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Scalable Downstream Strategies for Purification of Recombinant Adeno-Associated Virus Vectors in Light of the Properties

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Abstract: Recombinant adeno-associated virus (rAAV) vector is one of the promising delivery tools for gene therapy. Currently, hundreds of clinical trials are performed but the major barrier for clinical application is the absence of any ideal large scale production technique to obtain sufficient and highly pure rAAV vector. The large scale production technique includes upstream and downstream processing. The upstream processing is a vector package step and the downstream processing is a vector purification step. For large scale downstream processing, the scientists need to recover rAAV from dozens of liters of cell lysate or medium, and a variety of purification strategies have been developed but not comprehensively compared till now. Consequently, this review will evaluate the scalable downstream purification strategies systematically, especially those based on the physicochemical properties of AAV virus, and attempt to find better scalable downstream strategies for rAAV vectors.

Keywords: Clinical application, downstream purification, large scale, physicochemical properties, purification strategy, rAAV vector.

1. INTRODUCTION

Over the past ten years, data accumulated from many animal experiments and clinical researches for recombinant adeno-associated virus (rAAV) vector as one of the most promising vectors for gene therapy presented excellent safety records [1, 2]. The rAAV successfully delivered many different therapeutic genes for the treatment of different diseases, including hemophilia B [3, 4], Leber congenital amaurosis [5, 6] Parkinson's disease [7, 8], Duchenne muscular dystrophy [9, 10], cystic fibrosis [11, 12], rheumatoid arthritis [13] and lipoprotein lipase deficiency (LPLD) [14-16]. The European Medicines Agency (EMA) approved rAAV1 vector carrying human variant LPL S447X as the first rAAV-mediated gene therapy medicine for the treatment of patients with LDLD [17]. Hundreds of trials on rAAV vectors are currently ongoing, are under review or have been completed [18]. The clinical dosage of rAAV vectors ranges from 1×10^{12} DNase resistant particles (DRP)/kg (LPLD) [19] to 1×10^{13} DRP/kg (heart failure) for one patient [20]. The basic elements of drugs are safety, stability and effectiveness [21], with gene drugs not being exceptional. For investigation of a new drug, the US Food and Drug Administration (FDA) drafts guidance documents to describe an incremental approach for manufacturing controls [22]. In 2005, scientists compared the chromatography purification method in Adenovirus and rAAV and indicated that these methods were suitable for high-volume clinical grade vector production [23]. María Mercedes Segura showed that the nature of AAV virus had relationship with the purification method [24] but a comprehensive description of the specific properties of AAV with the scale purification is still awaited. The knowledge of the nature of AAV virus may help select better purification methods to obtain large amount of vectors influencing the selection of downstream processing, especially for scalable purification. So, this review focuses on correlating the advantages and disadvantages of currently scalable downstream purification methods, especially on the productivity and purity of rAAV vectors with the AAV viruses' physiochemical characteristics, and figures out two potentially ideal downstream purification strategies.

2. THE LARGE SCALE PURIFICATION PROCEDURE FOR rAAV VECTOR

The large scale production of the rAAV vectors includes upstream and downstream processing. Upstream processing includes cell culture, transfection or infection, and harvest. On the other hand, downstream processing includes cell crush, clarification and separation rAAV vectors [25].

2.1. The rAAV Upstream Processing

In order to achieve clinical-grade rAAV, we always need to culture ten to two hundred liters of cells using RB or reactor in current good manufacturing practice (cGMP) condition [26]. At present, there are three rAAV upstream processing methods, the helper virus-free plasmid transfection [27, 28], the recombinant baculovirus or other virus-infected production

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systems, and the packaging/producer cell lines [29]. For the triple plasmid transfection, the desired therapeutic transgene as inserted elements was flanked by AAV ITRs in a plasmid, the rep and the cap gene in trans were included in another plasmid, prototypically adenovirus E1, E2a, and E4 genes supporting for vector genome replication and packaging in a helper plasmid [30, 31]. The host cells (HEK293) provide E1a and E1b genes for AAV package [32]. Transfection methods use the coprecipitation strategies, such as calcium phosphate, polyethylenimine (PEI) or cationic lipid [31, 33]. Liu YL reported that the output of rAAV was over 1×10^{13} vector genome copy (VG) per roller bottle (RB) [34]. Thus, at least more than 20 L culture materials and hundreds of RB were needed to dispose for clinical grade application scale. (2) Many stable transfected cell lines expressing some requisite genes have been developed, and additional genes provided through the infection process, ultimately providing better scalability and cost-effectiveness vectors. For example, the baculovirus vector carrying AAV rep, cap and therapy genes were transfected into Spodoptera frugiperda (Sf9) cells for the production of rAAV vectors [26]. This method gave outstanding output and cost-effectiveness for serum-free suspended culture [35-37]. Till now, the output is about 2.0×10^{15} VG can be purified in 50 L reactor [38, 39]. Another concept put forward was that vaccinia virus (VV) containing the rep and the cap gene, and Ad/AAV containing therapeutic gene, were co-transfected with HeLa S3 cells to produce AAV vectors preventing wild type AAV contamination [40]. The yield per cell was similar to that from the triple plasmid transfection. About 1×10^{16} VG rAAV vectors can be purified from 100 L of suspension HeLa S3 cells. (3) The third method for large-scale rAAV production used rAAV packaging and producer cell lines. The primary advantages of this platform required only two biological reagents, the cell lines and a helper virus (such as wild adenovirus (wtAd)). The rep and cap genes are integrated into the host cell genome, which allows rAAV production in one step via infection with wtAd. Thorne reported that 250 L rAAV1 production process could readily supply highly pure vector in the order of 10^{16} DRP for clinical trials. There were no foreseeable impediments to scale up to commercial manufacture in 2000 L bioreactors or even larger [36]. It was also reported that nonclonal "masterwells" (MWs) producing the rAAV2 vector have been created that showed stable vector yields at $\geq 1 \times 10^5$ DRP/cell and stable ($\geq$ 60 passages) vector production. This system will be adaptable to large scale production of high quality rAAV vectors for clinical studies [41]. From these reports, we know that in order to achieve clinical-grade rAAV, huge volume of "raw material" should be handled in the upstream processing step. This means that the downstream purification technologies must match with the upstream processing.

