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Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield

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Conventional methods for rAAV purification that are based on cesium chloride ultracentrifugation have often produced vector preparations of variable quality and resulted in significant loss of particle infectivity. We report here several novel purification strategies that involve the use of non-ionic iodixanol gradients followed by ion exchange or heparin affinity chromatography by either conventional or HPLC columns. These methods result in more than 50% recovery of rAAV from a crude lysate and routinely produce vector that is more than 99% pure. More importantly, the new purification procedures consistently produce rAAV stocks with particle-to-infectivity ratios of less than 100, which is significantly better than conventional methods. The new protocol increases the overall yield of infectious rAAV by at least 10-fold and allows for the complete purification of rAAV in 1 working day. Several of these methods should also be useful for large-scale production.

Keywords: *adeno-associated virus; rAAV; gene therapy; vector production; vector purification*

Introduction

Recombinant adeno-associated virus (rAAV) has proven to be a useful vector for efficient and long-term gene transfer in a variety of tissues including lung,[1] muscle,[2–5] brain,[6] spinal cord,[7] retina[8,9] and liver.[10] rAAV vectors consist of a simple capsid with a single-stranded DNA genome and no viral coding sequences.[11,12] This vector system appears to elicit limited immune responses because of limited ability to transduce dendritic cells.[13] Currently, the first phase I clinical trials using recombinant AAV are under way for cystic fibrosis.[14,15]

rAAV is most often generated by cotransfection of rAAV vector plasmid and wild-type (wt) AAV helper plasmid into Ad-infected 293 cells.[16] Recent improvements in AAV helper design[17] as well as construction of non-infectious mini-Ad plasmid helper[18–20] have eliminated the need for Ad infection and improved the yield of rAAV per transfected cell in a crude lysate. Scalable methods of rAAV production that do not rely on DNA transfection have also been developed.[21–24] These methods, which generally involve the construction of producer cell lines and helper virus infection, are suitable for high-volume production. Despite these changes in the method of production, little progress has been made on the downstream purification of rAAV. A simple and rapid protocol that efficiently purifies high titer rAAV suitable for clinical studies remains to be established.

The conventional rAAV purification protocol involves the stepwise precipitation of rAAV using ammonium sulfate, followed by two, or preferably three rounds of CsCl density gradient centrifugation. Each round of CsCl centrifugation involves fractionation of the gradient and identification of active fractions by dot-blot hybridization or by PCR analysis. Not only does it require up to 2 weeks to complete, the current protocol often results in poor recovery and poor quality virus. The growing demand for different rAAV stocks often strains the limited capacities of vector production facilities. The need for a protocol that will substantially reduce the preparation time without sacrificing the quality and purity of the final product is clear.

We have developed an affinity purification procedure based on the recent observation that AAV binds to cell surface heparan sulfate proteoglycan.[25] To bind the virus to the affinity media efficiently, we have also introduced a new bulk purification technique that involves the use of the non-ionic gradient medium, iodixanol. This combination results in high recovery rates, improved viral infectivity and more rapid purification. We report here a novel method of rAAV purification resulting in higher yield and improved infectivity of viral particles (Figure 1).

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Results

Production of rAAV crude lysates

The primary production method used in these studies was calcium phosphate-mediated transfection using the rAAV vector plasmid pTR-UF5,[26] which contains the humanized *gfp* gene under control of the CMV promoter. Virus stocks were titrated by infectious center assay (ICA) as well as the green fluorescence cell assay (FCA)

based on GFP fluorescence (see Materials and methods for details). The helper plasmid, pDG, contains both the AAV genes (*rep* and *cap*), and only the adenovirus genes required for AAV propagation.[18] No replication-competent adenovirus has been detected with the use of this plasmid. To simplify the transfection protocol, the $CaPO_4$/DNA precipitate was left in the media for the entire incubation period of 48 h. This did not appear to compromise cell viability. The transfection efficiency routinely reached 60% as judged by GFP fluorescence (not shown). After harvesting the cells, virus was extracted by freezing and thawing the cells and clarified by low speed centrifugation. The use of sonication, microfluidizing and detergent extraction (for example, deoxycholate) did not appear to increase significantly the viral yield in initial experiments (data not shown).

It is also possible to concentrate and purify rAAV from the cell culture media apart from the material in the cell pellet. For bulk purification from media, we precipitated virus from the media with 50% ammonium sulfate. However, this procedure was felt to be unnecessary in light of the finding that at the time of harvest (48 h after transfection), the majority of the virus (about 90%) was found in the cell pellet, confirming previous observations.[18,19]

Characterization of the purified rAAV

An important index of virus quality is the ratio of physical particles to infectious particles in a given preparation. To characterize the purification steps and the quality of the virus obtained using different methods, we used two independent assays to titrate both physical and infectious rAAV particles. A conventional dot-blot assay (not shown) and QC PCR assay (Figure 2a) were used for physical particle titers. Infectious titers were determined by infectious center assay (ICA) and a fluorescence cell assay (FCA), which scored for expression of GFP (Figure 2b). In order to avoid adventitious contamination of rAAV stocks with wtAAV, the use of wtAAV was eliminated in the ICA (as well as all other protocols in the laboratory). This was made possible by the use of the C12 cell line,[24] which contains integrated wtAAV *rep* and *cap* genes, for both the ICA and the fluorescent cell assay. Adenovirus serotype 5 (Ad5), which was used to co-infect C12 along with rAAV, was titrated using the same C12 cell line in a serial dilution cytopathic effect (CPE)

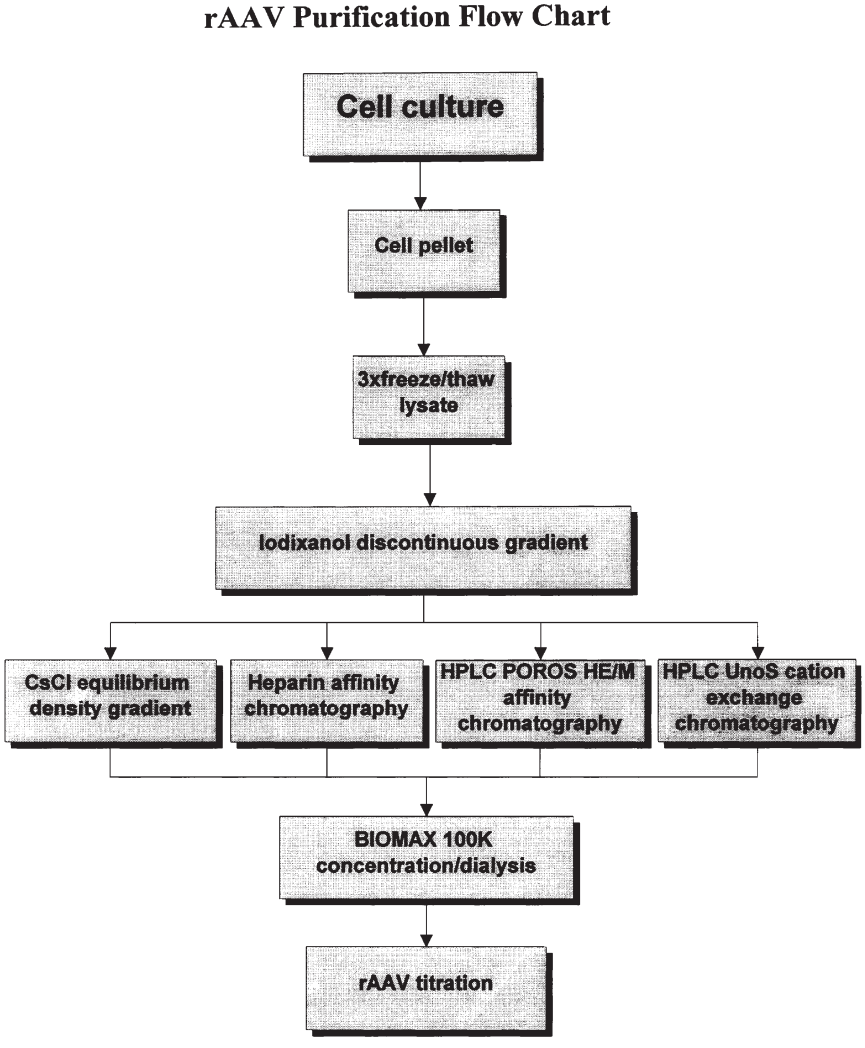

Figure 1 *The diagram illustrates the steps in rAAV purification that were investigated in this study.*

