HHS Public Access

Author manuscript

Mol Biotechnol. Author manuscript; available in PMC 2016 July 18.

Published in final edited form as:

Mol Biotechnol. 2016 January ; 58(1): 30–36. doi:10.1007/s12033-015-9899-5.

Large-Scale Production of Adeno-Associated Viral Vector Serotype-9 Carrying the Human Survival Motor Neuron Gene

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Abstract

Recombinant AAV (rAAV) vectors are a suitable vector for gene therapy studies because of desired characteristics such as low immunogenicity, transfection of non-dividing and dividing cells, and long-term expression of the transgene. In this study, the large-scale production of single stranded (ss) and self-complementary (sc) AAV9 carrying the human survival motor neuron (*SMN*) gene (AAV9-SMN) suitable for in vivo gene therapy studies of SMA was described. SMN cDNA has been cloned into pAAV-CB6-PI and pAAVsc-CB6-PI with and without its specific UTRs, respectively. Both plasmids bear CMV enhancer/beta-actin (CB) promoter, CMV IE enhancer, and polyadenylation signal sequences. 2.5 μg of constructed pAAV-CB6-PI-SMN and pAAVsc-CB6-PI-SMN cause to, respectively, 4.853- and 2.321-fold increases in SMN protein levels in transfected cells compared to untransfected cells. Ss and scAAV9-SMN vectors were also produced from these plasmids by transient transfection of HEK293 cells using $CaCl_2$ solution. The silver staining and electron microscopy analysis demonstrated good quality of both isolated vectors, ssAAV9-SMN and scAAV9- SMN, with the titers of 2.00E+13 and 1.00E+13 GC/ml. The results of this study show that, the plasmid containing UTR elements causes to twice more SMN gene expression in transfected cells. The quality control results show that both produced ss and scAAV9-SMN are suitable for in vivo studies.

Keywords

Adeno-associated viral vector; Electron microscopy; Gene therapy; Human embryonic kidney 293 cells; Spinal muscular atrophy (SMA); *Survival Motor Neuron* (*SMN*) gene; Transient transfection

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Compliance with Ethical Standards

Research Involving Human Participants and/or Animal Research did not involve Human Participants and/or Animal.

Conflict of interest None.

Introduction

Spinal muscular atrophy (SMA), an autosomal recessive neurodegenerative disorder, is the second most common cause of newborn mortality after cystic fibrosis in the west world (1). The most important symptoms of SMA are symmetrical muscle weakness and progressive paralysis (2). The homozygous mutations or deletions of *survival motor neuron* (*SMN*) gene are responsible for SMA disease (3). Currently, there is no curative treatment for SMA. Gene therapy offers new hopes for the treatment of SMA disease (4). In most of SMA gene therapy studies, adeno-associated virus (AAV) vectors have been used for introducing SMN gene to defective cells (5–8). Today, AAV with 12 serotypes and more than 100 variants, (9) which have been isolated from human and nonhuman primates, is a very exciting tool for gene delivery to mammalian cells. The AAV is a replication-defective virus which needs co-infection with another virus, such as adenovirus or herpes simplex virus, for efficient replication in host cells. The classic method for production of rAAV vectors is a triple transfection of HEK293 cells. In this method, all factors required for rAAV production are set in three plasmids; AAV trans-plasmid containing AAV cap and rep genes, AAV cis-plasmid containing gene of interest flanked by two ITRs in the ends, and a helper plasmid encoding adenovirus trans-acting helper factors (10). Special characteristics, such as low immunogenicity, site-specific integration of transgene into the host genome, transfection of non-dividing and dividing cells, and long-term expression of transgene, make the recombinant AAV (rAAV) suitable vector for gene therapy studies (11).

Here we describe a construction of cis-plasmid carrying SMN cDNA for the production of rAAV, which is planned for a further study on SMA gene therapy. We used pAAV-CB6-PI and pAAVsc-CB6-PI as the plasmid backbones for cloning of cis-plasmid containing SMN cDNA. PI as the plasmid backbones for cloning of cis-plasmid containing SMN cDNA.

