METHOD

Development of a high-yield, high-quality purification process for adeno-associated virus vectors that can be used in vivo without ultracentrifugation: Application to a lung endothelial cell-targeted adeno-associated virus

Yasunaga Shiraishi1,2 | Takeshi Adachi2 | Jose M. Cacicedo3 | Yasuo Ido2,4

1Division of Environmental Medicine, National Defense Medical College Research Institute, National Defense Medical College, Saitama, Japan
2Division of Cardiovascular Medicine, Department of Internal Medicine, National Defense Medical College, Saitama, Japan
3Department of Research and Development, ALPCO Diagnostics, Salem, New Hampshire, USA
4Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA

Abstract
Recombinant adeno-associated viruses (rAAVs) are useful vectors for expressing genes of interest in vivo because of their low immunogenicity and long-term gene expression. Various mutations have been introduced in recent years and have enabled high-efficacy, stabilized, and organ-oriented transduction. Our purpose for using rAAV is to express our target gene in the mouse lung to investigate pulmonary artery hypertension. We constructed a self-complementary AAV having mutant capsids with the ESGHGYF insert, which directs the vectors to lung endothelial cells. However, when this mutant virus was purified from the producing cells by the conventional method using an ultracentrifuge, it resulted in a low yield. In addition, the purification method using an ultracentrifuge is tedious and labor-intensive. Therefore, we aimed to develop a simple, high-quality method for obtaining enough lung-targeted rAAV. First, we modified amino acids (T491V and Y730F) of the capsid to stabilize the rAAV from degradation, and we optimized culture conditions. Next, we noticed that many rAAVs were released from the cells into the culture medium. We, therefore, improved our purification method by purifying from the culture medium without the ultracentrifugation step. Purification without ultracentrifugation had the problem that impurities were mixed in, causing inflammation. However, by performing PEG precipitation and chloroform extraction twice, we were able to purify rAAV that caused only as little inflammation as that obtained by the ultracentrifugation method. Sufficient rAAV was obtained and can now be administered to a rat as well as mice from a single dish: $1.50 \times 10^{13} \pm 3.58 \times 10^{12}$ vector genome from one φ150 mm dish (mean ± SEM).

KEYWORDS
adeno-associated virus, capsid modification, inflammation, purification method

Abbreviations: AAV, recombinant adeno-associated virus; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; NTRE, NFκB transcription responsive element; PAH, pulmonary arterial hypertension; PEG, polyethylene glycol; SLIC, sequence and ligation independent cloning; VP, viral protein.

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1 | INTRODUCTION

Recombinant adeno-associated virus (rAAV) has become the most convenient vector for in vivo research in both basic and clinical settings. Since its introduction, various improvements have been made by introducing mutations. The self-complementary mutations in the backbone of recombinant AAVs (scAAV)\textsuperscript{1,2} circumvents the slow expression of the gene of interest. Mutations in the capsid sequence in AAV2 allow retargeting to specific cell types.\textsuperscript{3,4} Another capsid mutation at threonine, serine, or lysine sites was found to improve the expression of the gene of interest by presumably stabilizing AAV from degradation.\textsuperscript{5,6}

Our research theme is to study pulmonary arterial hypertension (PAH) and elucidate its pathogenesis. We use mouse models for our research. Recently, a lung endothelial cell-targeted AAV capsid mutation for mice was developed,\textsuperscript{7} and we employed this capsid for our research. We purified this rAAV using a recently published ultracentrifuge-based method.\textsuperscript{8} However, this mutated rAAV had a significantly reduced viral yield compared to the original virus. In addition, purification using ultracentrifugation results in high viral loss and was labor-intensive.\textsuperscript{9}

To compensate for these problems, rAAV was purified by a non-ultracentrifugation method based on previous reports and administered to mice. However, inflammation was induced.

In this study, we developed a method to purify rAAV that induces only as much inflammation as rAAV purified by ultracentrifugation without using an ultracentrifuge. Furthermore, by introducing a mutation in the capsid of rAAV and optimizing the culture and purification conditions, we were able to obtain pulmonary vascular endothelium-directed rAAV with sufficient yield.

However, preparing sufficient and highly purified viruses for small animal experiments is still cumbersome and laborious.

2 | MATERIALS AND METHODS

2.1 | Reagent and materials

We used HEK293T (ATCC\textsuperscript{®} CRL-3216\textsuperscript{™}) to package the rAAV virus. The AAV plasmids kit was purchased from Stratagene (currently, Agilent Technologies). Real-time PCR reagent with intercalated dye (PowerUp SYBR Green Master Mix) was from Thermo Fisher Scientific, Inc. (USA). In some experiments, we used AAVpro\textsuperscript{®} Purification Kit Maxi (All Serotypes) (Takara Bio, Japan) to evaluate its performance. Oligonucleotides used to modify rAAV and capsid were purchased from Invitrogen, Eurofins, and Integrated DNA technologies. To manipulate plasmid sequence by PCR, Prime STAR Max DNA polymerase (Takara Bio, Japan) was used along with a homemade SLIC (sequence and ligation independent cloning) enzyme mix. All other reagents are described in the Supporting information S8.