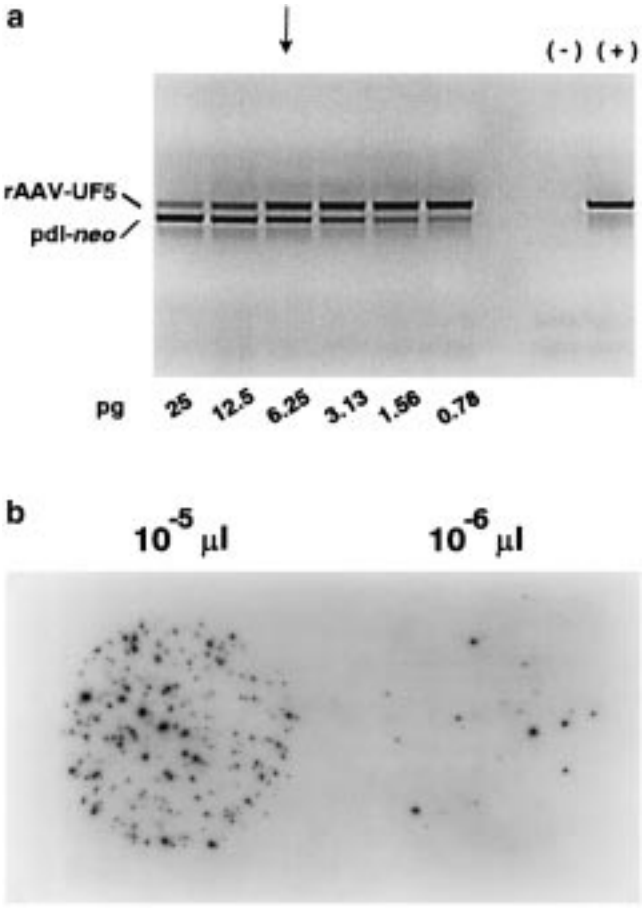

Figure 2 *Titering of rAAV stocks by the QC PCR assay (a) or infectious center assay (b). (a) Agarose gel electrophoresis of QC-PCR products (negative image). The top band is the product obtained from rAAV-UF5 viral DNA; the bottom band (pdl-neo) is the PCR product obtained from the standard competitor plasmid, which contains a small deletion in the region that is amplified. The numbers below each lane show the amount of input competitor DNA in each reaction. The viral DNA sample was diluted 1000-fold to achieve a template concentration which results in linear amplification of the input DNA. Symbols (–) and (+) indicate negative (no template) and positive (vector DNA without competitor) controls in the PCR reaction. The arrow indicates the concentration at which the target template and the standard template were equivalent. (b) Autoradiographic image of nylon filters containing products of an infectious center assay, in which C12 cells had been infected with rAAV-UF5 and Ad5 and hybridized with ^{32}P-labeled GFP probe. Numbers at the top show the respective dilutions of the rAAV used to infect the C12 cells. The assay was typically linear in the range of 10–200 spots.*

assay. The amount of Ad producing well-developed CPE in 48 h on C12 cells was used to provide helper function for both the ICA and FCA assays. Both physical particle titers and infectious titers, each obtained by two independent methods, were generally in agreement, differing in most cases by a factor of 2 or less (Table 1).

Iodixanol density step gradient

Early in our attempts to design an efficient purification method, it appeared that rAAV and cell proteins were forming aggregates in the crude cell lysate. Such complexes failed to display uniform biochemical properties, which made it difficult to develop a purification strategy, and led to poor recovery of the virus at all purification stages. Furthermore, this nonspecific interaction resulted in contamination with adventitious proteins even after several rounds of CsCl gradient centrifugation. Bulk purification of the crude lysate was, therefore, the most crucial step in rAAV purification.

To avoid aggregation, we investigated several alternative methods including the use of detergents and limited proteolytic digestion. Although these methods provided some improvement during subsequent purification, they resulted in preparations that were heat labile or sensitive to DNase (data not shown). Ammonium sulfate precipitation, which has been used successfully for concentrating virus, did not provide significant purification (data not shown). Furthermore, the residual ammonium sulfate in the protein pellet interfered with subsequent ion-exchange chromatography. Dialysis at this purification stage lead to the aggregation and precipitation of proteins and poor recovery of rAAV. We also tried the combination of $(NH_4)_2SO_4$ precipitation and hydrophobic interaction phenyl-sepharose chromatography. This approach also failed to produce a purified virus without substantial loss of viral infectivity (not shown). Tamayose *et al*[27] recently proposed cellufine sulfate chromatography as a method of purification and concentration of rAAV from crude lysates. We found, however, that cellufine sulfate fails to bind rAAV in crude lysates reproducibly (not shown). To circumvent these problems, we adapted a new strategy of purification based on density gradient centrifugation using iodixanol.

Iodixanol is an iodinated density gradient medium originally produced as an X-ray contrast compound for clinical use. Unlike CsCl and sucrose gradients commonly used for fractionating macromolecules, iodixanol solutions are made iso-osmotic at all densities. This property makes iodixanol a useful solvent for final purification and analysis steps. Because of its non-ionic and inert nature, electrophoretic analysis and viral infectivity assays can be carried out on gradient fractions directly in the presence of iodixanol. Since the viscosity of iodixanol solutions are also lower than sucrose of the same density, it is possible to use iodixanol fractions directly in subsequent chromatography purification steps without dialysis or dilution.

Because the apparent density of macromolecules in iodixanol is different from that in CsCl, the banding density of purified rAAV-UF5 was determined empirically by banding an aliquot of UF5 virus in a continuous iodixanol gradient. The density of rAAV-UF5 was found to be 1.266 g/ml (not shown), which is equivalent to a 50% solution of iodixanol. We chose to generate preformed step-gradients in order to expedite the centrifugation step. We used a 40% iodixanol (1.23 g/ml) step to accommodate rAAV/protein complexes trailing at slightly lower densities, followed by a 60% step that would presumably act as a cushion for any rAAV containing a full-length genome. To locate the 40% density step after centrifugation, we included phenol red dye (0.01 µg/ml) in the upper 25% and lower 60% density steps. The step gradient (consisting of 15, 25, 40 and 60% iodixanol) ran in a Ti70 rotor (Beckman, Fullerton, CA, USA).

Our initial attempts to purify rAAV using discontinuous iodixanol gradients were unsuccessful. As mentioned previously, rAAV aggregates with proteins in the cell lysate. This apparently changes its buoyant density and results in distribution of vector along the whole length of the gradient. To solve this problem, we modified the

Table 1 Summary of rAAV-UF5 purification

Experiment[d]		*Purification step*	*Particles by dot-blot* ($\times 10^{-11}$)[a]	*Particles by QC PCR* ($\times 10^{-11}$)[a]	*Infectious units by ICA* ($\times 10^{-9}$)[a]	*Infectious units by FCA* ($\times 10^{-9}$)[a]	*Particle-to-infectivity ratio*[b]	*Infectious units per cell*[c]	*Particles % recovery*[e]	*Infectious units % recovery*[e]
1	1	3 × freeze/thaw lysate	57	103	69	62.7	90.8	209	100	100
	2	Iodixanol	44	82	32.3	51	86	170	76	81
	3	Iodixanol/CsCl	5.7	2.5	4	3.6	158	12	(8.4)	7(6)
	4	Iodixanol/Heparin agarose	20	63	32	35	56	117	(35)	69(56)
	5	Iodixanol/HPLC POROS HE/M	15	16	12	20	73	67	(26)	40(32)
	6	Iodixanol/HPLC UNO S1	19	13	20	20	95	67	(33)	40(32)
2	7	2 × CsCl	7	6	4.8	2.9	241	1	—	—
3	8	$(NH_4)_2SO_4$/2 × CsCl	—	0.2	—	0.012	1667	—	—	—
	9	Iodixanol/Heparin agarose	—	1.0	—	1.5	67	—	—	—

[a]The yield of rAAV in each row is normalized to 3×10^8 cells (ten 15 cm plates).
[b]The particle-to-infectivity ratio was calculated using numbers obtained by dot blot assay and fluorescence assay.
[c]The yield per cell was calculated by using FCA.
[d]Experiment 1: The crude lysate (line 1) was produced by transfection of 293 cells with pDG and pTR-UF5 as described in Methods. The crude lysate was then purified by iodixanol step gradient centrifugation to produce the iodixanol fraction (line 2). The iodixanol fraction was then split into four equal parts and purified as described in lines 3–6. Experiment 2: A crude lysate was produced by transfection of 293 cells with pXX6, pACG and pTR-UF5 and purified by the conventional ammonium sulfate/cesium chloride protocol as described in Methods. Experiment 3: The crude rAAV stock was made by cotransfection of 293 cells with pDG and pTR-UF5 and purified by either ammonium sulfate and two cesium chloride gradients or by iodixanol/heparin agarose fractionation.
[e]Recovery of particles or infectious units for the particular step. Numbers in parenthesis are the overall % recovery at the indicated step in the purification. Infectious unit recoveries calculated from FCA assay, particle recoveries calculated from dot blot assay.

first iodixanol step (15%) so that it contained 1 m NaCl to destabilize ionic interactions between macromolecules. High salt was excluded from the rest of the gradient in order to band the virus under iso-osmotic conditions, hence allowing direct loading in subsequent chromatographic steps.

