchange chromatography

4.2.1. Resin-based supports

Ion-exchange chromatography (IEX) is the main alternative to affinity chromatography for the primary capture step and is the predominant technology used for polishing VVs, thanks to its versatility and applicability to a wide range of biomolecules. This technique capitalizes on the varying net surface charges of biomolecules and offers exceptional scalability. As mentioned in Section 4.1, VVs are more labile than proteins which incentivizes chromatographic methods with gentle elution conditions. IEX typically utilizes a change in salt concentration to elute the target, which, while causing some osmotic stress, is

generally much milder than the harsh, low-pH buffers common in affinity chromatography. The most common stationary phase employed in IEX is resin-based, but materials such as monoliths and membranes, described in Sections 4.2.2 and 4.2.3, are emerging as powerful alternatives. Table 2 provides a brief selection of results from studies utilizing these three supports and covers a broad range of viral vector targets. The technique's effectiveness hinges on the pH of the system relative to the isoelectric point (pI) of the biomolecule and the system's salt concentration. At the pI, there is no net charge, resulting in no interaction with the chromatography media. Biomolecules with a positive charge will be attracted to negatively charged cation exchange media if the pH is below the pI. Conversely if the pH is above the pI, the negatively charged biomolecules will interact with positively charged anion exchange media. This interaction, typically run at 0.5–1.5 pH units above or below the VV pI, can be disrupted by increasing ionic strength (or less commonly adjusting pH) to disrupt the electrostatic interactions and allow the salt ions to outcompete the VV for the functional groups on the ligand.

Anion-exchange chromatography (AEX) is the predominant form of IEX for viral purification and polishing as most VVs have a pI below 7 [165]. Moreover, IEX has fewer concerns regarding the leaching of immunogenic ligands and is lower cost than affinity based-methods. IEX can also be used in the polishing step after an affinity unit operation and is typically performed in bind and elute mode for capture, though it can also operate in flow-through mode. The flexibility of IEX is beneficial in developing continuous or semi-continuous chromatography, which can aid in process intensification. An example of this was developed by Silva et al. in 2020 [166]. There is limited literature on the purification of viral vectors in negative, flow-through mode. This method could be favorable for labile VVs, as no denaturing elution conditions would be required. To this end, Konstantinidis et al. (2023) utilized mixed-mode resin to operate a flow-through chromatographic system for viruses achieving yields ~50% and HCP LRVs 2.2–3 [167]. Additionally, it has been demonstrated that functionalized resins can bind HCPs while allowing target proteins to flow through, enabling continuous operations [75]. While IEX relies heavily on empirical data and heuristics for operation and optimization, efforts are being made to improve predictive modeling for more efficient scale-up and process development [168].

IEX boasts a powerful ability to separate empty from full capsids, which is a significant advantage over affinity chromatography, through the subtle manipulation of pH and salt concentrations in the buffer systems [169,170]. Full AAV capsids contain negatively charged single-stranded DNA (ssDNA), which results in a slightly lower pI of ~5.9 compared to empty capsids with a pI of ~6.3. A differential that IEX can exploit as seen in Fig. 4 [171,172]. This separation is important because full capsids are the carriers of therapeutic genetic material in gene therapy applications and higher full:empty ratios can increase the efficacy of the treatment. Conversely, empty capsids do not carry the therapeutic genetic payload, but do contribute to adverse immune responses. The separation is also critical for meeting regulatory recommendations, as evidenced by the FDA's Cellular, Tissue, and Gene Therapies Advisory Committee (GTGTAC) meeting on September 2nd-3rd, 2021, which emphasized the risks and toxicities of AAV vectors and the importance of characterizing and purifying empty, partially full, and full capsids [173]. This heightened scrutiny stems from the unexpectedly high levels of toxicity induced by gene therapy products, with empty and partially full capsids suspected as contributing factors [174]. Numerous studies in this review discuss the elimination of empty and partially full capsids and the optimization of purification schemes for this purpose. [11,47,101,169,175–177]

The purification step is often the bottleneck in the production of VV doses for clinical trials, necessitating extensive re-engineering of serotype-specific processes to meet stringent quality requirements. As the POROS CaptureSelect AAVX ligand was instrumental in enabling affinity platform purification, extensive research has been conducted to identify materials and develop methods for IEX platform purification [47,103,175,178–180]. Heldt (2019) demonstrated the feasibility of a serotype-independent platform approach to AAV purification using low pH and triton treatment during harvest, followed by cation-exchange chromatography (CEX) [141]. The POROS CEX optimized methods afforded yields of 21–61% with HCP and HC-DNA removal of 21–47% and 52–99%, respectively. AVB Sepharose provided comparatively lower yields of 13–24%, though higher HC-DNA removal of 93% and HCP removal of 100%. Despite lower HCP removal, the CEX method has broader applicability (e.g., AVB Sepharose cannot purify AAV8 or AAV9) and a 50-fold reduced cost compared to affinity chromatography. Nagase et al. (2022) presented a novel adsorbent – thermo-responsive mixed polymer brushes on silica resin – that allows for temperature-modulated elution of AAV [181]. This innovative approach showed yields of 81.4% under pure conditions with a temperature shift from 40 °C to 5 °C in PBS and infectious particles at high purities (based on SDS-PAGE gel) under competitive conditions with a BSA-spiked model fluid. This work addresses the challenge of optimizing elution conditions, which often prove excessively harsh for obtaining high infectious yields of the product.

The purification of the non-enveloped 70–100 nm AdV poses additional challenges, as it may prove too large for traditional AEX resins with small pores (~30–100 nm) [182]. Resin-based tentacle supports, such as Fractogel TMAE, Fractogel DEAE, and Fractogel DMAE, were designed to mitigate steric hindrances associated with conventional solid phases. Fractogel TMAE exhibited effective purification capabilities for AdV from HEK293 fluid, delivering high-resolution elution peaks, 91% purity, and 75% yield [142]. Furthermore it provided an acceptable virus particle-to-infectious virus ratio of 18.2 vp/IU, adhering to the FDA guidelines of <30 vp/IU. A notable example of a scalable AdV5 production process was reported by GE Healthcare in 2020, which employed Capto Q ImpRes resin for capture and Capto Core 700 for polishing [143]. This innovative approach demonstrated effective removal of HCP and gDNA below the LOD, with total protein of 10 μg/dose and a 3.9×10^{10} infectious virus titer representing a 43% recovery. In comparison, the reference process of capture with Q Sepharose XL followed by polishing with Sepharose 4 Fast Flow allowed higher levels of HCP (22 ng/mL), total protein (20 μg/dose total protein), and gDNA (LOD-3 ng/dose), with a slightly higher 4.4×10^{10} infectious virus titer (45% recovery). In addition to novel adsorbents, innovative methods have been developed such as periodic countercurrent chromatography (PCC) by Cytiva for continuous chromatography. PCC enables continuous movement of the mass-transfer zones in a ring of columns, facilitating binding closer to static binding capacity than dynamic binding capacity. Capto Q ImpRes IEX resin used in a PCC system provided impurity reductions for a OAd of over 80% and 70% for DNA and total protein, respectively, with recoveries ranging from 57% to 86% [144]. Another front in downstream advancements actually involves upstream process development where optimization can lead to improved IEX efficiency. Ruscic et al. (2016) deleted two negatively charged amino acids from an AdV which reduced AEX column retention time and can be used to modulate where the VV will elute compared to problematic HCPs [183]. The concept is comparable to scientists engineering the SPAFKPA epitope, mentioned in Section 4.1, into AAVs that do not already possess the epitope, thus allowing for off-the-shelf purification by AVB Sepharose

Much of the prior discussion has centered on AAV and AdV, however, LV vectors also play a crucial role in the VV arsenal, with numerous recent purification protocols developed [145,146,184,185]. LVs are particularly difficult targets, as they are extremely labile and known to lose infectivity under even mild conditions throughout downstream processing. Olgun et al. (2018) presented a complete, thorough method for LV purification published as part of the book Skin Stem Cells [145]. The method uses prefiltration to remove debris, ultracentrifugation, benzonase treatment, AEC with a HiTrap Q HP column, SEC with HiTrap desalting column, and finally a HiTrap Capto Core 700 polishing

Table 2
Comparative analysis of IEX methods for viral vector purification.

