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SHORT COMMUNICATION

Removal of Empty Capsids from Type 1 Adeno-Associated Virus Vector Stocks by Anion-Exchange Chromatography Potentiates Transgene Expression

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Production of recombinant adeno-associated virus (rAAV) results in substantial quantities of empty capsids or virus-like particles (VLPs), virus protein shells without the vector genome. The contaminating VLPs would interfere with transduction by competing for cell-surface receptors and, when administered *in vivo*, contribute to antigen load, which may elicit a stronger immune response. Density-gradient ultracentrifugation provides a means to separate VLPs from rAAV particles, but is not feasible for large-scale preparations of vectors. Since the compositions of the VLP and vector differ by the single-stranded DNA genome, we hypothesized that the isoelectric point of the vector may differ from that of the VLP. In an attempt to separate type 1 rAAV particles from VLPs by ion-exchange chromatography, we tested a number of buffer systems and found that trimethylammonium sulfate, or $[(CH_3)_4N]_2SO_4$, effectively separated rAAV1 particles from VLPs. The rAAV1-GFP chromatographically separated from VLPs induced stronger GFP expression in HEK293 cells than rAAV1-GFP contaminated with VLPs. The transduction of mouse muscles with rAAV1-SEAP (secreted form of alkaline phosphatase) isolated from VLPs also showed higher serum SEAP levels than rAAV1-SEAP with VLPs. These results suggest that chromatographic separation of rAAV1 from empty capsids increased the efficacy of rAAV1.

Key Words: AAV vector, empty capsid, antichaotropic ion, chromatography

Recombinant adeno-associated virus (rAAV) is one of the promising gene transfer vectors and efficiently transduces neurons, hepatocytes, and skeletal muscle in rodent, dog, and nonhuman primate models [1]. AAV vectors produced with serotype 1 capsid protein transduce skeletal muscles particularly well compared to serotypes 2, 4, 5, and 6 [2]. Obtaining clinically meaningful levels of a therapeutic protein depends on several factors, including the amount of particles administered. A human clinical trial using rAAV2 expressing coagulation factor IX (up to 10^{13} particles/kg) in hemophilia B patients has been conducted, resulting in a partial, but transient, amelioration of symptoms. Thus, extrapolating from these earlier studies, more than 10^{15} particles of rAAV2 would be required for the complete correction of hemorrhagic tendency [3]. Using serotypes with higher biological activities may reduce the dose; even so, large animal studies comparing the efficacy of rAAV1 and other serotypes indicated that large particle numbers of rAAV1 would still be required for human application [2,4].

rAAV is usually produced by plasmid transfection of HEK293 cells with two or three plasmids: AAV helper plasmid encoding *rep* and *cap* genes devoid of inverted terminal repeat (ITR) sequences, AAV vector plasmid harboring the therapeutic gene between the ITRs, and a plasmid containing a minimal set of adenovirus helper genes, E2A, VARNA, and E4orf6. Since the structural and nonstructural genes, as well as the *cis*-acting elements of AAV type 1 and AAV type 2, are highly conserved, it is possible to package the gene of interest between the type 2 ITRs into the coexpressed type 1 capsid [5], which is composed of VP1, VP2, and VP3 polypeptides with a stoichiometry of 1:1:10. In transfected HEK293 cells, the expression of the three structural proteins forms virus-like particles (VLPs) or empty capsids independent of vector DNA replication and packaging. The maturation of particles occurs during vector DNA replication and

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particles with vector genomes appear [6]. However, the fraction of VLPs that acquire vector DNA remains a minor component of the total particles in the cell. The ratio of empty to filled particles can range from 10:1 to 4:1 [7,8]. Without a DNA payload, the presence of VLPs in the rAAV stocks would diminish the effect by competing for cell-mediated processes, such as receptor binding and uptake, as well as providing a source of antigen that may elicit a stronger immune response *in vivo* against AAV vectors [9]. It is therefore desirable to eliminate empty capsids from rAAV vector stocks. The only established method to isolate rAAV from empty capsids is density gradient ultracentrifugation using CsCl or other density materials, which relies on the difference in the buoyant density between DNA-filled and empty particles. However, ultracentrifugation is not readily adaptable to the large-scale preparation of rAAV, especially for clinical grade material. In addition to density gradient centrifugation, other physicochemical differences may lead to exploitable processes for separating VLPs from vector particles. Although affinity column chromatography does not distinguish between vector and VLP, the process is scalable and also provides a higher level of purification than CsCl ultracentrifugation [7,10,11]. In addition, chromatography can preserve more infectious rAAV particles [10].