A typical gradient, before and after the 1 h centrifugation, is shown in Figure 3a, left and right tubes, respectively. A plot of the refractive index and density at the end of the run is shown in Figure 3b. rAAV is distributed through the 40% density step (Figure 3a, shown by the vertical bar, and Figure 3c). During a production run, the virus is typically recovered by inserting the syringe needle at about 2 mm below the 60%-to-40% density junction (indicated by the arrow) and collecting a total of 4 ml. In Figure 3, however, the gradient was collected by

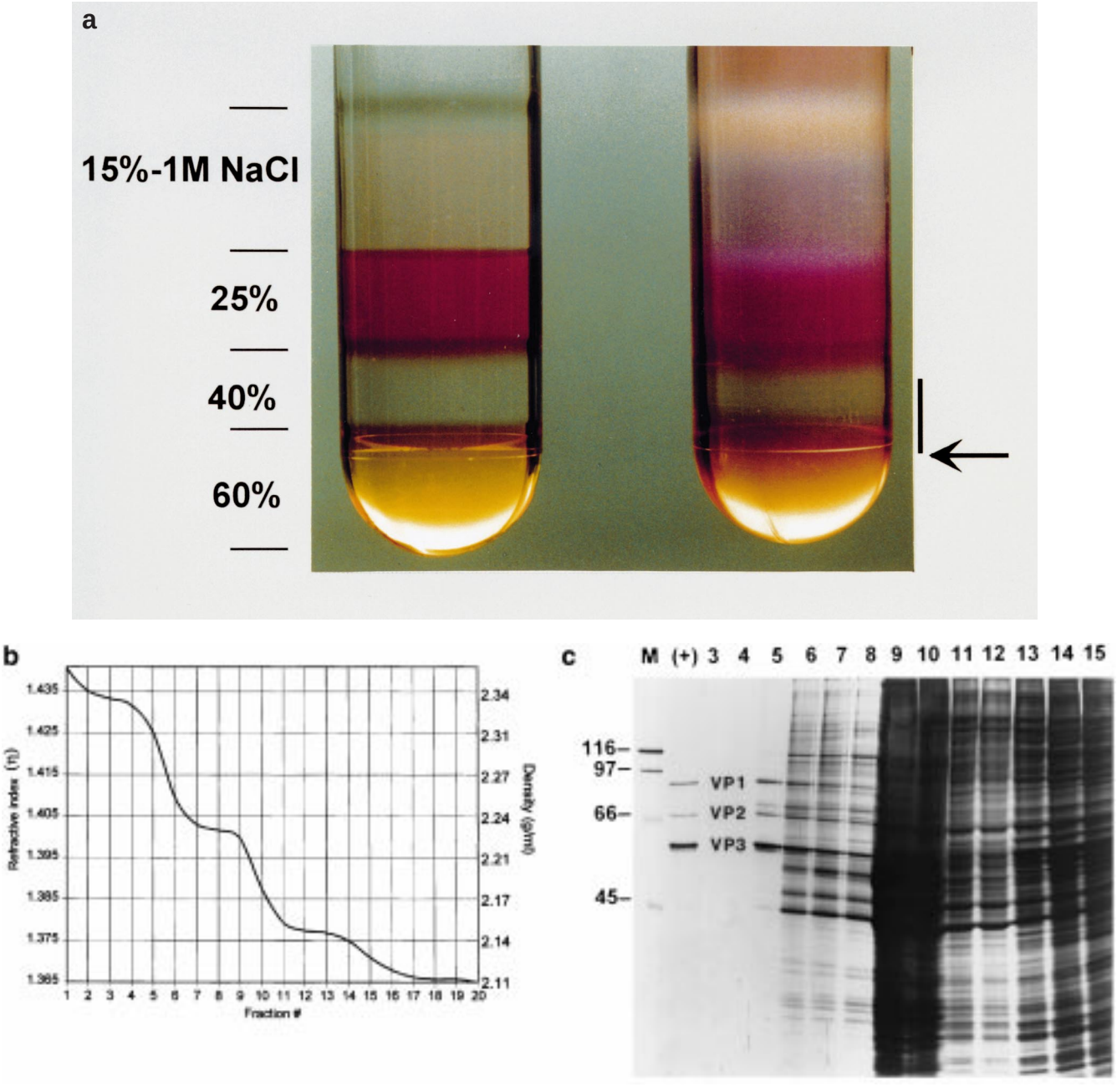

Figure 3 *Iodixanol step gradient for the purification of rAAV. (a) Preformed gradients shown before (left tube) and after (right tube) the 1 h spin. The red or yellow tinge in the 60 and 25% steps is due to the inclusion of phenol red in these steps. The positions of the density steps are shown on the left and the distribution of rAAV through the gradient is shown by the vertical bar. Typically, virus was collected by a needle puncture at the position on the side of the tube shown by the arrow. (b) A plot of the refractive index and density of 1 ml fractions collected from the bottom of the tube on the left in (a). The density of the respective iodixanol fractions could be derived using the formula: density (g/ml) = η3.362 – 2.483, where η is refractive index (Nycomed). (c) Silver-stained SDS/protein gel analysis of iodixanol fractions. rAAV was collected by dripping 1.0 ml fractions from the bottom of the gradient shown on the right in (a). Equivalent amounts of each fraction were then loaded on to a 12% SDS acrylamide gel and electrophoresed for 5 h at 200 V. The numbers on the top of the gel correspond to the fraction numbers; only fractions 3–15 are shown. VP1, VP2 and VP3 indicate the position of viral capsid proteins. Lane (+) contains purified rAAV virus as a positive control. The lane marked M contains protein standards whose molecular weight are shown on the left in kDa.*

dripping from the bottom and collecting 1 ml fractions for detailed analysis. The bulk of the rAAV virus bands within the 40% density step (Figure 3c, fractions 5–8). A heavy band that migrates at the 40%-to-25% density junction consists mostly of cellular proteins (Figure 3c, fractions 9 and 10) and contains less then 5% input rAAV, as judged by FCA. A small amount of rAAV also bands at the 40/60% junction (Figure 3b and c, fraction 5). Table 1 illustrates the recoveries that were typically achieved. Approximately 75–80% of the rAAV in the crude lysate is recovered in the iodixanol fraction (Table 1).

The nucleic acid/protein ratio in rAAV-UF5 is different from wtAAV because of the size of the DNA packaged: 3400 bases or approximately 73% wt size. Using the same protocol with no modifications, we have purified over 15 different rAAV vectors, in which the size of the packaged genome ranged from 3 to 5 kb. Regardless of the size, there is no substantial difference in the banding pattern of rAAV (not shown). Therefore, there is no need to modify the iodixanol step gradient to accommodate rAAV genomes in this size range.

