

TITLE OF THE INVENTION:

Use of Ion Concentrations to Increase the Packaging Efficiency of Recombinant Adeno-Associated Virus

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation of, and claims priority to, U.S. Patent Appln. Serial No. 16/511,612 (pending), which was filed on July 15, 2019, which application is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION:

[0002] The present invention is directed to methods for increasing the efficiencies with which recombinant adeno-associated virus (rAAV) are packaged, so as to increase their production titers. More specifically, the invention relates to a method for increasing the production titer of rAAV by transfected cells by increasing the ionic strength of the cell culture media through the administration of additional ions.

REFERENCE TO SEQUENCE LISTING

[0003] This application includes one or more Sequence Listings pursuant to 37 C.F.R. 1.821 et seq., which are disclosed in computer-readable media (file name: 2650-0002US_ST25.txt, created on July 15, 2019, and having a size of 38,334 bytes), which file is herein incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION:

I. Adeno-Associated Virus (AAV)

[0004] Adeno-Associated Virus (AAV) is a small, naturally-occurring, non-pathogenic virus belonging to the *Dependovirus* genus of the *Parvoviridae* (Balakrishnan, B. *et al.* (2014) “*Basic Biology of Adeno-Associated Virus (AAV) Vectors Used in Gene Therapy*,” Curr. Gene Ther. 14(2):86-100; Zinn, E. *et al.* (2014) “*Adeno-Associated Virus: Fit To Serve*,” Curr. Opin. Virol. 0:90-97). Despite not causing disease, AAV is known to be able to infect humans and other primates and is

prevalent in human populations (Johnson, F.B. *et al.* (1972) “*Immunological Reactivity of Antisera Prepared Against the Sodium Dodecyl Sulfate-Treated Structural Polypeptides of Adenovirus-Associated Virus*,” J. Virol. 9(6):1017-1026). AAV infect a broad range of different cell types (*e.g.*, cells of the central nervous system, heart, kidney, liver, lung, pancreas, retinal pigment epithelium or photoreceptor cells, or skeletal muscle cells). Twelve serotypes of the virus (*e.g.*, AAV2, AAV5, AAV6, *etc.*), exhibiting different tissue infection capabilities (“**tropisms**”), have been identified (Colella, P. *et al.* (2018) “*Emerging Issues in AAV-Mediated In Vivo Gene Therapy*,” Molec. Ther. Meth. Clin. Develop. 8:87-104; Hocquemiller, M. *et al.* (2016) “*Adeno-Associated Virus-Based Gene Therapy for CNS Diseases*,” Hum. Gene Ther. 27(7):478-496; Lisowski, L. *et al.* (2015) “*Adeno-Associated Virus Serotypes For Gene Therapeutics*,” 24:59-67).

[0005] AAV is a single-stranded DNA virus that is composed of approximately 4,700 nucleotides. The viral genome may be described as having a 5' half and a 3' half which together comprise the genes that encode the virus' proteins (**Figure 1**). The 5' half of the AAV genome comprises the AAV *rep* gene, which, through the use of multiple reading frames, staggered initiating promoters (**p5**, **p19** and **p40**) and alternate splicing, encodes four non-structural *Rep* proteins (**Rep40**, **Rep52**, **Rep68** and **Rep78**) that are required for viral transcription control and replication and for the packaging of viral genomes into the viral capsule (Lackner, D.F. *et al.* (2002) “*Studies of the Mechanism of Transactivation of the Adeno-Associated Virus p19 Promoter by Rep Protein*,” J. Virol. 76(16):8225-8235). The 3' half the AAV genome comprises the AAV capsid gene (*cap*), which encodes three capsid proteins (**VP**): **VP1**, **VP2** and **VP3**. The three capsid proteins are translated from a single mRNA transcript that is controlled by a single promoter (p40 in case of AAV2). The 3' half of the AAV genome also comprises the **AAP** gene, which encodes the AAV assembly-activating protein (**AAP**). Sixty VP monomers (comprising approximately 5 copies of VP1, 5 copies of VP2, and 50 copies of VP3) self-assemble around the AAV genome to form the icosahedral protein shell (**capsid**) of the mature viral particle (Büning, H. *et al.* (2019) “*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*,” Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Van Vliet K.M. *et al.* (2008) *The Role of the Adeno-Associated Virus Capsid in Gene Transfer*. In: DRUG DELIVERY SYSTEMS, Jain, K.K. (eds.), Meth.

Molec. Biol. 437:51-91). The AAV **AAP** protein is believed to be required for stabilizing and transporting newly produced VP proteins from the cytoplasm into the cell nucleus. The 3' half of the AAV genome also comprises the AAV **X** gene, which is believed to encode a protein that supports genome replication (Colella, P. *et al.* (2018) "Emerging Issues in AAV-Mediated In Vivo Gene Therapy," Molec. Ther. Meth. Clin. Develop. 8:87-104; Büning, H. *et al.* (2019) "Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors," Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Cao, M. *et al.* (2014) "The X Gene Of Adeno-Associated Virus 2 (AAV2) Is Involved In Viral DNA Replication," PLoS ONE 9, e104596:1-10).

