

Manufacturing of viral vectors: part II. Downstream processing and safety aspects

Manufacturing of viral vectors comprises the generation of these vectors, which then have to be purified in order to meet the quality attributes required for further use as gene delivery systems. The first part of this article deals with the production of the most important viral vectors used in gene therapy protocols. In the second part, we briefly review the most current methods used for the purification of viral gene therapy products focusing on four viral vectors that have been the most extensively used in clinical trials: adenoviral, adeno-associated viral and lentiviral vectors. Traditionally, γ -retroviral vectors were not purified and clarified vectors containing culture supernatant was directly used for *ex vivo* gene therapy. The final section of this article reviews some of the basic biosafety considerations specific to the respective viral vectors.

Viral vector purification: downstream processing

Purification of viral vectors for delivery of therapeutic genes has been relying on laboratory protocols, using essentially ultracentrifugation techniques developed to generate material in sufficient quantities to establish proofs of principles. However, to be used in clinical protocols, viral vector preparations need to comply with stringent standards that are forcefully scrutinized by regulatory agencies. In addition, to achieve large and multicenter clinical trials, scalable upstream and downstream processes need to be implemented. Consequently, the downstream processing steps are designed to achieve high recovery yields of viral vectors with defined critical quality attributes that may impact the safety and efficacy of the final product. Many current viral vector downstream processes have been largely adapted from methods originally developed for purification of recombinant proteins and, at some extent, viral vaccines. However, these approaches, although conceptually satisfactory, are not without serious challenges. [Table 1](#) shows some similarities of viral vectors and their surface characteristics. However, different

approaches have been taken to purify viral vectors by exploiting their size and surface characteristics, such as charge and hydrophobicity. The purification scheme also takes into consideration the reduction of host cell protein content. Host cell proteins in the final product are process-related impurities that are the remnants of the manufacturing method. Different media for cell culture, downstream processing units for purification and host cell lines used for production contribute different components in the cell harvest. In addition, it is essential to maintain viral delivery and gene expression functions as intact as possible throughout the sequence of purification steps. For example, the viral removal step or viral inactivation step, two common critical steps in recombinant protein manufacturing, are not, in general, a viable option in the case of viral vector manufacturing. Therefore, methods that guaranty the viral vector preparation safety without compromising their infectivity or functionality were developed. Although there may be a generic approach, for each vector type and serotype, a strategic design and step-by-step optimization of the purification process is crucial to maximize the yield and quality

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Key Terms

Adeno-associated viral vector: Viral vector based on adeno-associated virus (a member of the nonenveloped parvovirus family, which is composed of small viruses [20–25 nm] with a genome of a single-stranded DNA). This vector is nonintegrative.

Tangential flow filtration: Filtration process of solutions in which a semi-permeable membrane is used to separate molecules that are smaller than the pore size cut-off of this membrane. The bulk solution flows over and parallel to the filter surface, and under pressure, a portion of the water and small molecules is forced through the membrane filter.

Ion-exchange chromatography: Process that allows the separation of ions and polar molecules based on their affinity to the ion exchanger.

Size-exclusion chromatography: Chromatographic method in which molecules in solution are separated by their size and, in some cases, molecular weight.

Adenoviral vector: Viral vector based on adenovirus (a group of medium-sized [90–100 nm] nonenveloped icosahedral viruses, with a nucleocapsid surrounding a dsDNA genome). This vector is nonintegrative.

of the final product. Over the past 15 years, considerable efforts have been dedicated to develop purification strategies that meet the standards of current GMPs. The sequential downstream processing of viral vectors includes essentially the following steps (Figure 1):

- Harvesting of the viral particles from the cell culture will separate the cells from the cell culture. The nonenveloped viral particles such as adenoviral vectors and **adeno-associated viral vectors** (AAVs) are produced intracellularly, which will require a cell disruption step to release the viral particles and processing of the cell lysate or the whole cell culture. The primary recovery step includes breakage of cells by microfluidizer or detergents; may include the addition of nuclease (e.g., Benzonase™, EM Science, NJ, USA) to reduce host cell DNA and reduce the viscosity for subsequent steps; and may require the addition of chemical components (formulation) to avoid the formation of aggregates. Enveloped viral vectors such as γ -retro- and lentiviruses are released in the cell culture supernatant and only the supernatant is processed in this case. The crude viral harvest from either the cell lysate or the supernatant is further clarified to remove host cells and cell debris. This step is achieved at large scale by dead-end filtration, centrifugation or microfiltration. Clarified viral stock is generally reduced in volume, especially in the case of low production yield and diluted viral material. This step is completed by ultracentrifugation, precipitation or ultrafiltration by **tangential flow filtration** (TFF);

- Purification steps are used to separate the viral vector from the host cell and medium-related components. Depending on the nature of the viral vector, the type of feed and contaminants involved, more than one purification step may be required and would generally involve **ion-exchange chromatography** (IEC) or affinity chromatography. Further purification is achieved by polishing step(s) to achieve the specified viral preparation purity and eventually to remove defective or empty viral particles by using density gradient ultracentrifugation, hydrophobic interactions or **size-exclusion chromatography** (SEC). Please note that, for instance, IEC can also be a choice for separating full from empty particles in the case of AAV;
- A final concentration and buffer exchange step may be required to achieve a specified final product concentration and formulation.

This sequence of steps will involve different separation and purification techniques to maximize recovery yield and specific bioactivity while minimizing impurities and manufacturing costs. In addition, for each viral vector, a specific purification strategy needs to be defined and validated to achieve a set of critical quality attributes of the product, and meet safety and efficacy specifications for the intended use.

Adenoviral vector purification

Adenoviral vectors are large (65–80 nm), nonenveloped, double-stranded DNA viruses. Recombinant adenoviral vectors are produced intracellularly and released in the supernatant through cell lysis. Most of the reports on adenoviral vector processing in the current literature regard serotype 5. In general, these vectors are stable and they have traditionally been purified at laboratory scale, after freeze–thaw cycles of the cell lysate, by two or three rounds of cesium chloride (CsCl) density-gradient ultracentrifugation to achieve clearance of host cell nucleic acids, host cell proteins, unassembled adenovirus proteins, unpackaged viral DNA, and eventually, of products related with transgene expression [1,2]. An additional benefit of the density-gradient ultracentrifugation is the separation of empty capsids from functional recombinant adenoviruses providing a careful collection of the specific bands. Thereafter, the viral product is dialyzed or desalted into the formulation solution to achieve preparations with approximately 10^{12} viral particles/ml for early-stage clinical trials. The use of CsCl requires a long centrifugation time, recently, iodixanol medium has been proposed as an alternative and the separation of helper-dependent adenoviruses from helper adenoviruses has been

Table 1. Properties of adeno-associated viral, adenoviral and lentiviral vectors.