Stationary Phase	Purification Target	IEX product	Recovery	HCP Removal	DNA removal	Empty vs full metrics	Host Cell Line	Refs.
Resin	AAV2, AAV8, and AAV9	POROS HS-50	21–112%	21–70%	50–99%	–	Expi293, suspension-adapted HEK293, adherent HEK293	[142]
Resin	AdV5	Fractogel TMAE, DMAE, DEAE, or Q Sepharose XL	33–75%	74–91% purity (HPLC)	–	1.14 - 1.25 OD260/OD280	HEK293	[143]
Resin	AdV5	Capto Q ImpRes + Capto Core 700	43% IVP	<LOD	<LOD	–	HEK-293.2sus	[144]
Resin	AdV5	Q Sepharose XL + Sepharose 4 FF	45% IVP	22 ng/mL final	<LOD	–	HEK-293.2sus	[144]
Resin	OAd	Capto Q ImpRes	57–86%	70%	80%	–	A549	[145]
Resin	LV	HiTrap Q HP, HiTrap desalting, and HiTrap Capto Core 700	53%	90%	–	–	HEK293T	[146]
Resin	LV	DEAE-650C and Sepharose 6FF	20–40%	>99%	>99%	–	HEK293T	[147]
Resin	LV	Capto Q ImpRes, Macro prep high Q, Amino Sepharose 6 FF, or POROS 50D	~100%, 82%, 70–75%, or 85–90% respectively.	LRVs of 1.89, 2.51, 2.89, or 2.1, respectively	–	–	HEK293	[148]
Resin	Oncolytic measles virus	Eshmuno CPX	80.7%	80.5%	98.3%	–	Adherent Vero	[149]
Monolith	rAdV5	CIMmultus QA-1	34%	70% overall purity	–	–	HEK293F	[150]
Monolith	AAV8, AAV9	CIMmultus SO3 and CIMmultus PrimaT	100%	–	–	100% full	HEK293	[151]
Monolith	AAV5, AAV8, AAV6, AAV6 and AAV9	CIMmultus QA	72.7%, 81.7%, 79.9%, 83%, and 84.7%	–	–	1:4, 1:31, 1:18, 1:21, and 1:18 relative ratio of empty capsids to vector capsids	Sf9 and HEK293SF	[152]
Monolith	rAAV2	CIMmultus QA	>70%	–	–	>90% full	BHK	[153]
Monolith	Baculovirus	CIMmultus QA	20–99%	92–99%	52–62%	–	Sf9	[154]
Monolith	Measles Virus and Mumps Virus	CIMmultus QA or CIMmultus OH	1–27% and ~60%	11–77%	<LOD	–	Vero	[155]
Monolith	LV and OAd	DEAE and hydroxyapatite functionalized cellulose columns	OAd: 69% and 64% LV: 57%	~94%	55%	–	A549 cells for OAd and HEK293T cells for LV	[156]
Membrane	AdV5	Sartobind STIC and CaptoCore 700	~100%	99.999% (5 LRV)	99.99% (4 LRV)	–	HEK293	[157]
Membrane	ChAd63, ChAdOx1, and ChAdOx2 Simian adenoviruses	NatriFlow HD-Q, Mustang Q, or CIMmultus QA	76%, 83%, and 82%	85%, 90%, and 88%	<LOD	For Mustang Q: Empty capsid to VP ratios of 0.17, <0.1, and <0.1	HEK293T	[62]
Membrane	AdV5	Sartobind Q and Mustang Q	60% and 59%	Final concentration <100 μg/mL from 245 μg/mL initial concentration	85% and 78%	–	HEK293	[158]
Membrane	AAV2	Custom CEX-IDA followed by AEX-TEA	76%	99.95% (3.3 LRV)	99.994% (4.2 LRV)	0.42 VG/ capsids	Sf9	[159]
Membrane	LV	Cellulose nanofiber derivatized with RC quaternary amine	>90%	99%+ (2+ LRV)	0.4–1.1 LRV for fractions of main importance	–	HEK293T	[160]
Membrane	LV	Mustang Q and Sartobind Q	45% global	97%	95%+	–	HEK293T	[161]
Membrane	Newcastle disease virus	NatriFlow HD-Q	70%	97%	70%	–	DF-1 chicken embryo fibroblast cells	[162]
Membrane	Baculovirus	Sartobind Q, Mustang Q, ChromaSorb	<5%	~<LOD, with 28.3%, 54.8%, and 59.8% of HCP binding irreversibly to the membrane	77.1%, 85.9%, 99.1%	–	SF-21	[163]
Membrane	Orf virus	Sartobind S, Sartobind Q, Sartobind-PA, Sartobind Phenyl Pico, sulfated cellulose membrane adsorbers, CaptoCore 700, and	76–86%	~100%	82–95%	–	Vero cells	[164]

(*continued on next page*)

Table 2 (*continued*)

Stationary Phase	Purification Target	IEX product	Recovery	HCP Removal	DNA removal	Empty vs full metrics	Host Cell Line	Refs.
Membrane	γ-retrovirus (MLV origin)	regenerated cellulose membranes (Whatman RC60) Mustang Q	50%	99.698% (2.52 LRV)	99.383% (2.21 LRV)	–	EcoPack2	[165]

HPLC = high performance liquid chromatography. IVP = infectious virus particle. LOD = limit of detection. VG = viral genomes. MLV = murine leukemia virus.

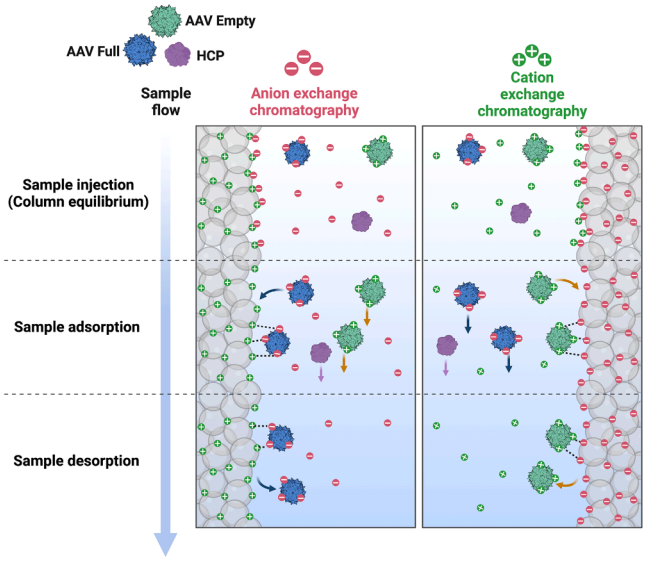


Fig. 4. A portrayal of IEX resins showing the separation of empty from full AAV based on capsid and resin charge.

column. This workflow provided a total vector recovery of 53% with contaminant protein removal of 90% and viral titers of 6.2×10^{10} TU/mL. Shaburova and Lanshakov (2020) utilized PEG 6000 precipitation and QA-derivatized IEX adsorbents to purify LV vectors encoding the brain neurotrophic factor BDNF with a titer of 1.12×10^{9} TU/mL [185]. Soldi et al. (2020) developed a research-scale method for producing and purifying lentiviral vectors for genetic engineering providing infectious titer of 10^{9} TU/mL and activity of 5×10^{4} TU/ng p24 with >99% removal of plasmid, DNA, and protein impurities and 20–40% recovery [146]. The purification train involves AEX capture with a DEAE-650C resin, concentration with TFF via 100 kDa hollow fiber or cassette (both performed comparably, and polishing and buffer exchange with gel filtration with Sepharose 6FF resin. Ghosh et al. (2022) investigated and optimized processes for LV purification using four IEX resins [147]. Capto Q ImpRes provided impressive infectious recoveries of ~100% and an HCP LRV clearance of 1.89. Other resins providing even higher HCP clearance, albeit at the cost of yield: Macro prep high Q offered a recovery of ~ 82% and HCP LRV of 2.51; Amino Sepharose 6 FF yielded 70–75% recovery and HCP LRV of 2.89; and Poros 50D achieved 85–90% recovery with an HCP LRV of 2.1. Niche VVs, such as oncolytic measles virus (MV), can also be purified by IEX. Eshmuno CPX in a CEX process for MV purification, provided reductions of 98.3% and 80.5% for total protein and DNA, respectively, along with a yield of 80.7% infectious particles [148].