2.2 | AAV vector construction

AAV-LacZ was a control vector that came with the kit, which has been modified to make a gateway compatible destination vector scAAV\textsubscript{173CMV_GW_SV40pA} (Figure 1A). The self-complementary mutation was made by removing AGGAACCCCTAGTGATGGAGTTGG from R-ITR. The original CMV promoter was replaced with a much smaller but equally effective truncated 173CMV promoter sequence (patent US20070098690A1). SV40ori sequence was inserted upstream of L-ITR to amplify plasmid DNA under Large-T antigen expressed in HEK293T cells. The full sequence is located in the Supporting information S1. In this study, we used mVenus protein as the gene of interest for expression. These cDNAs were subcloned into the pENTR1A vector (Invitrogen, USA) using PCR followed by SLIC reaction. Then, scAAV vectors were created by LR-reaction (Invitrogen, USA).

The C2 sequence in AAV2 RC plasmid was mutated to accommodate capsid stabilization and lung endothelial cell-targeted expression.\textsuperscript{7} For stabilizing mutations, we chose the Y730F and T491V mutations and incorporated them by PCR. In order to facilitate the site-directed mutation, the C2 sequence was subcloned into the pENTRD vector. The lung endothelial cell-targeting mutation consisting of 7 amino acids flanked by G and A (G-ESGHGYF-A) was inserted between R588 and Q589 by PCR. The wild type and stabilized mutant AAV\textsubscript{R32C2} plasmids made above had the lung endothelial cell-targeting mutation inserted between the BsiWI and XcmI cut sites.

2.3 | Animals

All experimental protocols were approved by National Defense Medical College Board for Studies in Experimental Animals (Institutional Animal Care and Use Committee: IACUC). Male and Female C57BL/6N mice were obtained from Japan SLC, Inc. (Japan). These mice were mated, and offspring mice of about 10 weeks of age were used. rAAV was retro-orbitally injected with 50 to 150μl of a viral preparation containing 1×10\textsuperscript{12} virus. Animals were maintained in a temperature-controlled facility on a 12-h light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.)
and fed with a regular chow diet (CLEA Japan, Inc.) ad libitum.

### 2.4 Brief description of the basal protocol of rAAV production and purification (φ150 mm)

Initially, the protocol was the same as the recently published protocol by Kimura et al.\(^8\) except for the virus production medium and duration of culture. HEK293T cells were seeded on one φ150 mm 0.001% collagen I coated dishes at 70% confluency and transfected with three plasmids\(^11,12\) (the AAV vector plasmid 30μg, Rep2-Cap plasmid 42μg, and the helper plasmid 42μg). After transfection, the cells were incubated with Advanced\(^\text{™}\) DMEM containing 1% FBS and 2mM glutamine (AAV production medium) for three days. Then, the cells and medium were collected, and AAV from the cells was extracted with the extraction buffer (Citrate buffer pH 4.9, 200 mM NaCl, 100 mM Sucrose). The extracted rAAV from cells and medium were PEG-precipitated overnight. The precipitated crude rAAV was dissolved with HNE buffer (100 mM HEPES, [pH 8.0], 150 mM NaCl, 20 mM EDTA),
and performed chloroform extraction. The top-layer was further purified using discontinuous iodixanol gradient ultracentrifugation. The two-phase extraction step described in the original protocol\textsuperscript{8} was omitted.

### 2.5 The detailed final protocol

A brief final protocol is described in the result section, and a detailed protocol with all the reagents was described in the Supporting information S8.

### 2.6 AAV titration

The virus titer was assessed by real-time polymerase chain reaction (qPCR) following the procedure described by Addgene (https://www.addgene.org/protocols/aav-titration-qpcr-using-sybr-green-technology/) using the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific: Waltham, USA) and SYBR Green Master Mix (#4367659, Thermo Fisher Scientific: Waltham, USA) following the manufacturer’s instructions. Primers at AAV ITR (Inverted Terminal Repeat), forward: GGAACCCCTAGTGATGGAGTT and reverse: CGGCCTCAGTGAGCGA used.\textsuperscript{13} The stock of standard was prepared with rAAV vector plasmid at $2.0 \times 10^9$ molecules/μL. Seven serial dilutions were made to make the standard curve, which was used to calculate the viral titer.

The viral number in organs was also measured by qPCR. DNA was extracted from the organs using the QIAamp kit (QIAamp DNA Mini Kit #51304, QIAGEN, Germany) as per manufacturer’s instructions. 46ng of total DNA in each organ was analyzed.

### 2.7 Silver stains of virus preparation samples

2× Laemmli sample buffer (#1610737, Bio-rad, USA) with 2-mercaptoethanol was added to rAAV solutions and boiled for ten minutes. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading $2.0 \times 10^3$ vg of each sample. The gel was stained by Silver Stain Kit (#1610443, Bio-Rad, USA) following the manufacturer’s instructions.

### 2.8 Detection and quantitative analysis of mVenus expression in mouse organs and HUVECs

Two months after injecting rAAV expressing mVenus with the capsid mutant targeting viral transduction to lung endothelial cells in mice, mVenus expression was assessed by fluorescence images using IVIS Imaging System (IVIS Lumina XR Series III, PerkinElmer, USA). The mice were sacrificed and the organs including: the heart, lung, liver, kidney, and spleen, were removed to capture the image. The excitation wavelength was set to 520 nm, and the detected wavelength was 570 nm. The software included in the IVIS imaging system was used to evaluate average radiant efficiency $[p/s/cm^2/sr]/[\mu W/cm^2]$ with ROI (Region of Interest) set to the lungs.