[0006] The above-described AAV gene-coding sequences are flanked by two AAV-specific palindromic inverted terminal repeated sequences (**ITR**) of 145 nucleotides (Balakrishnan, B. *et al.* (2014) "Basic Biology of Adeno-Associated Virus (AAV) Vectors Used in Gene Therapy," Curr. Gene Ther. 14(2):86-100; Colella, P. *et al.* (2018) "Emerging Issues in AAV-Mediated In Vivo Gene Therapy," Molec. Ther. Meth. Clin. Develop. 8:87-104).

[0007] AAV is an inherently defective virus, lacking the capacity to perform at least two critical functions: the ability to initiate the synthesis of viral-specific products and the ability to assemble such products to form the icosahedral protein shell (**capsid**) of the mature infectious viral particle. It thus requires a co-infecting "helper" virus, such as adenovirus (**Ad**), herpes simplex virus (**HSV**), cytomegalovirus (**CMV**), vaccinia virus or human papillomavirus to provide the viral-associated (**VA**) RNA that is not encoded by the genes of the AAV genome. Such VA RNA is not translated, but plays a role in regulating the translation of other viral genes. Similarly, the AAV genome does not include genes that encode the viral proteins E1a, E1b, E2a, and E4 of Ad; thus, these proteins must also be provided by a co-infecting "helper" virus. The E1a protein greatly stimulate viral gene transcription during the productive infection. The E1b protein block apoptosis in adenovirus-infected cells, and thus allow productive infection to proceed. The E2a protein plays a role in the elongation phase of viral strand displacement replication by unwinding the template and enhancing the initiation of transcription. The E4 protein has been shown to affect transgene persistence, vector toxicity and immunogenicity (see, Grieger, J.C. *et al.* (2012) "Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications," Meth. Enzymol. 507:229-

254; Dyson, N. *et al.* (1992) “*Adenovirus E1A Targets Key Regulators Of Cell Proliferation*,” *Canc. Surv.* 12:161-195; Jones N.C. (1990) “*Transformation By The Human Adenoviruses*,” *Semin. Cancer Biol.* 1(6):425-435; Ben-Israel, H. *et al.* (2002) “*Adenovirus and Cell Cycle Control*,” *Front. Biosci.* 7:d1369-d1395; Hoebe, R.C. *et al.* (2013) “*Adenovirus DNA Replication*,” *Cold Spring Harb. Perspect. Biol.* 5:a013003 (pages 1-11); Berk, A.J. (2013) “*Adenoviridae: The Viruses And Their Replication*, In: *FIELDS VIROLOGY*, 6th Edition (Knipe, D.M. *et al.*, Eds.), Vol. 2., Lippincott Williams & Wilkins, Philadelphia, pages 1704-1731; Weitzman, M.D. (2005) “*Functions Of The Adenovirus E4 Proteins And Their Impact On Viral Vectors*,” *Front. Biosci.* 10:1106-1117).

[0008] AAV viruses infect both dividing and non-dividing cells, and persist as circular episomal molecules or can be integrated into the DNA of a host cell at specific chromosomal loci (**Adeno-Associated Virus Integration Sites** or **AAVS**) (Duan, D. (2016) “*Systemic Delivery Of Adeno-Associated Viral Vectors*,” *Curr. Opin. Virol.* 21:16-25; Grieger, J.C. *et al.* (2012) “*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*,” *Meth. Enzymol.* 507:229-254). AAV remains latent in such infected cells unless a helper virus is present to provide the functions needed for AAV replication and maturation.

II. rAAV and Their Use in Gene Therapy

[0009] In light of AAV's properties, recombinantly-modified versions of AAV (**rAAV**) have found substantial utility as vectors for gene therapy (see, Naso, M.F. *et al.* (2017) “*Adeno-Associated Virus (AAV) as a Vector for Gene Therapy*,” *BioDrugs* 31:317-334; Berns, K. I. *et al.* (2017) “*AAV: An Overview of Unanswered Questions*,” *Human Gene Ther.* 28(4):308-313; Berry, G.E. *et al.* (2016) “*Cellular Transduction Mechanisms Of Adeno-Associated Viral Vectors*,” *Curr. Opin. Virol.* 21:54-60; Blessing, D. *et al.* (2016) “*Adeno-Associated Virus And Lentivirus Vectors: A Refined Toolkit For The Central Nervous System*,” 21:61-66; Santiago-Ortiz, J.L. (2016) “*Adeno-Associated Virus (AAV) Vectors in Cancer Gene Therapy*,” *J. Control Release* 240:287-301; Salganik, M. *et al.* (2015) “*Adeno-Associated Virus As A Mammalian DNA Vector*,” *Microbiol. Spectr.* 3(4):1-32; Hocquemiller, M. *et al.* (2016) “*Adeno-Associated Virus-Based Gene Therapy for CNS Diseases*,” *Hum. Gene Ther.* 27(7):478-

