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CONFERENCE PAPER

Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications

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In recent years, recombinant adenoviral and adeno-associated viral (AAV) vectors have been exploited in a number of gene delivery approaches. The use of these vectors in clinical gene transfer has increased the demand for their characterization, production and purification. Although the classical method of adenovirus or AAV purification by density gradient centrifugation is effective on a small scale, chromatographic separation is the most versatile and powerful method for large-scale production of recombinant adenovirus or AAV. This review describes different chromatographic modes for adenovirus or AAV purification and process development, as well as the utility of different purification steps for virus production. Advances in the development of viral vectors for gene therapy, such as the discovery of new AAV serotypes, adenoviral and AAV retargeting and improved production of helper-dependent adenoviral vectors, require further development of efficient purification methods.
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Adenoviruses and adeno-associated viruses as vectors for in vivo gene transfer

Gene therapy aims to treat both genetic and infectious diseases via introduction of new genetic material into the appropriate cells in the body. One of the greatest challenges of gene therapy is efficient transfer of genetic material into living cells. Use of recombinant viral vectors for gene delivery significantly improved this process. Two commonly used viral vectors capable of direct *in vivo* gene transfer are derived from adenovirus (Ad) and adeno-associated virus (AAV). The majority of the 987 worldwide gene therapy trials use viral vectors, 256 use Ad vectors (25.9%) and 25 use AAV vectors (2.5%).[1]

Human Ad comprise a group of double-stranded linear DNA viruses with broad tropism for a variety of vertebrate species. The molecular weight of the virus particle is approximately 2×10^8 Da with a diameter of 70–90 nm.[2] Ad contains an icosahedral capsid consisting of 252 protein subunits. Host cell transduction by Ad types 2 and 5 involves cell attachment mediated by the viral fiber protein binding to its high-affinity receptor (coxsackie-adenovirus receptor (CAR)).[3] Cell entry is then facilitated by association of the viral penton base protein with αvβ3 or αvβ5 integrin coreceptors. Approximately 50 serotypes of human Ad have been identified, but recombinant adenoviral (rAd) vectors for clinical applications are mainly derived from serotypes 2 and 5. This review describes production and purification of rAd derived from serotype 5. Serotypes 2 and 5 are associated with mild respiratory disorders, making them the first gene therapy vectors tested for the treatment of cystic fibrosis. The proportion of clinical use of rAd is second only to retroviral vectors.[1] Ad are highly advantageous for clinical applications that require transient transgene expression at a high level. The transient nature of rAd expression enduring up to several weeks is due to a combination of innate and adaptive immune host defenses deployed against the virus (reviewed by St George[4]). Ad facilitate local or systemic gene delivery and transduce dividing or nondividing cells of different lineages. Unlike retroviral and AAV vectors, the rAd genome is capable of incorporating large exogenous DNA fragments, but does not integrate into the host chromosome, providing an important safety advantage. Modern manufacturing methods allow for production of pure and concentrated Ad stocks required for human gene therapy.[4–6]

AAV is a small, nonenveloped, icosahedral virus 20–26 nm in diameter and with a linear single-stranded DNA genome of 4.7–6 kb.[7,8] Recombinant adeno-associated viral (rAAV) vectors as a class appear to be the most suitable for applications where persistent transgene expression is essential for achieving a therapeutic goal.[9,10] They have a broad host and cell lineage infectivity range for dividing and nondividing cells and are relatively nontoxic. rAAV have a remarkable safety profile; they have not been implicated as the etiological agent for any known disease in human, simian or other species. AAV vectors appear to elicit limited immune and inflammatory responses because of their limited ability to transduce dendritic cells[11] and because all viral

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genes are deleted from the rAAV genome. Nevertheless, development of neutralizing antibodies against rAAV capsid proteins partially limits clinical applications for rAAV vectors. Efficient *in vivo* gene transfer with rAAV requires an estimated multiplicity of infection between 10^3 and 10^5 rAAV vector particles per cell depending on the targeted cell type. A clinical dose in human comprises 10^{12} to 10^{14} rAAV vector particles depending on the required level of therapeutic protein expression.[12] Therefore, efficient scalable purification and concentration methods must be developed for production of high-titer and pure rAAV vectors that allow for adequate transgene expression with minimal immunogenicity.

The development of rAd packaging systems utilizing molecular cloning and DNA recombination in complementary producer cell lines preceded implementation of the rAAV vector technology. Advanced rAd production methods provided a platform for efficient realization of the rAAV technology. Helper Ad have been utilized to render host cells permissive during development of the early rAAV packaging systems.[13–15] Subsequently, more advanced helper-free rAAV production methods have been established.[16] The first attempts to purify rAAV vectors were based on a previously developed Ad purification method using a cesium chloride density gradient. More advanced rAAV purification approaches depend on the unique AAV properties such as specific affinity for target cell receptors. Ad and AAV are both nonenveloped DNA viruses with a protein capsid. Structural similarities shared by both viruses have facilitated parallel development of modern chromatographic purification methods for these viruses, although different chromatographic media and different separation strategies must be utilized for Ad and AAV. Purification and concentration of recombinant viral vectors present a number of challenges associated with the unique characteristics of virions including particle size and shape, physico-chemical properties, stability and binding to their receptors. The nature of contaminating substances reflecting the choice of virus production method must also be considered while choosing the purification strategy.

The initial Ad purification principle exploited the estimated density of hydrated Ad particles, which allowed for viral purification by density gradient ultracentrifugation. The main advantage of this method is efficient separation of the recombinant viral particles from defective virions and cellular debris. More advanced chromatographic Ad purification methods have since been developed. The ion-exchange-based approach takes advantage of retention of Ad particles, which have an acidic p*I* at physiological conditions, on an anion-exchange resin.[17–19] Efficient binding of Ad particles to charged divalent zinc ions has been exploited for developing Ad purification methods using immobilized metal affinity chromatography (IMAC). Importantly, the large size of Ad particles results in physical trapping of the virus in a column resin, becoming the source of significant viral loss during purification by some chromatographic methods. Since the Ad particles are considerably larger than protein components of the cell lysate, they can be isolated by size-exclusion chromatography. The relative fragility of the Ad particles must also be taken into account while choosing a chromatographic purification protocol. Rapid changes in ionic strength, as well as exposure to a pH greater than 8.0, can result in a conformational change associated with loss of activity, aggregation or disruption of viral particles. In some purification schemes, degradation of the virus due to osmotic shock was also observed.[18]