In vitro, mVenus was expressed in HUVECs using AAV6. mVenus positive cells were quantitatively evaluated using an all-in-one fluorescence microscope (BZ-X800, KEYENCE, Osaka, Japan) equipped with an image cytometer module (BZ-H4XI, KEYENCE, Osaka, Japan).

### 2.9 Western blotting

Western blotting was used to detect the target protein expression in the rAAV injected mice lungs. The lungs were homogenized with a Bead Homogenizer (Micro Smash-100R, TOMY, Japan) in RIPA Buffer (#9806, Cell Signaling Technologies, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich Corp, USA). After brief sonication, these samples were centrifuged at 10 000g for 10 min at 4°C. Standard SDS-PAGE and western blot were performed as before\textsuperscript{14} with Bio-Rad gel (#1658004JA, Bio-Rad, USA) and their system. Anti-GFP antibody (8371-2; BD Biosciences, USA) was diluted 1:1000 in TBS and incubated for overnight. They were then incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cell Signaling Technologies). The specific protein amount was detected after immersing in enhanced chemiluminescence (ECL) (Thermo Fisher Scientific Inc., Waltham, MA) and light emission was detected and captured by a LAS-3000 Imager (Fujifilm, Japan).

### 2.10 NFκB reporter gene assay

NFκB reporter and control pTAL (TATA-like) sequences were from the plasmid used previously.\textsuperscript{15} The sequences were sub-cloned to pENTR1A vector (Invitrogen) and the inserted sequences were transferred to homemade scAAV_GW vector by LR reaction (Supporting information S2).

The lungs of each rAAV-treated mouse were harvested 96h post-infection. The lungs were homogenized with a bead homogenizer (Micro Smash-100R, TOMY, Japan) in lysis buffer (25mM Tris-phosphate [pH 7.8], 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N’,N’-tetraacetic acid, 10% glycerol, 1% Triton X-100: 25 μl/1 mg lung).
These samples were centrifuged at 10,000g for 10 min at 4°C, and the supernatant was collected. ONE-Glo™ EX Luciferase Assay System (#E8110, Promega, Wisconsin, USA) was used as the luminescent reagent. We followed the product instructions and measured the luminosity by plate reader (#HH34000000, EnSight Multimode Plate Reader, Perkin Elmer, Massachusetts, USA).

The amount of virus in the lungs was measured by qPCR, and each luminosity was divided by the amount of virus. The luminescence produced by the rAAV with NTRE was normalized by the average value of luminescence produced by the rAAV without NTRE.

### 2.11 Statistical analysis

Results are presented as mean ± SEM. Student’s t-test, paired t-tests one-way ANOVA were performed by SAS ver9.4 university edition. Data were analyzed by the procedures UNIVARIATE, TTEST, and MIXED appropriately. p less than .05 was considered as a significance. In the graphed data, p values are denoted as * < .05, ** .01, and *** .001.

### 3 RESULTS

#### 3.1 Effects of capsid mutation for targeting lung endothelial cells and stabilization on rAAV titer

Lung endothelial cell targeted capsid was made as reported by Körbelin et al.3,7 The peptide sequence ESHGYYF was inserted at loop IV of VP1-3 of the AAV2 capsid (Figure 1A). We purified this rAAV using the recently published method with significantly less work and time than conventional methods8 and evaluated the yield compared to the original AAV2 capsid. This mutation reduced the total virus yield to less than half as compared to the original AAV2 (Ratio of the yield of lung targeted mutation to original AAV2: 0.402 ± 0.041 [mean ± SEM, n = 6, p = .0002]) (Figure 1B).

To compensate for the loss of virus yield caused by the lung endothelial targeted mutation, we examined the effect of capsid stabilizing mutations. AAV particles inside cells are postulated to be degraded through ubiquitin-proteasome pathway, and the phosphorylation of a specific serine, threonine, and tyrosine in the capsid may be responsible for degradation through this pathway.16–18 Several papers have reported that mutating these surface-exposed amino acids effectively increased transduction efficiency:4–6,19 Since AAV is produced intracellularly using HEK293T cells, some may be degraded by a similar pathway. Therefore, we hypothesized that these mutations may also suppress degradation during AAV production, resulting in increased production. Thus, we compared the yield of the stabilizing mutations with the yield of the non-stabilized (unmutated) capsid. Among reported mutations, we adopted the mutations in T491V and Y730F since they seemingly were most effective (Figure 1A). These stabilizing mutations increased AAV titer by 4.5-fold compared to non-stabilized lung targeted capsid (Ratio of the yield of stabilizing mutation to non-stabilized capsid: 4.496 ± 1.586 [mean ± SEM, n = 3 ~ 4, p = .029]) (Figure 1C). As shown later, these stabilizing mutations did not affect the functionality of lung endothelial cell target mutation. Therefore, these mutations are considered compatible.