496; Lykken, E.A. *et al.* (2018) “Recent Progress And Considerations For AAV Gene Therapies Targeting The Central Nervous System,” *J. Neurodevel. Dis.* 10:16:1-10; Büning, H. *et al.* (2019) “Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors,” *Mol. Ther. Meth. Clin. Devel.* 12:P248-P265; During, M.J. *et al.* (1998) “In Vivo Expression Of Therapeutic Human Genes For Dopamine Production In The Caudates Of MPTP-Treated Monkeys Using An AAV Vector,” *Gene Ther.* 5:820-827; Grieger, J.C. *et al.* (2012) “Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications,” *Meth. Enzymol.* 507:229-254; Kotterman, M.A. *et al.* (2014) “Engineering Adeno-Associated Viruses For Clinical Gene Therapy,” *Nat. Rev. Genet.* 15(7):445-451; Kwon, I. *et al.* (2007) “Designer Gene Delivery Vectors: Molecular Engineering and Evolution of Adeno-Associated Viral Vectors for Enhanced Gene Transfer,” *Pharm. Res.* 25(3):489-499).

[0010] rAAV are typically produced using circular plasmids (“**rAAV plasmid vector**”). The AAV *rep* and *cap* genes are typically deleted from such constructs and replaced with a promoter, a β -globin intron, a cloning site into which a therapeutic gene of choice (**transgene**) has been inserted, and a poly-adenylation (“**polyA**”) site. The inverted terminal repeated sequences (ITR) of the rAAV are, however, retained, so that the **transgene expression cassette** of the rAAV plasmid vector is flanked by AAV ITR sequences (Colella, P. *et al.* (2018) “Emerging Issues in AAV-Mediated In Vivo Gene Therapy,” *Molec. Ther. Meth. Clin. Develop.* 8:87-104; Büning, H. *et al.* (2019) “Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors,” *Mol. Ther. Meth. Clin. Devel.* 12:P248-P265). Thus, in the 5' to 3' direction, the rAAV comprises a 5' ITR, the transgene expression cassette of the rAAV, and a 3' ITR.

[0011] rAAV have been used to deliver a transgene to patients suffering from any of a multitude of genetic diseases (*e.g.*, hereditary lipoprotein lipase deficiency (**LPLD**), Leber’s congenital amaurosis (**LCA**), aromatic L-amino acid decarboxylase deficiency (**AADC**), choroideremia and hemophilia), and have utility in new clinical modalities, such as in interfering RNA (**RNAi**) therapy and gene-modifying strategies such as **Crispr/Cas9** (US Patents No. 8,697,359, 10,000,772, 10,113,167, 10,227,611; Lino, C.A. *et al.* (2018) “Delivering CRISPR: A Review Of The Challenges And Approaches,” *Drug Deliv.* 25(1):1234-1237; Ferreira, V. *et al.* (2014) “Immune Responses To AAV-Vectors, The Glybera Example From Bench To Bedside” *Front. Immunol.* 5(82):1-15),

Büning, H. *et al.* (2019) “*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*,” Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Rastall, D.P.W. (2017) “*Current and Future Treatments for Lysosomal Storage Disorders*,” Curr. Treat Options Neurol. 19(12):45; Kay, M. *et al.* (2017) “*Future Of rAAV Gene Therapy: Platform For RNAi, Gene Editing And Beyond*,” Human Gene Ther. 28:361-372; Berns, K. I. *et al.* (2017) “*AAV: An Overview of Unanswered Questions*,” Human Gene Ther. 28(4):308-313). More than 150 clinical trials involving rAAV have been instituted (Büning, H. *et al.* (2019) “*Capsid Modifications for Targeting and Improving the Efficacy of AAV Vectors*,” Mol. Ther. Meth. Clin. Devel. 12:P248-P265; Clément, N. *et al.* (2016) “*Manufacturing Of Recombinant Adeno-Associated Viral Vectors For Clinical Trials*,” Meth. Clin. Develop. 3:16002:1-7). The most commonly used AAV serotype for such recombinantly-modified AAV is **AAV2**, which is capable of infecting cells of the central nervous system, kidney, retinal pigment epithelium and photoreceptor cells. Another AAV serotype is **AAV9**, which infects muscle cells, also has been widely used (Duan, D. (2016) “*Systemic Delivery Of Adeno-Associated Viral Vectors*,” Curr. Opin. Virol. 21:16-25). AAV serotypes are described in US Patents No. 10,301,650; 10,266,846; 10,265,417; 10,214,785; 10,214,566; 10,202,657; 10,046,016; 9,884,071; 9,856,539; 9,737,618; 9,677,089; 9,458,517; 9,457,103; 9,441,244; 9,193,956; 8,846,389; 8,507,267; 7,906,111; 7,479,554; 7,186,552; 7,105,345; 6,984,517; 6,962,815; and 6,733,757.

III. Methods of rAAV Production

[0012] rAAV containing a desired transgene expression cassette are typically produced by human cells (such as **HEK293**) grown in either adhesion or suspension. Since, as described above, rAAV are defective viruses, additional functions must be provided in order to replicate and package rAAV.