To determine the resolving capacity of the iodixanol gradient an experiment was performed in which viral lysates obtained from 5, 10 or 15 plates (15 cm) were loaded on to separate gradients. This was equivalent to 1.6, 3.1 or 4.7×10^8 cells, respectively. After centrifugation, rAAV was recovered from each gradient as described in Materials and methods, and aliquots from each gradient were subjected to SDS-gel electrophoresis (Figure 4). The three viral capsid proteins VP1, VP2 and VP3 constituted the major protein species seen at all concentrations, suggesting that good separation of rAAV was achieved even at the highest concentration of lysate used. However, a significant increase in background contaminating protein

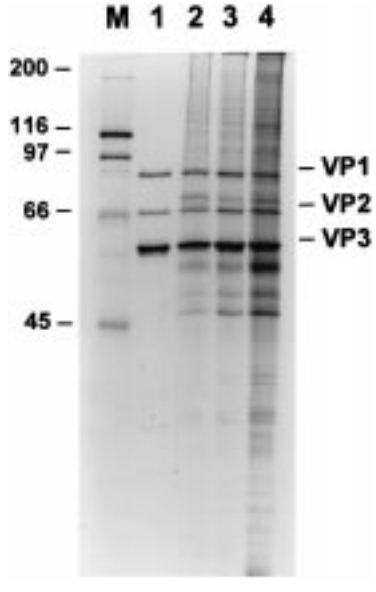

Figure 4 *Silver-stained SDS/protein gel analysis of the resolving capacity of an iodixanol step gradient. The virus lysate from 5, 10 or 15 dishes (lanes 2, 3 and 4, respectively) was purified on a single iodixanol gradient of the type shown in Figure 3a. After virus was collected, equivalent amounts of rAAV were loaded from each gradient on to a SDS-acrylamide gel as described in Figure 3. VP1, VP2 and VP3 indicate the position of the viral capsid proteins. Lane 1 contains purified virus as a positive control. The lane marked M contains standard proteins whose molecular weight are indicated on the left. See text for additional details.*

was seen when the lysate from 15 plates was used. Therefore, in further experiments we routinely loaded the lysate from only 10 plates per gradient. As a practical matter, this means that a 1 h iodixanol gradient run in a Ti 70 rotor would accommodate 2.5×10^9 cells from which approximately 10^{14} virus particles or 10^{12} infectious units could be purified.

Methods for separating adenovirus from rAAV

The production of rAAV by transient cotransfection with a mini-Ad plasmid is an efficient but laborious protocol. Although it eliminates the problem of removing Ad virus from the rAAV crude lysate, it requires up to 1 mg of plasmid DNA (combined), for transfection of 10 plates. For this reason, many laboratories continue to use adenovirus co-infection in lieu of the mini-Ad plasmids. Furthermore, infection of cells with Ad or herpes virus is likely to be more useful for large-scale production using suspension cell culture. The ideal production system would consist of a rAAV proviral cell line, induced to rescue and replicate by infection with a helper virus[22] (HSV, see accompanying article in this issue, pp 986–993 or rAd) carrying the rep and cap functions. For downstream purification the HSV helper could be separated from rAAV by simple filtration due to the considerable size difference between HSV and rAAV or by exposure to high-salt.[22] In the case of Ad, rAAV is usually separated by a combination of CsCl gradient centrifugation and heat treatment, both approaches suffering drawbacks. We investigated whether the iodixanol gradient would be effective in separating Ad from rAAV, and whether it could be combined with ion exchange chromatography (Figure 1) to separate rAAV and Ad without the use of heat inactivation.

To address this issue we prepared a vector, pTR-UF6, with a modified gfp cDNA that fluoresces blue. This construct is identical to pTR-UF5 except that the *gfp* cDNA contains a Tyr-145-Phe mutation in the pTR-UFB background described previously.[26] Use of pTR-UF6 along with a rAd-gfp vector (rAd-Uf7) allowed us to monitor infections with rAAV (pTR-UF6) and rAd (rAD-UF7) simultaneously by scoring for blue or green cells respectively (Figure 5). rAd-UF7 is a recombinant E1–E3-deleted Ad vector that contains the *gfp/neo* cassette from pTR-UF5. 293 Cells were cotransfected with pTR-UF6 and pDG, and simultaneously infected with the rAd-UF7 at a multiplicity of infection (MOI) of 10. Cells transfected with pTR-UF6 and infected with rAd-UF7 were processed through the iodixanol step exactly as described in Materials and methods for the purification of rAAV from cells transfected with the mini-Ad plasmid alone. The gradient was fractionated after puncturing the bottom of the tube and 25 μl aliquots from each fraction were subjected to the SDS acrylamide gel electrophoresis. The results of a Western analysis after detection with polyclonal anti-Ad antibody are shown in Figure 6. More then 99% of the Ad, as judged by the fluorescence assay, banded in the gradient with densities lower than 2.22 g/ml (fractions 9–15). rAAV, on the other hand, banded in fractions 5–8 (not shown, however, compare with Figure 3c), clearly separated from the Ad. The crude lysate contained 4.5×10^{10} p.f.u. of rAd-UF7 (as determined by the fluorescence cell assay). After the iodixanol gradient, the titer of the rAd-UF7 dropped to 4.2×10^8 p.f.u., approximately a 100-fold reduction (Table 2).

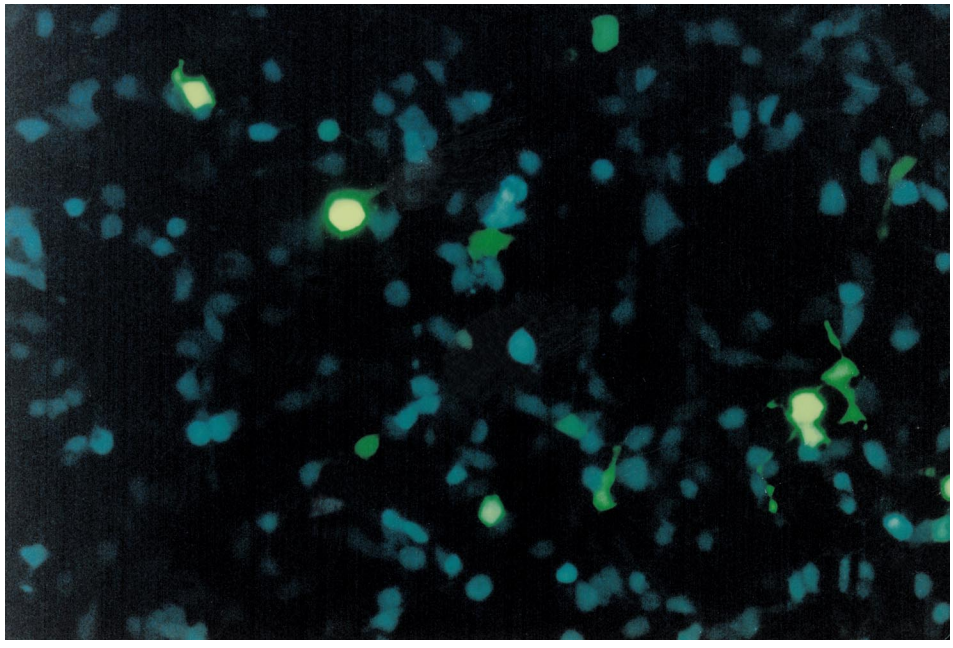

Figure 5 *293 Cells, infected with an rAAV-UF6 and rAd-UF7 mixture after an iodixanol purification step. Cells infected with rAAV fluoresce blue; cells infected with rAd (or both viruses) fluoresce green (see Materials and methods for details). The few green cells represent a 2-log reduction in rAd-UF7 contamination after iodixanol (Table 2).*

Heparin affinity chromatography

To purify rAAV further, we explored the use of column chromatography as a second step in purification following the iodixanol gradient. To compare the effectiveness of the various column chromatography steps, rAAV-UF5 was prepared from 1×10^9 cells as described in Materials and methods, using pDG helper plasmid. The crude lysate was purified using the iodixanol step gradient and virus-containing fractions were pooled. The pooled fractions were then split into equal portions and virus was purified using four different methods illustrated in Figure 1: (1) CsCl density gradient centrifugation; (2) heparin affinity chromatography; (3) HPLC heparin affinity chromatography; and (4) HPLC cation exchange chromatography. We monitored the purification steps by measuring rAAV titers, both physical and infectious, as well as protein concentration in virus samples generated by each purification step (Table 1, experiment 1, Figure 7). For purposes of comparison, a second batch of virus was purified by the commonly used method of ammonium sulfate precipitation followed by two consecutive CsCl gradients (Table 1, experiment 2).