#### 3.2 Optimization of cell culture conditions to increase rAAV yield

Next, we attempted to increase the yield of virus by optimizing the culture conditions and purification methods. The original AAV production and purification method was based on the recently published report by Kimura et al.8 Key points of their procedures are:

1. Virus was produced in HEK 293T cells with low glucose Dulbecco’s Modified Eagle Medium (DMEM) (Glucose: 5.5 mM) containing 1% or 2% fetal bovine serum (FBS),
2. Virus was collected from both cells and culture medium, and citrate buffer solution was used for extraction from cells, and
3. rAAV in medium and cells was first concentrated by polyethylene glycol (PEG) precipitation, then purified with chloroform extraction, 2-phase aqueous partitioning,10,20 and finally by iodixanol ultracentrifugation.

We investigated the conditions for rAAV production to increase the yield of rAAV. We modified the medium composition. (Details of the experiments are described in Supporting Information S3).

#### 3.2.1 Type of culture medium

It is known that the production of rAAV is reduced when the amount of serum is high.21 Advanced™ DMEM requires less serum to be added, and we have previously used Advanced™ DMEM for lentivirus production to obtain sufficient amounts of lentivirus (unpublished data). Thus, we used Advanced™ DMEM for AAV production. We compared these media to low glucose DMEM and
found Advanced™ DMEM was not inferior to low glucose DMEM (data not shown). We also examined the use of another serum-reduced medium, Gibco™ Opti-MEM™ I, and found no significant difference in virus yield from Advanced™ DMEM, but the yield tended to be lower (Supporting information S3A–C).

3.2.2 | Maintaining medium glucose levels

When the culture environment becomes acidic, the yield of the virus decreases.21 The cause of acidity is lactate produced by the glycolytic system, which is why the original method used a low glucose medium.8 We measured glucose concentration in the medium and found the glucose concentration was less than half within 12 h after the start of AAV production (25 mM → 11.5 mM ± 0.8 [mean ± SEM, n = 12]) (Supporting information S3D). It is assumed that a large amount of glucose is consumed through the pentose phosphate pathway to produce nucleic acids necessary for viral synthesis. Therefore, we added 1 M glucose every 12 h from the 2nd day of production (total of four times) to maintain the 25 mM glucose concentration. NaHCO3 was also administered every 12 h to correct the acidic environment. The amount of virus extracted from cultured cells was predominantly increased by the addition of glucose, but the amount of virus purified from the culture medium was not significantly different between the two. The total viral yield also tended to increase with the addition of glucose, but no significant difference was observed. The addition of glucose was considered desirable when purifying virus only from cultured cells (Supporting information S3E–G).

3.2.3 | Maintaining pH during virus production by adding NaHCO3 or KHCO3

We added 10 mM HEPES to the AAV production medium as the final concentration and measured pH every 12 h. The pH was maintained around 7.8 by adding 7.5% NaHCO3, as recommended for AAV production by Atkinson et al.21 We also evaluated KHCO3 instead of NaHCO3, but there was no improvement (Supporting information S3H–J).

3.2.4 | Effect of increased L-glutamine concentration

L-glutamine is an important amino acid as a culture medium component. The effect of increasing the concentration of L-glutamine was evaluated by comparing the original 2 mM vs 10 mM L-glutamine. Increasing L-glutamine in the medium had no effect on virus yield (Supporting information S3K–M).

Collectively, we modified the production methods as follows.

1. The composition of the rAAV production medium is Advanced™ DMEM with 25 mM glucose, 1% FBS, 2 mM glutamine, 10 mM HEPES, and Antibiotic-Antimycotic (1x).
2. Keep medium glucose concentration high by adding glucose every 12 h after 24 h.
3. Maintain the medium in alkaline conditions (pH = 7.8) by adding NaHCO3 every 12 h after 24 h.

3.3 | Improvement of purification method

Next, we attempted to improve the purification method. Purification of rAAV from both cells and culture medium requires more reagents due to the larger volume of solution to be handled. In addition, Takara Bio Inc. sells a kit (#6666 AAVpro® Purification Kit Maxi [All Serotypes]) for purifying rAAV from cells without using an ultracentrifuge. Considering the above, we compared purification from cells only in this comparison.

3.3.1 | Improvement of AAV cell extraction solution

The original protocol used a citrate buffer-based extractant for extraction of rAAV from cells.8 Compared to the extractant used in other reports,22,23 we decided to use an extractant suitable for the purpose of this study (113 mM citrate buffer pH 4.9 + 200 mM NaCl + 100 mM sucrose) (Supporting information S4 1). Details of the experiments are described in Supporting information S4.