Materials and Methods

Bacterial Strain

Escherichia coli DH5α strain (New England Biolabs) was used for plasmid cloning. Competent cells were prepared by calcium chloride (Heat Shock) method. 50 ng of plasmid DNA was added to 50 μl of the competent cells. 20 μl of bacterial suspension was transferred onto Terrific broth (TB) agar plate containing 100 μg/ml ampicillin (Sigma, A5354).

Plasmids

The pAAV-CB6-PI (4409 bp) and pAAVsc-CB6-PI plasmids (Gao's Lab., Gene Therapy Center, UMass Medical School, Worcester, MA, USA) were used in this study. The pAAVsc-CB6-PI plasmid bears engineered ITRs for scAAV vector. Plasmids carry Ampicillin Resistant gene for selection of transformed bacteria by ampicillin-containing medium. The complete SMN cDNA with its specific UTRs named as pCMV6-XL5-SMN (SC128237) was purchased from OriGene Company (Rockville, MD, USA). The SMN cDNA sequence was checked using DNA data bases (GenBank Accession No. NM_000344.2) to verify the sequence integrity.

Subcloning of Human SMN gene in pAAV-CB6-PI and pAAVsc-CB6-PI

The blunt-end ligation strategy was performed for construction of AAV Cis-plasmids carrying the SMN gene. The pAAV-CB6-PI and pAAVsc-CB6-PI plasmids were digested with EcoRI/KpnI and AgeI/SacI (New England Biolabs, MA, USA) restriction enzymes. The pCMV6-XL5-SMN plasmid was digested with NotI enzyme (New England Biolabs, MA, USA) to extract SMN cDNA with its UTRs from the original plasmid to be cloned into the pAAV-CB6-PI. For cloning SMN cDNA without its UTRs into pAAVsc-CB6-PI, the pCMV6-XL5-SMN was digested by BglII (New England Biolabs, MA, USA) restriction enzyme. All of the digested plasmids were run on the 1% agarose gel and 4393 bp of pAAV-CB6-PI, 4152 bp of pAAVsc-CB6-PI, 1629 bp of SMN cDNA with UTRs sequences, and 1037 bp of SMN cDNA without its UTRs were purified from the agarose gel using QIAquick gel extraction kit (Qiagen, Boston, MA, USA). The blunt-end ligation was done in 10 μl reaction using T4 DNA ligase (New England Biolabs, MA, USA). The 10 μl of ligation mixture was transformed to 100 μl of competent bacteria and cultured on TB-Amp plates. The plasmids were isolated from bacteria using QIAprep spin miniprep kit (Qiagen, Boston, MA, USA) according to the manufacturer's protocol. Constructed plasmids were checked with restriction digestion and sequencing analysis. The sequencing primers were shown in Supplementary Table 1. The integrity of AAV inverted terminal repeats (ITR) was determined by Sma I and AvaI digestions.

Transfection of HEK293 Cells with Constructed Plasmids

Low-passage HEK293 cells were inoculated into 6-well culture plates at a concentration of 2.5 X 10^5 cells per well, 24 h before transfection, and incubated in 37 °C with %5 CO_2 in a humidified atmosphere. 2.5 μg of constructed plasmids was used to transfection using lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol.

Total Cell Lysate Preparation and Western Blot Analysis

Forty-eight hours after transfection, cells were detached mechanically easily by forced pipetting and washed two times with ice-cold phosphate-buffered saline (PBS). The cells were collected by centrifugation at 1200x*g* for 10 min. The cells were lysed by adding 100 μl of ice-cold Ripa buffer (Thermo Scientific, MA, USA) to the pellets. The protein concentration of each sample was determined by BCA protein assay kit (Thermo Scientific, Pierce, MA, USA) according to the manufacturer's protocol. Twenty μg of reduced cell extracts were subjected to each well of 12 % SDS-PAGE gel. Then, separated proteins on the gel were transferred onto Protran (Whatman GmbH) nitrocellulose transfer membrane. After blocking by PBS-based Odyssey blocking buffer (LI-COR Biosciences, NE, USA), the membrane was incubated with 1:5000 diluted Purified Mouse Anti-SMN antibody (BD Biosciences, MA, USA) and 1:5000 diluted β-tubulin antibody (Abcam, MA, USA). The membrane was exposed to 1:15,000 IRDye 800CW Goat polyclonal Anti-Mouse IgG (H + L) seconder anti- body (LI-COR Biosciences, NE, USA) for 1 h. The membrane was visualized by odyssey infrared imaging system (LI-COR Biosciences, NE, USA).