Viral vectors	Size	Envelope	Stability	Net charge at neutral pH	Buoyancy density
AAV	~20 nm	No [†]	High [†]	Positive	1.39 in CsCl
AdV	~80 nm [†]	No [†]	High [†]	Negative [†]	1.34 in CsCl
LV	~100 nm [†]	Yes	Low	Negative [†]	1.16 in sucrose

[†]Similarities between different viruses.
AAV: Adeno-associated viral vector; AdV: Adenoviral vector; LV: Lentiviral vector.

demonstrated [3]. For large-scale operations, ultracentrifugation may still be an option where large-capacity ultracentrifuges are available as it is the case in many vaccine-manufacturing facilities. However, this approach remains capital investment intensive if considered in a new facility.

Scalable chromatographic purification techniques exploit size and surface charge (Figure 2) of the viral particle. In the case of adenoviral vectors, anion-exchange chromatography is the key purification step used in many of the industrial processes that have been described [4]. Prior to any processing, adenoviral vector needs to be released from the cells. For cell lysis, repeated freeze/thaw is not scalable, consequently other lysis methods including nonionic detergent addition (Tween-80 or Triton X-100), microfluidization and shear stress induced by a high agitation rate in the cell culture vessel or microfiltration devices have been applied for large-scale operations. Treatment with nucleases is the most common DNA clearance method employed for viral vector preparation. For adenoviral vector purification, Benzonase is often selected since it digests both DNA and RNA, and remains relatively stable in a variety of buffers. Clarification has been generally achieved by centrifugation, dead-end filtration or microfiltration, whereas the most common method for feed volume reduction and concentration of adenovirus with or without buffer exchanging is ultrafiltration.

For example, an IEC has been combined to SEC to achieve 99% purity and a typical overall recovery between 30 and 80% was obtained. More specifically, cell lysate is prepared by osmotic shock, treated with nuclease, centrifuged, conditioned and filtered before applying on to the ion-exchange column. The column eluate is concentrated by ultrafiltration before the SEC polishing step [5].

For the final formulation and characterization of adenoviral vector preparations, a valuable source of information can be found on the ISBioTech website [6], which describes the efforts dedicated by the scientific community to develop the Adenovirus Reference Material. This reference material consists of purified

adenovirus, type 5 (wild-type adenovirus, see 'ATCC VR-5') formulated as a sterile liquid in 20 mM Tris, 25 mM NaCl, 2.5% glycerol and pH 8.0 at room temperature, and stored frozen at -70°C [7].

AAV vector purification

There are over 100 different variant (naturally occurring or synthesized) capsid sequences of AAV. However, 12 AAV serotypes [8] isolated from human, simian and rhesus and cynomolgus monkeys have been studied intensively for their surface properties to target-specific organs and tissues. Having different surface properties for various serotypes adds complexity to the general scheme of AAV purification. Surface proteins of different AAV serotypes are different; therefore, the affinity chromatography method is specific to a particular serotype. In theory, IEC and hydrophobic interaction chromatography (HIC) can work for all serotypes but may have different binding and elution characteristics for each serotype. SEC may be utilized, in general, for all of the serotypes as a polishing step because AAV can remain in the exclusion range and elutes first followed by the smaller molecules as contaminants eluting later. Ultracentrifugation is a method that can be used for all serotypes because the virus can be separated on the basis of density, which is similar for all serotypes of AAV. Any of these methods has its advantages and disadvantages for small- or large-scale applications.

AAV is produced intracellularly in mammalian or insect cells. Normally, the cells are separated from the cell culture by low-speed centrifugation or TFF using microfiltration membranes before being resuspended in lysis buffer and treated to release AAV. By extending the harvest time for production, the amount of AAV released in the cell culture supernatant may be significant. In particular for the use of the HEK293-based

Key Term

Hydrophobic interaction chromatography: Chromatographic method in which molecules (e.g., proteins) in solution are separated by their relative hydrophobicity.