Heparinized supports have been successfully used for the purification of many heparin-binding macromolecules, including viruses such as CMV.[28] Heparin is the glycosaminoglycan moiety covalently bound to the protein core of proteoglycans (PG) found in mast cells. It is closely related to heparan sulfate (HS), the glycosaminoglycan (GAG) chain of the HS proteoglycan (HSPG). The latter has been shown to be a cell surface receptor that mediates AAV cellular binding.[25] Covalent binding of heparin molecules to the matrix through its reducing end mimics the orientation of the naturally occurring GAGs.[29] To take advantage of the structural similarities between heparin and HS, we tested heparin affinity chromatography to purify rAAV further.

Heparin is a heterogeneous carbohydrate molecule composed of long unbranched polysaccharides modified by sulfations and acetylations. The degree of sulfation strongly correlates with the virus-binding capacity of HS.[30] Therefore, we anticipated that heparinized matrices from different vendors would display different affinity towards rAAV. To develop the method, we tested several heparin ligand-containing media, including ACTI-Disk 50 (Arbor Technologies), which is a heparinized filter disk, three column chromatography media manufactured by Sigma (St Louis, MO, USA), Heparin-Agarose Type I, Heparin-Agarose Type II-S, Heparin Agarose Type III-S, and finally Affi-Gel Heparin Gel (Bio-Rad, Hercules, CA,

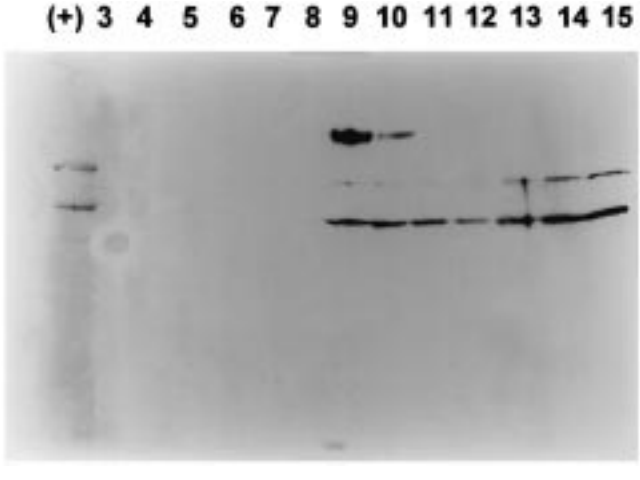

Figure 6 *Western blot analysis of the iodixanol gradient, fractionated exactly as described in Figure 3. The numbers on the top of the gel correspond to the fraction numbers. Lane (+) contains purified rAd positive control sample. The filter was probed with polyclonal anti-Ad antibodies. AAV fractions 3–8 show no reactivity with the anti-Ad antibody. Adenovirus capsid proteins are detected in fractions 9–15.*

Table 2 Comparison of Ad contamination as a function of purification method

Purification step	*rAAV titer (FCA)*	*rAd-UF7 titer (FCA)*
Crude	10^{10}	4.5×10^{1}
Iodixanol	8×10^{10}	4.2×10^{8}
POROS HE/M	3.4×10^{10}	8×10^{5}
$(NH_4)_2SO_4$/2 × CsCl	—	4.5×10^{8}

A crude rAAV stock was made by cotransfection of 293 cells with pDG and pTR-UF6 and co-infection with rAd-UF7. The rAAV was then purified by iodixanol/POROS HE/M fractionation or by ammonium sulfate fractionation followed by two consecutive CsCl fractionations. Ad and rAAV titers were determined by fluorescent cell assay (see Methods for details).

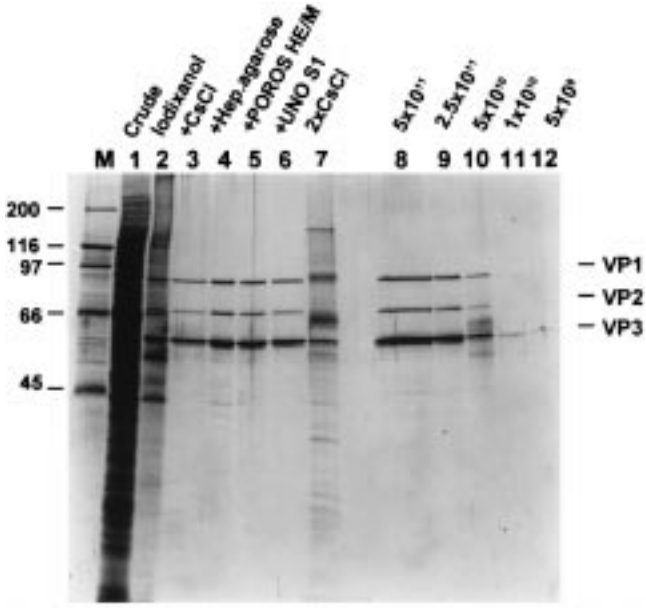

Figure 7 *Silver-stained SDS acrylamide gel electrophoresis of rAAV-UF5 at various stages of purification. VP1, VP2 and VP3 indicate the position of the three AAV viral capsid proteins. Lanes 1–6 are various fractions obtained from a single virus preparation that was processed according to the scheme illustrated in Figure 1. The titers and recovery at each step are summarized in Table 1, experiment 1. Lanes 8 to 12 contain serial dilutions of the UNO SI fraction shown in lane 5, illustrating that the UNO-S1 fraction is 99.9% pure. Lane 7 contains a separate rAAV-UF5 preparation that was purified by the conventional method of $(NH_4)_2SO_4$ precipitation followed by two CsCl gradients. The titers of this fraction are summarized in Table 1, experiment 2.*

USA). Although ACTI-Disk 50 filter disks were found to bind rAAV quantitatively, and to be useful for purifying rAAV (data not shown), the manufacturer discontinued this product, and thus, we did not pursue this approach. Affi-Gel Heparin Gel and Heparin Agarose Type III-S columns failed to bind at least 50% of the virus (not shown) and, therefore, were excluded from further consideration. Heparin-Agarose Type I and Heparin-Agarose Type II-S pre-packed 2.5 ml columns were efficient in both retaining and subsequently releasing rAAV. The Type II-S column, however, was found to be less selective, binding many cellular proteins along with the virus (not shown). The Heparin-Agarose Type I was the best among those tested in terms of binding specificity and virus recovery, and was used as the medium of choice.

Analysis of rAAV-UF5 purity at different stages of purification by silver stained SDS acrylamide gel electrophoresis is shown in Figure 7. The iodixanol-purified fraction prepared from cells transfected with pTR-UF5/pDG (lane 2) was directly applied to a Heparin-Agarose Type I column (lane 4) and eluted with 1 m NaCl as described in Materials and methods. The 1 m NaCl fraction contained 34.6% of the input rAAV (Table 1), which was more than 95% pure, as judged by silver stained SDS gel analysis (not shown). The heparin-agarose affinity fraction of rAAV was consistently more pure than virus purified by the conventional protocol using ammonium sulfate, followed by two rounds of CsCl gradient centrifugation (Figure 7, lane 7). These preparations also typically had the lowest particle-to-infectivity ratios (Table 1).

Purification of rAAV using HPLC chromatography

Two HPLC columns were found to be useful for purifying rAAV, UNO S1 and POROS HE/M heparin (Figure 8). UNO S1 column (Bio-Rad) is derivatized with strongly acidic, negatively charged SO_3 groups. POROS HE/M heparin (Boehringer Mannheim, Indianapolis, IN, USA) contains particles coated with a crosslinked polyhydroxylated polymer derivatized with heparin functional groups. Both columns were successful in removing most of the protein contaminants that remained in the iodixanol fraction yielding rAAV-UF5 that was comparable in purity to the virus obtained by conventional heparin agarose chromatography (Figure 7, compare lanes 5 and 6 with lane 4). In the case of the UNO-S1 purified virus, the product was more than 99% pure as judged by SDS acrylamide electrophoresis (Figure 7, see lanes 8–12). Curiously, two rAAV peaks were obtained during UNO

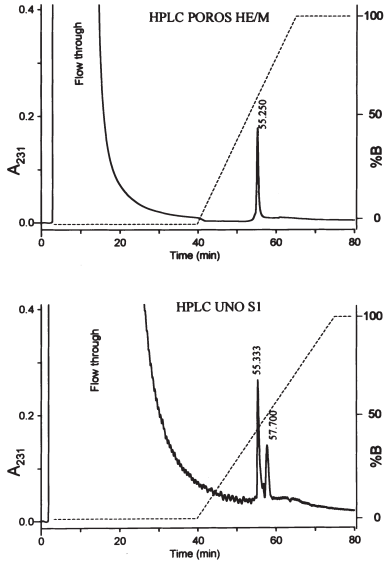

Figure 8 *HPLC purification of the iodixanol fraction of rAAV-UF5, monitored at 231 nm. (a) POROS HE/M chromatography, (b) UNO SI cation exchange chromatography. The axis on the right indicates the ratio of diluent B (loading buffer with 1 m NaCl), the dotted line indicates the shape of the gradient. Elution time is shown in minutes above the respective peaks.*

S1 fractionation (Figure 8b). Both peaks were found to contain rAAV that was indistinguishable both by SDS-gel electrophoresis analysis and by GFP fluorescence assay (not shown).