2.2. The rAAV Downstream Processing

The downstream processing begins from the cell or medium harvest. The diversity in impurities from various package systems affects the purification strategy. For instance, the empty particles of helper virus are inescapable in virus-free and virus-helper package system. Removal process of some related impurity becomes a challenge. In addition, the harvest fractions are significant differences in various systems. Vandenberghe [42] reported that several serotypes of rAAV were efficiently released into culture medium after 72 h transient transfection of HEK293 cells. Previous investigators indicated that the yield of rAAV2 vectors from the supernatant was about 20% with serum and 9% without serum, while 88% with serum or 68% without serum for rAAV6, and 51% for rAAV9 the presence of serum or over 85% for rAAV8 [43]. Lock [44] found that serotypes 6, 7, 8 and 9 could be released to the supernatant after five days of transfection which were about 61.5% to 86.3% of the total yield. Their studies also confirmed that extension of culture time could result in an increase in release of rAAV particles to the medium. An ideal ratio of yield/ medium for scalable rAAV vector manufacturing is about 3×10^{14} VG with 10 liters of medium, which is current good manufacture (cGMP) compatible [42]. As a consequence, a scalable purification processing takes into consideration a decrease in the volume of the medium or selects suitable chromatography to capture the rAAV in low concentration.

After crush and clarification, the "crude materials" contains rAAV vectors, cell host debris, protein, genomic DNA, serum protein, some elements of medium, helper DNA, or helper virus, and so on [45]. These impurities may poison cells, reduce transduction efficiency, even induce arouse systemic immune response or inflammatory response [27, 46]. Like other recombinant protein preparations, clinical grade rAAV vectors are scrutinized in stringent standards of purity, efficacy and safety by regulatory authorities. The rAAVs need to maintain viral activity as intact as possible throughout the purification process. Considering the complex and fragile structure of virus particles, scientists have developed many downstream processing strategies. So, it is important to think much more physico-chemical properties of rAAV for separation.

3. THE DOWNSTREAM PROCESSING STRATEGIES BASED ON THE rAAV PROPERTIES

3.1. The Density of AAV Virus and Ultracentrifugation (UC)

The density of rAAV is about 1.40 g/cm^3 which differs notably from those of contaminating cellular organelles (1.1 - 1.6 g/cm^3) [47], soluble proteins (about 1.3 g/cm^3), nucleic acids (1.7 - 2.0 g/cm^3) and even empty particles being lighter to about 1.32 g/cm^3 [24, 48]. Density gradient ultracentrifugation is a well-developed and the most widely utilized method for purification of rAAV in laboratory [24] because this method is suitable for all rAAV serotypes especially for removing empty particles. This technique separates virus particles from impurities in solution based on the shape, size and isopycnic point of rAAV virus. In general, the amount of impurities is fairly reduced after the first round of Cesium chloride (CsCl) ultracentrifugation. The higher levels of purity can be achieved by two or three rounds [27, 49] (Fig. **1**). However, this technology is time-consuming that needs more than 30 hours in two rounds of ultracentrifugation. Moreover, the rAAV containing solution should be split into many centrifuge tubes and then centrifuged repeatedly during processing. This is unsuited for the large scale rAAV downstream processing.

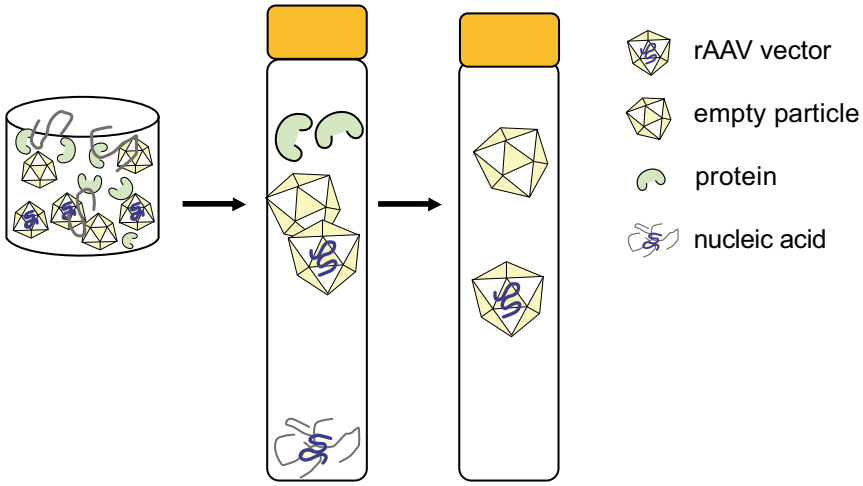

Fig. (1). Purification of rAAV virus by double rounds of CsCl density gradient ultracentrifugation. The rAAV virus, empty particles, nucleic acids or some cellular organelles could be separated by first round centrifugation, and further separated from empty particles by the second round of centrifugation.