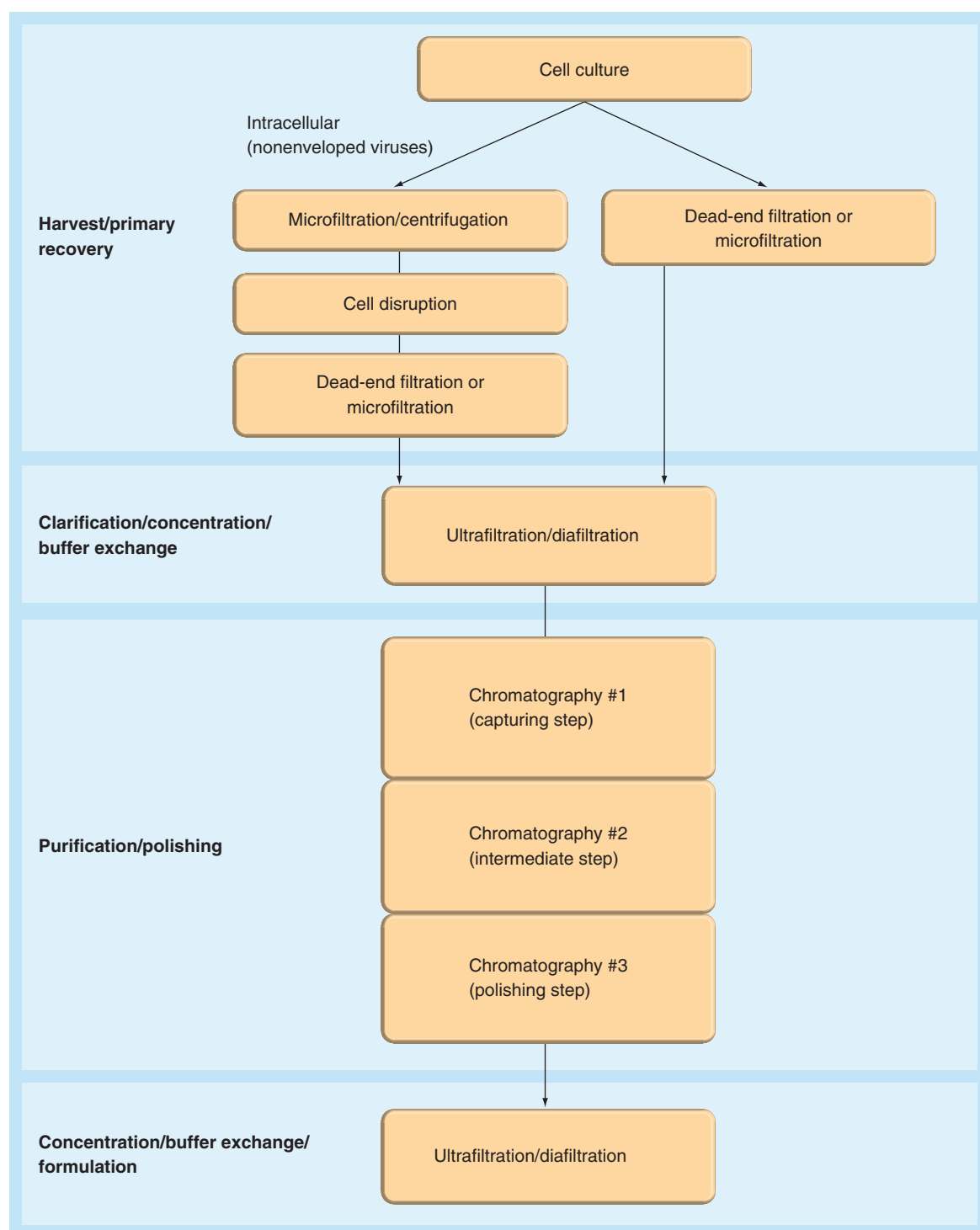


Figure 1. Virus purification for large-scale operations.

production, for several AAV serotypes including AAV1, AAV8 and AAV9, an abundant release of vector particles could be observed already 72 h post-transfection, signifying that the vectors can be directly harvested from the culture supernatant without a cell lysis step [9]. However, in the case of the Sf9/baculovirus system,

no such vector release has been observed [MERTEN O-W *ET AL.*, UNPUBLISHED DATA]. For all other serotypes or AAV production systems other than the transfection system based on the use of HEK293 cells, the whole cell culture is processed instead of the supernatant only. The primary recovery involves cell disruption to release

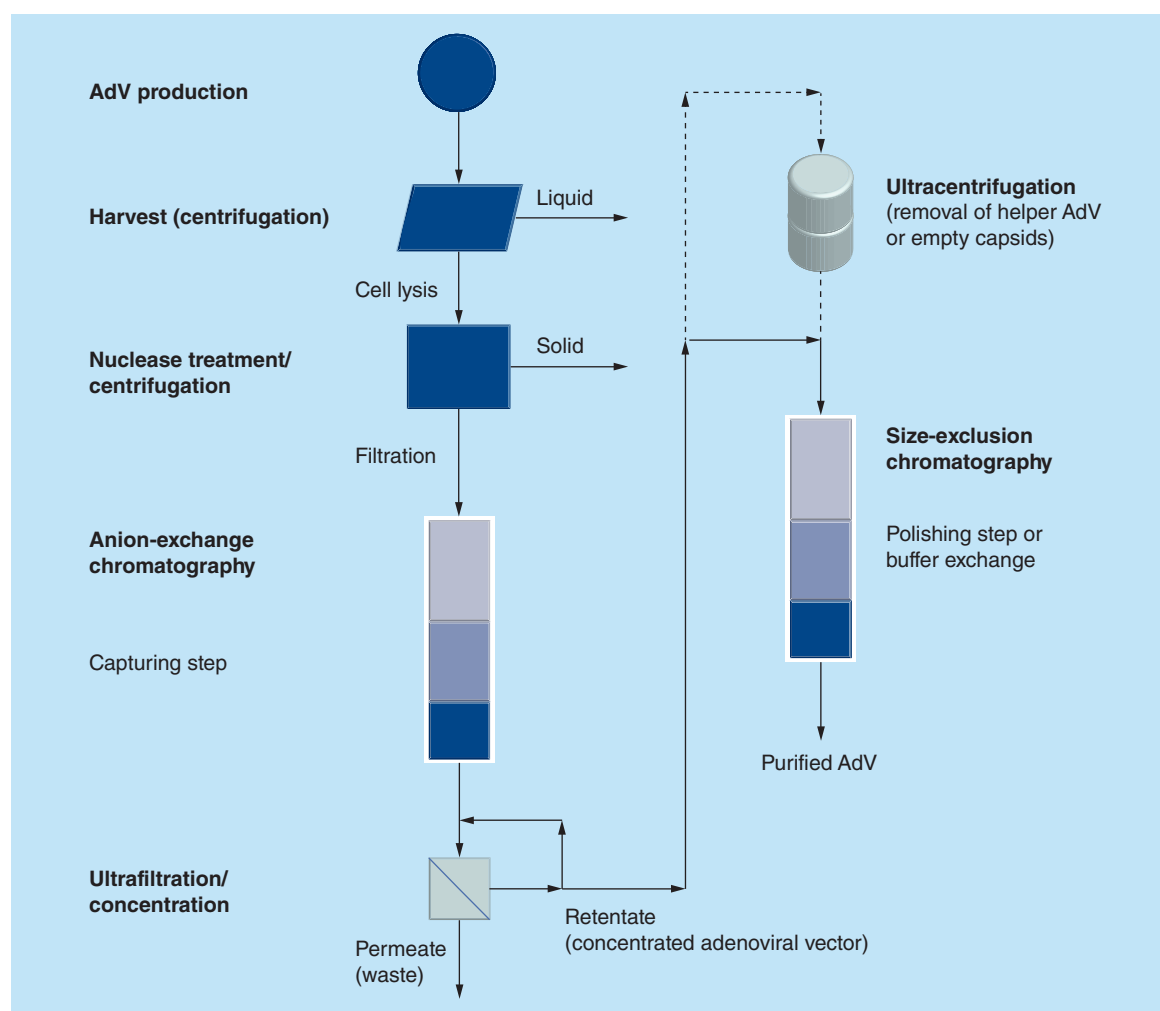


Figure 2. Adenoviral purification.

AdV: Adenoviral vector.

AAV and collection of supernatant containing AAV. Freeze–thaw is a common cell disruption technique that may be used to release AAV from mammalian or insect cells. Although not readily scalable, this technique is convenient at small scale. For large-scale operations, this step may be replaced by microfluidization. Detergents such as Triton X-100 (at 0.1–0.5%) may be effectively used to disrupt the cells instead of microfluidizer or repeated freeze–thaw cycles. Nuclease is usually added to digest the host cellular nuclear material during the extraction of AAV to avoid the formation of nucleic material–AAV complexes and to reduce the viscosity of the lysate.

The purification scheme may be complicated by the presence of helper viruses such as adenovirus or herpes virus if used in mammalian production methods, or baculoviruses if used to provide genomic material to insect cells. Helper, baculoviruses or contaminating viruses may be inactivated by raising the temperature to 56°C for 15 min.