3.3.2 | Purification method without ultracentrifugation process

Purification using an ultracentrifuge is labor-intensive and results in significant viral loss. Therefore, we compared the transduction efficiency of the purification method with and without ultracentrifugation at the end of the original method. Approximately half of the rAAV was lost by ultracentrifugation (Ratio of yield without and with ultracentrifugation: 2.17 ± 0.87 [mean ± SEM, n = 7, p = .028]) (Figure 2A). The degree of purification detected by silver staining was reduced without ultracentrifugation process (Figure 2B). We then administered rAAV to mice to
compare the two purification methods. Lung-targeted rAAV expressing mVenus, a fluorescent protein, was retro-orbitally administered to mice at $1 \times 10^{12}$ vg per one mouse. The mVenus expression was detected only in the lung by IVIS Imaging System (Figure 2C), and the average radiant efficiency of mVenus was higher for rAAV purified without the ultracentrifugation process (with ultracentrifugation: $1.96 \times 10^8 \pm 6.86 \times 10^7 \text{[p/s/cm}^2/\text{sr]/[\mu W/cm}^2\text{]}, without
ultracentrifugation: $3.43 \times 10^8 \pm 1.25 \times 10^8$ [p/s/cm²/sr]/[μW/cm²] [mean ± SEM, p < .0496]) (Figure 2D). The protein expression of mVenus was detected in the lungs by Western blot analysis (Supporting information S4 2C). The amount of virus in organs measured by PCR targeting the ITR of AAV was also highest in the lungs for both purification methods (Supporting information S4 2D). Microscopic examination of formalin-fixed lung tissue showed no obvious inflammatory changes due to rAAV administration (Supporting information S4 2E). In vitro analysis in which mVenus was expressed in human umbilical vein endothelial cells (HUVECs) using AAV6 (MOI: $1 \times 10^5$). Transfection efficiency was higher for rAAV purified without the ultracentrifugation step (Figure 2E,F). Based on these results, at least for “mVenus,” the exclusion of ultracentrifugation from the purification method did not inhibit expression; rather, transduction efficiency was high. Furthermore, higher yields could be obtained by omitting the ultracentrifugation step. However, it has been reported that some target genes are downregulated by low-grade inflammation caused by poorly purified AAV. Therefore, we sought a way to further improve the rAAV purification method.

To simplify and increase lung targeted stabilized AAV yield, we tried to purify rAAV without ultracentrifugation from cell extracts. Comparisons were made with and without the ultracentrifugation step in the same sample. (A) The viral yield was measured by real-time PCR using ITR-specific primers and divided by the mean of the ultracentrifugation group to obtain a ratio. The purification method without ultracentrifugation yielded approximately twice as much virus as the method with ultracentrifugation. Data from the same session are connected by bars. Paired t test was used to obtain statistics. Error bars represent SEM. n = 7 per group, *p < .05. (B) The degree of purification was detected by silver stain. Each $2.0 \times 10^{10}$ vg AAV was loaded into the well. The purity was low without the ultracentrifugation step. VP: viral capsid protein. (C) Representative figure of fluorescence degree detected by IVIS Imaging System. These mice were injected with lung-specific rAAV expressing mVenus through an orbital vein ($1 \times 10^{12}$ vg) and detected 2 months later. (D) Five sessions were performed, and the fluorescence intensity was quantitatively analyzed by the IVIS Imaging System. The fluorescence intensities were higher with rAAV purified without the ultracentrifugation step. Data from the same session are connected by bars. Statistical tests were performed with pared t-ttest. Error bars represent SEM. n = 5 per group, *p < .05. (E, F) In vitro analysis of transduction efficiency. M Venus was expressed in HUVECs using AAV6. (MOI: $1 \times 10^5$). M Venus positive cells were quantitatively evaluated using an all-in-one fluorescence microscope (BZ-X800, KEYENCE, Osaka, Japan). (F) shows the quantitative analysis. Transduction efficiency were high with AAV6 purified without ultracentrifugation process. Six sessions were performed and data from the same session are connected by bars. Statistical tests were performed with pared t-test. Error bars represent SEM. n = 6 per group, *p < .05.

### 3.4 Cleaner rAAV purification method

According to a previous publications and our own experience, rAAV is not only produced and found intracellularly, but it also migrates into the culture medium, and the amount of migration seems to be related to the duration of production time and type of the capsid. The original AAV2 capsid can hold the virus in the cells, and the reported yield from cell to the medium was 3:1 in 3 days production time. On the other hand, in the lung-specific AAV, a large amount of rAAVs was released from the cells into the cell culture medium. Such change can be explained by the loss of the heparin-binding sequence in the
mutant that can act as an anchor on the cell membrane. Therefore, we purified rAAV from cell culture medium using the purification method described above and compared it with rAAV purified from cells by silver staining. The rAAV extracted from the culture medium was found to have slightly reduced impurities compared to the rAAV extracted from the cells (Figure 3A). In order to obtain highly purified rAAV without the ultracentrifuge process, we thought it would be better to purify rAAV from culture medium only, rather than extracts from cells.

In the original protocol, the solution containing rAAV was concentrated by PEG precipitation to a volume...
suitable for ultracentrifugation. This procedure is quite standard for rAAV purification.\textsuperscript{8-10,20,27} After the first PEG precipitation, chloroform extraction was performed, followed by further PEG precipitation. (twice PEG precipitation procedure). Compared to the one-time PEG precipitation, the second pellet size was visibly smaller and easily solubilized in PBS (Supporting information S5A). Silver staining reduced the detection of impurities and clarified the virus proteins (VPs) by the twice PEG precipitation procedure (Figure 3B). Because lung-targeted rAAV is readily released from the cells into the cell culture medium, the final yield obtained from the culture medium by two PEG precipitation procedure was greater than the yield purified from the cells (Ratio of the yield to cell extraction: $6.181 \pm 2.417$ [mean $\pm$ SEM, $n = 5$, $p = .032$]) (Figure 3C). Functionality and infectivity were examined by IVIS Imaging System and PCR of AAV-ITR from tissues as described above. Fluorescence of mVenus was detected in a lung-specific manner and rAAV DNA was also present in the lung. (Supporting information S5B,C).