Preparation of ss and scAAV9-SMN Vector

The AAV9-SMN vector was produced by transient triple transfection of 293 cells using CaCl2 solution which previously described (10). Briefly, the HEK293 cells were cultured in 15-cm plate 1 day before transfection. The growth medium containing high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml) was used for HEK293 cell culture. Cells achieved to ~70–80 % density at the time of transfection. The DNA mixture was prepared for 40 plates by adding CaCl2 (2.5 M) 5.2 mL, pDF6 adeno helper plasmid 1040 mg, trans-plasmid 520 mg, cis-plasmid 520 mg, and the final volume reached to 54 ml by adding sterile Milli-Q water. Four 50-mL conical tubes each containing 12.5 ml of x2 HBS solution were prepared. 12.5 ml of DNA mixture was added to each x2 HBS, and incubated for 5 min at room temperature. 2.5 ml of the transfection cocktail was added to each plate and placed back in the 37 °C, 5 % CO2 incubator. 72 h after transfection, cells were detached by scraping and collected by centrifugation. The cell pellet was suspended in 14 ml of 50 mM Tris (pH 7.4), 1 mM MgCl2 buffer. Cells were sonicated twice, each for 1 min in an ice-water bath. 150 μl of Benzonase at the final concentration of 100 U/ml was added to the cell lysate and incubated for 20 min at 37 °C. 1.25 ml of 10 % deoxycholic acid was added and incubated for a further 10 min at 37 °C. The tube was immediately placed on ice for 10–20 min. Finally, cell lysate was centrifuged at 3000x*g* for 30 min at 4 °C, and the supernatant was collected. Isolated viral vectors were purified by cesium chloride gradient centrifugation and desalted by dialyze using Slide-A-Lyzer™ dialysis cassettes (Thermo Scientific) as described previously (10).

Quality Control of Produced Recombinant AAV9 Vectors

The titration of purified ss and scAAV9-SMN vector genome copy number were assessed by performing Real-Time qPCR after treatment of the purified vector solution by DNase I (Qiagene) to eliminate unencapsidated AAV DNA or contaminating plasmid DNA. After heat treatment to inactivate DNase I and releasing the vector genome by disrupting virus capsid, RT-qPCR was performed using primers and 6FAM fluorescent probe designed for polyA signal, and TaqMan Universal PCR Mix, no UNG (uracil N-glycosylase) (Applied Biosystems). 4–12 % SDS-acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA) was used to assess vectors purity, and transmission electron microscopy of negative-stained recombinant AAV virions was performed at Electron Microscopy Core, UMass Medical School, Worcester, MA for assessment of morphological integrity and full or empty. Those methods were previously described in detail by Gao and Sena-Esteves (10). The ratio of empty-to-full capsids was assessed by direct counting of the electron micrographs.

Statistical Analysis

The Western blot results were quantified via Student's t test using SPSS 15.0 software. The standard error of mean (SEM) for each group were evaluated by the same software. $P < 0.05$ was considered as significant. All experiments were repeated 3 times.

Results

SMN cDNA Sequence

The SMN cDNA sequence had three mutations at positions 496A>G, 871T>C, and 1155A>G (see Supplementary Fig. 1). The 496A>G and 871T>C mutations are silent mutations, and the last one is located in a non-coding 3′ UTR region. They had no effects on protein expression.

The genetic map of all plasmids is shown in Fig. 1. To verify the insertion of SMN sequence and integrity of the ITRs, the colonies carrying constructed plasmids were identified by digestions with different enzymes (i.e., Sam I, Ava I, NcoI/NaeI, SapI/SphI for pAAV-CB6-PI-SMN and AleI/NcoI or SmaI for pAAVsc-CB6-PI-SMN). The restriction enzyme digestion and sequencing analysis results confirmed that the related SMN sequences were correctly inserted into the backbone plasmid. Recombinant clones with the expected restriction banding pattern were subjected to sequencing analysis (see Supplementary Fig. 2).