All members of the *Parvoviridae* are structurally similar and have linear, single-stranded DNA genomes. It is possible that the presence of encapsidated DNA alters the isoelectric point (p*I*) of the AAV particles. We postulated that if the p*I* of rAAV differs from that of the empty capsid, then rAAV separation from empty capsid is possible using high-resolution chromatography.

For starting materials, we used empty and filled rAAV1 particles independently obtained by CsCl density ultracentrifugation and subsequent chromatography, as described in the Fig. 1 legend. We characterized each type of particle by density, DNA content, protein composition, and biological activity (data not shown). We confirmed the purity of rAAV or empty particles by silver staining of the samples resolved on an SDS–PAGE gel (Fig. 1A). We examined the elution profile of rAAV1 containing the green fluorescent protein (GFP) gene and type 1 empty capsid on a high-resolution anion-exchange column, Mini Q 4.6/50 PE (Amersham Biosciences, Piscataway, NJ, USA). Full rAAV particles, equivalent to 5×10^{10} vector genomes (vg), or an equivalent quantity of empty particles, were bound to the column in a low-salt buffer of 20 mM Tris–HCl (pH 8.4), 20 mM NaCl, and 4% glycerol; they were eluted with a linear 20–300 mM NaCl gradient at pH 8.4. Although there is overlap at the base of the peaks, Fig. 1A shows that the empty particles (broken line) eluted at a lower salt concentration than rAAV (solid line). While the resolution of the empty and filled particle fractions was not optimal, the ability to elute the two types of particles selectively was a very encouraging result. To increase the resolution of the eluted particle peaks, we surveyed an extensive range of elution buffers and found that the use of so-called antichaotropic ions, such as NH_4^+, $(CH_3)_4N^+$, PO_4^{3-}, and SO_4^{2-}, was capable of resolving rAAV from empty capsids better than using NaCl gradients. Fig. 1B shows a representative chromatogram of the mixture of rAAV1-GFP particles and VLPs eluted with a linear 20–300 mM Na^+ or $(CH_3)_4N^+$ gradient. Among the buffers we tested, $[(CH_3)_4N]_2SO_4$ or trimethylammonium sulfate most effectively separated the rAAV particles (F) from empty capsids (E). We also examined a weaker anion, $(C_2H_5)_4N^+$, for the separation of rAAV, which more efficiently separated the rAAV particles from empty capsids. However, the solution containing $(C_2H_5)_4N^+$ was viscous and disrupted the rAAV particles. NH_4^+ also isolated rAAV1 from empty particles. The ammonium ion, however, is volatile at high pH and the ammonium solution is not stable over time.

The pH of the buffers is also important for chromatography. The elution of rAAV and empty particles at different pH is shown in Fig. 1C. We loaded the mixture of purified rAAV and empty particles onto the column and eluted them with a linear 20–300 mM $(CH_3)_4N^+$ gradient at pH 7.5, 8.0, 8.5, or 9.0. The separation of the two peaks was better at pH 8.5 or 9.0 than at lower pH. Since the empty and filled AAV particles are unstable at a higher pH [12], we used buffers at pH 8.5 in the subsequent experiments.

Our final goal was to develop a chromatographic method for the purification of a large quantity of type 1 rAAV particles free of empty particles. We next tested the separation of approximately 10^{13} vg of rAAV1-GFP from empty particles. We produced rAAV particles and released them from HEK293 cells, as described in the legend to Fig. 1. After low-speed centrifugation, we again centrifuged the cleared cellular lysate for 10 min at 30,000*g* at 4°C and filtered the supernatant through 0.45- and 0.2-μm membrane filters. We diluted the lysate four times with a dilution buffer of 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$, and 4% glycerol and loaded it onto a 10-mm × 60-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 50-μm medium (Applied Biosystems, Foster City, CA, USA). rAAV1 was eluted with a linear 50–400 mM NaCl gradient (250 ml). We collected the fractions containing rAAV1 and diluted them threefold with the dilution buffer and loaded them onto the second anion-exchange column (5 mm × 10 cm) packed with POROS HQ 10-μm matrix (Applied Biosystems). The rAAV1 was again eluted with a linear 50–400 mM NaCl gradient (25 ml). We further purified the rAAV1 by gel filtration column chromatography, as described in the Fig. 1 legend. We mixed the fractions containing rAAV together and diluted them with 4 volumes of the dilution buffer and loaded them onto a high-resolution column (5 mm × 20 cm) filled with POROS HQ 10-μm equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted

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with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. A representative chromatogram (Fig. 2A) shows that the two peaks were observed as expected. The peak that appeared earlier or later corresponded to empty capsids or rAAV particles. The analysis of each fraction by Western blotting with an anti-type 5 VP antibody, which was cross-reactive with type 1 VP protein (middle), revealed that the first, larger peak contained much more AAV VP protein (fractions 19 through 22). The second, smaller peak also contained VP protein, although the amount was smaller (fractions 23 through 26). Quantification using real-time PCR indicated that the majority of rAAV vector genome was in fractions 23 through 26 (bottom), corresponding to the second peak fractions. Electron microscopy of a sample from the pooled peak fractions confirmed that the earlier peak corresponded to empty capsids and the later one corresponded to rAAV particles (insets in Fig. 2A). Since a single run was not sufficient to separate completely the empty from the full capsids, we repeated the high-resolution chromatography step. After the first separation, more than 90% of contaminating empty capsids was removed. The second run was able to eliminate empty particles further and we obtained a rAAV stock with less than 5% empty particles. Table 1 summarizes the recovery of rAAV1 particles after high-resolution column chromatography for the removal of empty particles. After two rounds of chromatography, we were able to recover approximately 50% of rAAV1-GFP.

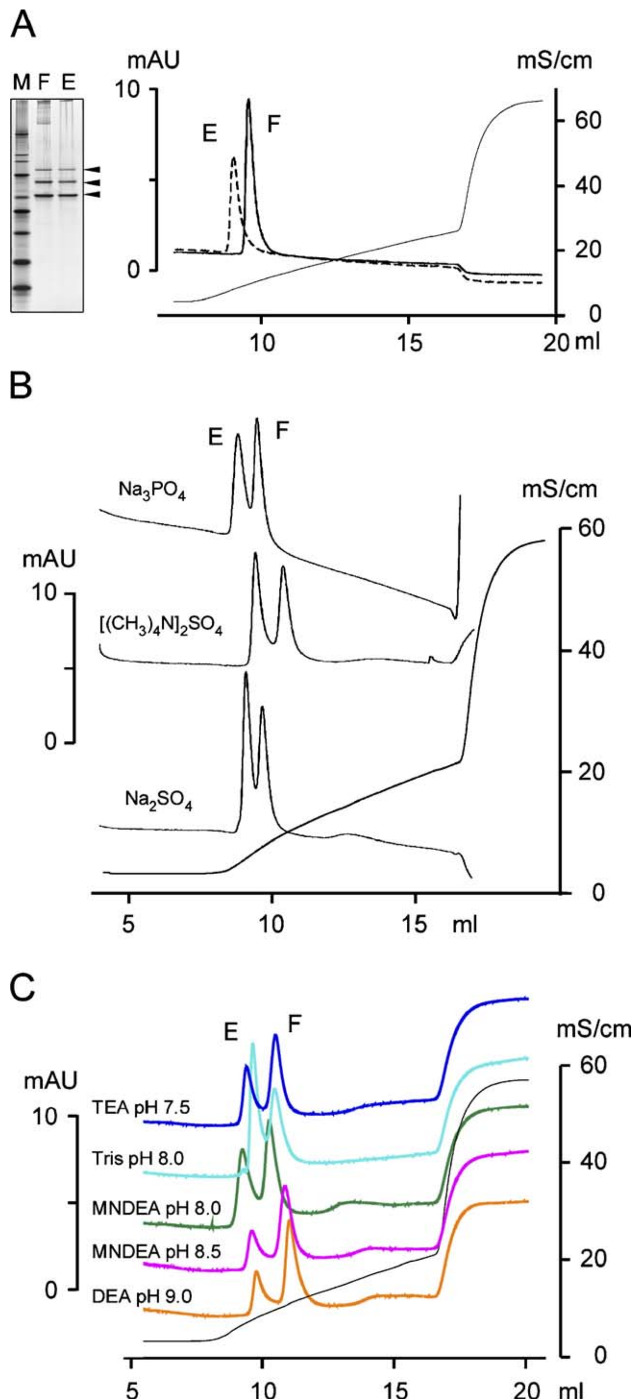

FIG. 1. (A) Elution profile of DNA-filled or rAAV1 and empty particles on a high-resolution anion-exchange column. For the production of rAAV1-GFP, HEK293 cells at 80% confluency (approximately 10^5 cells/cm^2) in a 225-cm^2 flask were cotransfected with 26.7 μg of an AAV vector plasmid harboring a humanized GFP gene (Stratagene, Palo Alto, CA, USA) under the control of the cytomegalovirus immediate early gene promoter (CMV) between the type 2 ITRs, 26.7 μg of an AAV1 helper plasmid carrying type 2 *rep* and type 1 *cap* genes [5], and 26.7 μg of an adenovirus helper plasmid using the calcium precipitation method. Two