3.2. The Size, Mass of AAV Virus and Size Exclusion Chromatography (SEC)

AAV virus belongs to parvovirus family and is about 20 nm in icosahedral diameter. The size is significantly larger than the protein impurities (< 5 nm), and smaller than the Adenovirus (about 80 nm). Furthermore, taking AAV2 as an example, VP1 (YP_680426.1), VP2 (YP_680427) and VP3 (YP_680428) are composed of 735, 598 and 533 amino acid, respectively [50, 51]. The ratio of VP1:VP2:VP3 of AAV2 has been estimated about 1:1:10 [52]. According to preliminary estimates, the mass of total rAAV capsid protein is over 600 kDa. The rAAV vector is about 170 kDa heavier than the empty particle because of 4.7 kb single strand DNA packaged. Thus, the rAAV vector can be embedded into appropriate aperture of SEC.

SEC, known as gel-filtration chromatography, is a method to separate large biomolecules by their size or weight in solution. Through this, the biomolecules could be separated from some other small size materials [53]. Superdex 200 has about 1.3×10^6 Da exclusion limit for globular proteins. It is significantly smaller than rAAV in weight. The viral particles are largely excluded from the Superdex. The majority of cellular proteins are retarded by the medium and emerge with a much greater elution volume [54]. Sephacryl S-300HR gels is also reported to remove some cell materials in rAAV purification [55]. The biological activity of the vectors can be preserved when various solutions pass through the filtration process. There is no significant loss because vector does not interact with the stationary phase. In addition, the SEC is also used in chromatography purification for buffer exchange [56]. Superdex 200, HW55, HW65 and HW75 resin could be used as buffer exchange for rAAV1 [57]. But, SEC is considered as a low resolution chromatography; in general, a 10% difference in molecular mass is needed for good separation. Therefore, this technology is often reserved for the final "polishing" step of a large scale [58].

3.3. The Isoelectric Point (*PI*) of AAV Virus and Ion Exchange Chromatography (IEX)

3.3.1. Anion Exchange Chromatography (AEX) and Canion Exchange (CEX)

The isoelectric point *(pI)* is a special pH value at which the net charge of protein or virus is zero. When the pH of solution is to equal to the *pI* of these biomolecule solutes, the latter have minimum solubility and precipitate out of the solution. Protein carries positive charge at a pH below the *pI*; on the contrary, it is a negative charge. Compared with the most contaminating impurities, rAAV viral particles contain multiple sites to bind anion exchangers, therefore anion exchange chromatography (AEX) is the widely used chromatography for rAAV purification [59, 60]. For example, Poros HQ [48, 55], Poros PI [61], Source 15Q [62], Q-Sepharose[xl] [48] or HiTrap Q [63], etc. were often used for purifying rAAV1, 2, 4, 5, 6 and 8, successfully (Table **1**).

The main challenge in downstream processing of viral vectors is how to separate the vectors from closely related impurities in structure such as empty capsids. Although the empty particles play an important role in gene therapy [64], some residue empty particles may induce a capsid-specific T-cell response [28]. Empty particles share the protein structure with the same capsid, but they contain no or little genome. They can be separated by two or three rounds of CsCl ultracentrifugation [27]. In comparison with proteins or nucleic acid impurities, the empty particles are even more difficult to separate. The mean calculated *pI* value of all the AAVs' empty capsids (pI_E) is about 6.3 because of high mass of VP3 in the capsid proteins (1:1:10 for VP1:VP2:VP3). The mean calculated *pI* value of AAV capsids packaged genomes (pI_F) (about 4.7 kb) is about 5.9 [65] (Fig. **2B**). When solution pH value is above the pI_E, the AEX should be chosen. The empty particles would be eluted earlier than packaged rAAV with increasing ion strength (Fig. **2A**) [43]. The separation rAAV vector from empty particles using a refined column AEX method has been demonstrated [48, 55]. On the contrary, when pH value is below the pI_F, rAAV