The rAAV is the smallest known particulate gene delivery vector. Unlike Ad particles, AAV virions are relatively stable to heat, nonionic detergents and proteolytic enzymes, making these viruses good candidates for efficient purification by chromatography. The charge of exposed protein moieties on the viral capsid allows purification by binding to ion-exchange chromatographic resins. Productive cell infection with wild-type AAV requires superinfection with a helper Ad or herpesvirus. In some rAAV vector production schemes, helper virus contamination presents an important safety issue for clinical gene delivery. Significant difference in the estimated densities of hydrated Ad and AAV particles (1.34 and 1.41 g/ml, respectively) allows for a separation of rAAV from a contaminating helper Ad by cesium chloride density centrifugation. However, this method may not completely eliminate Ad and is not suitable for the manufacturing scale. Relative AAV resistance to high temperature has been used for heat inactivation of the helper Ad prior to centrifugation. Aggregation of the rAAV particles with cellular proteins in the presence of cesium chloride presents further complications for this purification method, although another density separation medium, iodixanol, has been used successfully. Host cell tropism of the different AAV serotypes is defined by the structural determinants of viral capsid proteins. AAV particles attain their host cell attachment via specific receptor binding, whereas coreceptor recognition facilitates cell entry. Interaction of different AAV serotypes with cellular receptors has been exploited for developing rAAV purification methods by affinity chromatography.[20,21]

In this manuscript, we review different purification methods that have been originally implemented in different laboratories and their utility in producing large amounts of pure and concentrated viruses.

Adenovirus and adeno-associated virus packaging systems

In addition to the properties of the viral particle, the optimal virus purification strategy is determined by virus packaging methods, the producer cell type and cell culture conditions. Primary cell lysates contain variable amounts and composition of contaminants derived from packaging cells, culture medium and helper virus, as well as various chemical and biological additives such as detergents and endonucleases. Importantly, the virus production system must be compatible with the subsequent purification and recovery steps. Some of the virus production methods yield small amounts of recombinant virus. Other methods, although efficient, are difficult to scale up for commercial and clinical applications. Nonetheless, many small-scale purification schemes have been used as a foundation for developing more efficient and economical purification strategies on the industrial scale.

Generation of rAd vectors is accomplished by replacing the viral E1 and/or E3 genes with a transgene

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expression cassette. Construction of rAd genome can be achieved via homologous or Cre-mediated recombination in mammalian cells, or via reconstruction of full-length viral genome in bacteria (reviewed by Danthinne and Imperiale).[6] Lack of the viral E1 gene product renders rAd replication-deficient. Therefore, amplification of rAd particles is only possible in stable producer cell lines containing an integrated E1 transgene. The original versions of producer cell lines, such as the well-known HEK293 cells,[22] contain an E1 genomic region with significant sequence homology to Ad DNA. Thus, rAd packaging in HEK293 cells can lead to the occurrence of replication-competent adenovirus (RCA) via integration of the E1 gene into the recombinant virus genome via homologous DNA recombination. Contamination with RCA is unacceptable for clinical applications and must be avoided during production steps. In order to minimize the RCA occurrence, E1 complementing cell lines of the next generation, such as PER.C6 human embryonic retinoblasts,[23] N52.E6 human amniocytes[24] or HeLa-derived GH329 cells,[25] were established. These cells contain a minimal E1 gene fragment with no sequence overlap with the rAd genome.

Animal and human experimentation has revealed the main disadvantages of E1/E3-deleted rAd of the first generation. Namely, host infection with these vectors induces a robust cellular and humoral immune response resulting in significant inflammation at the infection site, elevated cytokine production and reduction in the level and duration of transgene expression. In addition, Ad of the first generation can only accommodate relatively small transgene expression cassettes. In order to correct both problems, rAd vectors of the second and third generations were developed by deleting additional viral genomic regions (reviewed by Volpers and Kochanek).[5] Helper-dependent adenoviral (HDAd) vectors of the third generation have all viral coding sequences deleted with the exception of the minimal *cis*-acting sequences required for virus propagation, inverted terminal repeats (ITR) and the packaging signal. These vectors are also called high-capacity Ad owing to their ability to accommodate up to 36 kb of exogenous DNA. HDAd have minimal toxicity and immunogenicity and high-level, long-term transgene expression *in vivo* due to the lack of viral gene expression.[26] HDAd are produced with a replication-deficient helper Ad providing the missing viral functions in E1 complementing cell lines. The approaches for reducing the fraction of contaminating helper virus include construction of helper viruses with impaired packaging function,[27] or recombinase-mediated excision of a packaging signal from the helper genome.[28,29] Another promising strategy for efficient production of helper-free HDAd vectors includes generation of stable packaging cell lines supplementing helper viral functions. Successful production of such cell lines has not been reported to date.

Three principal components are required for the production of an rAAV vector in cultured cells: an AAV vector genome containing a transgene expression cassette flanked by the *cis*-acting ITR sequences, AAV Rep–Cap proteins and a helper virus. The genes encoding AAV Rep and Cap proteins are usually provided in *trans* on a separate plasmid. Cell lines typically used for AAV production include HeLa or 293 cells[22] owing to their high transfection efficiency.

Standard protocols for helper virus-dependent generation of AAV vectors involve cell cotransfection with an rAAV genomic plasmid and a Rep–Cap complementing plasmid. In order to avoid formation of wild-type virus or replication-competent virus via homologous recombination between both plasmids, they do not share any identical DNA sequence.[30,31] Transfected cells must also be infected with a helper Ad or herpesvirus necessary for AAV replication and packaging. Alternatively, the minimal viral genes required for the helper function can be supplemented on a separate plasmid.[16,32,33] In the latter case, the rAAV production system requires simultaneous transfection with three plasmids. Since helper virus is not necessary for rAAV packaging via triple plasmid transfection, this system offers significant safety advantages for clinical use. However, efficient triple transfection is more laborious and difficult to achieve on a large scale compared to other rAAV packaging methods.

An alternative procedure to plasmid transfection is based on the use of packaging cell lines containing integrated Rep–Cap gene sequences, the rAAV vector genome or both.[34–36] Generation of stable packaging cell lines expressing high levels of Rep and Cap proteins is complicated by the well-documented strong cytostatic effect of the AAV Rep protein.[37,38] Stable packaging cell lines were created from HeLa cells by integration of the native AAV genome including a Rep–Cap gene cassette, but without both ITR sequences.[34,39,40] Induction of Rep and Cap proteins expression from a naturally silent native AAV promoter requires Ad E1 protein provided in *trans* from a helper virus.[39] Alternatively, Rep–Cap protein expression can be driven by a heterologous inducible promoter.[41] Some of the more advanced producer cell lines containing integrated Rep–Cap genes as well as AAV vector sequence do not require DNA transfection for the production of the rAAV. Infection of these cells with the helper virus renders them permissive.[39] Additional purification steps are required to obliterate the contaminating helper virus, complicating industrial applications of this packaging system.