days after transfection, the cells were pelleted by centrifugation and lysed in 2 ml (per 225-cm^2 flask) of lysis buffer (20 mM Tris–HCl (pH 8.4), 150 mM NaCl, 2 mM $MgCl_2$, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (Merck, Darmstadt, Germany), 60 U/ml benzonase (Merck)) and incubated at 37°C for 30 min. After low-speed centrifugation, solid CsCl was added to the lysate to produce a buoyant density of 1.36 g/cm^2 and the samples were centrifuged for 24 h at 36,000 rpm at 21°C in an SW40Ti rotor (Beckman Coulter, Fullerton, CA, USA). rAAV1-containing fractions were collected and spun once again. rAAV1-GFP was then loaded on a gel-filtration column (HiPrep 16/60 Sephacryl S-300 HR; Amersham Biosciences) preequilibrated with 50 mM Hepes (pH 7.4), 0.3 M NaCl, 2 mM $MgCl_2$ to eliminate further cellular contaminants. Type 1 empty capsids were also generated in 293 cells transfected with a type 1 AAV helper plasmid alone and purified as for rAAV particles except for the CsCl density of 1.30 g/cm^2. Their purity was confirmed by silver staining of the SDS–PAGE gel using the SilverQuest silver staining kit (Invitrogen, Carlsbad, CA, USA). Arrows indicate VP1, VP2, and VP3 polypeptides. Approximately 5×10^{10} vg of rAAV1-GFP (F) or an equivalent quantity of type 1 empty particles (E) was loaded onto a Mini Q 4.6/50 PE column (Amersham Biosciences) controlled by an ÄKTA FPLC system (Amersham Biosciences). The bound particles were eluted over 10 ml with a linear 20 to 300 mM NaCl gradient at pH 8.4. The profile is represented as the absorbance at 280 nm (mAU). Buffer conductance (mS/cm) is indicated by the thin line. M, molecular weight standards. (B) Chromatogram of the mixture of AAV1 and empty particles in antichaotropic buffers with a 20 to 300 mM Na^+ or $[(CH_3)_4N]_2^+$ gradient. The earlier elution from the column represents empty particles (E) and DNA-filled or rAAV1 (F) eluted at a higher salt concentration. (C) The effect of pH on the elution of rAAV1 and empty particles. A buffer of 25 mM triethanolamine (TEA) at pH 7.5, Trizma (Tris) at pH 8.0, *N*-methyldiethanolamine (NMDEA) at pH 8.0 or 8.5, or diethanolamine (DEA) at pH 9.0 with a 10 to 150 mM $[(CH_3)_4N]_2SO_4$ gradient was used for elution.