4.2.2. *Monolith-based supports*

Monoliths are a class of chromatographic supports that are based on a continuous, homogenous column which enables convective mass transport via channels. They are often used in polishing (including empty capsid removal), but have also proven robust in primary purification and analytical chromatography. Gagnon et al. (2020) showcased all three applications in a single study [186]. The monolith structure overcomes traditional resin issues, such as high void space, and membrane limitations, like low-binding capacity and peak broadening [187, 188]. They offer high flow-rates, binding capacities unaffected by flow rates, low-pressure drops, high resolution, and high porosity. When functionalized with traditional chromatographic moieties like affinity, ion-exchange (most common), and hydrophobic ligands, monoliths provide unique advantages, especially when processing large, diverse biomolecules like VVs, which may fail to enter resin pores [189,190]. However, monoliths may be more expensive than traditional resins, and their unique structure may present challenges in scaling up for large-scale manufacturing processes. Additionally, some monoliths may not be as chemically stable or easily regenerated as their resin counterparts. Most chromatography trials using monolithic carriers have focused on ion exchange chemistry. Lucero et al. (2017) found that a CIM QA-1 monolith provided higher recovery (34%) and purity (70%) than a Q-Seph AEC column for rAdV5 purification [149]. This approach was used as a basis to scale-up a GLP process for material production at the National Research Council of Canada for gene therapy targeting alcoholism in preclinical trials.

Monoliths have demonstrated excellent ability to separate empty capsids from full capsids. Typically, empty and full capsids are separated through IEX with shallow gradients or small steps in ionic strength buffer changes, using as little as 10 mM NaCl or 1 ms/cm per set point adjustment. Gagnon et al. (2021) developed a method to remove empty capsids from AAV preparations, successfully recovering 100% of full AAV9 capsids and completely eliminating empty capsids using a CEX-functionalized monolith followed by a multimodal, positively charged, metal ion affinity ligand PrimaT [150]. The column performed consistently across various metal ions and elution buffers. Joshi et al. (2021) established an AEX method capable of separating empty capsids and enriching AAV5, 6, 8, and 9 with a monolithic CIMmultus QA column [151]. Discontinuous gradient elutions of varying salts helped enrich AAV5 9-fold, with 80% of the preparation consisting of genome-containing capsids, while AAV6, 8, and 9 showed >90% vector enrichment. Dickerson et al. (2021) devised a novel, isocratic AEX method using the CIMmultus QA monolith to separate empty from full rAAV2 capsids, achieving recoveries >70% and >90% of full AAV particles [152].

Non-AAV- and -AdV-based vectors like LV [191,192] and baculovirus are also suitable for monolithic purification. Gerster et al. (2013) conducted a comprehensive analysis of baculovirus purification with monoliths [153]. A QA-functionalized monolith offered recovery of infectious virus from 20 to 99+% with total protein and DNA content reduced to 1–8% and 38–48%, respectively. Impressively, a 1 mL monolith provided an 82-fold volume reduction from 1150 mL cell culture supernatant with a 51-fold active virus enrichment. Measles virus (MeV), a promising VV with oncolytic properties, is not well suited for affinity purification due to 1) it being a particularly labile target with stability between pH 7–9, which precludes low-pH elution and 2) the low-production scale would make immunoaffinity ligands cost-prohibitive [193,194]. IEX and HIC monolithic columns are suitable though, as demonstrated by a QA monolith which achieved a 17% MeV recovery, with no infectious particles detected in the flow-through [154,195]. Interestingly, small channels (D = 1.4 μm) were ineffective for MeV purification, despite particle diameters being <400 nm. In contrast, large channels (6 μm) allowed efficient, flow-independent

recoveries up to 10 mL/min. HIC purification using CIM OH monolithic columns resulted in approximately 60% infective virus particle recovery, with DNA below LOD.

Monoliths are typically synthetic polymers (e.g., methacrylate), but Fernandes et al. (2015) developed 20 bio-based matrices, offering superior biocompatibility, biodegradability, and cost-effectiveness compared to traditional supports, which could be interesting for organizations seeking disposable, environmentally sustainable processes [196]. The use of novel manufacturing methods, such as additive manufacturing, present exciting research avenues for improving upon current constructs [155]. Moleirinho et al. (2021) leveraged this technology to create 3D-printed cellulose chromatographic columns functionalized with DEAE and hydroxyapatite, resulting in the successful purification of LV and OAd [155]. The unique flow-path design, based on the Schoen Gyroid, a triply periodic minimal surface with 300 μm channels and solid phase pores of 0.5–10 um, resulted in a dynamic binding capacity of 1.9×10^{10} vg/mL for OAd and 2×10^{9} particles/mL for LV. The purified yield was ~69% for OAd and 57% transducing units of LV.

4.2.3. Membrane-based supports

Membranes, like monoliths, are frequently employed in polishing processes and occasionally in primary purification applications. They offer numerous advantages over traditional resin chromatography, such as higher throughput due to faster flow-rates with convective driven mass transfer, single-use designs, and the absence of small pores which large viral particles may not enter [197]. However, compared to resins membranes can have more peak broadening, difficulties scaling-up, and limited compatibility with chemicals and ligands for functionalization. Membranes are typically grafted with ion-exchange moieties such as those found on quaternary ammonium ion (QA) ligands which have given rise to products like Sartobind Q (Sartorius Stedim Biotech, Germany) and Mustang Q (PALL, Life sciences, USA).

These membranes have demonstrated success in VV purification, with yields and purities dependent on ligand density and membrane structure [156,198]. Hydrogel-grafted membranes showed AdV5 recovery in the 50–90% yield range with medium ligand density (2.4 μmol/cm^2) and flow-through percentages of 3.6–13%, 1.8–6.1%, and 1.0–2.7% for ligand densities of 1.7, 2.4 and 3.3 μmol/cm^2, respectively. For directly grafted membranes the percentage of viral particles detected in the flow through was 25% for high (4.5 μmol/cm^2) ligand densities and up to 60–70% for low (0.5 μmol/cm^2) and medium densities. Most membrane-based systems operate in bind-and-elute mode, though Nestola et al. (2015) also developed a flow-through platform with anion-exchange polyallylamine membranes (Sartobind STIC) that was used for the purification of Ad5 [156]. They achieved high purities with HCP and DNA LRVs of 5 and 4, respectively, with close to 100% recovery. NatriFlo HD-Q is a unique microporous hydrogel membrane structure that, in 2019, was demonstrated for the first time in a viral vector purification scheme and was compared to a Mustang Q membrane and a CIMmultus QA-1 monolith [62]. For the purification of simian adenovirus, NatriFlo Q provided a recovery of 76%, HCP reduction of 85%, and $DBC_{10\%}$ of 6×10^{13} VP/mL of bed, while Mustang Q membranes and CIM QA monoliths provided recoveries of 83 and 82%, HCP reductions of 90 and 88%, and $DBC_{10\%}$ of 10×10^{13} and $>4 \times 10^{13}$ VP/mL of bed, respectively. Kawka and colleagues (2022) compared Sartobind Q with Mustang Q for purification of AdV5 from HEK293 clarified cell lysate [157]. Mustang Q achieved a yield of 59% with a reduction in DNA by 78% and protein impurities below 100 ug/mL. Similarly Sartobind Q achieved a yield of 60% with a reduction in DNA by 85% and protein impurities below 100 ug/mL. Fan et al. (2022) utilized a custom, quaternary amine functionalized nonwoven membrane to purify AAV2 from SF9 cell lysate [158]. The membrane achieved a high binding capacity (9.6×10^{13} vp/mL) at a 1 min residence time and outperformed commercial membranes by affording a high productivity of 2.4×10^{13} capsids/(mL.min) and HCP LRV ~1.8. Cellulose-nanofibers have been gaining attention for their accessibility to large viral vectors and high scalability [159,199]. A protocol based on nanofiber IEX by Ruscic et al. (2019) provided an HCP LRV of 2, 100 fold concentration of LV, and >90% yield of functional LV with an impressive 100 cv/min flow-rate. The flow-scheme for these membrane-based technologies needs to be empirically optimized. One optimization could involve the use of laterally-fed flow to reduce dead-volume and establish uniform flow patterns within the devices [200].