From a practical point of view, the HPLC Heparin column was easier to use because it allowed for a higher back pressure and, therefore, higher flow rates. It also removed iodixanol in the flowthrough much faster (30 min *versus* 45 min, Figure 8a and b). Finally, it performed consistently, producing essentially identical chromatograms for as many as 10 different virus runs (the maximum tried).

Separation of rAd and rAAV by heparin chromatography

Having established that both the UNO-S1 and POROS HE/M columns could be used successfully to purify rAAV, we then asked whether they also would separate adenovirus from AAV in preparations grown in the presence of Ad virus. To this end, the rAAV-UF6/rAd-UF7 mixture (described above) was purified by iodixanol gradient centrifugation and then subjected to HPLC POROS HE/M affinity chromatography under the conditions described in Material and methods. The majority of the contaminating rAd-UF7 was found in the flowthrough (data not shown). The peak of rAAV-UF6 contained 8×10^5 p.f.u. of rAd, as compared with 3.4×10^{10} IU of rAAV-UF6 particles (Table 2). Thus, the rAd titer in the mixed stock was decreased from 4.5×10^{10} in the crude lysate, to 4.2×10^8 in the iodixanol fraction, to 8×10^5 after the HPLC heparin affinity step (compare with rAAV-UF6 titer of 3.4×10^{10} of infectious particles). The same degree of purification was achieved with conventional chromatography using Heparin-Agarose Type I (not shown). In contrast, UNO S1 cation exchange chromatography failed to separate rAd and rAAV. Using a conventional CsCl purification method, 1% of the original infectious Ad remains in the rAAV stock even after two successive rounds of equilibrium centrifugation (Table 2).

Iodixanol plus CsCl density gradient

We also compared the use of an iodixanol step gradient followed by a CsCl gradient with the conventional use of two consecutive CsCl gradients (Figure 7 and Table 1). The iodixanol plus CsCl protocol produced rAAV with purity that was comparable with iodixanol followed by column chromatography. Both methods produced rAAV that was significantly more pure than virus that had undergone only two consecutive CsCl gradients (Figure 7, compare lane 7 with lanes 3–6). It is also worth noting that the rAAV produced by conventional CsCl purification (see Materials and methods) generally had higher particle-to-infectivity ratios (200–1000) than the novel method we describe here (Table 1). Furthermore, rAAV that had undergone even one CsCl centrifugation (Table 1, row 3) had a higher particle-to-infectivity ratio than virus that had not been exposed to CsCl (Table 1, rows 4–6). These observations suggest that treatment with CsCl leads to reduced viral infectivity.

Taken together, our data shows that a combination of iodixanol plus heparin affinity chromatography (either heparin agarose or heparin HPLC) has unique advantages as a method for purifying rAAV. To compare this method directly with the current method for rAAV purification, we prepared a crude rAAV virus stock and compared the two methods of purification side by side with the same starting material, ie ammonium sulfate fractionation followed by two CsCl gradients *versus* iodixanol fractionation followed by heparin agarose chromatography (Table 1, experiment 3 and Figure 9). A significant increase in recovery of vector was seen with the iodixanol/heparin protocol, resulting from an approximately five-fold higher recovery of vector particles and over a 100-fold increase in infectivity. Expressed as the ratio of infectious particles to total particles, the virus prepared by CsCl centrifugation had a significantly higher ratio than virus prepared by the iodixanol protocol, approximately 1700 *versus* 67 (Table 1). Furthermore, as expected the virus prepared by the conventional CsCl method was significantly less pure than that prepared by iodixanol/heparin (Figure 7, compare lanes 4 and 7, and Figure 9, compare lanes 4 and 7).

Discussion

The history of rAAV as a gene delivery vector is not without controversy. While some investigators in the field

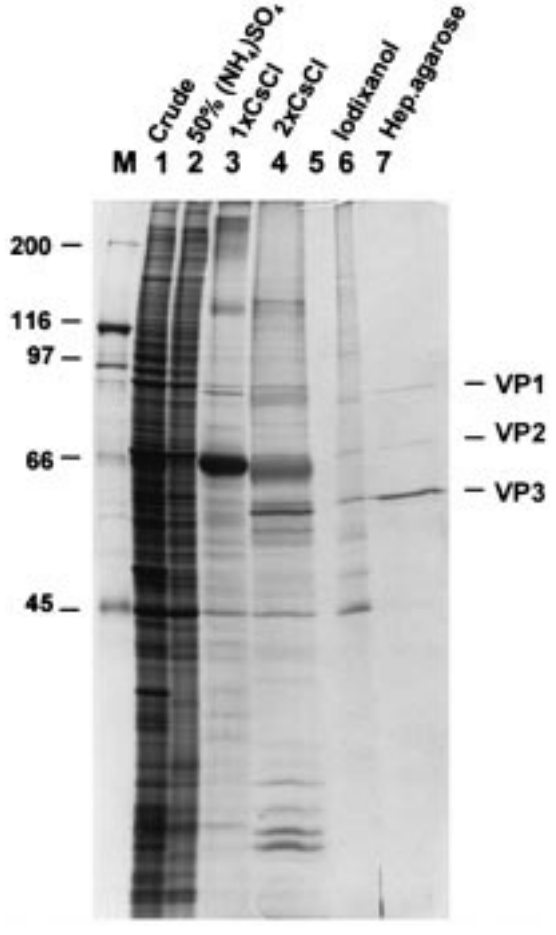

Figure 9 *Comparison of the ammonium sulfate/cesium chloride and iodixanol/heparin agarose purification methods. Twenty plates of 293 cells were transfected with pDG and pTR-UF5. The crude freeze–thaw lysate was then split into two equal parts and purified by either ammonium sulfate fractionation (50% $(NH_4)_2SO_4$) and two rounds of cesium chloride density gradient centrifugation ($1 \times$ CsCl and $2 \times$ CsCl), or by sequential fractionation on iodixanol and heparin agarose. Equivalent amounts of each fraction were loaded on to an SDS polyacrylamide gel for analysis. Molecular weight markers (M) in kDa and the position of the viral capsid proteins are also indicated.*

report efficient rAAV-mediated transduction, others have found a strong dependence of transduction upon Ad helper virus contaminants,[31] wtAAV contaminants[12,32] or the growth state of the cells being transduced.[33] Crude rAAV preparations have also been considered as a source of protein for artifactual transduction.[34] Some of the variability in rAAV transduction *in vivo* is undoubtedly due to the intrinsic properties of the target cells. Some targets, for example, do not have the high affinity heparin proteoglycan receptor[25] and others may be incapable of efficiently synthesizing the transcriptionally active form of the rAAV genome.[31,35] However, much of the variation is also due to the methods used for purifying rAAV and the contaminants that are present in the final preparation. In general, there has been a correlation between the success of AAV vectors and the ability to generate high-titer virus free of contaminants. Under optimal conditions, as few as 10–40 infectious particles of rAAV have been found to be sufficient to transduce one cell *in vivo*.[6,7,9]

During the course of these studies at least three problems were routinely encountered during purification of rAAV using conventional cesium chloride centrifugation methods. First, rAAV is often nonspecifically bound to cellular protein and helper Ad virus. These associations lead to aggregation of rAAV and lower yields, as well as virus preparations that are less pure. Often the final stock is contaminated with cell or serum proteins, which may compromise subsequent interpretation of the data by triggering an immune response. Second, the conventional purification method often produced virus preparations with particle-to-infectivity ratios of greater than 1000. rAAV can be prepared with particle-to-infectivity ratios as low as 20:1.[12] A ratio of a 1000:1, therefore, means that as much as 98% of the vector particles are nonviable. Third, the conventional purification methods take up to 2 weeks to complete and often result in a substantial loss yield. The purpose of this study, therefore, was to develop a fast, simple and reproducible rAAV purification protocol, that is amenable to scale-up and yields stocks of high titer and purity.