[0013] Typically, rAAV are produced by transiently transfecting cells with an rAAV plasmid vector and a second plasmid vector that comprises an AAV helper function-providing polynucleotide that provides the **Rep52** and **Rep78** genes that are required for vector transcription control and replication, and for the packaging of viral genomes into the viral capsule (**Rep40** and **Rep68** are not required for rAAV production) and the *cap* genes that were excised from the AAV in order to produce the rAAV. The

second plasmid vector may additionally comprise a non-AAV helper function-providing polynucleotide that encodes the viral transcription and translation factors (E1a, E1b, E2a, VA and E4) required for AAV proliferation, so as to comprise, in concert with the rAAV, a **double plasmid transfection system** (Grimm, D. *et al.* (1998) “*Novel Tools For Production And Purification Of Recombinant Adeno-Associated Virus Vectors*,” Hum. Gene Ther. 9:2745-2760; Penaud-Budloo, M. *et al.* (2018) “*Pharmacology of Recombinant Adeno-associated Virus Production*,” Molec. Ther. Meth. Clin. Develop. 8:166-180).

[0014] However, it has become increasingly common to clone the AAV helper function-providing polynucleotide (which provides the required *rep* and *cap* genes) into an “**AAV helper plasmid**,” and to clone the non-AAV helper function-providing polynucleotide (which provides the genes that encode the viral transcription and translation factors) on a different plasmid (*i.e.*, an “**Ad helper plasmid**”), so that such plasmids, in concert with an rAAV plasmid vector, comprise a **triple plasmid transfection system (Figure 2)**. Use of the triple plasmid transfection system has the advantage of permitting one to easily switch one *cap* gene for another, thereby facilitating changes in the rAAV’s serotype. The use of helper plasmids, rather than helper viruses, permits rAAV to be produced without additionally producing particles of the helper virus (François, A. *et al.* (2018) “*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*,” Molec. Ther. Meth. Clin. Develop. 10:223-236; Matsushita, T. *et al.* (1998) “*Adeno-Associated Virus Vectors Can Be Efficiently Produced Without Helper Virus*,” Gene Ther. 5:938-945).

[0015] The transient transfection of plasmid DNAs comprising an rAAV plasmid vector, a plasmid vector providing AAV helper functions *rep* and *cap* genes, and a plasmid vector providing non-AAV helper functions into HEK293 cells by calcium phosphate coprecipitation has become the standard method to produce rAAV in the research laboratory (Grimm, D. *et al.* (1998) “*Novel Tools For Production And Purification Of Recombinant Adeno-Associated Virus Vectors*,” Hum. Gene Ther. 9:2745-2760). However, the use of such a calcium phosphate-mediated transfection process with suspension-cultured transfected mammalian cells requires media exchanges, and is thus not considered ideal for the large-scale rAAV production that is

required in order to produce therapeutic doses of rAAV (Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” *Hum. Gene Ther.* 21:1259-1271). For this reason, polyethylenimine (**PEI**), has been used as a transfection reagent and has been found to provide yields of virus that are similar to those obtained using calcium phosphate-mediated transfection (Durocher, Y. *et al.* (2007) “*Scalable Serum-Free Production Of Recombinant Adeno-Associated Virus Type 2 By Transfection Of 293 Suspension Cells*,” *J. Virol. Meth.* 144:32-40).

[0016] rAAV may alternatively be produced in insect cells (*e.g.*, sf9 cells) using baculoviral vectors (see. *e.g.*, US Patents No.: 9,879,282; 9,879,279; 8,945,918; 8,163,543; 7,271,002 and 6,723,551), or in HSV-infected baby hamster kidney (**BHK**) cells (*e.g.*, BHK21) (François, A. *et al.* (2018) “*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*,” *Molec. Ther. Meth. Clin. Develop.* 10:223-236). Methods of rAAV production are reviewed in Grieger, J.C. *et al.* (2012) “*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*,” *Meth. Enzymol.* 507:229-254, and in Penaud-Budloo, M. *et al.* (2018) “*Pharmacology of Recombinant Adeno-associated Virus Production*,” *Molec. Ther. Meth. Clin. Develop.* 8:166-180.

IV. Methods of rAAV Purification and Recovery

[0017] After production, rAAV are typically collected and purified by one or more overnight CsCl gradient centrifugations (Zolotukhin, S. *et al.* (1999) “*Recombinant Adeno-Associated Virus Purification Using Novel Methods Improves Infectious Titer And Yield*,” *Gene Ther.* 6:973-985), followed by desalting to form a purified rAAV production stock. Titers of 10^{12} - 10^{13} infectious rAAV capsids/mL are obtainable.