Membranes can effectively capture and polish all types of VVs, not just AdV and AAV. For instance, LV membrane-based AEX purification is commonly used, and can achieve high process recovery (~90%), purity (HCP and DNA removal of 97% and 90%, respectively), and infectious titer (2.1×10^{4} TU/ng of p24) [159,160,201,202]. Moreira et al. (2021) presented a comprehensive method for scalable LV purification with 4 unit operations that can be completed in 5 h with Mustang Q and Sartobind Q membranes [160]. The process affords 45% recovery yield and with ~5.9 μg HCP per 10^{9} TU with the IEX membranes removing 97% of HCPs. Newcastle disease virus, of the family *Paramyxoviridae,* can also be purified via AEX, as Santry et al. (2020) demonstrated with a NatriFlo HD-Q membrane achieving yields of 70% with protein and DNA reductions of 97% and 70%, respectively [161]. Grein et al. (2012) purified a recombinant baculovirus with three different membranes, including Sartobind Q, Mustang Q, and ChromaSorb, achieving DNA reductions of 77.1%, 85.9%, and 99.1% and HCP reductions of below LOD, 7.62×10^{-4} g/g, and 5.97×10^{-2} g/g, respectively [162]. The membranes achieved almost complete binding of the baculovirus, however, they struggled to elute the virus, generating yields of <5%. Orf virus is another promising vector, but most research on it has focused on upstream development rather than downstream purification, resulting in methods that provide insufficient purity for gene therapy applications. Lothert et al. (2020) addressed this issue by developing a two stage process (either AEX or steric exclusion chromatography (SXC) followed by SEC or HIC) with recoveries of 76–86%, DNA reductions of 82–95%, and total removal of cellular proteins [163]. Membrane-based Mustang Q AEX has also been used to purify γ-retrovirus (MLV origin) with 50% yields and LRVs of 2.52 and 2.21 for HCPs and DNA, respectively [164]. Overall, membranes functionalized with varying ligands and operating under a diverse set of purification strategies can separate a wide variety of VVs for gene therapy.

4.3. Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) serves as a versatile technique for both capturing and polishing, although it is not as widely employed as affinity chromatography or IEX. HIC capitalizes on the differences in hydrophobicity between intact AAV capsids, amongst other VVs [43], and impurities. A primary concern with HIC is the osmotic stress resulting from rapid changes in high ionic strength buffers; therefore, it is essential for process development to incorporate an analytical evaluation of denaturation [203]. McNally et al. (2020) used a Sartobind Phenyl-ligand HIC membrane to capture 76–100% of AAV1, AAV5, AAV8, and a novel AAV "mutant C" serotype [46]. This research also reported the first dynamic binding capacity of a non-affinity adsorber for AAV, with the membrane displaying a $DBC_{10\%}$ of 2×10^{13} and 1×10^{13} capsids/mL of membrane for AAV8 and AAV-MutC, respectively. The use of lyotropic salts facilitated phase separation of dsDNA-containing insoluble material, reducing dsDNA levels by more than 90%. The AAV-MutC demonstrated a recovery rate of 90%, HCP reduction of 90%, and dsDNA reduction of 80%.

4.4. Size exclusion chromatography

Size exclusion chromatography (SEC) offers a mild, isocratic separation technique, making it an appealing choice for small-scale viral vector purification. Unlike bind and elute methods, SEC operates in flow-through mode, ensuring that viral vectors remain unaffected by

varying pH and salt concentrations. Key drawbacks of SEC include its low-throughput nature, inability to separate empty from full capsids, and potential to induce shear stress depending on the column and flow rates. Although SEC is primarily employed as a polishing step in smaller-scale processes, often following affinity chromatography or IEX capture steps [204], it can also function as the primary purification method [205]. For instance, Heider et al. (2017) achieved impressive LV recoveries of 24–57% and a purity increase of up to 60-fold using SEC [206]. Similarly, Nestola et al. (2014) utilized a two-column, quasi--continuous, simulated moving-bed process with Sepharose 4 FF to purify AdV5 via SEC, achieving an 86% yield, 6-fold productivity improvement, and substantial DNA and HCP reductions of 90% and 89%, respectively [207]. In another innovative application, James et al. (2016) developed a high throughput, rapid (<3 h) in-slurry pull-out method using Capto Core 700, which delivered reovirus purity and infectivity equivalent to a CsCl gradient [208]. This method successfully eliminated the ultracentrifugation bottleneck and removed confounding contaminants such as proteases and cytokines.

4.5. Steric exclusion chromatography

Steric exclusion chromatography (SXC) is a new method developed in 2012 [209] for VV and large protein purification which works by exploiting the unique physicochemical properties of large biomolecules and the principle of steric exclusion. The key component in this method is PEG, a long-chain polymer that is chemically inert to hydrophilic surfaces and used at high concentration. When mixed with the VV solution and loaded onto a hydrophilic, hydroxyl-functionalized monolithic column, PEG-induced phase separation begins to occur. The phase separation of PEG effectively excludes large biomolecules, such as VVs, from the bulk fluid driving them to a separate, concentrated phase associated with the column, while smaller impurities remain in the PEG-rich phase. A key benefit of this method is the extraordinarily gentle elution, which occurs by simply removing PEG from the buffer system. SXC can also be used as an analytical tool for determining the purity of AAV preparations [108,115]. Marichal-Gallardo et al. (2021) developed a unique, serotype-independent AAV capture step involving membrane-based SXC [209], where the virus particles bound to the surface of the membrane as they became preferentially hydrated in the presence of PEG [14]. A mixture of PEG and cell culture supernatant was fed to a single use, 1 μm pore size, stacked cellulose membrane unit and provided yields of >95%, and impurity reduction >80%. Lothert et al. (2020) seek to replace AEX as the predominant mode for baculovirus due to the technique's lack of robustness for broad purification of genetically modified virus particles and implement SXC for platform purification [36]. The optimal stationary phase was determined to be cellulose with a binding capacity of 5.08×10^7 pfu/cm^2, mean yields of 91% ± 6.5%, 99% protein removal, and 85% DNA removal (without nuclease treatment) with elution occurring via PBS without PEG. Hydrosart cellulose membranes from Sartorius Stedim Biotech GmbH were tested and optimal viral retention and recovery was observed at 8% PEG 8000. It was also observed that using PEG of a higher MW required a slower flow rate. However, for removal of DNA and viruses, lower concentrations and lower MW of PEG led to better purity. Following three batch runs, a mean DNA removal of 84.9% ± 13.75% was observed. Viral inactivation due to SXC was low, as flow cytometry control experiments showed a GFP fluorescence in 2.9% of cells post-SXC and 2.4% of cells pre-SXC. Lothert et al. (2020) in another paper, demonstrated highly efficient Orf virus purification using SXC as a capture step for two genotypes, recovering more than 90% in the elution fraction with $DBC_{10\%}$ of 2.01×10^8 IU/mL and 4.01×10^8 IU/mL [164]. Additionally, protein removal was over 98%, with remaining DNA levels of 24% and 19% compared to the feed.

5. Non-chromatographic purification

5.1. Centrifugation

The production of viral vectors in large quantities has only recently become a high priority. As such, most original [210] and ongoing small-scale purification methods, such as ultracentrifugation, are still in use. Dominant methods include cesium chloride (CsCl) and iodixanol gradient centrifugation, which subject the clarified cell culture fluid to centrifugal force, causing the viruses to coalesce into a purified band based on their buoyant densities. CsCl operations generally require more processing steps and exhibit higher toxicity than iodixanol, with conflicting reports on which method can provide highest purity. One clear benefit is that in AAV preparations, CsCl can provide <1% empty capsids compared to ~20% with iodixanol [176]. Sucrose gradients are another less commonly used option, though can offer more benign biocompatibility than CsCl and is generally easier to use. In-depth techniques for AAV iodixanol gradient centrifugation are detailed by Kohlbrenner et al. (2017) [211] for AAV9 purification and Burger et al. (2016) for rAAV which yielded 300 μL of 5×10^{12} to 1×10^{13} genome copies/ml viral preparation [212]. Additional studies by Blessing et al. (2018) found higher transduction efficiency with iodixanol compared to affinity chromatography [101] and by Hashimoto et al. (2017) which found iodixanol provided superior infectious yields compared to CsCl [213]. Similarly, detailed methods for AAV and AdV CsCl gradient centrifugation are well-documented in studies by Wada et al. (2023) [214], demonstrating a large-scale 1-L system, and the research by Sayedahmed et al. (2019) [215].