Protein Expression

To determine the amount of SMN protein expressed by different constructed cis-plasmids, HEK293 cells were transfected with 2.5 μg of each plasmid. As shown in Fig. 2, untransfected cells have a basal level of SMN expression as expected, however, transfected cells by pAAVsc-CB6-PI-SMN and pAAV-CB6-PI-SMN showed 4.853-fold and 2.321-fold higher SMN expression levels than untransfected cells ($P < 0.001$), respectively.

Quality Control of Isolated ss and scAAV9-SMN Vectors

The viral titer was assessed by RT-PCR as a 1.00E+13 and 2.00E+13 GC/ml for scAAV9-SMN and ssAAV9-SMN vectors, respectively. As Fig. 3 shows, the only three capsid proteins, VP1, VP2, and VP3 were detected by silver-stained SDS polyacrylamide gel. These bands were compared to highly purified rAAV2-CMW-lacZ preparation with known virus titer (1.00E+13 GC/ml) as a reference standard. The electron microscopy images, as shown in Fig. 4 demonstrated pure viral vector with few empty capsids.

Discussion

The increasing use of rAAV vectors for clinical gene therapy studies couraged us to describe our simple and effective protocol for producing rAAVs. The constructed plasmids in this study, pAAV-CB6-PI-SMN and pAAVsc-CB6-PI-SMN, are the cis-plasmids bearing the SMN gene necessary for AAV vector production. The SMN expression in HEK293 cells transfected with constructed plasmids was investigated using Western blot analysis. After that the produced cis-plasmids were used to ss and scAAV9-SMN vectors by triple transfection method of HEK293 cells.

AAV vectors, especially self-complementary rAAVs, have limited capacity for carrying the desired gene (AAV have ~4.7 kb genome in long) into the host genome (12). Generally, transgenes have been cloned into AAV cis-plasmid without its UTR elements. Since the UTRs have important roles in gene expression, their absence would cause low expression of

the transgene. For solving this problem and induction of transgene overexpression in mammalian cells, cis-plasmid normally bears very strong interval promoter like CMV, enhancer, and polyA signal (10). Here, SMN gene was cloned with its specific UTRs into pAAVsc-CB6-PI-SMN, but this gene was cloned into pAAV-CB6-PI-SMN without the SMN gene's UTRs. Both plasmids contain similar elements necessary for gene expression such as CMV promoter, poly A signal, and one small intron. The Western blot results showed the increased expression of SMN protein in transfected cells (Fig. 2) but the cells transfected with cis-plasmid containing SMN cDNA and its specific UTRs (pAAVsc-CB6-PI-SMN) shown twice more SMN protein expression. These results in accordance with the results of Miao et al. showed that plasmid containing 3′ UTR gave ~2-fold higher expression than those containing the bovine growth hormone polyadenylation signal (bGH P(A)) alone (13). Erhardt and Kay similarly demonstrated that plasmid containing gene's UTRs led to factor IX expression in high level for over 9 weeks in vivo, while vectors lacking this element expressed 40-fold less (14). It means that not only strong promoter and poly A signal are necessary, but also gene's specific UTRs should be cloned along with transgene amid to optimal gene expression for a long time. Since transcription is tightly coupled with posttranscriptional processing and gene's specific element like UTRs, TATA box, and poly A signal are necessary for transcription, it has been considered that improvement of AAV viral vector systems help to expression of a transgene for a longer period of time in clinical gene therapy studies.

The major capsid proteins of AAV virions are VP1, VP2, and VP3. The purified AAV vectors separated on SDS polyacrylamide gel and visualized by silver stain normally should show just those three capsid proteins in 87, 73, and 62 kDa at a ratio of 1:1:18, respectively. The semi quantitative estimate of virus particle concentration could be assayed by comparing these bands to reference standard with known virus titers (10). As Fig. 3 shows that only three viral capsid protein bands related to the VP1, VP2, and Vp3 were visualized in this study. This means that the isolated virus preparations are pure. Fig. 4 shows Transmission electron microscopy imaging of ss and scAAV9-SMN. Some virus particles with darkly stained center are empty or partially packaged capsids. Empty particles appear when transgene cannot be completely packaged into assembled capsids (etc. because of large size of transgene). In this study, the purity of isolated vectors was demonstrated by the silver staining and electron microscopy analysis. The majority of AAV9 virions were full particles and a low number of empty capsids was seen in electron micrographs of ss and scAAV9-SMN vectors. As shown in Fig.4, the Transmission electron micrographs related to scAAV9-SMN vectors contain less empty capsids compared to ssAAV9-SMN micrographs.