The two major rAAV production methods described above are based either on a triple transfection of HEK293 cells or helper Ad infection of HeLa cell-derived stable packaging cell lines. Because stable expression of the E1 protein in the Rep–Cap packaging cell line would suppress cell growth via induction of Rep protein expression, E1 protein function must be provided in *trans* to render cells permissive for the rAAV production. Therefore, it is impossible to use replication-deficient E1/E3-deleted helper Ad with the producer cell line. On the other hand, using RCA is also undesirable owing to a high level of helper virus contamination. To resolve this problem, several efficient and scaleable production systems utilizing infection of stable packaging cell lines with hybrid viruses have been developed.[42,43] One of these rAAV packaging systems exploits herpes simplex virus (HSV)/AAV hybrid vectors. In this method, HEK293 cells are coinfected with two hybrid HSV-based viruses: the first virus containing an AAV Rep–Cap gene cassette and the second one containing the rAAV vector genome.[42] The main disadvantage of this method is the necessity to inactivate and remove the contaminating HSV from the purified rAAV fraction. Another alternative hybrid vector system explores the advantage of

rAAV production in insect cells grown in suspension. Sf9 cells are coinfected with three recombinant baculoviruses containing the rAAV genome, Rep protein and helper viral proteins.[43]

Virus harvesting and initial purification steps

Because Ad and AAV are produced in cultured cells, highly developed cell culture techniques are necessary for efficient virus production. While adherent stock cultures are used for small-scale production, large-scale applications require cell lines adapted to suspension culture and, preferably, serum-free conditions.[44,45] Cultures of attachment-dependent cells, which cannot be adapted to suspension, require the use of microcarriers in the bioreactor. The first step of Ad and AAV purification involves harvesting of the infected cells. Infected cells can be collected with trypsin or allowed to detach from the surface. Cells grown in suspension are recovered by centrifugation or filtration. In cases where Ad-infected cells are cultured until full lysis, cell suspension, cell debris and supernatant are collected together. Detailed time-course analysis for recombinant virus accumulation inside cells *versus* supernatants is usually performed to optimize harvesting.[19,44] In a standard Ad production protocol, cultured cells are infected with an amplified seed viral stock for 2–4 days followed by lysis of infected cells by freezing/thawing. Purification from a cell pellet has the advantage of allowing easy virus particle concentration for further purification steps by low-speed cell centrifugation. Alternatively, cell infection with Ad can be continued for 5 days until full cell lysis, providing a significant increase in the virus to contaminating DNA and viral infectious particles to total viral particles ratios.[19]

For a small-scale production, three cycles of freeze/thaw are sufficient to release the virus from infected cells. For a manufacturing scale, cells can be lysed by sudden pressure drop resulting from microfluidization, crossflow filtration or osmotic shock. Cell lysis results in release of large amounts of host cell DNA and RNA, as well as nonencapsulated viral DNA. DNA and RNA must be digested with nuclease in order to remove unwanted nucleic acids, reduce the viscosity of the cell lysate and avoid aggregation complicating further purification steps. Benzonase is the nuclease of choice, because it is available in quantities sufficient for commercial virus purification. However, removing the residual Benzonase from cell lysates can be difficult.[46] In addition to a nuclease, some purification methods require detergents and protease treatment.[20,36,46–50] Some of these additives are hard to eliminate and therefore cannot be used for commercial manufacturing. In contrast to Ad, the relative stability of the AAV particle to heat, mild proteolytic digestion and nonionic detergents allows it to withstand robust purification procedures, thus facilitating scaled-up production. Clarification of the treated lysate is achieved by a combination of filtration and centrifugation. Centrifugation is used to remove cellular debris, but it has to be brief to minimize the loss of viral particles due to sedimentation. Similarly, clarification by filtration has to be optimized to minimize loss of the viral product. Both steps can significantly improve the performance of chromatography in terms of purity and product yield.

When rAAV Rep–Cap packaging cell lines or Ad–AAV hybrid viruses are used for virus production, it is important to ensure that the final product is free of contaminating Ad, since even minor contamination with heat-inactivated Ad can result in a local inflammatory response at the virus injection site. Helper Ad can be removed by heat treatment at 56°C for 1 h, although this step is unsuitable for commercial manufacturing. Ad contaminants can be removed during subsequent chromatographic purification steps by employing columns with high binding capacity and selectivity for Ad.[47] Such columns, commonly used for Ad purification, can be incorporated into AAV purification schemes.

Purification by ultracentrifugation

The first methods for Ad and AAV purification were based on CsCl density gradient centrifugation rather than on chromatographic techniques. CsCl salt forms a density gradient when subjected to a strong centrifugal field. When the viruses are centrifuged to equilibrium in a CsCl salt, they are separated from contaminants and collected in bands on the basis of their buoyant densities.

The general strategy for rAd purification by ultracentrifugation begins with infected cell lysis and DNA digestion. The viral lysate is applied to a continuous CsCl step gradient ranging from a density of around 1.4 g/ml at the bottom of the tube to 1.25 g/ml in the top layer. Because the Ad particle has a buoyant density of 1.34 g/ml, it separates from the contaminating proteins and collects as a band in the middle of the gradient after the first round of centrifugation. The collected Ad band is mixed with 1.35 g/ml CsCl solution and subjected to the second round of isopycnic gradient ultracentrifugation. Standard purification of different AAV serotypes is also performed via CsCl gradient centrifugation. rAAV vectors with a genome length equal to the wild type will band at a density of 1.41 g/ml. If the rAAV production method employs a helper Ad, then density gradient centrifugation allows for a partition of rAAV from the Ad helper. For animal experimentation, rAAV purification should include multiple CsCl gradient centrifugation steps. Density centrifugation permits separation of assembled Ad or AAV virions from their empty particles, a benefit that is difficult to achieve with other separation techniques.