Ultracentrifugation (UC) is useful for all VVs including LV and NDV. Gandara et al. (2018) used serial ultracentrifugation to produce LV functional titers of 10^7 to 10^{10} particles/mL [216]. Benskey and Manfredsson (2019) detail a protocol for ultracentrifugation at 80,000 x g for two hours that is versatile and results in LV vector titers of 2×10^{12} vg/mL [217]. Santry et al. (2017) produced a comprehensive method for producing and purifying pre-clinical grade high titer NDV for use in mice experiments [218]. Purification begins with typical clarification, depth filtration, and TFF. At this point sucrose gradient purification is used to collect the virus which will generally be between the 40% and 50% sucrose layers after ultracentrifugation at 120,000 g for 3.5 h at 4 °C. The sample is buffer exchanged and concentrated into PBS to provide viral titers of $2–5 \times 10^9$ PFUs/mL in 2–6 mL from 500 mL of allantoic fluid with a recovery of ~60%. Alphaviruses are another class of viral vectors for gene therapy [219], but have typically been used on smaller scales. So far, they predominantly have been purified by centrifugation [220–222].

There are many methods tweaking the traditional ultracentrifugation protocols. Hudry et al. (2016) found that low-speed centrifugation followed by a simple ultracentrifugation step to collect pelleted exo-AAV9 provided sufficient results for mice CNS transduction, thus dramatically lowering the cost and complexity for research-grade material [223]. Kikusui et al. (2018) went a step further and found that for transfection of mouse neurons, the supernatant from a simple centrifugation step of 2 × 16,000 g for 10 min provided adequate infectivity [224]. Iodixanol gradient separations are often criticized for long processing times. Buclez et al. (2016) improved this by eliminating the 15% iodixanol gradient phase, using TFF instead [225]. This adjustment led to a doubling of viral vector suspension capacity and enabled the purification of several liters of crude lysate in a day. The refined method preserved AAVs' functionality and significantly reduced UC time from 80 to 20 min. Jiang et al. (2014) offered an alternative to the high-speed UC method used for LV concentration, using a low speed (≤10,000 g) sucrose gradient process, which delivered an 85.6 ± 0.07% recovery, superior purity (determined via SDS-PAGE), and higher transduction efficiency of 185.8 ± 23.7% compared to the 90,000 g UC protocol. Papanikolaou et al. (2013) simply used 100 kDa MWCO ultrafiltration for LV concentration, and obtained a final functional titer equal to 7.2 ×

10^8 TU/ml vs 3.5×10^8 TU/ml derived from the UC process with purity analysis detecting albumins in both products [226]. CsCl density gradient purification typically involves >100,000 g x 1 h. Nasukawa et al. (2017) optimized the method to allow for the same purification levels of UC at general centrifugation levels of 40,000 g x 2hr [227].

In an effort to streamline rAAV purification and make it cost-effective and accessible to novice users requiring only lab-scale quantities, Chen et al. (2020) developed a two-step process capable of purifying infectious rAAV serotypes 1, 5, 6, 8, and 9 [228]. After centrifugation to remove cellular debris, crude rAAV particles were pelleted with a 40% sucrose cushion overnight at 100,000 g which eliminated the need for fraction collection. After resuspension and dilution, centrifugation at 500 g was completed to remove debris and final concentration with a 100 kDa MWCO protein concentrator was completed. The purity was comparable to commercial rAAV, with high yields from low-starting amounts, thus enabling rapid production and purification of new rAAV constructs.

5.2. Aqueous two-phase systems

Aqueous two-phase systems (ATPs), a method for separating compounds that dates back to 1896, have recently been utilized for viral vector purification. In ATPS, the two phases are typically created using a water-based solution with varying concentrations of polymers and salts. The primary separation mechanisms involve surface tension, virus surface hydrophobicity, and biomolecular charge [229]. In 2020, a three-phase system was employed for the first time to purify AAV from 90% of cellular impurities, as well as to separate empty from full capsids in a scalable manner [230]. However, predicting how molecules will separate into the phases is challenging. Therefore, empirical evidence is necessary to optimize the system, taking into account variables such as polymer molecular weight, concentration, pH, temperature, and hydrophobicity. The tie-line length (TLL), a thermodynamic parameter, is an essential variable that describes the system and influences effective separation, depending on the component ratios [231]. Typically, the virus is recovered from the PEG-rich phase and would need to be diafiltered before proceeding to further downstream processing steps. Importantly, the process is serotype-independent, which aids in platform purification [232–234], and can operate in continuous mode [235]. Equipment from the chemical industry can be readily repurposed for ATPS further reducing cost and barriers to entry [50,236]. Additionally, ATPS have demonstrated success in purifying other biomolecules, providing a foundational knowledge base which can be reapplied to viral vectors [237–239]. Kimura et al. (2019) combined PEG precipitation and ATPS in an innovative approach to purify AAVs in a serotype-independent manner, yielding a highly infectious recovery of 10^{10}–10^{11} vg/uL [232]. Similarly, Arden and Metzger (2016) reported a serotype-independent AAV purification method that yielded $>2 \times 10^{13}$ vg/mL [234]. Guo et al. (2012) presented an AAV8 ATPS purification process for *in vivo* work with 95% recovery and higher purity than standard CsCl gradient purification [233].

Many non-AAV vectors can also be purified with this technique, including porcine parvovirus (PPV), HIV-VLP [240,241], M13 bacteriophage [242], Human B19 parvovirus-like particles [243,244], and foot-and-mouth disease [245]. Turpeinen et al. (2021) developed a novel, continuous ATP-based purification technique for viral vectors, demonstrating a PPV recovery of 90% with 96% and 89% DNA and protein removal, respectively [235]. HIV VLPs were also recovered at a staggering 99% with decent DNA removal of 73% and high purity based on SDS-PAGE. This method holds great promise for upscaling, while matching recoveries and purities of existing batch mode techniques. Joshi et al. (2021) utilized osmolytes to drive partitioning and achieved 100% recovery of infectious PPV and 92% for HIV-VLP, essentially complete clearance of HCPs, and high DNA clearance rates (61–91%) [240]. This work is a great improvement upon their previous 2019 paper that achieved PPV recoveries of 79% and more limited DNA removal [231]. Gonzalez-Mora et al. (2017) used ATPS for the purification of M13, an *E. coli* specific bacteriophage, achieving a recovery of 83.3% [31]. Effio et al. (2015) reported a delicate balance between purity and yield loss in ATPS purification of Human B19 parvovirus-like particles [243]. A novel two-step process involving batch ATPS and precipitation provided a 64% yield with a 99.8% DNA removal and 90.6% purity. Du et al. (2019) purified Foot-and-Mouth Disease (FMDV) with a multiple-stage, highly scalable, ATPS system, achieving a 72% recovery [245]. There are other precipitation based methods involving chloroform [246] and mannitol flocculation for serotype independent, scalable purification of AAV [247]. The latter was demonstrated in a study by Heldt et al. (2018) which achieved recoveries of 58–96% for enveloped and non-enveloped particles and high protein reductions up to 80%, though low DNA removal [247].