[0018] Because rAAV infection does not cause a cytopathic effect, plaque assays cannot be used to determine the infectious titer of an rAAV preparation. Infectious titer is thus typically measured as the median tissue culture infective dose (**TCID₅₀**). In this method, a HeLa-derived AAV2 *rep*- and *cap*-expressing cell line is grown in a 96-well plate and infected with replicate 10-fold serial dilutions of the rAAV preparation, in the presence of adenovirus of serotype 5. After infection, vector genome replication is determined by quantitative PCR (qPCR) (Zen, Z. *et al.* (2004) “*Infectious Titer Assay*

For Adeno-Associated Virus Vectors With Sensitivity Sufficient To Detect Single Infectious Events,” Hum. Gene Ther. 15:709-715). Alternatively, the infectious titer of an rAAV preparation can be measured using the infectious center assay (ICA). This assay uses HeLa *rep-cap* cells and Ad, but, after incubation, involves transferring the cells to a membrane. A labeled probe that is complementary to a portion of the employed transgene is used to detect infectious centers (representing individual infected cells) via hybridization. Although more widely used, the TCID₅₀ assay has been reported to lead to a higher background than the ICA and to overestimate vector infectivity relative to the ICA (François, A. *et al.* (2018) “*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls,*” Molec. Ther. Meth. Clin. Develop. 10:223-236). Methods of producing and purifying rAAV are described *inter alia* in US Patents No. 10,294,452; 10,161,011; 10,017,746; 9,598,703; 7,625,570; 7,439,065; 7,419,817; 7,208,315; 6,995,006; 6,989,264; 6,846,665 and 6,841,357.

[0019] As discussed above, multiple rounds of overnight cesium chloride gradient centrifugation are typically employed in order to produce rAAV in the research laboratory. However, prolonged exposure to CsCl has been reported to compromise the potency of rAAV plasmid vectors (Zolotukhin, S. *et al.* (1999) “*Recombinant Adeno-Associated Virus Purification Using Novel Methods Improves Infectious Titer And Yield,*” Gene Ther. 6:973-985). Additionally, such gradients have a limited loading capacity for cell lysate, and thus limit the amount of rAAV that may be purified. Although an isotonic alternative gradient medium, iodixanol, has been used to purify rAAV plasmid vectors, iodixanol shares the same loading capacity drawback as CsCl for rAAV production.

[0020] In order to overcome such gradient-specific constraints, researchers have developed ion-exchange chromatographic methods, affinity purification methods, and antibody-affinity based methods of rAAV purification (Auricchio, A. *et al.* (2001) “*Isolation Of Highly Infectious And Pure Adeno-Associated Virus Type 2 Vectors With A Single-Step Gravity-Flow Column,*” Hum. Gene Ther. 12:71-76; Brument, N. *et al.* (2002) “*A Versatile And Scalable Two-Step Ion-Exchange Chromatography Process For The Purification Of Recombinant Adeno-Associated Virus Serotypes-2 And -5,*” Mol. Ther. 6:678-686; Zolotukhin, S. *et al.* (2002) “*Production And Purification Of*

Serotype 1, 2, And 5 Recombinant Adeno-Associated Viral Vectors,” Methods 28:158-167; Davidoff, A.M. *et al.* (2004) “*Purification Of Recombinant Adeno-Associated Virus Type 8 Vectors By Ion Exchange Chromatography Generates Clinical Grade Vector Stock*,” J. Virol. Methods 121:209-215; Smith, R.H. *et al.* (2009) “*A Simplified Baculovirus-AAV Expression Vector System Coupled With One-Step Affinity Purification Yields High-Titer rAAV Stocks From Insect Cells*,” Mol. Ther. 17:1888-1896; Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” Hum. Gene Ther. 21:1259-1271). Unfortunately, however, such chromatography-based purification methods are generally unable to separate vector-related impurities, such as empty capsids from fully functional vector particles. Thus, despite its drawbacks, CsCl gradient centrifugation remains the best characterized method for removing empty particles from rAAV vector preparations.

[0021] It has been observed that rAAV of various serotypes is released to the supernatant in both calcium phosphate- and PEI-transfected cultures (Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” Hum. Gene Ther. 21:1259-1271; US Patents No. 6,566,118 and 6,989,264, and US Patent Publication US 2005/0266567). US Patents No. 6,566,118 and 6,989,264, and US Patent Publication US 2005/0266567 disclose that high titers of recombinant AAV vectors are released into the supernatant of cell suspensions if the culture medium had been formulated to initially comprise an osmolarity of between about 100 mOsM to about 650 mOsM using NaCl (*i.e.*, 50-325 mM NaCl) and other, but unspecified, salts, mannitol or glucose, or by manipulating the conductivity of the culture medium to be at least about 5mS, using an ionic solute such as Na⁺ or K⁺. An initial osmolarity of 300 mOsM (150 mM) NaCl was found to be optimal. Adamson-Small, L. *et al.* (2017) similarly demonstrated that 60-90 mM sodium chloride in the production medium resulted in a significant increase in rAAV9 transducing units and capsid proteins under infection conditions in which increased sodium chloride was present 4-6 hr post-transduction (WO 2017/112948; Adamson-Small, L. *et al.* (2017) “*Sodium Chloride Enhances Recombinant Adeno-Associated Virus Production in a Serum-Free Suspension Manufacturing Platform Using the Herpes Simplex Virus System*,” Hum. Gene Ther. Meth. 28(1):1-14).