First we established a method of bulk purification which allows for rapid processing to subsequent steps. The bulk purification method used was the iodixanol step gradient. Iodixanol is an X-ray contrast reagent and, as such, it has been subjected to rigorous screening and clinical testing. It is nontoxic to cells; indeed, cells can be grown in up to 30% iodixanol for several days with no subsequent effect on viability. Iodixanol proved to be an excellent bulk purification method that accomplished three things. Crude lysate was purified by at least 100-fold and when Ad helper was present, Ad contamination was reduced by a factor of 100. The virus was concentrated in a non-ionic and relatively non-viscous medium that could be loaded on to virtually any kind of chromatographic matrix. Finally, for reasons that are not clear, iodixanol prevented rAAV aggregation and the associated loss of virus that accompanies most other bulk purification and column chromatography methods that we tried. Typically, 70–80% of the starting infectious units are recovered following iodixanol gradient fractionation (Table 1) and unlike other purification methods, this step was more reproducible.

Following iodixanol gradient fractionation, rAAV was apparently sufficiently free of cellular protein (Figure 7) such that it displayed reproducible chromatographic behavior during subsequent purification. Two types of columns have been identified that are capable of purifying rAAV approximately 10- to 100-fold, heparin sulfate and sulfate cation exchange resins. Both types of material could be used successfully in the HPLC format and displayed recoveries of 40–70% (Table 1). By contrast, CsCl purification of the iodixanol fraction resulted in the recovery of as little as 7% of the starting infectious units. Therefore, we have identified final production methods that increase the yield of infectious rAAV by at least 10-fold in this step.

More important, however, is the fact that neither iodixanol fractionation nor column chromatography on heparin or cation exchange resins had a significant effect on the particle-to-infectivity ratio of rAAV. In contrast, the use of CsCl gradients generally had the detrimental effect of increasing the particle-to-infectivity ratio. If CsCl were the only method used for purification, the increase could be dramatic. The particle-to-infectivity ratios of rAAV that had been purified by iodixanol and heparin affinity ranged from as low as 26 (data not shown) to 73 (Table 1). The particle-to-infectivity ratio of rAAV that had been purified by iodixanol and CsCl was approximately 158. Finally, virus that had been purified only by ammonium sulfate fractionation and sequential CsCl centrifugation had particle-to-infectivity ratios of 241 to 1600 (Table 1).

In conclusion, we have investigated several methods for rAAV purification and developed novel methods for producing pure, high-titer rAAV that are significantly better in yield and quality of material produced than the conventional methods currently in use. One of these methods, a simple iodixanol step gradient followed by a conventional Heparin agarose column has consistently resulted in overall recoveries of greater than 50% of the starting material, and produces virus that is better than 99% pure, with particle-to-infectivity ratios less than 100:1. Furthermore, the method allows the purification of rAAV in 1 day. The iodixanol/heparin method is easily adapted to most molecular biology or biochemistry laboratories and is suitable for the production of preclinical grade vector for virtually all applications. Additionally, most if not all of the purification steps we have identified here can be adapted for high volume clinical grade vector production. The use of such techniques should make broader application of rAAV feasible in the near future.

Materials and methods

Cells

Low passage number (passage 29–35) 293 cells were propagated in DMEM/10% FBS. The C12 cell line[24] was maintained in the presence of 0.5 mg/ml G418, while the Cre8 cell line[36] was propagated in DMEM supplemented with 200 μg/ml G418.

Construction of recombinant plasmids

The construction of pTR-UF5 was described previously.[6] To produce the enhanced blue fluorescent mutant[37] of gfp, we introduced the Tyr145Phe mutation into the pTR-UFB background[26] using the Quick Change site-Directed Mutagenesis kit (Stratagene). The resulting plasmid was termed pTR-UF6. To construct the recombinant adenovirus vector, rAd-UF7, we substituted the expression cassette from pTR-UF5 for the CMV promoter fragment

in pAdlox and rAd-UF7 was prepared as described by Hardy *et al*.[36] QC-PCR standard template p*dl-neo* was constructed as described previously.[7] The primers used to detect rAAV were: 5′-TATGGGATCGGCCATTGAAC-3′ and 5′-CCTGATGCTCTTCGTCCAGA-3′.

Production of rAAV

All stages of rAAV purification were carried out in the designated area of the University of Florida Vector Core Lab physically separated from the rest of the facility. The virus-containing reagents were handled exclusively in bio-safety cabinets. Only Quick-Seal tubes (Beckman) were used to purify the virus.

To produce rAAV, a triple cotransfection procedure was used to introduce a rAAV vector plasmid (pTR-UF5 or pTR-UF6) together with pACG2 AAV helper plasmid and pXX6 Ad helper plasmid[19] at a 1:1:1 molar ratio. Alternatively, rAAV vector plasmid was cotransfected with the helper plasmid pDG carrying the AAV *rep* and *cap* genes, as well as Ad helper genes required for rAAV replication and packaging.[18] Plasmid DNA used in the transfection was purified by a conventional alkaline lysis / CsCl gradient protocol. The transfection was carried out as follows: 293 cells were split 1:2 the day before the experiment, so that when transfected the cell confluence was about 75–80%. Ten 15-cm plates were transfected as one batch. To make $CaPO_4$ precipitate 180 μg of pACG2 were mixed with 180 μg of pTR-UF5 and 540 μg of pXX6 in a total volume of 12.5 ml of 0.25 m $CaCl_2$. Alternatively, 0.7 mg of pDG and 180 μg of pTR-UF5 were mixed in the same volume. The old medium was removed from the cells and the formation of the $CaPO_4$ precipitate was initiated by adding 12.5 ml of 2 × HBS pH 7.05 (pre-warmed at 37°C to the DNA-$CaCl_2$ solution. The DNA was incubated for 1 min, and transferring the mixture into pre-warmed 200 ml of DMEM-10% FBS then stopped the formation of the precipitate. Twenty two milliliters of the media were immediately dispensed into each plate and cells were incubated at 37°C for 48 h. The $CaPO_4$ precipitate was allowed to stay on the cells during the whole incubation period without compromising cell viability. Forty-eight hours after transfection cells were harvested by centrifugation at 1140 *g* for 10 min; the medium was discarded unless specified otherwise. Cells were then lysed in 15 ml of 0.15 m NaCl, 50 mm Tris HCl pH 8.5 by three freeze / thaw cycles in dry ice-ethanol and 37°C baths. Benzonase (Nycomed Pharma, pure grade) was added to the mixture (50 U / ml, final concentration) and the lysate was incubated for 30 min at 37°C. The lysate was clarified by centrifugation at 3700 *g* for 20 min and the virus-containing supernatant was considered the crude lysate.

Conventional CsCl purification protocol

For comparison purposes, rAAV was purified by conventional cesium chloride centrifugation essentially as described previously[38] with the following modifications. The virus pellet after the second ammonium sulfate cut was resuspended in a total of 39 ml of 1.37 g / ml CsCl / PBS and centrifuged for 18 h in a Beckman 60 Ti rotor at 255 600 *g* at 15°C. The gradient was fractionated from the bottom of the tube and aliquots of the middle 10 fractions were screened for rAAV by PCR. Positive fractions were pooled, diluted to 13 ml with the CsCl solution of the same density and centrifuged in an 80 Ti rotor at 391 600 *g* for 3.5 h at 15°C. After fractionation of the gradient, the positive fractions were identified by PCR and pooled. The virus was then concentrated and dialyzed using the ULTRAFREE-15 centrifugal filter device BIOMAX-100K (Millipore).