6. Viral clearance, formulation, and filling

6.1. Viral clearance

Traditional methods of viral clearance for biologics manufacturing often involve a blend of the main purification methods previously detailed, along with additional unit operations. These include low pH treatment, solvent or detergent inactivation, viral filtration, and heat treatment [248–250]. However, viral clearance for viral vectors poses a challenge: it can be difficult to develop a method that effectively differentiates the target from adventitious and contaminant viruses [250]. For AAV products, there are more viral clearance strategies available compared to LV, as AAV is a smaller, non-enveloped virus [251]. These include detergent-based viral clearances that disrupt the lipid membrane of enveloped viruses. Furthermore, AAV's stability in thermal and low-pH conditions introduces additional viral clearance methods. Its small size also enables filtration through 35 nm nanofilters, retaining larger viruses. LV, which is 80–100 nm and enveloped, is sensitive to heat, pH, and detergents. Thus, the primary two viral clearance methods for LV are the purification steps already used (e.g., AEX) and nanofiltration, where LV is retained while smaller viruses pass through. However, nanofiltration can result in the loss of significant quantities of viral vectors. It's also important to clear and quantify infectious helper viruses–those used to deliver functions necessary for producing replication-defective recombinant AAV vectors–for patient safety. Ye et al. (2014) developed a process that achieves a HSV LRV of 14.04 in AAV production via detergent lysis of cell harvest followed by two column chromatography steps (CIM Q monolith and AVB Sepharose) [252].

6.2. Formulation and filling

The final stage of the downstream process involves formulation and sterilization for filling. Formulation typically involves concentration and diafiltration, often through TFF, to achieve a therapeutic titer and introduce the final buffer system containing stabilizing excipients. The chosen formula depends on the route of administration (e.g., intrathecal, intravenous, subretinal), but typically involves PBS, though other biological buffers such as HEPES and PIPES are commonly used [253] and may offer advantages over PBS. For example, tris-based systems might result in lower capsid titer loss [254]. Stabilization is often enhanced with sugars like sucrose [255] and trehalose. There's also evidence indicating that reducing sugars, like lactose, and proteins such as human albumin [256] can improve stability [257]. Rodrigues et al. (2019) published a comprehensive review of formulation strategies that discusses buffers, lyo/cryo protectants, surfactants, and tonicity agents as well as the advantages and drawbacks of various storage mechanisms, such as freezing [258] and lyophilization [259,260]. The membranes and operating conditions used for diafiltration can significantly impact vector titers, as they tend to be less shear-resistant than many biologics in similar fill/finish unit operations [53].

Sterilization, usually the last step, involves 0.22 μm filtration into a

sterile vial, or other suitable container, performed under aseptic conditions [261]. In rare cases, the purification process for larger viral vectors like HSV can bypass sterile filtration if validation can prove a closed, aseptic system [262,263]. A primary concern of the sterilization step is yield loss, which can range from 30 to 70%, but certain buffer manipulations can help reduce this loss [264].

7. Analytical technologies and techniques for characterizing viral vector preparations

The meticulous characterization of VVs is a crucial step in their production process, with the U.S. FDA imposing rigorous testing requirements to ascertain product safety, purity, potency, stability, and identity [265–267]. These overarching parameters are further delineated into critical quality attributes (CQAs) which encompass, but are not limited to, quantification of residual HCP and DNA, empty vs partially full vs full capsid ratios, infectivity, vector genome levels, and capsid titer as seen in Table 3. Several analytical methodologies, originally developed for broader applications, have been successfully repurposed for viral vector characterization without requiring major modifications. For instance, HCP enzyme-linked immunosorbent assays (ELISAs) are examples of such techniques. Nonetheless, the specificity and complexity of viral vectors often necessitate the development of bespoke analytical technologies, which, in turn, can present unique challenges. These include differentiating and quantifying capsids that are empty, partially filled, and fully loaded.

The thrust of recent advancements in the field has been towards a paradigm shift from labor-intensive, low-throughput techniques like ultracentrifugation to rapid, high-precision technologies that can be seamlessly integrated into QC workflows, such as liquid chromatography-mass spectrometry (LC-MS). Arguably the most significant opportunity lies in the development of inline and online tools to replace measurements currently requiring offline methods. Fig. 5 provides a brief overview of future opportunities in research and development, spanning from analytical systems to purification technologies, weaving together multiple themes from this review. The development of multifaceted technologies capable of simultaneously assessing multiple vector characteristics is also receiving significant attention [268]. For instance, the technique proposed by McIntosh et al. (2021) elegantly couples SEC with UV, RI, and MALS to assess capsid size, integrity, aggregation, capsid content, total capsid levels, and genome titers [269]. For further insights into the analytical landscape of rAAV-based gene therapies, consider consulting the comprehensive review by Gimpel et al. (2021) [270] which offers an in-depth exploration of techniques. This can be supplemented by a review of chromatography based analytical tools for viral nanoparticles by Kramberger et al. (2015) [271], an extensive overview of characterization techniques of super large proteinaceous particles by Yang et al. (2020) [272], and a highly detailed report AAV analytical techniques by Kohlbrenner and Weber (2017) [211].

7.1. Purity

The assessment of VV purity is arguably the most intricate among all critical quality parameters, given the myriad potential impurities. These contaminants originate from both process-specific (e.g., organic solvents, detergents, cellular residual proteins [273], and nucleic acids) and product specific (e.g., empty vs partially full vs full and intact capsids) sources. As gene therapy progresses beyond preclinical and clinical stages, purity criteria such as the quantification of high-risk HCPs are anticipated to become increasingly stringent.

Process-related impurities are typically identified through an array of techniques such as ELISA, LC-MS/MS, AP-MS, qPCR, PicoGreen assay, and various nucleic acid detection methods. Non-protein impurities, like iodixanol, are generally characterized via HPLC and MS [274]. For protein-based impurities, many insights from mAb production can be

Table 3
Analytical technologies and techniques for characterizing viral vector preparations.

Purity		Identity		Stability		Potency		Safety	
Capsid characterization: analysis of empty vs partially full vs full capsids	• SV-AUC • AEX-HPLC • TEM • CD-MS • HPLC dual-wavelength UV A280/A260	**Capsid Identification:** serotype, capsid integrity, and packaging ratios	• Western Blot • ELISA • SDS-Page • LC/MS • Differential Scanning Fluorimetry	**Particle size distribution and capsid integrity**	• Multi-Angle Static Light Scattering (FFF-MALS) • Multi-Angle Dynamic Light Scattering (MADLS) • Nanoparticle Tracking Analysis (NTA) • Tunable Resistive Pulse Sensing (TRPS) TEM • AUC • Size Exclusion Chromatography (SEC-MALLS)	**Functional viral titer**	• Plaque-Forming Units (PFU/mL) • Infectious units (IFU/mL) • Transducing Units (TU/mL) • Viral plaque assay Immunofluorescence foci assays • TCID50 • LD50 • EID50	• Endotoxin • Adventitious agent • Mycoplasma • Replication-competent virus analysis.
HCP evaluation	ELISA	**Vector genome identity**	• PCR • Genome sequencing			**Total viral titer**	• qPCR • ddPCR		
Protein-specific information	MS	**Vector's isoelectric point**	• Chemical Force Microscopy (CFM) • Capillary Isoelectric Focusing						

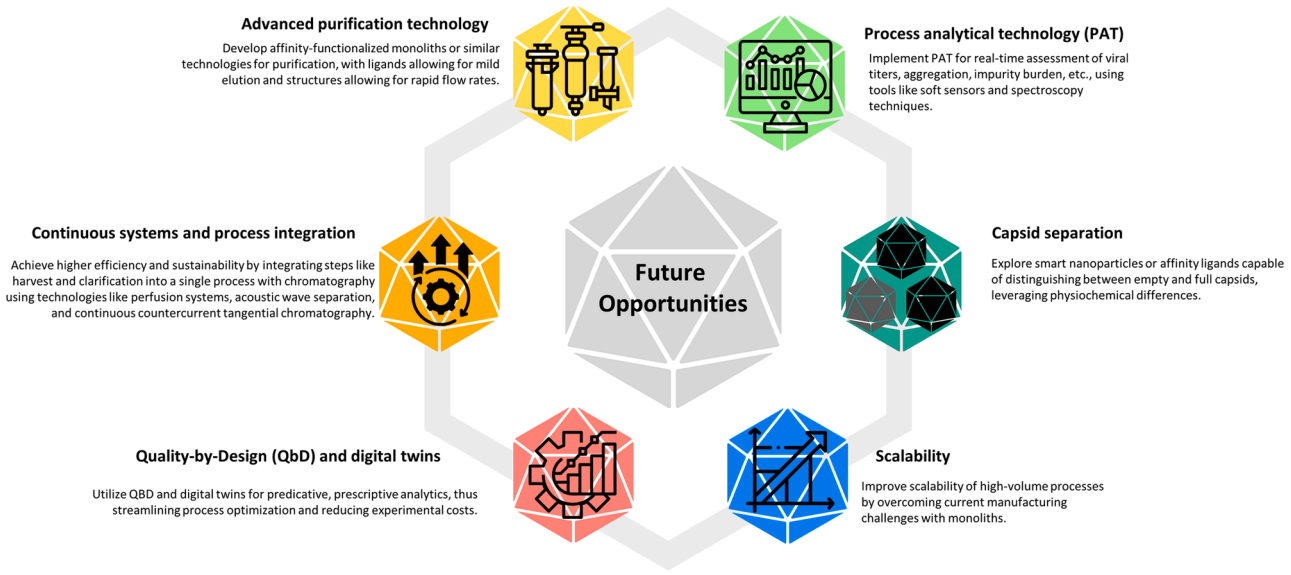


Fig. 5. A summary of six avenues for possible future research and development for downstream processing of viral vectors and associated examples.