AAVs are widely accepted as in vivo gene transfer vectors due to their mild inflammatory profile.\textsuperscript{28} However, common impurities found during purification of AAV vectors, which include host cell proteins and DNA, can affect innate and acquired immunity.\textsuperscript{29} Therefore, we investigated whether our purification method without the ultracentrifugation step (twice PEG precipitation procedure) induces inflammation compared to the conventional purification method including the ultracentrifugation step. The transcriptional activity of NFκB is known to be elevated in inflammation. Therefore, we designed a reporter gene assay system based on the NFκB transcription responsive element (NTRE) that drives the expression of firefly luciferase. Thus, we created an rAAV for NFκB detection that targets the lung and administered it to mice. First, to confirm function, these rAAVs purified by ultracentrifugation were injected into mice and then LPS (lipopolysaccharide), an inflammation-inducing substance, was administered intraperitoneally and luminescence of firefly luciferase was detected. Indeed, a higher degree of luminescence was observed in the LPS-treated group compared to non-treated group, and there was no luminescence detected in the lung without rAAV, suggesting that the lung-specific AAV-induced NFκB reporter gene assay was functional (Supporting information S7). Next, we purified these rAAVs by the twice PEG precipitation procedure and administrated to mice and compared their luminescence in the lungs with that of those purified by the ultracentrifugation process. To correct for differences in transduction efficiency, the degree of luminescence was divided by the number of AAVs measured by PCR in the ITR and normalized by the degree of luminescence attributable to rAAVs omitting only NTREs. The degree of luminescence was higher when purified by the twice PEG precipitation procedure than when purified by ultracentrifugation, suggesting that the degree of purification was insufficient, and inflammation was induced by rAAVs purified with the twice PEG precipitation procedure (Figure 3D). Inflammation may be induced by contamination of DNA from the virus-producing host cells. Therefore, we treated with endonuclease (Benzonase\textsuperscript{®}) and made a comparison, however there was no significant difference (Supporting information S6).

Then, we added an additional chloroform extraction to the twice PEG precipitation procedure (twice PEG + chloroform procedure). Silver staining with the addition of chloroform extraction similarly detected distinct AAV proteins, but no clear improvement over twice PEG precipitation procedure (Figure 3B). On the other hand, the degree of inflammation detected by NFκB reporter gene assay was improved to the same level as the purification method including the ultracentrifugation process. (Figure 3D).

With the twice PEG + chloroform procedure, the final yield was significantly higher than the original procedure purified from the cell and culture medium including ultracentrifugation step (the original procedure: $6.52 \times 10^{12} \pm 1.95 \times 10^{12}$ vg, the twice PEG + chloroform procedure: $1.50 \times 10^{13} \pm 3.58 \times 10^{12}$ vg [per one 150mm dish, mean $\pm$ SEM, $n = 9 \sim 10$, $p = .037$]) (Figure 3E).

### 3.5 Final protocol

In summary, the brief final protocol is presented in Figure 4A, and the detail of the protocol is described in the Supporting information S7. By omitting the ultracentrifugation step, we no longer need an ultracentrifuge machine and have been able to reduce the cost of ultracentrifugation. (tubes and iodixanol [OpitPrep: #1114542 Abbott Diagnostics Technologies AS]). We employed a stabilized rAAV capsid and produce rAAV in an optimized environment. Then, by purifying rAAV from the culture medium alone by two times PEG precipitation and chloroform extraction, we were able to produce rAAV with a degree of purification comparable to that of the conventional purification method by ultracentrifuge. The final yield of rAAV particles was much higher than that of the conventional method without stabilizing mutations and without optimization of production (non-stabilized lung targeted rAAV purified by previous ultracentrifugation procedure: $2.17 \times 10^{11} \pm 9.61 \times 10^{10}$ vg, stabilized lung targeted rAAV purified with the twice PEG + chloroform procedure: $1.50 \times 10^{13} \pm 3.58 \times 10^{12}$ vg [per one 150mm dish, mean $\pm$ SEM, $n = 4$ and10 respectively, $p = .007$]).
Other serotype AAVs could be purified from medium with the twice PEG + chloroform procedure. (AAV2: $4.2 \times 10^{12} \pm 3.7 \times 10^{12}$ vg, AAV2 [stable modification]: $5.1 \times 10^{12} \pm 3.8 \times 10^{12}$, AAV8: $7.7 \times 10^{12} \pm 6.2 \times 10^{12}$ from one φ150 mm dish) (Table 1).

Animal studies of pulmonary vascular pulmonary hypertension often use rat models rather than mice. Rats require a higher amount of AAV than mice. We developed a rat lung-specific AAV and confirmed that the target gene is expressed specifically in the rat lung by a purification method using twice PEG + chloroform procedure (Figure 4B).