Purification of Ad or AAV by CsCl density gradient is easy to perform and yields highly pure viral preparations suitable for research applications.[51] Although this technique is extremely useful for production of small-scale viral lots, its main disadvantage is the limited capacity of laboratory centrifuges, prohibiting large-scale applications such as large animal models and clinical trials. In addition, extensive dialysis of viral preparations is required due to CsCl toxicity. Other disadvantages of this purification method include variable quality of vector preparations, significant loss of infectivity and aggregation in storage. Moreover, elimination of residual helper Ad from rAAV stocks requires a purification methodology superior to cesium chloride ultracentrifugation.

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More recently, iodixanol has been used as an alternative density medium for small-scale rAAV purifications. Iodixanol has a much lower toxicity compared to CsCl; it also prevents aggregation of rAAV particles and does not reduce infectivity.[49,50,52] The recently described multistep AAV purification methods employ a discontinuous iodixanol gradient centrifugation as a prepurification step, followed by an affinity virus purification by a heparinized support matrix chromatography[50] or ion-exchange chromatography.[52] The resulting rAAV stocks have significantly higher purity compared to purification via two rounds of CsCl gradient. The 50% overall rAAV recovery is also significantly improved compared to the 10–12% recovery from CsCl ultracentrifugation.

Purification by ion-exchange chromatography

Column chromatography is the most powerful and versatile method for Ad and AAV purification. The yield, purity and biological potency of the final viral product resulting from chromatography-based purification schemes surpass that of conventional CsCl purification. The modes of chromatography applicable to these viruses include ion exchange, affinity, gel filtration and hydrophobic interaction. A series of optimized chromatographic steps is required for obtaining virus of high yield and purity. Two-step purification protocols, including two chromatographic steps or a combination of chromatography with ultracentrifugation/filtration, are optimal for Ad or AAV vectors. Because many chromatographic elution buffers used for Ad or AAV purification procedures are not suitable for *in vivo* manipulations, additional purification steps such as dialysis or concentration may be necessary.

Purification by ion-exchange chromatography is based on the net charge of proteins on the exterior of the viral capsid. The net charge of the surface proteins depends on the pH of the exposed amino-acid groups. Ad particles have an acidic p*I* and a net negative charge at neutral pH. As such, a number of anion-exchange adsorbents have been used successfully for the chromatographic purification of Ad.[17–19] The resolution of Ad structure by X-ray crystallography[53] indicates that functional groups of the proteins II, III, IIIa, IV and IX mediate the interaction between viral particles and the anion exchanger. Hexon protein (II) may mediate the strongest interaction, since it has a net negative charge of 24.8 at pH 7.0 and it is highly abundant in the particle. A trend was found between the charge of the hexon L1 loop of different Ad serotypes and their binding to anion exchanger,[17] indicating that hexon L1 loop is very likely to be a major determinant of the strong interaction of the Ad particle with the adsorbent. Owing to its strong negative charge, Ad particle binds to an anion-exchange resin at high salt concentration, whereas most proteins elute from the column under the same conditions, allowing for an efficient separation from proteins. On the other hand, DNA has higher charge density than Ad, and therefore requires higher salt concentrations for elution from an anion-exchange column. The initial chromatographic column should have high virus binding capacity and selectivity to allow substantial viral purification from large amounts of starting material. Initially, viral binding to an ion-exchange resin must be tested within the range of pH and salt concentrations. These conditions have to be explored carefully since there is a fine balance between recovery and purity of the eluted sample.

Modern chromatographic techniques for Ad purification were originally described by Huyghe *et al*.[18] This purification scheme, developed for the Ad 5 vector, used a Fractogel DEAE-650 M (DEAE: diethylaminoethyl; EM Science, Gibbstown, NJ, USA) anion-exchange column as a first separation step. Given that the fragile Ad particle is only stable at a narrow pH range around 7, the column was buffered with 50 mM Hepes, pH 7.5. Infected cell lysate was loaded onto the column in 350 mM NaCl, eliminating the majority of cell-derived contaminants in the flow-through fraction. Elution with a 300–600 mM linear NaCl gradient yields a well-resolved rAd peak at 450 mM NaCl. Virus recovery from an anion-exchange column can be as high as 99%, but it is usually lower since part of the peak is not collected to improve viral purity. The virus purification procedure was completed by IMAC. The overall virus yield and infectivity were superior relative to CsCl gradient purification (Table 1). In a different report, a number of anion-exchange adsorbents were evaluated for their performance in Ad particle purification.[17] The chromatographic performance of Q Sepharose XL (Amersham Biosciences, Piscataway, NJ, USA) was significantly better compared to all other matrices tested, including Source 15 Q (Amersham Biosciences) and Fractogel TMAE (EM Science). A two-step chromatographic procedure (Source 15 Q–Q Sepharose XL) is also applicable to fiber-modified and helper-dependent recombinant viruses. An overall recovery rate of highly purified viral particles prepared from low-titer crude lysates exceeded 50%.[17]

Although the first-generation purification protocols are effective in replacing traditional Ad purification by density, they are not sufficient for gene therapy applications owing to their limited purification scale of 10^{13} input viral particles per run. An improved process offered by Green *et al*[19] is scalable to 10^{15} input particles per run and is highly reproducible. This method employs tandem column chromatography incorporating two matrices: DEAE-Fractogel 650 M (EM Science) anion-exchange capture step and a chromatographic polishing step utilizing PolyFlo resin (Puresyn Inc., Malvern, PA, USA). PolyFlo is a chemically inert, nonporous polymer used in a flow-through mode in the Ad purification scheme. Virus eluted from the DEAE column is loaded on the PolyFlo column under conditions prohibiting intact virus binding to the column resin, but favoring binding of host and unincorporated viral proteins.[19] The major advantage of this chromatographic process is its 100-fold scale-up potential compared to the previously described protocols[17,18] without compromising biological activity, recovery and purity of the recovered virus (Table 1).