extrapolated to HCP analysis in VV processes [275]. While ELISAs are the primary modality for HCP evaluation, they suffer from incomplete coverage of all process-specific impurities [276], particularly those of low MW (<25 kDa) [277], and they cannot quantify individual proteins [278]. MS, on the other hand, offers protein-specific information enabling a highly precise evaluation of HCPs and is an orthogonal test frequently combined with ELISA and 2D gel electrophoresis. An early exemplification of such an approach is provided by Dong et al. (2014) who identified the 14 viral and cellular proteins most commonly co-purified with AAV from HEK293 cells via GeLC-MS [279].

Process residual proteins originate from viral production cells and growth media (e.g., fetal bovine serum (FBS)) which contains bovine serum albumin, growth factors, and more. Nucleic acid residues come from production cells, host cell DNA and RNA, and plasmids. To contextualize the complexity of the fluids, a Phase 1 trial can be completed with 10^{15} vector particles, 6.5 mg of AAV capsid protein, and roughly 3.5 mg of vector DNA [71]. Purifying these components from a cell culture fluid containing approximately 4 g of non-vector protein (comprised of 1 g FBS protein, 3 g HEK293 HCPs from 10^{10} cells), and 350 mg of non-vector nucleic acids (originating from production plasmid DNA (30 mg) and HEK293 cellular nucleic acids (320 mg)) poses a significant challenge. The main challenge for AAV purification is to provide a 10^4–10^5 fold reduction of impurities while maintaining a yield of vector ≥50%.

Beyond HCPs and nucleic acids, product-related impurities also warrant attention. Post-translational modifications (PTMs) of rAAVs such as acetylation, phosphorylation, and glycosylation are becoming a focal point of concern due to their potential to provoke immunogenic responses by altering activity, aggregation, stability, and antigen presentation [280–282]. Other impurities such as residual host cell DNA, RNA, and plasmids can lead to genotoxicity. Comprehensive discussions on this subject can be found in the work of Rumachik et al. (2021) which develops a method for characterizing capsid PTMs and vector impurities [283], and Wright (2014), who provides an in-depth review of various impurities in AAV products and distinguishes between impurities and contaminants. The latter is specifically attributed to adventitious agents, such as microbial species, inadvertently introduced during the manufacturing process [71]. One such impurity is the encapsidation of DNA that deviates from the recombinant genome. To address this, Penaud-Budloo et al. (2017) innovated a single-strand virus sequencing (SSV-Seq) protocol, offering an advancement over the conventional qPCR method [284]. They identified baculovirus expression vector-derived DNA accounted for less than 2.1% of DNA in rAAV stocks with Sf9- derived DNA accounting for less than 0.03% of total reads using next-generation sequencing.

Capsid characterization poses significant challenges due to the subtle differences in potential protein and content profiles. The analysis of empty vs partially full vs full capsids stands out as a particularly arduous task, often requiring orthogonal tests to validate results [285,286]. Characterization hinges on techniques such as A280/A260 measurements, AEX-HPLC, CD-MS [287], TEM, and AUC [288]. AEX, while a useful tool in this matter, suffers from a limited ability to resolve partially filled or overpacked capsids from full capsids due to their similar net-charges, leading to co-elution even when applying an exceedingly shallow conductivity gradient. TEM, despite being a powerful technique, is expensive and requires skilled operation, with these challenges exacerbated when identifying partially filled capsids. Sedimentation velocity (SV)-AUC requires a significant sample volume (400–500 μL at 5×10^{12} vg/mL) and is low-throughput, requiring approximately 6 h to process a mere 3–7 samples. An expedient method to discern the packaging ratio of capsids involves HPLC analysis by dual-wavelength UV monitoring at 260 nm (nucleic acid detection) and 280 nm (protein detection). Gagnon et al. (2021) observed that full capsids exhibited an A260/A280 ratio of 1.31 versus 0.64 for empty capsids. Full capsid peak area estimations were calculated as 85%, 78%, and 76% by monitoring UV signal, intrinsic fluorescence, and light-scattering, respectively [289]. Overall, HPLC can be a superior choice for AAV quantification compared to qPCR, as it circumvents the need for pre-treatment of samples with DNase I and proteinase K, which are not fully effective in lysing capsids as only 50% were lysed after 24 h. Analytical AEX, due to the shift in the overall capsid charge proportional to the DNA packaging level, can be employed to separate empty from full capsids. Wang et al. (2019) and Fu et al. (2019) devised AEX methodologies for the quantification of empty vs full capsids, presenting a more accessible alternative for quality control labs compared to traditional methods [290,291]. Both relied on CIMac AAV full/empty-0.1-mL columns from BIA Separations and necessitated ≤20 μL of sample at a titer of $2.7–5 \times 10^{11}$ vg/mL with a sensitivity down to 2.9% empty capsids. Further, Li et al. (2020) capitalized on capillary isoelectric focusing to analyze the ratios of empty, partial, and full capsids, as well as for distinguishing serotypes [292].

7.2. Identity

Every viral vector preparation must undergo rigorous identity testing to ensure that both the encapsulated genetic material and the final capsid align with the intended final product. To verify the vector genome identity, conventional methods such as PCR and genome sequencing are deployed. The identification of the capsid, however, presents a more intricate challenge given the broad array of serotypes, capsid integrity factors, and packaging ratios. Typical techniques for this analysis are ELISAs, western blot immunoblot, and MS. To bolster the reliability of these results, secondary confirmation is sought from techniques such as SDS-PAGE, LC-MS [293], and differential scanning fluorimetry [293–296]. It is worth noting that differential scanning fluorimetry can discern serotypes varying by even a single amino acid residue through the differentiation of melting temperatures in ~1 h using only 10^{11} particles. The vector's isoelectric point, an inherent property reflecting its charge state, can also shed light on serotype and packaging ratios. This metric can be determined by techniques such as novel single-particle chemical force microscopy (CFM) that requires just 150 μL at a titer of 10^8 MTT_{50}/mL or capillary isoelectric focusing as described above [292,297].

7.3. Stability

Stability studies play a pivotal role in ensuring patient safety and drug efficacy. Over the course of storage, transportation, and patient administration, the profile of the drug (e.g., infectivity and aggregate formation) may change, thus necessitating these studies. Primarily, stability investigations focus on bioactivity and aggregation under diverse conditions, such as fluctuations in pH and temperature [296, 298–300]. For instance, a study by Potter et al. (2014) subjected rAAV9 vectors to buffers spanning pH 2.5 to 8. The observations indicated no loss in infectivity after 2 h, but a tenfold reduction after 24 h, with the most notable decrease in the pH 5–6 range. Beyond infectivity studies, visualization techniques have become a staple tool for assessing stability. These techniques range from the traditionally employed TEM [301], size exclusion chromatography (SEC-MALS), and analytical ultracentrifugation (AUC), to more recently developed and implemented techniques. The latter category includes field flow fractionation with multi-angle static light scattering (FFF-MALS), multi-angle dynamic light scattering (MADLS), nanoparticle tracking analysis (NTA), and tunable resistive pulse sensing (TRPS). For example, Dobnik et al. (2019) used TEM to visualize individual particles of AAVrh.10hCLN2, classifying them as empty, full, damaged, aggregated, or of atypical size [302]. The researchers succeeded in correlating TEM observations with variations in titers as determined by qPCR or ddPCR, with the latter demonstrating enhanced precision with average coefficient of variations ~6% compared to 16% for qPCR.