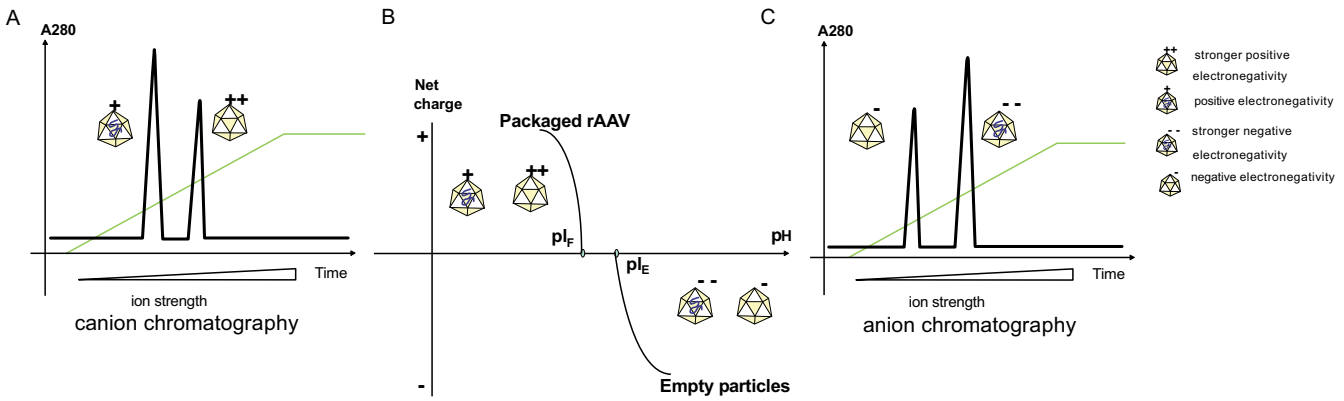

Fig. (2). The rAAV separation by ion exchange chromatography. (**A**) Separation of the packaged rAAV and the empty particles by CEX. The rAAV virion is positively charged when pH value is below the pI_F. The electronegativity of the empty particles is stronger than that of the packaged rAAV. The packaged vector will be eluted earlier than the empty particles with increasing ion strength. (**B**) Isoelectric points of the packaged rAAV and the empty particles. The mean calculated *pI* value of AAV capsids packaged genomes (pI_F) is lower than the value of empty capsids (pI_E.). (**C**) Separation of the packaged rAAV and the empty particles by anion exchange chromatography. The empty particle is negatively charge when pH value is over the pI_E. The electronegativity of the empty particles is smaller than that of the packaged rAAV. The empty particles will be eluted earlier than the packaged vector with increasing ion strength.

virion is positively charged, favoring the selection of CEX. The empty particles would be eluted late than packaged rAAV with increasing ion strength (Fig. **2C**) [43]. Poros HS is a successful resin for purification of rAAV2 [48] and rAAV9 [66].

IEX chromatography has the following advantages: (1) the purity of rAAV will be more than 98% through 2 or 3 kinds of resins [48, 66]; the purity and yield of the vectors purified by the methods would be stable between every batch [23]; (2) this purification processing can be handled by automation equipments and become labor-saving; (3) the resins can be regenerated for recyclable usage; (4) the vector interaction with resins is a dynamic process in mild salt buffer at suitable pH, which maintains the activity of rAAV as much as possible; (5) The scale is easily adjustable according to the quantity. But, there are some disadvantages: (1) some resins are still unsuitable for some serotype rAAV; such as rAAV9 does not efficiently bind some AEX (Poros 50PI, Poros 50HS, Poros 50D) under low salt loading conditions at pH 8.5 [66]. It is hard work to establish an optimal method for all rAAV serotypes. (2) Every resin has its lifetime. There occurs loss of resolution, binding capacity, and recovery, increased pressure drop, and "Ghost" peaks during blank gradient runs when it is used repeatedly. (3) The high concentrations of salt (1 - 2 M NaCl), acid (0.5 - 1.0 M HCl) or alkali (0.5 - 1.0 M NaOH) for resin regeneration will change into the industrial waste. (4) The resin may be polluted by some organic materials, such as bacteria, when stored in bad condition.

3.3.2. Dual Ion-exchange Adsorptive Membranes (DIAM)

Membrane absorbers, such as dual ion-exchange absorptive membranes (DIAM), are more attractive than traditional bead-based chromatography in viral purification processing because the binding sites of the membrane adsorbers are all exposed to the virus or biomolecules [43, 67]. The binding sites of the membrane absorbers exposed to the molecules are shorter in diffusion distances than bead ligands in the columns packed. Therefore, the purification processing is much easier than the packed-bed chromatography. Okada reported that pure rAAV vector (less than 1% empty particles) obtained by membrane adsorbers is an effective gene transfer *in vivo* or *in vitro* [43, 68]. Empty particles and other impurities can be cleared and high purity rAAV vectors should be obtained from clarified lysate in small or large scale. In comparison to classic resin-based chromatography, DIAM saves time during equilibration, sample loading, washing or elution.

3.3.3. Convective Interaction Media (CIM)

CIM monoliths, as chromatographic supports, have been successfully applied to purify large bio-molecules, such as viruses, virus like particles or plasmid DNA [69]. Compared with classic globular-based chromatographic support residing in their structures, this virus purification and concentration procedure can be improved by methacrylate monoliths. The large flow-through channels are able to form convective mass transport in molecules leading to dynamic binding or separation capacity [70, 71]. Lock [72] loaded full or empty rAAV8 vector preparations on CIM-QA disk, then washed them with a 50 - 150 mM NaCl gradient elution, recovering more than 99% of the loaded rAAV8. Furthermore, this method can be used to separate empty particles and quantify the rAAV8 particle numbers according to the peak area. However, for an entire quantification reason, highly purified vectors are required, because such an assay is always interfered by the protein contaminants. It may be a candidate for scale purification in polish step.