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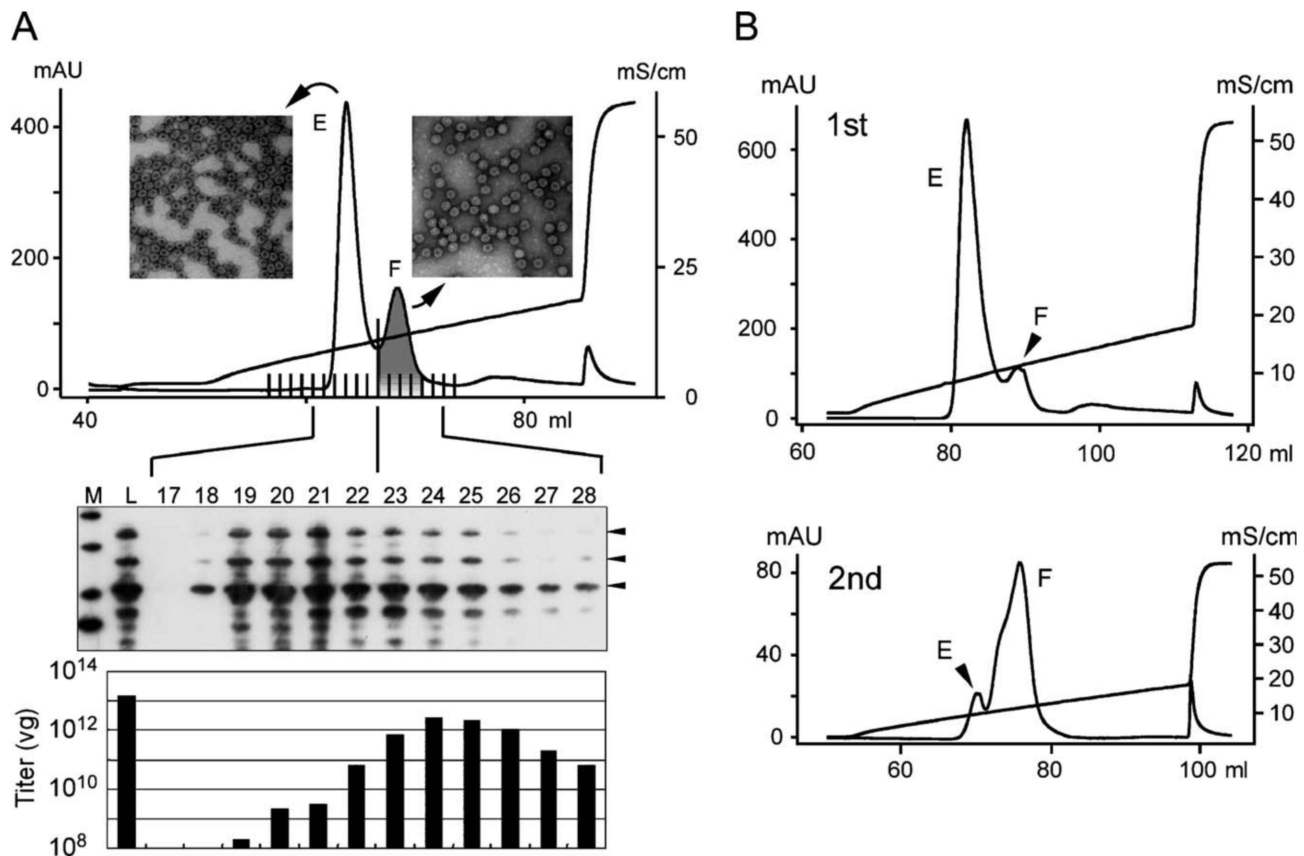

FIG. 2. (A) A representative chromatogram of a rAAV1-GFP preparation. Approximately 10^{13} vg of vector particles were generated and purified as described in the legend to Fig. 1 and finally loaded onto a 5-mm × 20-cm Tricorn column (Amersham Biosciences) packed with POROS HQ 10-μm matrix (Applied Biosystems) equilibrated with 25 mM *N*-methyldiethanolamine (pH 8.5) and 10 mM $[(CH_3)_4N]_2SO_4$. Bound viral particles were eluted with a 10–125 mM $[(CH_3)_4N]_2SO_4$ gradient over 38 ml at a flow rate of 0.5 ml/min. F and E indicate filled and empty particles, respectively. Electron microscopy of negatively stained samples from each peak is shown as an inset. After 1-ml fractionation samples were analyzed on a 4–12% NuPAGE gel (Invitrogen), the separated proteins were transferred to a Durapore membrane (Millipore, Bedford, MA, USA) and incubated with a rabbit polyclonal anti-type 5 VP antibody. After incubation with a secondary anti-rabbit immunoglobulin G labeled with horseradish peroxidase (Pierce, Milwaukee, WI, USA), chemiluminescent signals were detected using the SuperSignal West Pico Chemiluminescent substrate (Pierce) (middle). The fraction number is indicated above each lane. VP1, VP2, and VP3 capsid proteins are indicated by arrows. A sample from each fraction was also analyzed by real-time PCR to quantify the GFP vector DNA using a primer set specific to the CMV promoter, as previously described [15]. M, molecular weight standard; L, loaded sample. (B) An example of separation of rAAV1-GFP from empty particles by two runs of the high-resolution column chromatography. The first run was able to eliminate more than 90% of the contaminating empty capsids (E) from rAAV1-GFP (F). Reloading of the eluate from the first run further removed the contaminating empty particles.