Development of an integrated process is required for an optimized Ad production on a larger scale (20–100 l) using suspension cultured HEK293 cells in serum-free medium.[45] The efforts of a large-scale production are focused on optimization of operating conditions such as rate and mode of feed of the suspension culture bioreactor, infection time, harvest time and shear force reduction. Ion-exchange chromatography on Fractogel

Table 1 Efficiencies of Ad chromatographic purification steps

Study	Chromatographic purification steps	Column type	Purification scale	Purity (%)	Yield (VP, %)	Yield (IU, %)	VP/IU ratio
Huyghe *et al* (1995)	1. Anion exchange	Fractogel DEAE-650 M	10^{13} input viral	92	67	49	82
	2. Affinity	IZAC column with zinc/glycine system	particles	98	47	44	88
	Final product				32	22	
Blanche *et al* (2000)	1. Anion exchange	Q Sepharose XL	10^{13} input viral		81		
	2. Anion exchange	Source 15Q	particles				
	Final product			99		40	25
Green *et al* (2002)	1. Anion exchange	Fractogel DEAE-650 M	10^{14} input viral		73	75	
	2. Proprietary resin	PolyFlo	particles		84	94	
	Final product				55	57	
	1. Anion exchange	Fractogel DEAE-650 M	10^{15} input viral		73	90	
	2. Proprietary resin	PolyFlo	particles		78	97	
	Final product				47	54	
Kamen and Henry (2004)	1. Anion exchange	Fractogel EMD DEAE-650 M	20 l scale suspension		80		
	2. Size exclusion	Sephacryl S-400 HR	culture production				
	Final product			99			

Viral particles (VP): measured by HPLC analytical anion-exchange assay; infectious units (IU): measured by tissue culture infectious dose (TCID50) assay; purity: determined by SDS-PAGE and Western blot analysis or by integration of HPLC chromatogram at 260 nm.

EMD DEAE-650 (EM Science) was selected as a first purification step and size-exclusion chromatography on Sephacryl S-400HR (Amersham Biosciences) as a polishing step for batches up to 100 l. This purification scheme requires viral particle concentration by ultrafiltration before loading on a size-exclusion column. However, the filtration step can lead to significant viral loss due to aggregation of highly concentrated particles in the vicinity of the membrane. The tandem ion-exchange/size-exclusion chromatographic process recovers 99% pure Ad from a large-scale suspension production. The authors suggest further process optimization to avoid losses during the ultrafiltration step.

Ion-exchange chromatography is also a desirable method for AAV purification. Purification of AAV2, AAV4 and AAV5 vectors using ion-exchange resins has been reported by several groups.[36,44,54,55] AAV2 binding to cation-exchange resin at a physiological pH can be utilized as a first purification step. The AAV packaging system based on the Rep–Cap cell line B50 and an Ad–AAV hybrid virus was chosen to establish a robust, scaleable column purification process for the AAV2 vector suitable for commercial applications.[36] The authors used a POROS 20 HE (PerSeptive Biosystems, Framingham, MA, USA) cation-exchange column for the first separation step and a POROS 50 PI (PerSeptive Biosystems) anion-exchange column for the polishing step. AAV bound to the first column was eluted with 400 mM NaCl directly onto the POROS 50 PI column. The first chromatographic step was insufficient for complete elimination of contaminating helper Ad, although 99.9% of Ad contaminants were eliminated. The pH of the AAV eluate was adjusted to 5.5 before loading to the anion-exchange column, promoting binding of Ad and cellular contaminants to the POROS 50 PI resin, while allowing AAV to flow through the column, followed by AAV concentration to yield final purified product. Purified AAV2 vectors had a higher degree of purity, recovery rate and biological potency than those purified by the conventional CsCl gradient centrifugation method (Table 2).

Another AAV2 purification method adaptable for commercial production involves 293 cell cotransfection with two plasmids and a helper Ad.[47] Cell lysates pretreated with trypsin and nuclease were purified via two-step ion-exchange chromatography using ceramic hydroxyapatite (Bio-Rad Laboratories, Hercules, CA, USA) and DEAE-Sepharose (Bio-Rad Laboratories) followed by affinity chromatography on cellufine sulfate (Amicon, Billerica, MA, USA). DEAE ion-exchange chromatography was included in the purification scheme in order to eliminate contaminating Ad, resulting in removal of more than 99% of helper virus after three subsequent chromatographic steps. The purity of the final rAAV was greater than 90%, with approximately 30% recovery of rAAV infectious particles (Table 2). Substitution of cellufine sulfate column with a recently described heparin affinity virus purification method may allow further improvement of rAAV particle recovery.[20,21]

Anion-exchange chromatography in combination with gel filtration was recently reported for AAV5 purification.[44] AAV5 particles exhibited favorable binding and elution profiles when applied to Mono Q quaternary ammonium (Q), DEAE or diethylaminopropyl (ANX) substituted anion-exchange chromatography media at pH 6.0. Mono Q (Amersham Biosciences) was superior to the other tested resins in virus binding capacity, resolving capability and high flow rate, and therefore was chosen for the first purification step. Ion-exchange chromatography was followed by gel filtration on Superdex 200 (Amersham Biosciences) column. This combination has allowed rAAV5 purification almost to homogeneity (Table 2).

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Table 2 Efficiencies of AAV chromatographic purification steps

Study	*AAV serotype*	*Purification steps*	*Column type*	*AAV purity*	*Yield (VP, %)*	*Yield (IU, %)*	*VP/IU ratio*	*Helper Ad (%)*
Tamayose *et al* (1996)	AAV2	Sulfonated cellulose	Cellulofine sulfate	+	Nearly 100		500	
Zolotukhin *et al* (1999)	AAV2	Iodixanol					86	1
		Iodixanol/CsCl			76	81	158	1
		Iodixanol/heparin	Heparin agarose type I	++	8.4	6	56	<0.01
		Iodixanol/HPLC heparin affinity	POROS HE 1/M		35	56	73	<0.01
		Iodixanol/HPLC cation exchange	UNO-S1	++	26	32	95	NA
Clark *et al* (1999)	AAV2	HPLC heparin affinity	POROS HE/M		73		50	<0.02 specific Ad proteins[a]
Anderson *et al* (2000)	AAV2	Heparin affinity	POROS HE/P		35		50	
Gao *et al* (2000)	AAV2	1. Cation exchange	POROS 20 HE		90–95			
		2. Anion exchange	POROS 50 PI		60–70			
		Final product		++			4–40	
O'Riordan *et al* (2000)	AAV2	Three-step ion exchange	Ceramic hydroxyapatite			88		
			DEAE Macroprep			60		
			Cellufine sulfate			48		
		Final product		++		30		
Auricchio, O'Connor *et al* (2001)	AAV2/5	Mucin affinity	Mucin Sepharose	+			180–1600	
		CsCl density gradient		+			288–928	
Auricchio, Hildinger *et al* (2001)	AAV2	Heparin affinity	Heparin agarose	++			10	
Potter *et al* (2002)	AAV2	Streamline heparin affinity	Streamline Heparin			56[b]		
		Hydrophobic interaction	Phenyl-Sepharose			48[b]		
		Heparin affinity	Heparin matrix			27[b]		
		Final product		++			6	
Zolotukhin *et al* (2002)	AAV1	Iodixanol/cation exchange	Q-Sepharose	++				
	AAV2	Iodixanol/cation exchange	Q-Sepharose	++			20	
	AAV5	Iodixanol/cation exchange	Q-Sepharose	++				
Brument *et al* (2002)	AAV2	Cation exchange/anion exchange	SP Sepharose HP/Source 15 Q	++	44		150	
	AAV5			++	33		300	
Kaludov *et al* (2002)	AAV2	Anion exchange	POROS PI	++	9	>90	848	
	AAV5	Anion exchange	POROS PI	++	15	>90	36 904	
	AAV4	Anion exchange	POROS-HQ	++	11	>90	2248	
Smith *et al* (2003)	AAV5	Anion exchange/gel filtration	Mono Q HR/Superdex 200	++	37		2690	