3.4. Capsid Structure and Affinity Chromatography (AC)

3.4.1. Heparin Affinity Chromatography (HEAC)

Thirteen AAV serotypes have been discovered from primate or other animal species [73-78], and expanded the AAV's tissue tropism [79-82]. The surface structure of the

capsid protein is an essential component in the binding of the AAV to the target cell [83]. The scientists have found that heparin sulfate proteoglycan (HSPG) is one receptor of AAV2 [84, 85]. The AAV2 heparin binding sites and neighboring basic residues are located on a trimer of VP3 subunits in the capsid surface. The K531, K532, R484/R487 and R585/R588 [86, 87] are all important residues on rAAV2 capsid surface. Hence, heparin-based affinity chromatography is successfully used to purify rAAV2 vectors [88] (Table **1**). The rAAV6 has heparin binding capability but rAAV1 does not have although there is only six amino acid residues difference [89]. Wu reported that the heparin binding ability of rAAV6 could be attenuated by AAV6-K531E mutation. On the contrary, mutant AAV1-E531K impaired heparin-binding ability of rAAV1 like rAAV2 and rAAV6. Similar phenomenon was found on rAAV8, which also conferred heparin binding by rAAV8-E533K mutant [90]. The same mechanism, a single-step mucin type I-S sepharose has been chosen to purify rAAV5 vectors [91]. Generally, HEAC is more suitable for rAAV2 purification in large scale.

3.4.2. Immunoaffinity Chromatography (IAC)

The different serotype rAAV vector has different capsid protein structure. Feasible interactions between viral surface antigens and immobilized antibodies make IAC separate the vectors. This technique separates rAAV vectors from other impurities with a specific monoclonal antibody [92]. For example, AVB Sepharose has been used for the purification of both rAAV1 and rAAV2. The AVB Sepharose medium is able to recognize rAAV1 to rAAV8, its linear flow-rate is up to150 cm/h, and the vector binding capacity is over 10^{12} particles per ml medium [93]. Previous investigations have revealed that rAAV capsid ORF following VP1 could be engineered into a small biotin acceptor peptide (BAP), BAP-modified rAAV capsid proteins were then biotinylated metabolically by packaging cells during vector production, and could be quickly and separated by commercially available monomeric avidin affinity chromatography [94]. For solid phase rAAV capsid-binding studies, heptapeptide ligands can recognize rAAV serotype 8 capsids. Pep8-based affinity column chromatography can selectively recognize rAAV8 capsids, but not rAAV 1, 2, 5, 6 or 9. Another advantage of the IAC is that it can recognize different rAAV serotypes by immobilized antibodies. The disadvantages include (1) it is considered a luxury to use monoclonal antibody to separate rAAV from hundreds of liter cell lysate or medium [95], (2) the stringent elution conditions are always needed to break the strong antibody–antigen interactions, including low pH (such as, pH2.7 in AVB chromatography, pH2.2 in Pep8-agarose), high salt, denaturing agents or others, and (3) the low stability of the ligands to sanitizing agent is unsuitable for to large-scale processes.

3.4.3. Immobilized Metal Affinity Chromatography (IMAC)

Recombinant DNA technology can insert metal-tag into the relevant gene. The modified rAAV vectors display affinity tags on their capsid protein surface generating their purification ability by affinity chromatography. Hexahistidine affinity tag (His_6) has been inserted into the rAAV capsid protein and the virus can be purified by the immobilized nickel ions metal affinity chromatography [96]. Previous report showed that insertion of an His_6-tag at rAAV2 VP3 capsid protein did not impair the infectivity of rAAV2 particles [97]. Koerber reported that engineered rAAV2 and rAAV8 His6-containing mutants could be purified by single-step Ni-NTA chromatography and successfully used *in vitro* and *in vivo* [96]. IMAC has broad applicability in the protein purification field because of its low cost, minimal product loss, mild solution conditions and high loading capacity. But the inserted metal-tag in capsid protein may impair the rAAV tissue tropism. If cutting the tag, another purification step may make a loss of the vector.

3.4.4. Apatite Chromatography

Apatite chromatography media is composed of an inorganic salt chromatography. Hydroxyapatite, (CHT) defined as mixed mode ion-exchange resin possesses both positively charged groups (calcium ions) and negatively charged groups (phosphate group) [98]. These charged sites are distributed regularly throughout the crystal structure of the matrix. The rAAV particles dominantly interact through canion exchange via the phosphate groups or metal affinity via the calcium atoms. Canion exchange occurs when rAAV capsids' amino groups interact electrostatically with the negatively charged phosphates. Calcium affinity occurs via interacting with carboxyl clusters of rAAV vector surface protein and phosphoryl groups on genome (Fig. **3**). The calcium affinity interaction is 15 and 60 times stronger than ionic interactions and classical metal-affinity effect, respectively. Specific binding through calcium affinity may adsorb more strongly as the ionic strength increased due to ionic shielding of the charge repulsion from the CHT phosphate sites [99]. Zhou reported that CHT was an optimal column for capturing rAAV9 with approximately 90% vector yield, and more than 98% purity of vector could be obtained through combining with canion exchange resin (Poros 50HS). The binding capacity of CHT is about 27 ml cell lysate (about 54 mg total protein) per ml media [66]. It is reported that the bind capacity of apatite ranges from 1.2×10^{12} DRP per ml media to 1.5×10^{14} DRP per ml media. More than 900 ml column CHT chromatography was packaged for rAAV1 purification [57]. Alternatively, chromatography method should be used to separate rAAV from Ad based on the differences in