[0022] Lock, M. *et al.* (2010) disclose a PEI transfection-based- and supernatant harvest-based-technique for facilitating the recovery of rAAV particles (Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” Hum. Gene Ther. 21:1259-1271). The method is based on the observation that rAAV belonging to AAV serotypes other than AAV2 were released primarily into the culture medium of calcium phosphate-transfected cells and were not retained in the cell lysate. As such, Lock, M. *et al.* (2010) discloses that for such rAAV serotypes, the production culture medium represents a relatively pure source of rAAV plasmid vector that possesses a lower level of cellular contaminants and that these factors improve the loading capacity and resolution of purification gradients. In the disclosed method, rAAV, including rAAV belonging to AAV2 serotype, were transfected into HEK293 cells using calcium phosphate. Seventy-two hours (or 120 hours) post-transfection, serum-free media was added and the incubation was continued for an additional 28 hours. Benzonase®, a genetically engineered endonuclease that degrades all forms for DNA and RNA, was then added to the culture supernatant. After 2 hours, NaCl was added to 500mM and the incubation was resumed for an additional 2 hr before harvesting the culture medium. The clarified medium was then concentrated 125-fold by tangential flow filtration (TFF), and the rAAV was purified using iodixanol step gradients. The method could be employed with AAV of serotypes AAV1, AAV6, AAV7, AAV8, and AAV9. Use of the high-salt incubation of Lock *et al.* (2010) is disclosed to lead to a further 20% release of rAAV6 and rAAV9 plasmid vectors to the culture medium (relative to the methods of US Patents No. 6,566,118 and 6,989,264 and US Patent Publication US 2005/0266567), but was seen to have elicited little change with respect to other serotypes. Although the average overall yields of rAAV8 and rAAV9 were high (2.2×10^{14} genome copies), yields of other rAAV serotypes were significantly lower (*e.g.*, 6.7×10^{13} genome copies for rAAV6). Although the estimated purity of the produced rAAV exceeded 90%, between 35% and 50% of the produced rAAV8 and rAAV9 were lost in the processing steps, and 80-85% of the produced rAAV6 were lost in processing, and rAAV2 were mostly retained within the cells and not released into the culture medium.

[0023] Provision of salt has also been used to permeabilize cells in order to more easily measure transgene-associated gene expression. Thus, for example, During, M.J.

et al. (1998) used a “release buffer” containing 135 mM NaCl, 3 mM KCl, 1.2 mM CaCl₂, 1.0 mM MgCl₂, 10 mM glucose, 200 mM ascorbate and 2 mM sodium mono- and dibasic phosphate buffered to pH 7.4 to promote the release of dopamine from HEK 293 cells that had been transfected with an rAAV expressing human tyrosine hydroxylase (TH) and aromatic amino decarboxylase (AADC) (During, M.J. *et al.* (1998) “*In Vivo Expression Of Therapeutic Human Genes For Dopamine Production In The Caudates Of MPTP-Treated Monkeys Using An AAV Vector*,” *Gene Ther.* 5:820-827).

[0024] However, despite all such prior successes, a need remains to develop methods capable of addressing problems that presently limit the applicability of rAAV to gene therapy (Grieger, J.C. *et al.* (2012) “*Adeno-Associated Virus Vectorology, Manufacturing, and Clinical Applications*,” *Meth. Enzymol.* 507:229-254; Kotterman, M.A. *et al.* (2014) “*Engineering Adeno-Associated Viruses For Clinical Gene Therapy*,” *Nat. Rev. Genet.* 15(7):445-451; Kwon, I. *et al.* (2007) “*Designer Gene Delivery Vectors: Molecular Engineering and Evolution of Adeno-Associated Viral Vectors for Enhanced Gene Transfer*,” *Pharm. Res.* 25(3):489-499; Naso, M.F. *et al.* (2017) “*Adeno-Associated Virus (AAV) as a Vector for Gene Therapy*,” *BioDrugs* 31:317-334). Such problems include:

(1) The Limited Tissue-Specific Tropism of rAAV: One such problem reflects the limited tissue-specific tropisms of AAV and rAAV. The use of multiple helper plasmids, encoding capsid proteins of differing serotypes (*i.e.*, “mosaic” capsids) has been exploited as a way to increase the range of tissue types that can be infected by rAAV (Hauck, B. *et al.* (2003) “*Generation And Characterization Of Chimeric Recombinant AAV Vectors*,” *Mol. Ther.* 7:419-425; Rabinowitz, J.E. *et al.* (2004) “*Crossdressing The Virion: The Transcapsidation Of Adeno-Associated Virus Serotypes Functionally Defines Subgroups*,” *J. Virol.* 78:4421-4432; Lisowski, L. *et al.* (2015) “*Adeno-Associated Virus Serotypes For Gene Therapeutics*,” 24:59-67).