Ultracentrifugation

The method that has been extensively used to purify viruses is based on CsCl isopycnic density-gradient ultracentrifugation. The soluble CsCl salts form a density gradient when spun at high centrifugal force and the virus particles accumulate at similar densities and stay in equilibrium. The virus is thus separated from the contaminants present in the solution. The CsCl density gradient usually takes 24 h per run, further development in ultracentrifugation techniques led to the use of iodixanol instead of CsCl and the centrifugation time was reduced to less than 2 h. If AAV is produced with adenovirus as a helper virus, it is easily separated by CsCl and iodixanol density-gradient ultracentrifugation. The recombinant AAV and the AdV helper band at their buoyant densities of 1.41 and 1.34 g/ml, respectively.

Although the ultracentrifugation method is serotype independent, it remains labor intensive. This method is limited to laboratory-scale production and the avail-

able capacity of laboratory ultracentrifuges. High-capacity ultracentrifuges may be considered for large-scale operation; however, they are associated with high capital and facility investments. To achieve an acceptable level of purity, several rounds of density-gradient ultracentrifugation may be required.

The iodixanol-based ultracentrifugation [10] employs discontinuous density gradients (containing 15, 25, 40 and 60% iodixanol) that include 1 M NaCl in the layer containing 15% iodixanol as an alternative to the CsCl ultracentrifugation technique. The advantage of using the iodixanol technique is less centrifugation time compared with the CsCl method. Figure 3 shows a schematic that has different volumes of each layer to extend the viral vector separation zone. Fractions may be taken to identify the empty versus genome-containing capsids. To reduce the contaminated proteins, the cell lysate may be treated with nuclease (e.g., Benzonase) to avoid nucleic acid–protein aggregation, cell debris may be removed, or viral particles may be precipitated with PEG prior to loading to the gradient [11].

The viral particles can also be purified by sucrose cushion (e.g., using 20% sucrose) that would allow only virus particles being heavier than other proteins to pass through the sucrose cushion while retaining the host cell- and medium-related contaminants. The virus is collected in the pellet at the bottom of the centrifuge tube, which is resuspended in the buffer of choice. Some viruses such as AAV tend to aggregate at higher concentrations; therefore, these situations must be avoided.

Chromatography purification

Chromatography is a well-established scalable process and has been adapted to separate contaminant proteins present in the virus solution based on physicochemical properties such as affinity to a ligand, net charge, hydrophobicity and size. Since AAV has different serotypes, the surface characteristics are different for each type. Here, we describe different methods employed for AAV purification.

Affinity chromatography

AAV2 binds to the cell surface via the heparin sulphate proteoglycan receptor. Similar binding was observed for AAV serotypes 3 and 6. Therefore, heparin-affinity chromatography was successfully applied to purify these serotypes [12]. Similarly, the AAV serotypes 4 and 5 [13] show binding to 2,3-linked sialic acid, therefore mucin-affinity chromatography is employed to purify these serotypes. The binding domains of other serotypes have not yet been identified; therefore, affinity chromatography is limited to only certain serotypes. In general, the heparin purification is carried out by load-

ing on an equilibrated heparin column (e.g., POROS HE/M) at low or no salt buffer. After washing the unbound material, the elution is carried out by continuous or step gradient with 0.5 M NaCl concentration. The eluted fractions containing AAV are pooled. The affinity chromatography based on heparin or mucin usually needs a subsequent polishing step that may be IEC or SEC to obtain a high purity AAV preparation.

Further development of fusing histidine (His₆) or endogenous biotinylation sequences on the capsid surface has led to the development of the metal affinity and avidin affinity chromatography method as a universal method for the purification of different serotypes [14,15]. The concept has been applied with great success to purify AAV serotypes 1, 2, 3, 4, 5 and 8. It remains to be evaluated for all AAV serotypes whether the histidine or biotinylated fusion protein has any detrimental effect in clinical studies due to reduced transduction efficiency or tissue tropism. Monoclonal antibody A20 that was generated against AAV2 has been used in affinity columns [16] that recognize only assembled AAV2 particles, thus it can be successfully used to eliminate unassembled capsid proteins and adenovirus present in cell lysate if used as a helper virus in the production step. AVB Sepharose High Performance medium (GE Healthcare Lifesciences, Little Chalfont, Buckinghamshire, UK), has antibody fragments against AAV from llamas, which are produced in yeast and coupled to *N*-hydroxysuccinimide-activated Sepharose High Performance [17]. This has been shown to purify AAV serotypes 1, 2, 3, 5, 6 and 8.

Peptide-based affinity purification was evaluated for AAV8 vector. Heptapeptide motive GYVSRHP was identified, which selectively recognized AAV8 without any cross-reactivity with other serotypes (evaluated for serotypes 1, 2, 5, 6, and 9) [18], and thus could be valuable alternatives to antibody-based affinity chromatography of AAV vectors.

Affinity chromatography can be easily scalable, but the cost of the resin is high, therefore multiple batches of purifications may be applied. Affinity chromatography would need an extra step to eliminate or reduce the leaked ligand that coelutes with viral vectors. This method is serotype specific and therefore its use is limited to selected serotype purifications.

Ion-exchange chromatography

IEC is a simple and cost-effective technique to purify different serotypes of AAV. The method takes advantage of minor differences in net surface charge properties of viral particles and proteins present in the solution at a given pH. It can be used to separate AdV when present in cell culture. AdV is a negatively charged vector near neutral pH, whereas AAV is charged posi-

tively. Anion or cation exchangers can be used to bind AdV or AAV, respectively. In one method, a POROS 20HE (cation) column (Applied Biosystems, CA, USA) was used to bind AAV but eluted fractions containing AAV coeluted AdV as well. Therefore, a second column POROS 50PI (anion) was added to the process to bind the contaminants and residual AdV present in the eluted fractions from the first column, whereas, AAV remained in the flow through [19]. The buffer concentration and pH along with the salt type and concentration (ionic strength) are evaluated in IEC to bind AAV serotypes effectively to the type of resin selected. A general protocol for IEC using anion or cation resin involves loading at low salt buffer conditions (normally less than 150 mM NaCl) and after washing the unbound proteins, a gradient in step or continuous mode is generated towards higher salt concentration (normally up to 500–1000 mM NaCl). The eluted fractions are identified for AAV and pooled for further purification. One IEC step is not sufficient to purify AAV serotypes. The eluted AAV is further processed by another IEC, HIC or SEC. A slightly higher negative charge due to the presence of DNA in full capsids compared with empty capsids made it possible to utilize IEC to separate empty capsids from the AAV preparation. This method utilized a high-resolution column packed with POROS HQ (anion exchanger of 10 μm matrix) running twice to purify AAV1. The first run eliminated more than 90% of the empty capsids, and the second run further removed empty capsids [20]. Another method first utilized POROS 50HS (cation exchanger) and the eluted material was separated by Q-Sepharose anion exchanger (GE Healthcare Lifesciences) to purify AAV2 vectors [21]. This method was able to partially separate empty capsids of AAV6; therefore, it suggested that separation of empty capsids of different serotypes need to be optimized by selecting different elution conditions, choice of salts and type of resins.