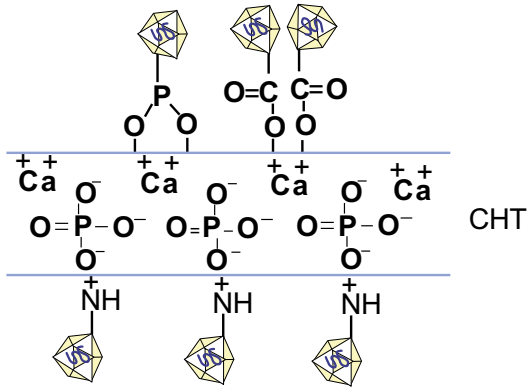

Fig. (3). The mechanism of CHT chromatography separation of rAAV vector. Calcium affinity occurs via interactions with carboxyl clusters of rAAV vector surface protein and phosphoryl groups on genome, while canion exchange occurs via rAAV capsids' amino groups with phosphates.

chemical and physical properties between the various viruses. It is reported that Adenovirus can be cleared for about 4 log reduction value (LRV) clearance [57] by CHT resin. CHT purification strategy is scalable, robust and fit for cGMP manufacturing of rAAV vectors or other serotypes.

3.4.5. Hydrophobic Interaction Chromatography (HIC)

Hydrophobic amino acids of proteins are usually far away from molecular surfaces. The sufficient exposure of hydrophobic groups allows protein to interact with hydrophobic ligands on media. HIC is an excellent purification step after ammonium sulfate precipitation or high salt elution in IEX. During HIC, target protein bound to the column in high ionic strength buffer (typical 1 to 2 M ammonium sulfate) is eluted by decreasing the salt concentration [100]. The rAAV is no exception. It can be precipitated by ammonium sulfate [101]. Then, the rAAV solution is loaded onto HIC and HIC butyl (C4) and HIC hexyl (C6) were found to be suitable for rAAV1 purification. But, low infectivity of recovery is the main problem due to disruption of viral particles at the high salt concentrations. This purification has low resolution for rAAV purification because some other proteins are resolved in the elution condition. In addition, the high salt solution may increase the risk of rAAV aggregation [57], so it is not widely used for rAAV purification.

3.5. Others

Tangential Flow Filtration (TFF) is an essential concentration method in rAAV downstream processing. It is usually used to reduce solution volume at least two-fifty folds and improve virus titer and save time [102]. The mass of rAAV virus is over 600 kDa which enables to enrich the retentate fraction (such as 100 kDa MWCO), while salt; water and small molecular weight protein pass through the membrane (Fig. **4**). As reported, a 130 fold concentration could be achieved using 100 kDa MWCO at a pressure of 10 - 12 psi without significant loss of vectors [44, 103]. There is not any phase change for ultrafiltration process that may be helpful in maintaining virus activity in contrast to other concentration methods. TFF can be used at any step of purification. It is desirable for buffer exchange before running the next purification process. Ultrafiltration is a choice of method for large-scale concentration of rAAV particles, since gentle volume reduction manifests in a relatively short time. Moreover, membrane process is easily scaled up and handled for cGMP manufacturing. Ultrafiltration has some purification effect when choosing suitable molecular weight cut-off (MWCO) to hold back big cell fraction and discharge low molecular protein. More importantly, ultrafiltration can be executed in a variety of filtration devices, such as centrifugal filtration, flat-sheet cassette devices or hollow fibers.

As analyzed above, we know that the selection of optimum rAAV purification strategies is dependent heavily on the features of the vector properties. Investigators have been looking for balance between purity and yield because every purification step may suffer loss. Usually more than one purification step is needed to get the desired purity. Fortunately, rAAV particles are physically and chemically very robust. Many methods have been reported in describing purification of various serotypes of rAAV. Recovery of the vectors from the cell lysate relies on its density or interaction with chromatographic media.

4. PROPOSED PROTOCOLS FOR SCALABLE DOWNSTREAM PURIFICATION OF rAAV VECTORS

In the present study, a multi-step purification protocol has been developed, in general combining membrane-based, several chromatographic and centrifugal process techniques. An optimum purification strategy in downstream processing usually includes the concentration, vector purification step and polish step (Fig. **5**).

4.1. Capture Chromatography Combined with Separation Chromatography

Nowadays, it is difficult to obtain sufficiently pure rAAV vectors to meet clinical requirement in one step purification. Generally, there are two column chromatography steps. The first column acts as capture chromatography to catch rAAV from raw materials. The second column acts as separation chromatography to isolate rAAV from the elution fraction. Combination of the same or different kinds of chromatography in series has been reported, such as AC combining with AEX [108], AEX with CEX [48, 61-63], IEX with HIC or SEC [54, 55], DIAM [43, 111], apatite with CEX [66], HIC or SEC [57] (Table **1**).