Complete removal of empty capsids from full ones is difficult and remains as a desirable feature of purification methods. The CsCl gradient method is known as the best method to remove empty capsids from full ones (15). rAAV-SMN vectors produced in this study demonstrated a desirable quality. Therefore, it is considered that it can be used for further in vivo studies of SMA which are planned by the authors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by University of Massachusetts Medical School (an internal grant), National Institutes of Health (R01NS076991-01, 1R21DA031952-01A, 2P01HL059407, 1P01AI100263-01), the Will Foundation, Jacob's Cure, NTSAD Foundation, Canavan Foundation, and partial support from a grant from the National High Technology Research and Development Program ("863" Program) of China (2012AA020810). G.G. is one of the founders of Voyager Therapeutics and holds equity in the company. G.G. is an inventor on patents with potential royalties licensed to Voyager Therapeutics and other pharmaceutical companies.

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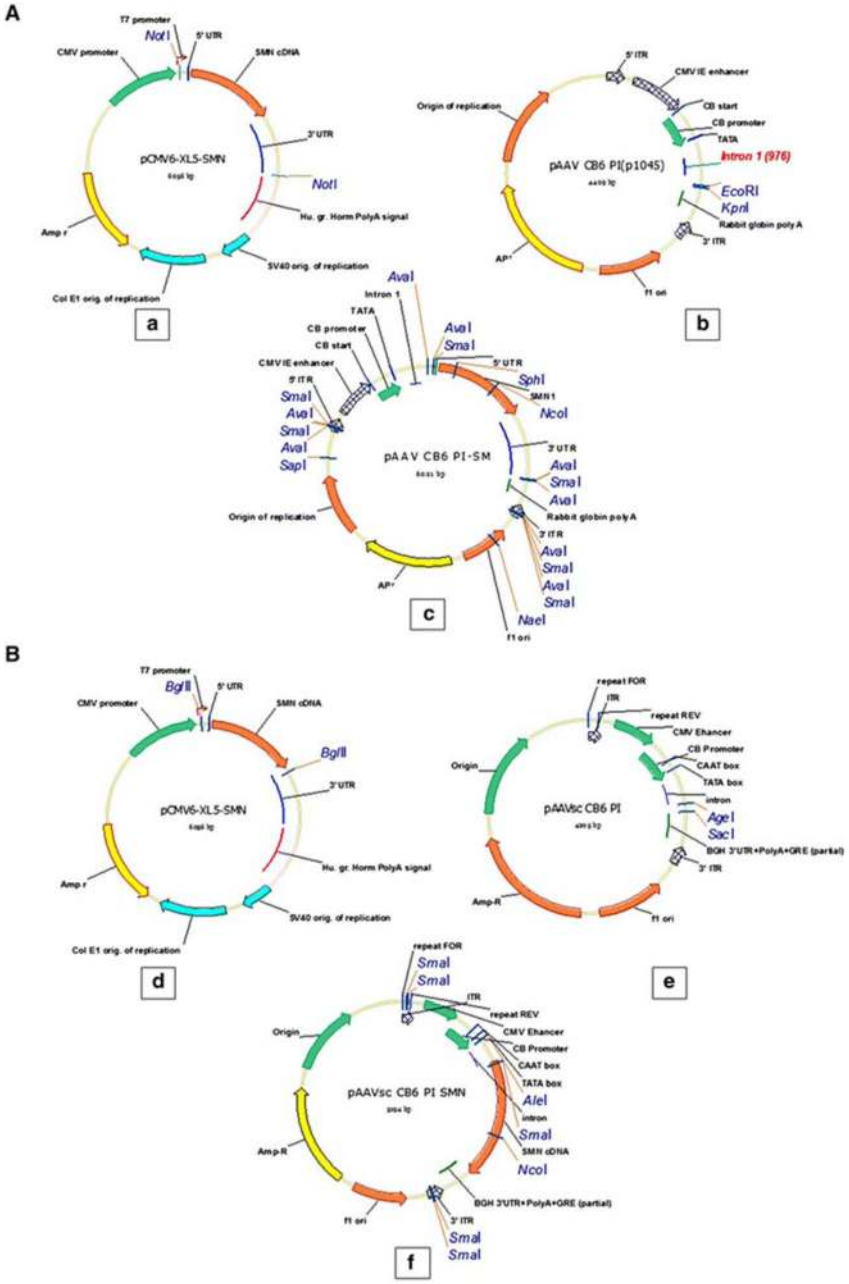