Iodixanol density gradient

The typical discontinuous step gradient was formed by underlayering and displacing the less dense cell lysate with iodixanol, 5,5′[(2-hydroxy-1–3-propanediyl)-bis (acetylamino)] bis [N,N′-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide], prepared using a 60% (w / v) sterile solution of OptiPrep (Nycomed). Specifically, 15 ml of the clarified lysate were transferred into Quick-Seal Ultra-Clear 25 × 89 mm centrifuge tubes (Beckman) using a syringe equipped with a 1.27 × 89 mm spinal needle. Care was taken to avoid bubbles, which would interfere with subsequent filling and sealing of the tube. A variable speed peristaltic pump, Model EP-1 (Bio-Rad), was used to underlay in order: 9 ml of 15% iodixanol and 1 m NaCl in PBS-MK buffer (1 × phosphate-buffered saline (PBS), 1 mm $MgCl_2$, and 2.5 mm KCl); 6 ml of 25% iodixanol in PBS-MK buffer containing Phenol Red (2.5 μl of a 0.5% stock solution per ml of the iodixanol solution); 5 ml of 40% iodixanol in PBS-MK buffer; and finally, 5 ml of 60% iodixanol in PBS-MK buffer containing Phenol Red (0.01 μg / ml). Tubes were sealed and centrifuged in a Type 70 Ti rotor (Beckman) at 350 000 *g* for 1 h at 18°C. The purpose of the Phenol Red was to distinguish the alternating density steps. Four milliliters of the clear 40% step was aspirated after puncturing the tubes on the side with a syringe equipped with an 18-gauge needle with the bevel uppermost. A similar amount is removed as 0.75 to 1 ml fractions upon harvest. The iodixanol fraction was further purified using each of the methods described below.

Purification of iodixanol fraction using CsCl gradient centrifugation

The rAAV-containing iodixanol fraction was further purified using a conventional CsCl gradient. To form the gradient, 4.5 ml of virus in iodixanol were mixed with 35 ml of CsCl (1.37 g / ml in PBS), transferred into a Quick-Seal 25 × 89 mm centrifuge tube (Beckman) and centrifuged in a Type 60 rotor at 214 800 *g* overnight at 18°C. The gradient was processed as described above.

Purification of iodixanol fraction using heparin affinity chromatography

Binding, washing and elution conditions were identical for all heparin-ligand affinity media used in the process development. Typically, a pre-packed 2.5 ml Heparin-Agarose Type I column (Sigma) was equilibrated with 20 ml of PBS-MK under gravity. Alternatively, the columns were placed inside 15 ml screw cap conical tubes (Sarstedt) and spun in the low speed centrifuge Type J6-HC (Beckman) at 50 *g* for 5 min. After each spin the flow-through was discarded and fresh buffer was added to repeat the washing three more times. The rAAV iodixanol fraction was then applied to the pre-equilibrated column under gravity, and the column was washed with 10 ml of PBS-MK buffer either under gravity or in the spin column mode. rAAV was eluted with the same buffer containing 1 m NaCl under gravity. After applying the elution buffer, the first 2 ml of the eluant were discarded,

and the virus was collected in the subsequent 3.5 ml of elution buffer. Conventional Heparin columns that were not prepacked were loaded and eluted in a similar manner.

Alternatively, the prepacked Heparin-Agarose columns were placed into screw-type valves of the Visiprep Solid Phase Extraction (SPE) Vacuum Manifold (Supelco). The manifold valves were equipped with disposable Teflon valve liner guides, designed to eliminate the possibility of cross-contamination from one sample to the next in the same manifold port. Each guide was placed into 15 ml screw cap conical tube (Sarstedt) used as the collection vessel. This arrangement ensures that all surfaces that come in contact with the sample can be replaced following each chromatography. Chromatography was performed with a house vacuum attached to the manifold's vacuum gauge. Precise flow control through each column was provided by rotating the independent, screw-type valves built into the cover. Up to 12 samples could be purified simultaneously using the 12-Port Model manifold.

For ACTI-Disk 50 filter disk chromatography, binding of rAAV (in 40% iodixanol fraction) was performed in the upward fashion, ie the flow of the solution was directed against gravity from the bottom part of the filter assembly towards the top using a peristaltic pump. Once applied, the filter assembly was turned upside-down and chromatography was resumed in a regular downward fashion with gravity.

Purification of iodixanol fractions using HPLC chromatography

System Gold (Beckman) hardware installed inside a biosafety cabinet was used to purify the iodixanol fraction further. Only biocompatible polyetheretherketone (PEEK) tubing and fittings were used to process the samples. Chromatography was monitored at 231 nm. The virus in 4 to 5 ml of iodixanol was directly loaded on to a column using a 5 ml injection loop. When the volume of the sample exceeded 5 ml, multiple successive injections were performed, each followed by washing with 5 ml (injection loop dwell volume) of mobile phase.

Two types of HPLC columns were used. The UNO S1 cation exchange column (Bio-Rad) (bed volume 1.3 ml) was pre-equilibrated with PBS-MK buffer. The virus sample was loaded at 0.5 ml/min and the column was washed with PBS-MK buffer until the iodixanol-induced absorption was reduced to near background levels. A 0–1 m gradient of NaCl in PBS-MK was applied over 36 min (15 column volumes) and the virus was eluted as a double UV absorption peak. The chromatography conditions for the POROS HE/M heparin column (Boehringer Mannheim) (bed volume 1.7 ml) were essentially the same as described for the UNO S1 column, except that a 0–0.5 m Na_2SO_4 gradient in PBS-MK buffer was used (15 column volumes) at a flow rate of 1 ml/min. A single UV absorption peak of a virus was eluted.

Concentration of rAAV

The virus was concentrated and desalted by centrifugation through the BIOMAX 100 K filter (Millipore) according to the manufacturer's instructions. The high-salt buffer was changed by repeatedly diluting the concentrated virus with the Lactated Ringer's solution and repeating the centrifugation.

Quantitative competitive PCR (QC-PCR) assay for determining rAAV physical particles

The purified viral stock was first treated with DNaseI to digest any contaminating unpackaged DNA. Ten microliters of a purified virus stock was incubated with 10 U of DNase I (Boehringer) in a 100 μl reaction mixture, containing 50 mm Tris HCl, pH 7.5, 10 mm $MgCl_2$ for 1 h at 37°C. At the end of the reaction, 10 μl of 10× Proteinase K buffer (10 mm Tris HCl, pH 8.0, 10 mm EDTA, 1% SDS final concentration) was added, followed by the addition of 1 μl of Proteinase K (18.6 mg/ml, Boehringer). The mixture was incubated at 37°C for 1 h. Viral DNA was purified by phenol/chloroform extraction (twice), followed by chloroform extraction and ethanol precipitation using 10 μg of glycogen as a carrier. The DNA pellet was dissolved in 100 μl of water. The PCR reaction mixtures each contained 1 μl of the diluted viral DNA and two-fold serial dilutions of the internal standard plasmid DNA pdl-*neo*. The most reliable range of standard DNA was found to be between 1 and 100 pg. An aliquot of each reaction was then analyzed by 2% agarose gel electrophoresis, until two PCR products were resolved (Figure 2a). The analog image of the ethidium bromide stained gel was digitized using an ImageStore 7500 system (UVP). The densities of the target and competitor bands in each lane were measured using the ZERO-Dscan Image Analysis System, version 1.0 (Scanalytics) and their ratios were plotted as a function of the standard DNA concentration. A ratio of 1, at which the number of viral DNA molecules equals the number of competitor DNA molecules was used to determine the DNA concentration of the virus stock.

Infectious center assay/Fluorescent cell assay

A modification of the previously published protocol[12] was used to measure the ability of the virus to infect C12 cells, unpackage, and replicate. Briefly, the C12 cells were plated in a 96-well dish at about 75% confluence and infected with Ad5 at a MOI of 20. One microliter of serially diluted rAAV was added to each well, and the cells were incubated for 42 h. Cells infected with rAAV-UF5 were visually scored using a fluorescence microscope. Blue fluorescence induced by rAAV-UF6, as well as rAd-UF7-induced green fluorescence in the same field (Figure 5) was monitored as described previously.[26] High sensitivity CHROMA filter No. 41012 High Q FITC LP was used to monitor green fluorescence of rAAV-UF5. To calculate the titer by hybridization, cells were harvested and processed essentially as described previously.[12] An example of such an assay is shown in Figure 2b.

Protein concentration and acrylamide gel electrophoresis

The protein concentration in rAAV samples was determined using the NanoOrange Protein Quantitation Kit (Molecular Probes). The fluorescence in the sample was measured using a Model TD-700 Fluorometer (Turner Designs). To estimate the purity of various virus fractions, virus was electrophoresed on 12% SDS acrylamide gels for 5 h at 200 V under standard buffer conditions and visualized by silver staining.

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