We assessed the biological activity of the rAAV1-GFP isolated by column chromatography. We infected HEK293 cells with rAAV1-GFP samples, before or after chromatographic removal of empty particles, at the particle per cell numbers indicated (Fig. 3A). Seven days after transduction, we examined the cells under a fluorescence microscope. To quantify the GFP fluorescence, we also analyzed the cells by flow cytometry as described [13]. The analysis gave the percentage positive cells and the average GFP fluorescence, which refers to the average fluorescence intensity in the subpopulation of GFP-positive cells. The fluorescence volume represents a summation of GFP fluorescence within the subpopulation of GFP-positive cells, which was calculated to be equal to the fraction of GFP-positive cells in the sample population times the mean fluorescence intensity. When HEK293 cells were infected with either rAAV1-GFP at more than 10^4 vg per cell, both vectors transduced almost all the infected cells. However, the volume of GFP

TABLE 1: Recovery of rAAV1-GFP after removal of empty capsids

Preparations	Load	After 1st run (%)	After 2nd run (%)
#1	1.2×10^{13}	7.6×10^{12} (63.3)	5.0×10^{12} (41.7)
#2	3.3×10^{13}	2.4×10^{13} (72.7)	1.7×10^{13} (51.5)
#3	1.3×10^{13}	8.6×10^{12} (66.2)	6.8×10^{12} (52.3)

Number of rAAV1 particles was determined by real-time PCR. The percent recovery was calculated by dividing the number of rAAV1 particles loaded onto the first high resolution column by the number of rAAV particles recovered after chromatography.

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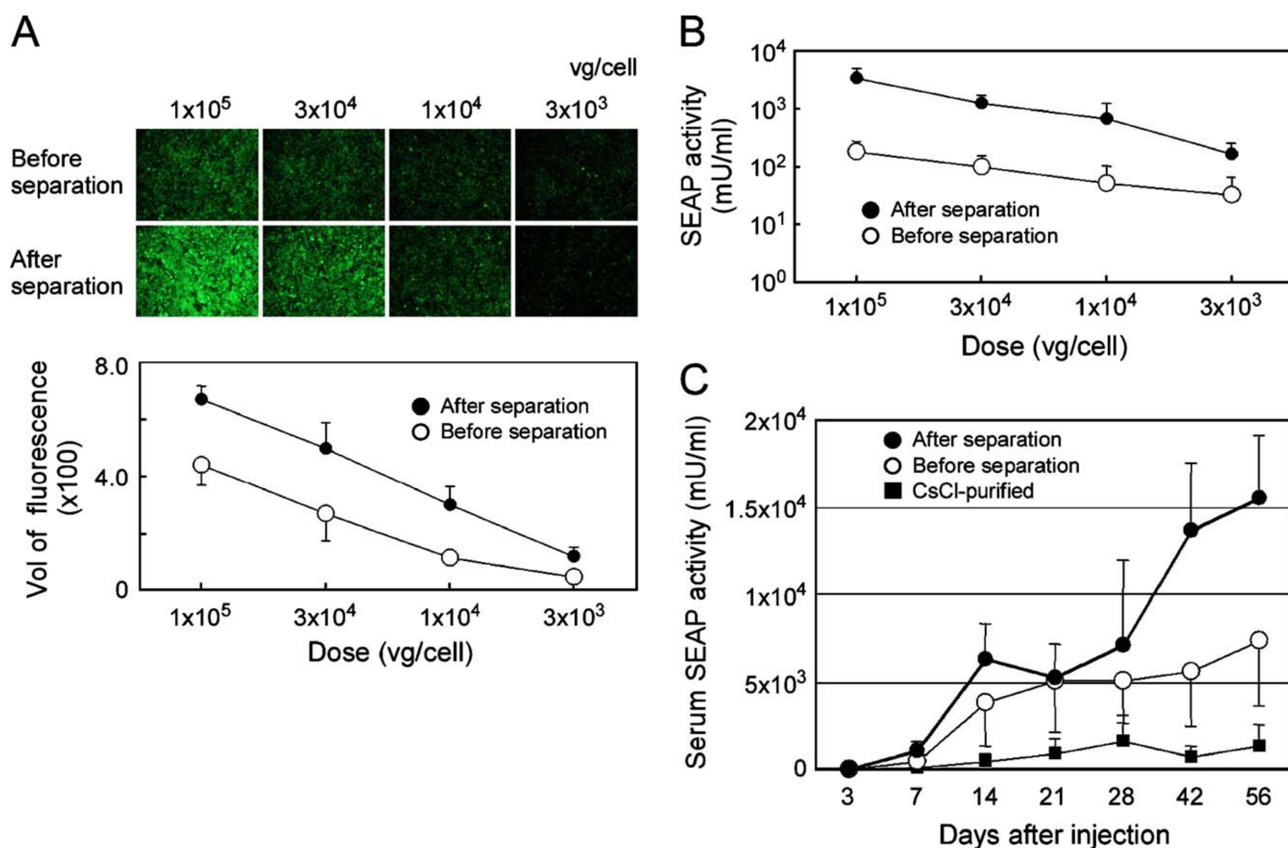