Different workers have used different resins to demonstrate AAV purification by IEC. In the literature, it can be found that Q (most often used) and diethylaminoethanol-based anion exchangers, and S, SP, CM, SO_3^- and cellulose sulphate (also referred as affinity resin)-based cation exchangers have been used to purify not only AAV2, but also serotypes 1, 4, 5 and 6.

Size-exclusion chromatography

Since AAV is a large particle (20 nm, 3.6×10^6 Da) compared with the contaminants present in the cell lysate, SEC can be utilized for its purification. Mainly, it is used as a polishing step where process volume has been significantly reduced from the previous step. This method not only removes small-molecular-weight

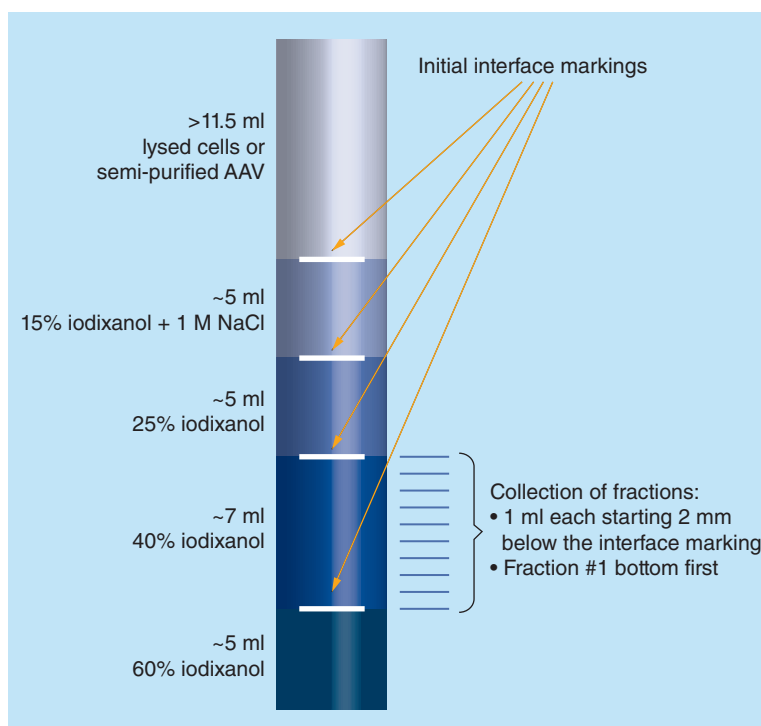


Figure 3. Iodixanol gradient used for the purification of adeno-associated viral vectors.

Different layers of iodixanol consisting of different densities reported by percentage iodixanol concentration in each layer are used. Lines are marked to identify the interfaces of different densities before the start of ultracentrifugation. The 15% iodixanol-containing layer also contained 1 M NaCl to avoid AAV interactions with cellular proteins and nuclear material. AAV: Adeno-associated viral vector.

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contaminants present in the solution, but this gentle process simultaneously gives a high yield and allows a buffer exchange to the final formulation. The size exclusion for globular proteins on Superdex 200 (GE Healthcare Lifesciences) is 1.3×10^6 Da, therefore AAV, regardless of its serotype, elutes in the void volume [17,22]. The proteins that are generally of low molecular weight elute at higher elution volumes. The capacity of the load is optimized based on the separation of AAV and contaminants. It is possible to load as high as 30% column volume for SEC when used as a polishing step in AAV purification scheme.

Hydrophobic interaction chromatography

HIC separates molecules based on the differences in hydrophobicity of proteins and virus particles. A high salt concentration (normally ammonium sulphate) is required to make hydrophobic protein surfaces, thus minimizing their exposure to the solvent and increasing the binding affinity towards the resin. The elution to a lower salt concentration results in separation from other contaminants. This step is usually added after the IEC step where the eluted material is already at a

Key Terms

γ -retroviral vector: Viral vector based on mouse leukemia virus (a member of the enveloped retrovirus family, which is composed of medium-sized [80–120 nm] enveloped viruses, with two ssRNA genomes). This vector is integrative.

high ionic strength. The use of Toyopearl Butyl 650 M (Tosoh Biosciences, Stuttgart, Germany) has been used for the purification of AAV2 [23]. The use of HIC to purify different serotypes of AAV has not yet been documented.

Further purification steps

Tangential flow filtration

TFF may be employed before any chromatography steps. It can be used efficiently for buffer exchange between different steps and concentration to reduce operation volumes. Normally, a >500 K molecular weight cut-off (or microfiltration membranes) can be used to remove cellular debris, whereas a 100 K molecular weight cut-off [24] can be used to retain AAV to concentrate and remove low-molecular-weight contaminants.

Other considerations for the purification steps

Recombinant AAV tends to form aggregates in the cell lysate during primary recovery from cell paste. This results in reduced recovery during the filtration step of clarification of cell lysate. Addition of 150 mM NaCl or 37.5 mM MgSO₄ increases the ionic strength sufficiently to avoid binding to cell debris or other cellular proteins released after cell lysis [23]. In the case of AAV2 vectors, it could be shown that isotonic formulation with elevated ionic strength (e.g., use of citrate buffer instead of a buffer system based on monovalent salts) could prevent aggregation during concentration and long-term storage [25].

Downstream processing of retroviral vectors

Due to the relatively fragile nature of retroviral vectors, the development and set up of downstream processing protocols is much less straightforward than for adenoviral and AAV vectors. Whereas at a research level, purification protocols based on different purification schemes including chromatography and membrane-based methods have been developed for γ -retro- and lenti-viral (LV) vectors [26], such protocols have only found application for manufacturing of LV vectors because, in general, they are generated via tri- or quadri-transfection of HEK293 or HEK293T cells (for details, see part I of this article [27]) requiring several purification steps for removing residual plasmids. Since murine leukemia virus (MLV)-based retroviral vectors are generated using stable producer cell lines and they have been mostly used for thera-

peutic *ex vivo* applications, the requirement for a high-performing purification protocol was considered of lower importance. In addition, no real scalable purification schemes of gibbon ape leukemia virus (GaLV) or amphotropic-pseudotyped γ -retrovirals have been developed mainly due to relative fragility of these vectors (e.g., towards shear stress). Thus, no real purification protocols have been set up for the manufacturing of clinical γ -retroviral vector lots.