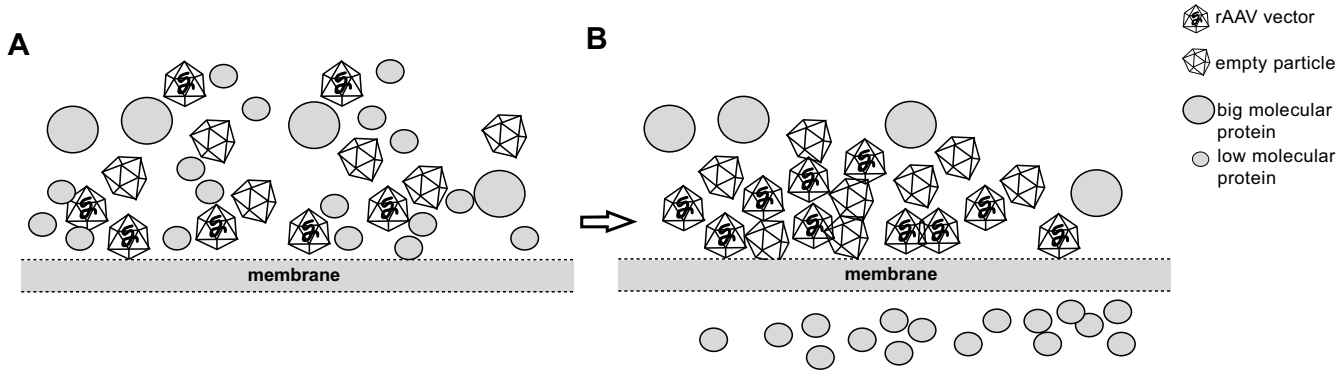

Fig. (4). TFF concentration of rAAV. (**A**). The cell lysate contains rAAV, empty particles, large molecular protein or low molecular materials. (**B**). After TFF, the low molecular materials get through the membrance, while the vector or large molecular proteins are concentrated.

Table 1. Purification rAAV in deferent method.

rAAV serotype	Purification steps and type				Purity	Scalability	References
	1st	2nd	3rd	Type			
All serotype	CsCl	CsCl	CsCl	UC/UC/UC	< 90%	No	[104]
	CsCl	CsCl	/	UC/UC	> 98%	No	[27]
rAAV1	Poros 50HQ	Poros 10HQ	Sephacryl S-300 HR	AEX/AEX/SEC	> 95%	Yes	[55]
	Iodixanol	HiTrap Q	/	UC /AEX	> 95%	No	[63]
	AVB sepharose HP	/	/	IAC	> 90%	Yes	[93]
	CsCl	Mustang S	Mustang Q	UC/ DIAM	> 99%	No	[43]
	CHT	AEX	HIC/SEC	Apatite/ AEX/ HIC/SEC	> 95%	Yes	[57]
BAP rAAV1	Monomeric avidin agarose	/	/	IAC	> 90%	No	[94]
rAAV2	Poros 20PI	/	/	AEX	> 90%	Yes	[61]
	Poros 50HQ	Poros 50HQ	/	AEX/AEX	> 90%	Yes	[48]
	Poros 50HS	Poros 50HS	/	CEX/CEX	> 90%	Yes	
	Poros 50HS	Q-Sepharose xl	/	CEX /AEX	> 90%	Yes	
	SP Sepharose HP	HiTrap Q	/	CEX/AEX	> 98.5%	Yes	[62]
	SP Sepharose HP	Source 15Q	/	CEX/AEX	> 98.5%	Yes	
	Poros 20 HE	/	/	HEAC	> 99%	Yes	[61]
	Iodixanol	HiTrap Q	/	UC/AEX	> 99%	No	[63]
	Iodixanol	heparin	/	UC/HEAC	> 99%	No	
	Sulfonated cellulose	/	/	AC	/	No	[105]
	Iodixanol	UNO-S1	/	UC/CEX	> 90%	No	[106]
	Iodixanol	Poros HE	/	UC/HEAC	> 90%	No	
	HPLC heparin	/	/	HEAC	> 90%	Yes	[107]
	Poros HE	/	/	HEAC	> 98%	Yes	[88]
	Poros 20 HE	Poros 50 PI	/	HEAC /AEX	> 95%	Yes	[108]
	CHT	DEAE Macroprep	Cellufine sulfate	Apatite /AEX/AC	> 90%	Yes	[109]
	Heparin	/	/	HEAC	> 90%	Yes	[110]
	Heparin	Phenyl-Sepharose	Heparin	HEAC/HIC/HEAC	> 99%	Yes	[63]
	AVB sepharose HP	/	/	IAC	> 90%	Yes	[93]
	Mono Q HR	Superdex 200	/	AEX/SEC	> 97%	Yes	[54]
His6rAAV2	Ni-NTA agarose	/	/	IMAC	> 90%	No	[96]
rAAV4	Poros PI	Poros HQ	/	AEX/AEX	> 90%	Yes	[61]
	Poros HQ	/	/	AEX	> 90%	Yes	
rAAV5	Mustang S	Mustang Q	/	DIAM	> 90%	Yes	[111]

(Table 1) Contd….

rAAV serotype	Purification steps and type				Purity	Scalability	References
	1st	2nd	3rd	Type			
	Mono Q HR	Superdex 200	/	AEX/SEC	> 97%	Yes	[54]
	Iodixanol	HiTrap Q	/	UC/AEX	> 99%	No	[63]
	SP Sepharose HP	Source 15 Q	/	CEX/AEX	> 98.5%	Yes	[62]
	Poros PI	/	/	AEX	> 99%	Yes	[61]
	mucin coupled Sepharose	/	/	AC	> 90%	No	[91]
rAAV6	Poros 50HQ	Poros 50HQ	/	AEX/AEX	> 90%	Yes	[48]
rAAV8	SP Sepharose HP	Source 15Q	/	CEX/AEX	> 98.5%	Yes	[62]
	CsCl	Mustang S	Mustang Q	UC/DIAM	> 99%	No	[43]
	Sephacryl S-300 HR	Poros 50HQ	/	SEC/AEX	98%	Yes	[28]
	Pep8-agarose	HiTrap Q		IAC/AEX	> 90%	No	[112]
His6rAAV8	Ni-NTA agarose	/	/	IMAC	> 90%	No	[96]
rAAV9	CHT	Poros 50HS	CsCl	apatite /CEX/UC	> 99%	Yes	[66]