7.4. Potency

Potency is a measure of the concentration and activity of the therapeutic agent, ensuring consistent dosing regimens. Determination of potency hinges on two central attributes: the total viral titer and the functional (or infectious) viral titer. Analytical techniques for lentivirus bear similarity to those employed for AAV. Perry and Rayat's (2021) review paper on lentivirus bioprocessing dedicates a section to vector characterization and quality control [22]. Quantification is typically achieved through p24 ELISA [303], qPCR [304], or gene transfer assay (GTA). Transduction assays, another quantification tool, use flow cytometry, but require 2–4 days to obtain a result which is too long for process control in large scale production. In response to this, Transfiguracion et al. (2020) developed an IEX-HPLC method for the quantification of lentivirus in clarified supernatant [305]. The method showed linearity in the concentration range of 3.13×10^8 to 1.0×10^{10} TP/mL. The upper limit of detection could not be determined due to lack of samples with higher lentivirus titer. The presence of DNA contaminants can also be assessed by the ratio OD260/280, with values lower than 1 indicating lack of nucleic acid contaminants. When lentivirus titers were determined with HPLC, there was no statistical difference from a p24 ELISA test, but the values were higher than those determined by qPCR and GTA. Therefore, HPLC is not able to distinguish between functional and non-functional lentivirus particles. Moreover, SDS-PAGE and Western blot had to be used to identify which peak in the HPLC chromatogram corresponded to the lentivirus target. As an advantage, HPLC is cheaper and faster than the other methods used for lentivirus quantification.

7.4.1. Total viral titer

Ascertaining the physical titer, expressed as viral particles per milliliter (VP/mL), relies on several well-established methodologies, such as qPCR [306]—applicable even in crude lysates [307]—and ddPCR [308]. As the next-generation analytical tool, ddPCR garners preference for its superior precision and reproducibility, particularly in the face of contaminants and impurities [309,310].

7.4.2. Functional viral titer

The functional viral titer typically takes the form of plaque-forming units per mL (PFU/mL) or infectious units per mL (IFU/mL) for AV and AAV vectors. LV and retrovirus vectors, on the other hand, are reported as transducing units per mL (TU/mL). Essential tests for assessing infectivity encompass viral plaque assay, 50% tissue culture infectious dose ($TCID_{50}$), 50% lethal dose (LD_{50}), 50% egg infectious dose (EID_{50}), and immunofluorescence foci assays [311]. While traditional assays are often slow and labor-intensive, real-time measurements have been developed using impedance-based bio-sensing techniques, as described in a proof-of-concept study by Charretier et al. (2018) [312]. They found that 96% of titers were determined within the same range of variability ($\pm$ 0.3 $\log_{10}$ $CCID_{50}$/mL) seen with traditional $CCID_{50}$ assays while being 3.5x lower cost and 5x less labor-intensive. Furthermore, Francois et al. (2018) delved into the methods for discriminating between infectious and non-infectious rAAV8 vectors. They discovered the most reliable measurements hinged on vector genome replication or transgene expression, with the infectious center assay (ICA) emerging as the most selective method [313]. They utilized a VP1-deficient AAV8 vector to serve as negative control and found ICA showed the modified vector was 1000 fold less infectious, while the $TCID_{50}$ titration method showed a 6-fold decrease in infectivity, demonstrating ICA to be the more discriminating method for AAV8.

7.5. Safety

Once the identity, potency, purity, and stability are determined, the final preparation's safety is analyzed. This includes sterility, endotoxin, adventitious agent, mycoplasma, and replication-competent virus analysis. Aggregate analysis, typically reliant on dynamic light scattering or visualization methods, is found under the stability section.

Despite the low risk of replication-competent viral vectors, due to their engineering to be non-replicating, testing is still mandatory. Such testing can be accomplished through techniques like southern blotting and qPCR, which detect the rep and cap sequences [314]. Notably, the FDA has recently issued comprehensive guidance for industry on the testing retroviral vector-based human gene therapy products for replication-competent retrovirus (RCR). This guidance elaborates on the required assays and the extent of testing needed [315]. They no longer recommend RCR testing on working cell banks, but recommend testing for RCR with sufficient vector (defined as ensuring a 95% probability of detection of RCR if there is a 1 RCR present per dose equivalent) to demonstrate <1 RCR per patient dose and all retrovirus transduced cell products (1% or 10^8 cells).

Sterility from bacteria and fungi is typically determined over a two-week period by observing turbidity of a solution prepared by inoculation

or membrane filtration of the product (USP<71>, EP 2.6.1, JP 4:06). Adventitious agent testing and clearance should adhere to the current guidance for viral vaccines, which provides the most applicable framework for viral vectors (EP 2.6.16). A comprehensive review of adventitious agents in viral vaccines has been completed by Klug et al. (2016) [316]. Several LV papers have looked at adventitious agent analysis as well [317,318]. Current FDA regulations stipulate less than 5 endotoxin units/kg (EP 2.6.14, USP<85>). Endotoxins are potent pyrogens which can cause fever and sepsis even with minor exposures and are part of the cell membrane of Gram-negative bacteria [319]. The rabbit pyrogen test is being phased out, but Kondratova et al. (2019) provide details on the removal of endotoxin from rAAV samples while also describing the widely used Limulus Amebocyte Lysate (LAL) assay as a suitable test for product release [320,321]. Another test that holds promise is the recombinant Factor C (rFC) assay [322]. Standard tests for mycoplasma can be found in FDA guidance [323] and European Medicines agency (EMA) literature (EP 2.6.7), or can be purchased from commercial manufacturers [324]. Dreolinini et al. (2020) developed a rapid and sensitive mycoplasma screening technique based on nucleic-acid amplification. This technique, already in use for clinical cell therapy products, boasts a 10 CFU/mL detection level and is significantly faster than the typical 28 days required for other tests [325].

8. Conclusion

The opportunity set for gene therapies is vast, requiring a diverse repertoire of viral vector platforms which can be further engineered to account for the specific nuances of each targeted gene. The purification of such a diverse set of viral vectors remains a bottleneck and key challenge in further R&D and scale-up. Additionally, product related impurities, like empty capsids, present an additional layer of complexity as they closely resemble the product, but are a profound risk factor contributing to dose-dependent toxicity. Fortunately, there are some conserved properties across all viral vectors and within each species that can allow for rational design of downstream purification processes using the methods reported here. Recent successes of novel methods like steric exclusion chromatography and supports like monoliths and membranes may reduce processing times by taking advantage of the size properties of viral vectors. Further development should be completed on continuous chromatography which may allow for process intensification and reduced costs, which is important for enabling access to these therapies. Another opportunity for future work is in the development of computational approaches to rapidly identify the recommended purification techniques for VVs. This could be through either modeling the purification process to predetermine optimal processing conditions (e.g., buffers) or through capsid design and engineering to allow for compatibility with off-the-shelf purification methods. As the field of gene therapy is on the brink of unprecedented advancements, the continuous evolution of the downstream purification toolbox for viral vectors is more important than ever.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used OpenAI GPT-4 in order to assist with grammar and word choice selections. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

CRediT authorship contribution statement

Ryan Kilgore: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Arianna Minzoni:** Writing – original draft, Writing – review & editing. **Shriarjun Shastry:** Data curation, Writing – original draft, Writing – review & editing. **Will Smith:** Data curation, Writing – original draft, Writing – review & editing. **Eduardo Barbieri:** Data curation, Writing – original draft. **Yuxuan Wu:** Data curation, Writing – original draft, Writing – review & editing. **Jacob P. LeBarre:** Data curation, Writing – original draft, Writing – review & editing. **Wenning Chu:** Writing – original draft, Writing – review & editing. **Juliana O'Brien:** Data curation, Writing – original draft. **Stefano Menegatti:** Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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