(2) The Prevalence of anti-rAAV Immune Responses: A second such problem reflects the fact that 30-80% of humans have been naturally exposed to AAV infection (mainly AAV2) and 20-67% of humans harbor titers of neutralizing anti-AAV capsid antibodies in their blood and other bodily fluids (Liu, Q. *et al.*

(2014) “*Neutralizing Antibodies Against AAV2, AAV5 And AAV8 In Healthy And HIV-1-Infected Subjects In China: Implications For Gene Therapy Using AAV Vectors*,” *Gene Ther.* 21:732-738; Vandamme, C. *et al.* (2017) “*Unraveling the Complex Story of Immune Responses to AAV Vectors Trial After Trial*,” *Hum. Gene Ther.* 28(11):1061-1074). The presence of these antibodies attenuates the effectiveness of rAAV therapy by preventing transgene expression. Synthetic polymer conjugates (e.g., polyethylene glycol (PEG)) have been used as a means for shielding rAAV from neutralizing antibodies (Le, H.T. *et al.* (2005) “*Utility Of Pegylated Recombinant Adeno-Associated Viruses For Gene Transfer*,” *J. Control. Release* 108:161-177; Lee, G.K. *et al.* (2005) “*PEG Conjugation Moderately Protects Adeno-Associated Viral Vectors Against Antibody Neutralization*,” *Biotechnol. Bioeng.* 92:24-34). The use of rAAV having alternative serotypes or mutated non-immunogenic capsids has also been pursued (Smith, J.K. *et al.* (2018) “*Creating An Arsenal Of Adeno-Associated Virus (AAV) Gene Delivery Stealth Vehicles*,” *PLoS Pathog.* 14(5):1-6).

(3) The Limitation of rAAV Packaging Capacity: The packaging efficiency of rAAV has been found to significantly decrease beyond 5 kb, with larger genomes being encapsidated with 5' truncations (Wu, Z. *et al.* (2010) “*Effect Of Genome Size On AAV Vector Packaging*,” *Molec. Ther.* 18:80-86; Ghosh, A. *et al.* (2007) “*Expanding Adeno-Associated Viral Vector Capacity: A Tale Of Two Vectors*,” *Biotechnol. Genet. Eng. Rev.* 24:165-177; McClements, M.E. *et al.* (2017) “*Adeno-associated Virus (AAV) Dual Vector Strategies for Gene Therapy Encoding Large Transgenes*,” *Yale J. Biol. Med.* 90:611-623).

(4) The Limitations of Large-Scale Manufacturing Technologies: The ability to manufacture rAAV in amounts sufficient for use in large-scale therapy has been a barrier to the successful application of the technology, with process yields ranging from below 5% to below 30% (Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” *Hum. Gene Ther.* 21:1259-1271).

These problems are, in some cases, inter-related. For example, the presence of empty particles in the final product exposes the recipient of the vector to a large source of AAV antigen that can lead to unwanted immune responses and toxicity. Thus,

improved methods for increasing packaging efficiency and obtaining high production titers are of great importance.

[0025] The present invention is directed to improved methods for increasing the efficiency of rAAV packaging by altering the concentration of ions in a culturing medium during the production of rAAV.

SUMMARY OF THE INVENTION:

[0026] The present invention is directed to methods for increasing the efficiency with which recombinant adeno-associated virus (rAAV) are packaged, so as to increase their production titers. More specifically, the invention relates to a method for increasing the production titer of rAAV by transfected cells by increasing the ionic strength of the cell culture media through the administration of additional ions.

[0027] In detail, the invention provides a method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), wherein the method comprises the steps:

- (A) culturing cells that have been transfected with the rAAV in an initial culture medium for an initial period under conditions sufficient to permit the production of rAAV, wherein the cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing polynucleotide;
- (B) changing the ionic strength of the culture medium after the initial period by adding one or more ions other than Na⁺ to the culture medium; and
- (C) continuing the culturing of the cells to thereby produce a production titer of with the rAAV that is greater than a titer obtained in the absence of step (B).

[0028] The invention additionally provides the embodiment of such method wherein each of the added ion(s) is provided in an amount sufficient to increase the concentration of such ion in the initial culture medium by from about 10 mM to about 80 mM.

[0029] The invention additionally provides the embodiment of such methods wherein the production titer is at least 50% greater than the titer obtained from a similarly conducted cell culturing in the absence of the step (B).

[0030] The invention additionally provides the embodiment of such methods wherein the rAAV comprises a transgene cassette that encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.

[0031] The invention additionally provides the embodiment of such methods wherein the rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of such serotypes.

[0032] The invention additionally provides the embodiment of such methods wherein the rAAV belongs to the rAAV2, rAAV5, or rAAV9 serotype, or to a hybrid of the serotypes.

[0033] The invention additionally provides the embodiment of such methods wherein the added ions comprise one or more of K^+ , Ca^{++} , or Mg^{++} .

[0034] The invention additionally provides the embodiment of such methods wherein the added ions comprise one or more of CO_3^- , HCO_3^- , HPO_4^- , PO_4^- , SCN^- , SO_4^- , HSO_4^- , and Cl^- .

[0035] The invention additionally provides the embodiment of such methods wherein the added ions comprise one or more of acetate, aspartate, bipthalate, bitartrate, butoxyethoxy acetate, caprylate, citrate, dehydroacetate, diacetate, dihydroxy glycinate, d-saccharate, gluconate, glutamate, glycinate, glycosulfate, hydroxymethane sulfonate, lactate, methionate, oxalate, phenate, phenosulfonate, propionate, propionate, saccharin, salicylate, sarcosinate, sorbate, thioglycolate, and toluene sulfonate.