**TABLE 1** The rAAV yield compared by different purification methods and type of capsid

| Purification methods | Source of extraction | Capsid type | Vector genome converted to one φ150 mm dish (mean ± SEM) | Reference |
|----------------------|----------------------|-------------|----------------------------------------------------------|-----------|
| Ammonium sulfate precipitation → iodoxanol gradient ultracentrifugation | Cell | AAV2 | $4.7 \times 10^{10} \pm 1.5 \times 10^{10}$ | Hermens et al. [34] |
| PEG precipitation → chloroform extraction → aqueous two phases partitioning | Cell and Medium | AAV8 | $1.1 \times 10^{11} \pm 1.8 \times 10^{10}$ (mean ± SD) | Guo et al. [10] |
| AAVpro® Purification Kit Maxi/ Midi kit (Takara Bio, Japan) | Cell | All serotypes | $1.3 \times 10^{11} \sim 1.3 \times 10^{12}$ (maximum) | Reply from the company |
| Our methods | Medium | AAV2 | $4.2 \times 10^{12} \pm 3.7 \times 10^{12}$ | |
| Our methods | Medium | AAV2 (stable modification) | $5.1 \times 10^{12} \pm 3.8 \times 10^{12}$ | |
| Our methods | Medium | AAV8 | $7.7 \times 10^{12} \pm 6.2 \times 10^{12}$ | |
| Our methods | Medium | Lung specific capsid (stable modification) | $1.5 \times 10^{13} \pm 3.6 \times 10^{12}$ | |

**Figure 4** Final protocol. (A) The brief final protocol. (B) Representative figure of fluorescence degree detected by IVIS Imaging System. The rats were injected with lung-specific rAAV expressing mVenus through their tails ($1 \times 10^{13}$ vg) and detected 1 month later.

**4 | DISCUSSION**

Recombinant AAVs are now used for human gene therapies.30–32 For clinical use, rAAV is purified by ion-exchange chromatography.33 Although this method assures its purity, it is very costly. For small-scale production used in animal studies, the current popular methods employ purification with iodoxanol density gradient ultracentrifugation.34,35 The compound has been popular because of no or low toxicity. Our basal protocol developed by our colleague in Boston University, Prof. Bachschmid, also adopted iodoxanol gradient ultracentrifugation to produce...
high titer rAAV from both cells and medium for in vivo use. However, the ultracentrifugal process requires special machinery to perform, and the technique is cumbersome. In this study, we tried to omit the ultracentrifuge step. Guo et al. also reported a simple method to purify AAV without using ultracentrifugation. They reported that PEG precipitated AAV (AAV6-GFP, AAV8-GFP, AAV9-GFP) followed by chloroform extraction and aqueous two-phase partitioning was purer than CsCl gradient purification. Takara bio Inc. offers a kit for rAAV purification from cells without the ultracentrifugation step. This kit does not publish images of silver staining, but from our own experience with it, the isolated rAAV contained more impurities than the purification method that includes the ultracentrifugation process (data not shown). When we purified rAAV extracted from cells with citrate buffer using only one PEG precipitation and chloroform extraction, it appeared to contain a higher level of contaminating proteins as measured by silver stain. However, when it came to mVenus, the transduction efficiency and functionality appeared to be rather higher than the ultracentrifugation method. Although this mechanism needs to be investigated in the future, PEG can promote fusion of lipid membranes because it is both hydrophilic and hydrophobic. Therefore, it may promote fusion and penetration of lipid-coated viruses. The hepatitis B virus is known to promote infection of cell culture systems by PEG. The mechanism is reported to be that PEG enhances the binding of HBV to cells by strengthening its interaction with heparan sulfate proteoglycans, thereby increasing the efficiency of infection. A similar mechanism may be occurring in the AAV.

The ultracentrifugation process may not always be necessary; however, impurities are thought to cause inflammation in vivo and some target genes may be adversely affected by impurities. Kimura et al. indicated that the addition of an iodixanol density gradient ultracentrifugation step is still necessary to obtain sufficient purity and express the target gene. To begin with, AAV has a relatively low immune profile, induces only a limited inflammatory response, and is considered a suitable vector for clinical application. However, some human clinical trials have reported that AAV-induced immunity may affect gene expression. Furthermore, it has been reported that inflammation suppresses transgene expression not only in higher primates but also in mice. Immune responses are thought to be elicited against the AAV vector component, the transgene product, or both, but may also be elicited by the adulterants contained. Hence, impurities that may cause inflammation should be reduced as much as possible.

NFκB is one of the transcription factors that play a central role in immune responses, we used NFκB reporter gene assay to evaluate the degree of inflammation caused by purified rAAV. Purification of rAAV from cell culture medium originally had fewer impurities detected by silver staining than purification from cell extracts. The addition of the second PEG precipitation (twice PEG precipitation procedure) allowed the detection of distinct VPs in the silver staining, however, NFκB was activated in the NFκB reporter gene assay. The addition of a second chloroform extraction (twice PEG + chloroform procedure) further confirmed that NFκB was not activated to the same degree as the conventional purification method by ultracentrifugation. It is believed that host cell DNA remains during rAAV purification and causes inflammation in the organism to which it is administered. Therefore, as an alternative method, endonuclease (Benzonase) was added to the rAAVs purified with the twice PEG precipitation procedure and degrade the nucleic acids. However, no significant effect of degrading nucleic acids was observed. In silver staining, there was no significant difference in purity with or without the addition of a second chloroform extraction. The purification method that eliminated ultracentrifugation increased the transduction effect of the target gene. Elucidation of the mechanism and identification of impurities that may cause inflammation are future issues.