In the following, downstream protocols traditionally used for the purification of γ -retroviral and LV vectors are briefly presented.

'Purification' of MLV vectors

As above mentioned, in general, MLV vector preparations have not been specifically purified for clinical *ex vivo* applications because these vectors are generated using stable vector producer cell lines. The only key step consisted of a validated filtration/clarification step (e.g., Reeves and Cornetta [28]) allowing the removal of eventually detached producer cell lines. Since continuous cell lines of more or less tumorigenicity (e.g., CRIP, PG13, HEK293 based; for more details, see Stacey and Merten [29]) are used for vector production, this clarification has to be efficient for removing any eventually present producer cell. In addition, cell debris and other particulate material have also got to be removed.

Different groups have approached this essential point differently. Whereas, Reeves and Cornetta [28] used a filtration cascade consisting of dual inline filters (40/150 μ m) followed by a 20 μ m filter and at the end a Sepacell filter (a depth filter; Asahi Kasai Medical, Tokyo, Japan), Eckert *et al.* [30] used a 0.45 μ m filter. The filtration cascade used by Reeves and Cornetta [28] was validated for the retention of producer cells and used for the filtration of clinical batches by Przybyłowski *et al.* [31].

A further option to reduce the presence of serum- and/or medium-derived contaminants is the use of low-serum or serum-free media for the production phase after termination of biomass generation allowing thus the reduction of medium-derived protein contaminants in the vector preparation. This approach was used by Eckert *et al.* [30], Schilz *et al.* [32] or Wikström *et al.* [33].

However, it should be kept in mind, that in the case that MLV vectors are to be used for *in vivo* applications, real downstream processing protocols have to be set up because any contaminant having a potentially negative effect on the treated person has to be removed. In this context, it could be shown, for instance, for LV vectors, that crude or only centrifuged (concentrated and only partially purified) preparation can generate an immune response [34], in addition, such vector preparations are generally less efficient than purified preparations [34–36]. In general, such protocols have been developed and tested at a

research level for the purification of MLV vectors (e.g., Segura *et al.* [26]) and, generally, they are very similar to those used for the purification of LV vectors at a large scale (see below).

Purification of LV vectors

As described in part I of this paper [27], LV vectors are produced with transfection methods, thus a downstream processing protocol is required in order to get rid of contaminating DNA (derived from the plasmids but also from the lysed producer cells), and foreign serum- and producer cell-derived proteins.

The presently used LV vectors are essentially pseudotyped with the glycoprotein of the vesicular stomatitis virus (VSV-G) envelop protein, which renders the vector rather resistant to shear and centrifugation stress. Thus, traditional small-scale purification methods are often based on ultracentrifugation of vector supernatant. Vector particles are both pelleted and then resuspended in a small volume allowing over 100-fold concentration. Although high vector concentrations can be achieved, long process times can lead to partial vector inactivation and these purification methods cannot be scaled up to large-scale processes. In addition, such methods do not lead to pure vector preparations and cell- and culture medium-derived contaminants can be co-concentrated potentially leading to adverse effects or reduced vector efficacy when such preparations are used *in vivo* [34,35]. Thus, purification methods are required for removing any contaminants of potential adverse effects when the vector preparations are used *in vivo*. In addition, in view of clinical and industrial applications, such purification methods should be scalable. In most of the cases, process steps traditionally used in the biotechnology industry have been developed and put together for getting to a highly performing downstream processing protocol, including filtration/clarification, IEC, concentration/diafiltration using TFF, DNase step for the degradation of residual DNA, a polishing step (often SEC) and final sterile filtration [37].

VSV-G pseudotyped LV vectors are purified using anion-exchange chromatography, whereby large-scale purification protocols make use of classical low-pressure column chromatography [38] or membrane-based IEC [39,40]. Such membrane cartridges (e.g., Mustang Q) (PALL Corp., NY, USA) allow the purification of up to 1500 l/d when used as the first concentration step in a protocol [39]. In general, the vectors are eluted by a linear [41] or step salt gradient [38]. Scale-up is straightforward, leading to pure vectors (e.g., removal of 99.9 and 98.9% of contaminating protein and DNA, respectively [38]), but the vector recovery is not as efficient as for ultracentrifugation.

In order to concentrate the bulk product (supernatant or an intermediate product), TFF systems are employed allowing also buffer exchange (diafiltration). At a small scale or at later stages of an industrial downstream processing protocol, centrifuge-based disposable devices [41,42] and at a larger scale, hollow fiber or flat membrane cartridges are used [38–39,43–44]. TFF devices have also been applied for the concentration and diafiltration of LV vectors pseudotyped with other envelope proteins than VSV-G [45,46]. However, this has only been performed at small scale. The molecular weight cut-off of the membranes can be as high as 750 kDa (which is equivalent to 50 nm), due to the size of the vector particle ranging from 100 to 120 nm.

Due to the low volume ratio (volume of the preparation to be treated/volume of the SEC column), SEC is generally employed as the polishing as well as formulation step at the end of the downstream processing protocol. It leads to an efficient removal of all contaminants smaller than the pore size of the chromatographic matrix, thus to a further and efficient purification. Ideally, the pore size is chosen to exclude only the viral vector particles (e.g., pore size > 500 kDa) and to allow the retention of all contaminants as well as buffer components. In addition, the use of modern 'multimodal' supports, such as the Capto Core 700 support from GE Healthcare Lifesciences, adds to the traditional size exclusion step an anion-exchange chromatography step allowing a further considerable reduction of DNA contamination. The major drawback of SEC is the dilution of the vector preparation by a factor of at least 3. Most of the actual large-scale purification protocols have adopted SEC as a final polishing step [38–39,41].

A supplementary process step is a DNase (Benzonase or Pulmozyme® [Genentech, CA, USA]) treatment during the upstream or often downstream processing phase for removing cellular and plasmid-derived DNA contaminants. This step increases the overall biosafety feature of the process because the reduction of the size of cellular or plasmid DNA leads to a reduced risk of transfer of oncogene sequences to the target cells. Such a DNA degradation step has been implemented for all large-scale downstream processes.

Other process steps include clarification after harvesting (a single 0.45 µm filtration step [44] [as for the preparation of MLV vector preparations]) or a cascade of membranes with different pore sizes [47] going for instance from 0.8 over 0.65 to 0.45 µm and in most of the cases a final sterile filtration of the vector preparation before further use.