Fig. 1.

Genetic and restriction map of the constructed plasmids. **A** (*a*) pCMV6-XL5-SMN digested with NotI, (*b*) pAAV-CB6-PI was digested with EcoRI and KpnI, to construct (*c*) pAAVsc-CB6-PI- SMN. **B** (*d*) pCMV6-XL5-SMN was digested with Bgl II, (*e*) pAAVsc-CB6-PI was digested with AgeI and SacI, to construct (*f*) pAAVsc-CB6-PI-SMN. The restriction enzymes used for investigation of the accuracy of SMN insertion into constructed plasmids are shown in the parts c and f. A small intron sequence was inserted between promoter and transgene to help to mRNA splicing of the transgene. ITR sequence is necessary for packaging of the transgene into virions and its integration into the host genome

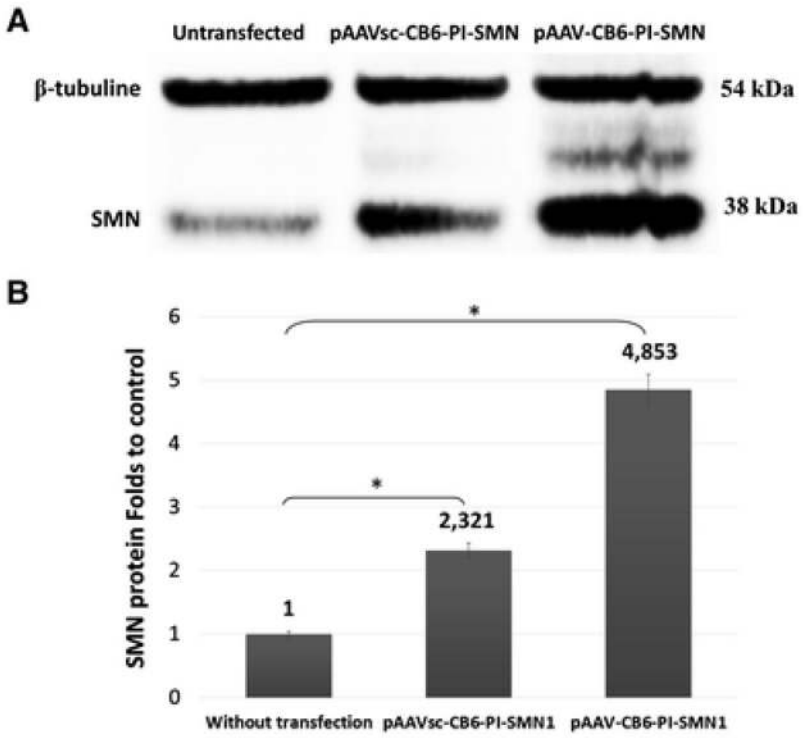

Fig. 2.