We employed currently known mutations to increase yield. Our scAAV vector has an SV40 origin sequence. In lentivirus production, this insert is known to increase the virus yield with a large-T antigen-expressing cell-line, such as HEK293T cells. We have not systematically tested this effect. However, SV40 large-T mediated AAV amplification method was reported previously. Taking this into account, we transfected the rAAV vector plasmid, Rep-Cap plasmid, and Helper plasmid in a 5:7:7 ratios. In addition to this mutation, we found that Y730F and T491V mutations increased the virus yield. This effect has not been reported previously, but yields also increased with the original AAV2 capsid. However, the increase in yield was more pronounced with the pulmonary vascular-directed capsid, and this effect may be unique to this capsid. Therefore, further studies are needed.

In our method, we also examined the cell culture conditions. Among them, maintaining the glucose concentration of the culture medium was effective in part. The method we based our method on, at Boston University, showed that low glucose DMEM (glucose: 5 mM) increased rAAV production compared to high glucose DMEM (glucose: 25 mM). They speculated that high glucose promotes acidification due to excessive glycolysis, which inhibits rAAV production. We confirmed that HEK293T cells consume a lot of glucose during rAAV production by measuring the glucose concentration of the culture medium. Glucose consumption was particularly high as the...
medium became more acidic, and we hypothesized that the cells’ glucose metabolic flow rate was increased because the pentose phosphate pathway is required to supply deoxyribose in order to make rAAV nucleosides. We thought that as long as we maintained the pH around 7.8, we could further increase the production of rAAV by adding glucose and maintaining the concentration. The pH was adjusted by adding NaHCO₃. Maintaining the glucose concentration in the medium significantly increased the yield of rAAV by cell extraction, and the total yield of cell extraction and cell culture medium tended to be higher, although it did not reach significance. We aim to elucidate the pathogenesis of pulmonary vascular pulmonary hypertension, a progressive disease. Rats, not mice, are often used as animal models for pulmonary hypertension. Although pulmonary hypertension is induced in mice under hypoxic conditions, it is reversible and differs from the pathophysiology in humans. Although a large amount of rAAV is required for rats, the purification method used in this study allows us to obtain a relatively large amount of virus, which can be used for research using rats.

The purification method could be selected on a case-by-case basis depending on the intended use of rAAV. For example, if inflammation is not a concern in in vitro experiments, a two-fold PEG precipitation procedure can be used, whereas if inflammatory effects are a concern in vivo, a higher purification from cell culture medium with the twice PEG + chloroform procedure is recommended. To increase yield as much as possible at the expense of purity loss, it is recommended to purify from both cells and cell culture medium by adding sugar during rAAV production.

It is known that loop IV of VP1-3 of the AAV2 capsid determines organ-directedness, and since mutations occur in the heparin-binding sequence that acts as an anchor on the cell membrane, more rAAV is expected to be present in the cell culture medium during production. This was also the case for the pulmonary vascular-specific capsid in this study. Further organ-specific rAAV will be developed in the future, and their kinetics in production will need to be verified, but it is thought that rAAVs will be present in large amounts in cell culture media based on the same mechanism. We believe that the purification of organ-specific rAAV can be achieved without the use of an ultracentrifuge by the method described in this study.

5 | CONCLUSIONS

With the improvements made in this study, we were able to obtain highly purified lung-targeted rAAV from a single 150 mm DISH in sufficient quantity for animal experiments in mice. This method saves on labor and cost.

AUTHOR CONTRIBUTIONS

Yasunaga Shiraishi and Yasuo Ido conceived and designed the experiments; Yasunaga Shiraishi performed the research and acquired the data; Yasunaga Shiraishi and Yasuo Ido analyzed and interpreted the data and performed data statistical analyses; Yasunaga Shiraishi, Yasuo Ido, and Takeshi Adachi contributed to funding acquisition. Yasunaga Shiraishi and Jose M. Cacicedo contributed to manuscript writing. All authors have reviewed and approved the final manuscript.

ACKNOWLEDGMENTS

We thank our animal institute members for their assistance with animal care and those of the Central Research Laboratory of the National Defense Medical College for pathological assistance. The current study was supported by the JSPS KAKENHI Grant Number JP18K08120 to Yasuo Ido and JP20K17204 to Yasunaga Shiraishi.

DISCLOSURES

All the authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Yasunaga Shiraishi, upon request.

ORCID

Yasunaga Shiraishi https://orcid.org/0000-0001-5901-4026

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**How to cite this article:** Shiraishi Y, Adachi T, Cacicedo JM, Ido Y. Development of a high-yield, high-quality purification process for adeno-associated virus vectors that can be used in vivo without ultracentrifugation: Application to a lung endothelial cell-targeted adeno-associated virus. *The FASEB Journal*. 2022;36:e22653. doi: 10.1096/fj.20220840RR