FIG. 3. (A) Transduction of HEK293 cells with rAAV1-GFP chromatographically separated from empty particles. 293 cells were infected with rAAV1-GFP before or after column chromatography intended to separate empty particles at the doses indicated. The GFP-expressing cells were analyzed by flow cytometry. The volume of GFP fluorescence was obtained by calculating (the fraction of GFP-positive cells) × (the average GFP fluorescence). Data represent means and standard deviation of experiments performed in triplicate. (B) The SEAP activity of the culture supernatant after infection of HEK293 cells with rAAV1-SEAP contaminated with or without empty particles. HEK293 cells were infected with type 1 SEAP vector before or after chromatographic removal of empty capsids at doses ranging from 1×10^5 through 3×10^3 vg per cell in triplicate. Results are expressed as means ± SD. (C) The serum SEAP levels after injection of rAAV1-SEAP into mouse muscles. A total of 10^{10} vg of rAAV1-SEAP particles before or after high-resolution chromatography or rAAV1-SEAP purified by CsCl ultracentrifugation was injected into mouse tibialis anterior muscles in triplicate and blood was taken from 3 through 56 days after injection.

fluorescence obtained by rAAV1-GFP separated from empty capsids was larger than that by rAAV contaminated with empty particles. We also infected HEK293 cells with rAAV1 expressing the human secreted alkaline phosphatase (SEAP). We excised the SEAP gene from pSEAP2-Basic (Clontech, Mountain View, CA, USA) with *Nru*I and *Sal*I and blunt-ended the resulting 1.8-kb fragment and inserted it between the type 2 ITRs. We used the resulting plasmid for transfection of HEK293 cells and purified rAAV1-SEAP as described above. We measured the SEAP activity in the culture supernatants 1 week after infection by using the SEAP Report Gene Assay (Roche Diagnostics, GmbH, Penzberh, Germany) according to the manufacturer's instructions. The rAAV1-SEAP separated from empty particles induced higher SEAP levels than rAAV1-SEAP contaminated with empty capsids at the doses tested (Fig. 3B). These results suggested that contaminating empty capsids interfered with the transduction of HEK293 cells by rAAV1.

To investigate the efficacy of rAAV1 *in vivo*, we injected rAAV1-SEAP (10^{10} vg) into mouse tibialis anterior muscles in triplicate. We used rAAV1-SEAP before chromatographic separation of empty capsids and CsCl-banded rAAV1-SEAP as controls. Fig. 3C shows the time course of the serum SEAP levels after the injection of SEAP vectors. rAAV1-SEAP purified by anion-exchange chromatography induced the highest levels of serum SEAP activity. The rAAV1-SEAP purified by column chromatography, but contaminated with empty particles, expressed lower levels of SEAP. CsCl-banded SEAP vector showed the lowest level, although the difference in the SEAP activity among the three groups was not statistically significant due to the small number of animals employed. The serum SEAP level at 56 days postinjection with the rAAV1 vector from which empty capsids were removed by chromatography was 10 times higher than that with the rAAV-SEAP from which empty capsids were excluded by CsCl ultracentrifugation, which may be due

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to the impurity and/or the damage of CsCl-purified rAAV1 [14]. These results again indicated that the removal of empty particles from rAAV1 stocks by chromatography potentiated the SEAP expression in the muscles.

In summary, we report here a method for the selective removal of empty capsids from type 1 AAV vector. The chromatographic separation obtained pure rAAV1 stocks contaminated with less than 5% empty capsids. This method can remove empty capsids without the loss of the efficacy of rAAV1 and is easily scalable to a large volume. It will be useful for the purification of large quantities of rAAV1 for large-animal or human applications.

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