As already mentioned, recently developed large-scale purification protocols make use of chromatographic

and membrane-based purification steps. Generally, after clarification by microfiltration, vector containing supernatant is chromatographed using anion-exchange chromatography, followed by a concentration/diafiltration step and eventually a SEC step. Different protocols have inserted a Benzonase step either before or after the anion-exchange chromatography step. Sterile filtration is in most of the cases performed before fill and finish [37].

Table 2 presents downstream processing protocols used for the large-scale purification of vector preparations for clinical applications. As far as published, the performances are as follows: the concentration factor is ranging between ten- and 50-fold and the overall process yields range from 16 to 40% for the protocols, making use of an IEC step. The final vector concentrations are comparable and are ranging from 1 to 2×10^9 infective particles/ml when applied for *ex vivo* gene therapy purposes [37]. In the case of *in vivo* administration (for the treatment of the CNS or the retina) concentrations beyond 10^{11} RNA copies per ml are required [40]. For more detailed information on the overall purification of γ -retroviral and LV vectors as well as isolated purification steps (different types of chromatography, membrane-based purification principles and so on), the reader is referred to the reviews by Segura *et al.* [26] and Rodrigues *et al.* [48], and with respect to details on downstream processing of LV vectors at a large scale to Schweizer and Merten [37].

Biosafety of viral vectors

The use of viral vectors for treatment of human patients bears a number of vector-specific health risks. A major safety issue is due to the derivation of several viral vectors from human pathogens such as adenovirus, herpes virus, poxvirus and measles virus, or even from ebola virus or lentiviruses such as HIV-1 or -2. Accordingly, the occurrence of replication-competent parental virus in vector preparations has to be strictly avoided to prevent virus-specific pathogenicity. Most vectors are designed to be replication-incompetent by deletion of viral genes required for virus replication. Ideally, the vector particle does contain exclusively the gene coding for the transgene, which is transferred into the target cell and expressed there. In theory, this is realized in retroviral (including LV) vector systems, since only the transfer vector RNA carries the signal required for packaging into vector particles. However, since the vector producer cells carry all viral genes coding for the components of the vector particle, unintended recombination events may occur, which, in the worst case, may result in formation of replication-competent infectious parental virus released from the producer cells. For retroviral vectors, this problem appears to be solved, since modern split-packaging systems (where the viral genes are separated on different plasmids without sequence overlaps) are used, where the probability of recombination events is extremely low. Nevertheless, the occurrence of replication-competent retroviruses (RCRs) or lentivi-

Table 2. Principle process steps of large-scale downstream processing protocols for the purification of VSV-g (glycoprotein of the vesicular stomatitis virus) pseudotyped lentiviral vectors (for clinical purposes).

Company/ institution	Process steps in chronological order						Ref.
VIRxSYS (MD, USA) [†]	Filtration [‡]	Q IEC chromatography (capsule)	Concentration/diafiltration	DNase [§]	Diafiltration	Sterile filtration [‡]	[39]
Oxford Biomedica (Oxford, UK) [†]	Clarification	DNase [§]	Anion EX membrane chromatography	Concentration/diafiltration	Sterile filtration	Aseptic hollow-fiber ultrafiltration/concentration	[40]
Généthon (Évry, France) [†]	Clarification (0.45 μ m)	DNase [§]	DEAE-IEC chromatography	Concentration/diafiltration	SEC (formulation)	Sterile filtration	[38]
Beckman Research Institute (CA, USA)	Clarification (0.45 μ m)	DNase [§]	Ultrafiltration/diafiltration (500 kDa)	High-speed centrifugation	Resuspension and removal of particulate material	No sterile filtration (semi-closed process)	[44]
Bluebird Bio (MA, USA) [†]	Clarification [‡]	IEC chromatography [‡]	Concentration/diafiltration [‡]	Sterile filtration [‡]			[49]

[†]These companies/institutions use an IEC chromatography step in their downstream processing protocol.
[‡]No details are available on these process steps (e.g., with respect to the clarification step, the pore size/exclusion size was not communicated).
[§]For the schemes presented, Benzonase™ (EM Science, NJ, USA) was chosen.
 DEAE: Diethylaminoethanol; EX: Exchange; IEC: Ion-exchange chromatography; SEC: Size-exclusion chromatography.

ruses (RCLs) has to be monitored very carefully using methods with high sensitivity at different levels of the manufacturing process, for example, in the master and working cell banks, the end-of-production cells and of course in the vector preparation itself. The most sensitive methods for RCR or RCL testing are cell culture based, where the test article is co-cultured with a cell line that is highly permissive for the respective potential RCRs or RCLs [50,51]. The same is true for all vector types derived from other viruses. For adenoviral vectors, prevention of replication-competent adenoviruses is more challenging due to the greater genome length and since the vector particles usually contain a number of viral genes in addition to the transgene, thus enhancing the recombination risk. Although a low number of replication-competent adenoviruses may be acceptable for some clinical applications due to the relative mild pathogenicity of adenoviruses, further efforts are ongoing to enhance vector safety by successive deletion of more viral genes, minimizing sequence overlaps and development of enhanced packaging cell lines complementing for viral gene functions. Remarkably, the possibility of recombination between vector sequences and endogenous or exogenous viruses, for example, after superinfection of transduced cells, has also to be taken into account in addition to recombination events between vector components.

In rare cases, replication-competent viral vectors are used, since gene transfer efficiency is significantly higher compared with a one-step infection by replication-incompetent vectors. Mainly in tumor therapy, so-called 'conditionally replicating viruses' are used, which are capable for exclusive replication in the specific target tissue, for example, in tumors [52]. This attenuation is often achieved by deletion of a pathogenicity factor and has to be demonstrated not only during product characterization, but also in batch release.

Another vector-specific safety concern is the induction of immune reactions against vector components. This risk came apparent when the first person died on a human gene therapy trial. The patient, Jesse Gelsinger, had been treated with a high dose of an adenoviral vector infused into the liver [53]. The reason for his death was very probably a severe immune response leading to failure of the lung and other organs. Again, in further vector developments, the deletion of more viral genes, which are transferred by the vector, will lower the risk of immune reactions and contribute to vector safety. As for most medicinal products, release testing for clinical grade vectors also includes sterility testing, endotoxin testing and testing for adventitious viruses.