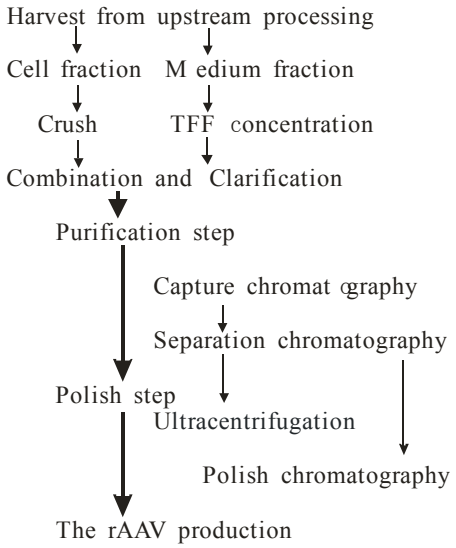

Fig. (5). The flow chart of rAAV vector in downstream processing.

For the capture chromatography step, the selection of suitable binding and elution buffer should be based on the following considerations: (1) the media has suitable binding and elution ability with rAAV when vector containing solutions passes though. It does not require too severe conditions for loading, washing and elution. (2)The media has higher flow rates and significant gains in throughput and productivity when scaling up to production. (3) The media needs high capability to bind rAAV as much as possible. (4) And possesses some intensity to bear high pressure during purification processing. (5) The cost of media must be considered in large-scale. Some resins reported are perfect in downstream process. Poros HQ, a common industrial resin is used because of its high resolution, high binding capacity, and the high flow rate gains in throughput and productivity when scaling up to production. POROS HQ is deemed to be the most widespread of chromatography which has been used for purification for rAAV2 [61], rAAV4 [61], rAAV5 [61], rAAV6 and rAAV8 [28], and has proved appropriate for a large-scale, cGMP-compatible rAAV production. CHT as an industrial resin is reported that the recovery is more than 90% in the assistance of PEG [57, 66].

Most contaminants are removed after the capture chromatography step the purpose of second chromatography is to get purer vector. Unlike capture chromatography, there are more choices for chromatography. The principle for the second chromatography media choice is based on two features: (1) one is the efficiency; it refers to the location of the maximum concentration of a peak. The ideal situation has sharp peaks resolved. Peaks in which the maxima are widely disengaged still may be so broad that the vector is incompletely resolved. Plate height and number of theoretical plates determine the efficiency of the column. Improving column efficiency is to increase the number of plates and decrease the plate height [113]. (2) The other is resolution. Efficiency is important to achieve satisfactory resolution. The vector peak and the adjacent peaks must be disengaged. Such disengagement depends on the vector property and the selectivity of the stationary and mobile phases. Various chromatographies have been used to separate rAAV vector, for example, Poros HS [66], Source 15Q [62], Poros HQ [55], Q-Sepharose[xl], Superdex 200 [54] and Phenyl-Sepharose [63]. Poros HS can be used not only as the first column for rAAV2 purification but also as the second column for rAAV9. As high as over 1×10^{14} VG per ml resin binding capacity, more than 98% purity and 94.4% of vector yields can be obtained. In downstream processing, more than two columns for purification are required in order to get higher purity. For instance, an rAAV1 purification strategy has been developed in which CHT acted as a capture resin; anion exchange resin was used for further separation, which was followed by HIC and SEC for polish steps [57].

4.2. Ultracentrifugation or Polish Chromatography as Polish Step

The aim of the polish step is to separate the related impurity, such as empty particles. In early times, CsCl or iodixanol step gradients centrifugation acted as the first step followed by anion or AC for rAAV1, 2, 5 purification [63, 106]. If ultracentrifugation is adopted in the first step, it would limit the scale-up in downstream processing. Ultracentrifugation could be introduced as a polish step to remove the empty particles at the end of a standard chromatography process. Jingmin Zhou [66] reported that the rAAV empty particles could be removed by CsCl when the samples had been separated and concentrated by IEX or apatite chromatography. When the only aim of ultracentrifugation is to separate empty and rAAV vectors, it will be extremely effective and easily handled. Sometimes, even the third or fourth chromatography is required as polish chromatography for rAAV purification. CIM, DIAM, SEC or TFF may be chosen for separation related impurity or changing the buffer for storing rAAV.

5. CONCLUSION

The rAAV is an excellent vector for gene therapy human disease. FDA-approved processes for purification of rAAV products suitable for clinical trials rely upon commercial scale processes [22]. The optimal purification processing overcomes the bottleneck for further human therapy studies and pharmaceutical preparation from rAAV vector production. It is expected that, these well-established and practical downstream purification methods would promote the coming of another era of AAV vector based gene therapy.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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