Viral particles (VP): measured by dot-blot analysis or quantitative competitive PCR; infectious units (IU): measured by infection center assay; purity: determined by SDS-PAGE and Western blot analysis; purity scale: '+', comparable to CsCl purification, '++', significantly higher than CsCl purification; helper Ad: measured by fluorescent cell assay.
[a]Estimated by Western blot analysis.
[b]Represents a single run.

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Ion-exchange chromatography permits development of universal purification protocols that can be used for different AAV serotypes. A two-step chromatographic procedure involving a cation-exchange SP Sepharose HP (Amersham Biosciences) chromatographic column followed by an anion-exchange Source 15 Q (Amersham Biosciences) column was successfully used for purification of AAV serotypes 2 and 5.[54] Salt concentration and pH adjustments were necessary for optimization of binding of the viral particles to both chromatographic resins. The first purification step was carried out at pH 6.5 in 100–150 mM NaCl. During the second chromatographic step, the pH of the loading sample was increased to 8.2 in 20–50 mM NaCl in order to improve weak AAV2 and AAV5 binding to anion-exchange resins at neutral pH. Virus elution from both columns was performed with a linear salt gradient. The average recovery of infectious AAV particles was 44 and 33% for rAAV2 and rAAV5, respectively (Table 2). Similarly, a combination of ion-exchange chromatography and filtration was used for purification of rAAV serotypes 2, 4 and 5.[55] The viral lysates produced by a triple plasmid transfection method were subjected to freezing–thawing cycles, solubilized with octylglucopyranoside (OGP) and applied onto the weak anion-exchange POROS PI (PerSeptive Biosystems) column. To optimize virus binding and recovery, pH and salt concentration were adjusted for each serotype. The viral eluate was further purified and concentrated by filtration through a high molecular weight retention filter Centriplus 100 (Millipore, Billerica, MA, USA) capable of eliminating the majority of the contaminating proteins. This purification method is easily adaptable to other AAV serotypes and yields viral particles with purity of more than 90% (Table 2).

Purification by affinity chromatography

Affinity chromatography is capable of separating viral particles from protein and DNA contaminants based on a reversible interaction between the viral capsid and a specific biological ligand or receptor coupled to a chromatographic matrix. This technique is ideal for a capture or intermediate step in a purification protocol and can be used whenever a suitable specific ligand or receptor is available. Purification by affinity chromatography is attractive due to its high selectivity, hence high resolution, and high virus binding capacity leading to a considerable recovery of biologically active material concentrated up to several thousand-fold.

IMAC can be used for Ad vector purification. Ad purification by IMAC is based on efficient binding of viral particles to charged zinc ions covalently linked to the column with a chelating ligand such as iminodiacetic acid. Virus elution is achieved either by changing pH, or by addition of imidazole or glycine, which compete for column binding sites. IMAC was successfully used as a polishing step in an Ad purification scheme utilizing DEAE chromatography as the initial virus purification step.[18] Because IMAC can tolerate high-salt conditions, a DEAE virus eluate containing 450 mM NaCl was directly loaded under iso-osmotic conditions onto a TosoHaas AF chelate 650 M (Tosoh Bioscience LLC, Montgomeryville, PA, USA) immobilized metal affinity resin charged with divalent zinc. After loading, the salt concentration was gradually decreased to 150 mM in order to avoid virus degradation due to osmotic shock. Contaminants that copurified with Ad in the DEAE step were recovered in the IMAC flow-through fractions prior to Ad elution with 0–500 mM glycine step gradient.

The first affinity purification procedure developed for the AAV vector employed the negatively charged cellulose affinity medium cellulofine sulfate (Seikagaku-kougyo Corp., Tokyo, Japan).[56] Selective binding of AAV2 to the matrix allowed purification and concentration of the viral particles. However, insufficient purity and yield of recovered rAAV fraction precludes cellulofine sulfate affinity chromatography as a single-step AAV purification technique, although it can be utilized as a virus concentration step (Table 2). An alternative AAV2 affinity purification approach is based on the recognition of assembled AAV2 vector particles by a monoclonal antibody A20, allowing separation of unassembled capsid proteins.[32]

The discovery that heparan sulfate proteoglycan mediates AAV2 cell entry[57] facilitated the development of several AAV2 purification protocols based on heparin affinity.[20,21,48,58] A single-step high-performance liquid chromatographic (HPLC) purification scheme described for AAV2 used heparin column POROS HE/M (PerSeptive Biosystems).[20] The recovery of rAAV2 from the crude lysate exceeded 70% (Table 2). An alternative single-step heparin affinity purification of AAV2 by gravity flow does not require any special equipment.[21] The purity of AAV2 produced by this method is comparable to the HPLC-purified vectors. AAV2 vectors purified on heparin columns possess higher biological potency when compared to virus purified by cesium chloride density gradient, which can cause virus inactivation by aggregation. Nonetheless, heparin affinity chromatography is not highly specific for AAV2 and therefore using this virus purification step alone is problematic. Introduction of additional purification steps is necessary to reduce contamination with cellular proteins that bind to heparin.[20,21,50] An improved AAV2 purification method combines iodixanol density ultracentrifugation with subsequent heparin affinity purification.[50] Another method utilizing POROS HE/P (Boehringer Mannheim, Indianapolis, IN, USA) heparin affinity column introduces an additional purification of the virus eluate including trypsin digestion and extraction with an organic solvent 1,1,2-trichlorotrifluoroethane (Arklone, BDH, New York, NY, USA).[48] Many current large-scale AAV2 purification schemes adopt two chromatographic steps including a heparin affinity resin followed by polishing on an ion-exchange column.