Western blot results of transfected HEK293 cells with constructed plasmids. **A** Protein levels relative to SMN and b-tubulin. **B** Both pAAVsc-CB6-PI-SMN and pAAV-CB6-PI-SMN transfected cells showed 4.853-fold and 2.321-fold higher SMN expression levels than untransfected cells. *Error bars* denote SEM. $*P < 0.001$

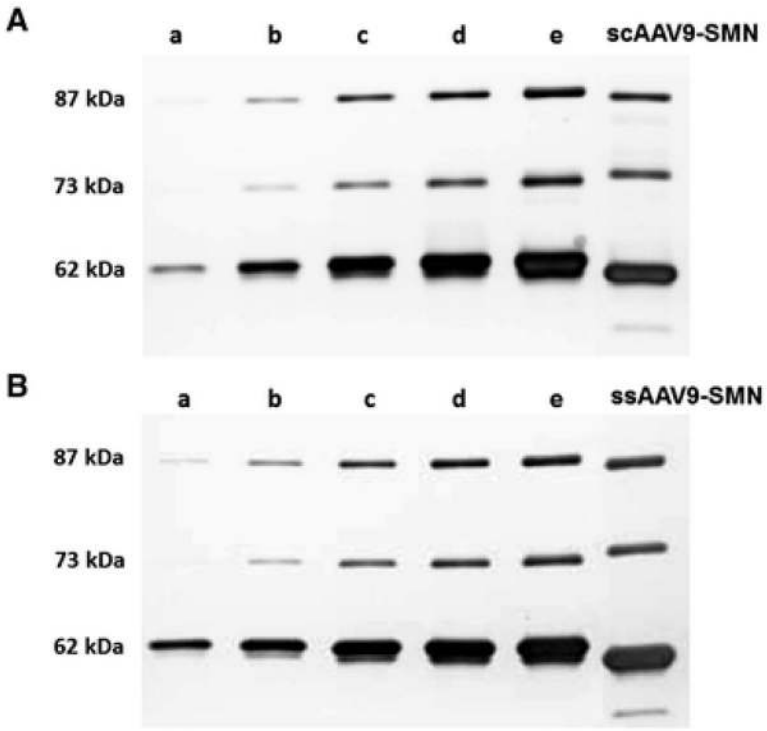

Fig. 3.

Silver-stained SDS-PAGE of AAV preps. **A, B** are silver staining results of scAAV9-SMN and ssAAV9-SMN, respectively, with rAAV2-CMW-LacZ (1.00E+13) as a reference standard. *a*, *b*, *c*, *d*, *e*, in **A, B** are 0.5, 1, 2, 3, 4 μl of rAAV2-CMW-LacZ, respectively. 2 μl of each isolated viral vector was loaded onto an SDS-PAGE gel. The VP1, VP2, and VP3 capsid proteins were detected by silver staining examination

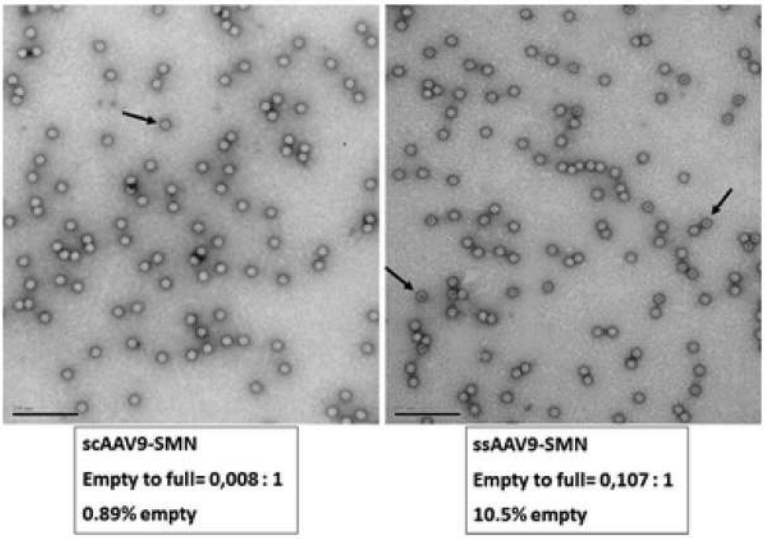

Fig. 4.

The electron microscopy images (*scale bar*, 200 nm) of isolated vectors. The ss and scAAV9-SMN vectors were negatively stained with uranyl acetate and controlled by transmission electron microscopy. As the images demonstrate the majority of virions are full particles. The ratio of empty-to-full particles and the percentage of empty particles was shown. The scAAV9-SMN was approximately 12 times better packaged than ssAAV9-SMN. *Arrows* indicate partially packaged or empty viruses