Beside the viral genes, the therapeutic gene may reveal toxicity, which often can be minimized by optimization of the vector design. For example, many vectors are capable of gene transfer into a wide range of target cell types, which may lead to transgene expression in unintended tissues. The development of targeting vectors, which reveal a narrowed target cell spectrum, for example, by modification of the viral envelope protein responsible for binding to the target cells [54], will also enhance safety in gene therapy. For example, an AAV vector that was modified to target exclusively Her2-expressing cells and carried a suicide gene efficiently inhibited tumor growth in mice, whereas treatment with an AAV wild-type vector carrying the same transgene resulted in heavy liver failure [55]. Other approaches comprise the use of tissue-specific promoters allowing gene expression exclusively in the intended target cell population.

The most prominent vector-specific safety issue is the oncogenic potential of integrating viral vectors, which is due to integration of retroviral vector DNA into the target cell chromosome. Integration may lead to destruction of cellular genes or to alteration of expression of genes adjacent to the integration site and result in oncogene activation or inactivation of tumor suppressor genes. Unfortunately, this risk did realize in very promising human gene therapy trials, when patients were treated for severe combined immunodeficiency (SCID-X1), a monogenic inherited disease caused by mutation of the gene coding for a component of the IL-2 receptor, resulting in failure of T cells and natural killer cells to differentiate [56]. After introduction of the correct version of this gene into their bone marrow cells using a γ -retroviral vector, a dramatic clinical improvement could be observed since the patients' immune system was almost completely restored. However, five out of 20 patients treated were diagnosed with leukemia due to γ -retroviral vector-mediated **insertional mutagenesis**. In all cases, overexpression of a proto-oncogene adjacent to the integrated vector was observed. Similar cases of insertional mutagenesis occurred in clinical trials for treatment of chronic granulomatous disease or Wiskott-Aldrich syndrome [57].

To increase the safety of retroviral vectors, a number of strategies are under development. The most important one is the development of self-inactivating vectors [58]. Self-inactivating transfer vectors encompass an

Key Term

Insertional mutagenesis: Mutation caused by the insertion of new genetic material into a normal gene, a potential problem related to the use of integrative viral vectors.

intact 5' LTR, whereas an internal deletion is introduced into the promoter/enhancer region of the 3' LTR. As the 3' LTR is used as the template for reverse transcription, the resulting viral DNA will encompass the inactive 3' LTR on both ends. Thus, no functional retroviral LTR promoter is integrated into the host cell DNA and cannot enhance the transcription of adjacent cellular genes, whereas the transgene is expressed by an internal heterologous promoter. Another strategy to prevent modulation of the expression of cellular genes flanking the integrated vector is the introduction of so-called 'insulator sequences' [59] into the transfer vector.

The maximal vector copy number is a critical safety parameter because a higher number is directly associated with a higher risk for vector integration into a 'sensible' gene (e.g., the promoter region of an oncogene or a gene associated with proliferation). The broadly accepted rule is that oncogenic transformation of primary cells requires up to six independent but cooperating genetic lesions [60,61], which seems to be relatively elevated; however, the risk of multiple insertions can be estimated using Poisson distribution [62]: a mean vector copy number of one (transduction efficiency: ~63%) still means that approximately 41% of the transduced cells contain between two and five vector copies per cell. In addition, it could be shown for three published leukemia cases related to insertional mutagenesis [63,64] that a single insertion was present in the target cells, which led to the activation of proto-oncogenes.

This clearly indicates that for safety reasons the mean vector copy number should be as low as possible, which is typically in the range of 1–2 copies per target cell. In *ex vivo* gene transfer, the copy number can be influenced by the transduction conditions, mainly by the amount of vectors per target cells used.

Furthermore, great efforts are being carried out to develop retroviral vectors with the capacity for site-specific integration [65]. The possibility to target retroviral vector integration to intended safe sites within the host genome, which is hopefully reached in the near future, would significantly enhance the safety of these vectors.

To avoid insertional mutagenesis, LV vectors have been generated, which are integration-deficient due to a modification of the integrase gene [66]. The genomes of such vectors persist extrachromosomally as episomes, which can serve as a template for efficient gene expression. Nevertheless, whereas the risk of insertional mutagenesis is minimized, these vectors lost the possibility for long-term gene expression since stable integration of the transgene is lacking. They can only be used for transient gene expression in mitotic cells or long-term gene expression in some quiescent cells,

until future developments, such as the development of replicating LV episomes, will allow maintenance also in dividing cells.

Conclusion & future perspective

With the development and the marketing authorization of the first gene therapy vector for the treatment of a rare disease, downstream processing of viral vectors has practically reached the same level with respect to process technology as that of other more traditional biological products such as recombinant proteins. Although not all process steps, in particular those that are standard wise used for the inactivation and/or removal of potential adventitious viruses, can be implemented (as for enveloped vectors) or can only be partially implemented (as for adenoviral or AAV vectors) for the purification of viral vectors, as is also the case for the purification of live-attenuated viral vaccines, the process standards as well as associated quality control have to be the highest possible level; and up now, no adverse or severe adverse events have been observed due to insufficient quality of the transferred viral vector. Although several types of viral vectors, in particular, enveloped viral vectors pseudotyped with envelope proteins different from the VSV-G protein, such as RD114-TR or GALV-TR pseudotyped LV vectors, cannot be purified for the moment, the development of novel purification devices, including new chromatographic supports, ligands or membrane materials, as well as the use of more adapted purification conditions in combination with stabilizing additives will pave the way towards the large-scale manufacturing of these vectors and their clinical use (for *ex vivo* as well as *in vivo* applications). This concerns also biosafety issues specific for the use of viral vectors. Based on knowledge gathered in the last 10–15 years, the use of viral vectors (*in vivo* and *ex vivo*) becomes safer due to the improvement of vector constructs, use of advanced vector generations and so on. Based on these achievements, future developments and improvements in viral vector technology, the use of viral vectors will become a standard medication for rare and more frequent diseases.

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Executive summary**Viral vector purification: downstream processing**

- The review presents an update on the present situation of purification of viral vectors for gene therapy purposes; detailing the essential differences between adenoviral, adeno-associated viral, γ -retroviral and lentiviral vectors and actual downstream processing methods used.
- Due to their protein structure and thus their improved stability, adenoviral and adeno-associated viral vectors can be relatively easily purified.
- The development of purification protocols for enveloped viral vectors (retroviral vectors in a larger sense) is more difficult due to their increased sensitivity to adverse environmental conditions, including shear stress, extreme pH values and so on.
- For lentiviral vectors, only glycoprotein of the vesicular stomatitis virus pseudotyped vectors have been developed and are presently used in the clinics.

Biosafety of viral vectors

- Based on better understanding of vector integration and immune reactions, but also due to improvements in vector constructions and also improved purification protocols, the biosafety of the different viral vectors has significantly improved over recent years.
- Since this is an ongoing development, biosafety of viral vectors will further improve in the future.

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