Development of new recombinant gene therapy vectors based on other AAV serotypes brought more attention to the AAV manufacturing process. Many AAV serotypes, such as AAV1, 4 and 5, fail to bind heparin columns efficiently, thus precluding application of the same production process to all serotypes.[59] New purification methods were therefore required for new AAV vector types. The observation that 2,3-linked sialic acid is required for AAV4 and AAV5 cell entry[60,61] led to the development of a single-step affinity chromatography for purification of AAV5.[62] The affinity matrix for capture of AAV5 particles consisted of a sialic acid-rich protein called mucin that was covalently coupled to CNBr-activated Sepharose (Amersham Biosciences). The purity of the recovered AAV5 is similar to that achieved by CsCl ultracentrifugation (Table 2). A role of PDGFR-alpha and

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PDGFR-beta as cellular receptors for AAV-5 has been established recently,[63] but receptor specificities for other AAV serotypes remain to be discovered. Hence, chromatographic affinity purification approaches for different AAV serotypes will require further development.

Purification by size-exclusion and hydrophobic interaction chromatography

The large size of viral particles is a feature that can be used for separation of mature viruses from partially assembled capsids, proteins and DNA fragments on a size-exclusion column. Size-exclusion chromatography, also called gel filtration, is the method of choice for purification of many viruses, including tick-borne encephalitis virus and retrovirus.[64,65] It can also be used as a polishing step for Ad purification.[45] A few disadvantages are associated with the use of size-exclusion chromatography for virus purification. First, due to constraint of column size, the viral lysate must be concentrated before loading, leading to the loss of virus. Second, it is difficult to separate virus particles from high molecular weight contaminants that coelute with the virus in the excluding volume of the column. Despite these limitations, size-exclusion chromatography offers the advantage of being a gentle purification method with a high yield and the possibility of exchanging the buffer to the desired formulation. For example, gel filtration chromatography on Superdex 200 (Amersham Biosciences) was used as a second step for AAV5 purification following ion-exchange column chromatography.[44] The exclusion size for globular proteins on Superdex 200 is approximately 1.3×10^6 Da, whereas the molecular weight of assembled AAV particles is about 3.6×10^6 Da. Thus, AAV elutes in the void volume, while greater elution volumes are required for the majority of the proteins.

Hydrophobic interaction chromatography is based on binding of the viral capsid proteins to the column matrix through hydrophobic interaction in an aqueous solvent. A salt at high concentration, such as ammonium sulfate, is used as a solvent to promote clustering of hydrophobic protein surfaces, thereby minimizing their exposure to solvent. However, high salt may destabilize viral particles, resulting in viral degradation. Purification of Ad vectors by hydrophobic interaction chromatography on Toyopearl butyl 650 M (Tosoh Bioscience) or Toyopearl phenyl 650 M (Tosoh Bioscience) yields a low recovery ranging from 5 to 30% and results in viral degradation.[18] AAV purification scheme, consisting of three sequential chromatographic purification steps, utilizes chromatography on phenyl-Sepharose (Amersham Biosciences) in a flow-through mode as a second purification step.[49] Binding of AAV serotypes 2, 4 and 5 to some hydrophobic resins has been evaluated;[55] however, purification of AAV particles by hydrophobic interaction chromatography has not yet been described.

Analytical chromatographic methods for process development and process control

Selection of an appropriate combination of chromatographic purification steps demands quick analytical methods that are able to assess the quantity and purity of virus following each step. Analytical ion-exchange HPLC serves this purpose and provides information about purity, particle integrity and quantity of Ad and AAV vectors during all stages of the production process. Analytical HPLC is performed by injecting small samples of virus at different production stages onto a small high-resolution HPLC column under high pressure facilitating rapid run times. The result of the analytical run is judged by the shape and resolution of the eluted virus peak that is quantified against a standard curve.

Analytical HPLC is one of the most useful techniques for analysis of both pure and crude samples of Ad[17,66–69] and AAV.[70] HPLC using anion-exchange column Source 15 Q (Amersham Biosciences) for analysis of Ad particles in crude lysates[66] offers a significant advantage over the semiquantitative and time-consuming assays available previously.[71] However, the sensitivity of Source 15 Q HPLC is not sufficient for very crude and very dilute virus preparations, and requires digestion of host nucleic acids for accurate readings.[66] Anion-exchange HPLC using Q Sepharose XL (Amersham Biosciences) for Ad sample analysis offers a 10-fold greater sensitivity compared to Source 15 Q, as well as a lack of host nucleic acid interference.[17] An analytical assay for AAV2 has been developed based on a strong cation-exchange resin TSK gel SP-NPR (TosoHaas, Montgomeryville, PA, USA).[70] This method reduces the assay time to less than 20 min and can be applied to a broad range of sample conditions, as well as to quantification of AAV vectors.

Another analytical chromatographic method called reversed-phase HPLC (RP-HPLC) can be used for composition analysis of the viral particle proteins.[72] In this assay, the virus is irreversibly denatured by an organic solvent acetonitrile. Hydrophobic interaction of the exposed regions of the denatured viral proteins with the hydrophobic surface of the resin mediates binding to the reverse-phase column. The chromatogram resulting from protein elution with increasing concentrations of acetonitrile allows evaluation of the quantity and quality of viral particles. During characterization of the purified virus fraction, it is important to assess the presence of empty viral capsids that are potentially immunogenic during gene therapy treatments.[73] The fraction of empty capsids is affected by cell infection and culture conditions, as well as by the subsequent purification scheme. Empty capsids can be efficiently separated from the packaged virus by conventional CsCl density gradient centrifugation. While some chromatographic steps for Ad purification, such as DEAE-Fractogel chromatography, are not suitable for capsid elimination, other chromatographic methods, including immobilized zinc-chelating or hydrophobic interaction chromatography, can substantially reduce contamination with empty viruses.[69] Purification of rAAV particles by ion-exchange chromatography cannot discriminate between full and empty AAV capsids.[49,54] A method for quantification of Ad capsids by RP-HPLC is based on measuring the amounts of capsid protein VIII precursor. This protein is present in empty capsids but not in intact Ad particles.[69]

Perspectives for the purification of adenoviral and adeno-associated viral vectors

In recent years, a significant effort is underway to develop helper-dependent, fully deleted Ad vectors

and retargeted Ad vectors intended to achieve greater persistence of transgene expression and lower vector-induced toxicity in clinical applications. Broader applicability of these vectors will require the development of production and purification schemes that are sufficient to supply large amounts of clinical grade Ad. A variety of approaches have been developed in the last few years to retarget Ad vectors away from the primary CAR receptor to new tissue- or cell-specific receptors. The goal is to restrict viral transduction to the tissue of interest and to minimize the immune response to the virus. Strategies to alter Ad tropism are based on modification of the viral capsid proteins. Tropism-modified Ad vectors can be generated by complexing Ad particles *in vitro* with bispecific conjugates that introduce novel tropism or by genetic engineering of the viral capsid proteins to contain cell-targeting ligands. These genetic modifications can change the affinity of the virus to various chromatographic matrices. In addition, the new viral binding properties might be considered in the purification scheme. For example, one of the early modifications to the fiber was the addition of an oligolysine motif to the N-terminus of the fiber protein, providing the virus with an affinity for polyanions such as heparin sulfate.[74] Ad fiber can be engineered to express histidine repeats at the C-terminus to serve as a receptor binding ligand.[75] Histidine repeats on the protein surface confer affinity to metal ions and this interaction is employed for protein purification by affinity chromatography. Protein tag–ligand, receptor–ligand and some other typical biological interactions that are frequently used in protein affinity chromatography could provide the basis for virus purification. For example, the fiber capsid protein was genetically fused to a biotin acceptor peptide (BAP).[76] Ad particles bearing BAP were metabolically biotinylated during vector production by the endogenous biotin ligase in 293 cells to produce covalently biotinylated virions. The resulting biotinylated vector can be retargeted to new receptors by conjugation to biotinylated antibodies using tetrameric avidin. The authors demonstrated that such metabolically biotinylated Ad could be affinity purified from crude lysate on a monomeric avidin column by elution with biotin. The virus yield was 17% of the input virus; the limited virus recovery was due to the competitive binding of the free biotin and biotinylated fiber proteins in the lysate. This problem could be overcome by using an additional purification step before monomeric avidin column purification.

Lately, HDAd have been used for a variety of applications where persistent transgene expression is required, and they have demonstrated clear advantages over the first-generation Ad.[26] However, it remains difficult to produce and purify HDAd in clinically relevant quantities. All of the current HDAd systems have two major limitations, which slow progress in the field: (a) the current production systems allow generation of only limited amounts of virus and (b) contamination with helper virus. The second problem was traditionally addressed by physical separation of the HDAd from the helper Ad by CsCl ultracentrifugation based on the differences in the genome size of the two viruses. While this technique could significantly reduce the level of helper Ad contamination, it cannot be used for clinical grade large-scale production. Modern chromatographic purification methods developed for the first-generation rAd are difficult to apply to the helper-dependent virus because of helper virus contamination. A future goal for the helper-dependent system is to minimize helper virus by developing packaging complementing cell lines and by targeted recombinase-mediated deletion of the packaging signal on helper virus.

HDAd vector production schemes that provide increased yields, minimal level of helper virus and minimal level of RCA have been described in several studies.[29,77,78] Sandig *et al*[77] addressed the problem of helper Ad contamination by minimizing the homology between the packaging signal (ψ) of helper Ad and HDAd, thus reducing the probability of homologous recombination. Palmer *et al*[78] reversed the orientation of ψ with respect to HDAd, making the genome of recombinants too large to be packaged and also selected a producer cell line with higher intracellular Cre levels for efficient ψ excision. Umana *et al*[29] report 100% of ψ excision with FLPe recombinase compared to 80% with Cre and claim that this will allow large-scale production of HDAd vectors using column chromatography-based virus purification. Chromatographic purification procedures applied to first-generation Ad vectors by several groups[17–19] will need further development to obtain HDAd vectors in the large-scale amounts that are required for clinical trials.

New generations of rAAV vectors, based on the new AAV serotypes or on receptor-targeted capsid mutants, have recently been investigated. Most of the initial studies concerning rAAV production and purification procedures were performed with rAAV2. However, rAAV2 vectors do not efficiently transduce some therapeutically interesting targets, for example, muscle fibers and hepatocytes.[79] Another disadvantage of AAV2-mediated gene transfer in clinical studies is the presence of neutralizing antibodies in humans.[80] It is possible that alternative serotypes of AAV could circumvent these problems. Seven other serotypes have thus far been described, denoted AAV1 to AAV8. Unique cell-type specificities for the different AAV variants have been reported.[80] For example, AAV serotype 8 can transduce the murine liver 10–100 times more efficiently than other serotypes.[81] rAAV produced from serotypes 1 and 5 transduce skeletal muscle much more efficiently than AAV of serotype 2.[82,83] Discovery of new AAV serotypes could allow further improvements in cell transduction efficiencies as well as broaden the range of somatic host cell types. Screening human and non-human primate tissues has revealed over 40 novel AAV variants, most of which have been derived via genomic recombination at the capsid protein regions between different parental serotypes.[81]

Designing AAV capsid mutants that allow the virus to enter previously nonpermissive cells is another way to generate AAV with improved cell transduction efficiencies. Targeting of several cell-specific receptors by inserting corresponding ligands into the virus capsid proteins resulted in the enhancement of cell transduction efficiency.[84,85] Pseudotyping rAAV vector, that is, using an alternative serotype capsid to package the rAAV vector genome, can also substantially increase the vector transduction efficiency and broaden the host range for rAAV-mediated gene transfer (reviewed by Flotte).[9]

Utilization of emerging alternative serotypes and receptor targeted capsid mutants demands the develop-

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ment of efficient methods for their production and purification. Affinity purification schemes have been developed for only a subset of the AAV serotypes (eg, heparin resin for AAV2 and mucin resin for AAV-5). The receptor specificity for other AAV serotypes is still unknown and cannot be utilized for purification. It is possible that some of the current ion-exchange chromatographic purification procedures might be applied to the production and purification of other AAV serotypes. It is important to continue developing universal purification schemes, because it allows the direct comparison of AAV of different serotypes in *in vivo* models. Several groups have attempted to develop a purification procedure that would be applicable for several serotypes. A chromatographic procedure has been described for AAV serotypes 2, 4 and 5 based on a single ion-exchange resin purification.[55] A two-step ion-exchange scalable chromatographic procedure with very little modification can be used for purification of AAV serotypes 2 and 5,[54] and the authors claim that this method could be applied for AAV1 and AAV4 particles as well. Equally important is the direct comparison of different purification methods with respect to the same batch of produced virus since this may illustrate some basic principles of purification.

Chromatographic resins and equipment continue to evolve. Improvements in chromatography and advances in our knowledge of viral structure and function will result in improvements in the purification methods. Additionally, many of the purification strategies that we describe in this review can be adapted for high-volume clinical grade vector production. The use of such techniques will make broader application of recombinant gene therapy vectors feasible in the future.

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