[0036] The invention additionally provides the embodiment of such methods wherein the added ions comprise K^+ and CO_3^- .

[0037] The invention additionally provides the embodiment of such methods wherein the cells are human embryonic kidney cells, baby hamster kidney cells or sf9 insect cells.

[0038] The invention additionally provides the embodiment of such methods wherein the cells are HEK293 human embryonic kidney cells.

[0039] The invention additionally provides the embodiment of such methods wherein the cells are BHK21 baby hamster kidney cells.

[0040] The invention additionally provides the embodiment of such methods wherein the initial culture medium is Dulbecco's Modified Eagle's Medium or Dulbecco's Modified Eagle's Medium supplemented with serum.

[0041] The invention additionally provides a pharmaceutical composition that comprises:

- (A) a preparation of recombinantly-modified adeno-associated virus (rAAV) produced by any of the above-described methods, wherein the rAAV comprises a transgene cassette that encodes a protein, or a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, and wherein the pharmaceutical composition contains an effective amount of the rAAV preparation; and
- (B) a pharmaceutically acceptable carrier.

[0042] The invention additionally provides a preparation of recombinantly-modified adeno-associated virus (rAAV) produced by any of the above-described methods, wherein the rAAV comprises a transgene cassette that encodes a protein, or a transcribed nucleic acid, or the above-described pharmaceutical composition for use in the treatment of a genetic or heritable disease or condition.

BRIEF DESCRIPTION OF THE DRAWINGS:

[0043] **Figure 1** provides a schematic genetic map of the wild-type (**Wt**) AAV genome.

[0044] **Figure 2** provides a schematic of the structural domain of the wild-type AAV2 genome **(1)**, a recombinant AAV (rAAV) **(2)**, complementing “AAV helper plasmid” **(3)** and an adenovirus helper plasmid (“Ad helper plasmid”) **(4)**. The wild-type (Wt) AAV2 **(1)** is composed of AAV-specific palindromic inverted terminal repeated sequences (ITR), a 5' half containing genes that encode the **Rep** proteins and a 3' half containing genes that encode the **Cap** proteins. The rAAV **(2)** is formed by replacing the **Rep**- and **Cap**-encoding genes of the wild-type (Wt) AAV2 **(1)** with a transgene cassette that comprises a promoter (**Pro**), the exogenous transgene of interest, and a polyadenylation site (**pA**). In order to produce the rAAV **(2)**, a complementing “AAV helper” plasmid vector **(3)** and an adenovirus helper plasmid vector (Ad helper plasmid) **(4)** are provided. The complementing AAV helper plasmid **(3)** provides **Rep** and **Cap** proteins. The Ad helper plasmid **(4)** provides adenovirus proteins E1a, E1b, E2a, VA and E4.

[0045] **Figure 3** shows a map of the AAV helper plasmid vector **pAAV-RC2**.

[0046] **Figure 4** shows a map of the non-AAV helper plasmid vector **pHelper-Kan**.

[0047] **Figure 5** shows a map of the rAAV plasmid vector **pAV-CMV-EGFP**.

[0048] **Figure 6** shows a map of the rAAV plasmid vector **pAV-TBG-EGFP**.

[0049] **Figures 7A-7C** show the effect of cation and cation concentration on the production of rAAV by transfected cells. **Figure 7A** shows the extent of expression of the enhanced green fluorescent protein (**EGFP**) in the transfected cells and the titering of the rAAV stocks using the infectious center assay. Stocks were produced by growing transfected HEK293 cells in Dulbecco's Modified Eagle's Medium in the presence of additionally added NaCl, KCl, CaCl₂ or MgCl₂. The additional concentration of such provided salt is 0, 20, 40, 60, 80 or 100 mM. **Figure 7A** shows the infectious center assay. **Figure 7B** is a graph of the fold-change in the titers of AAV vectors and salt concentration. **Figure 7C** is a graph of the fold-change in Total Genomes (**TG**) of AAV as a function of cation and cation concentration. The concentration shown in the Figure is the concentration increase in the culturing medium provided by the addition of such salts.

[0050] **Figures 8A-8B** show the effect of cation and cation concentration on the production of rAAV stocks. **Figure 8A** shows the extent of expression of the enhanced green fluorescent protein (EGFP) in the transfected cells and the titering of the rAAV stocks using the infectious center assay. Stocks were produced by growing transfected HEK293 cells in Dulbecco's Modified Eagle's Medium in the presence of additionally added 12 salts. The additional concentration of such provided salt is 40, 50, 60 or 70 mM. **Figure 8A** shows the infectious center assay. **Figure 8B** is a graph of the fold-change in the titers of AAV vectors and salt concentration. The Figure shows the fold-change in rAAV titer for rAAV that were produced in the presence of different anions and differing additionally provided concentrations of such anions. The concentration shown in the Figure is the concentration increase in the culturing medium provided by the addition of such anions.

[0051] **Figures 9A-9B** demonstrate that the provision of KHCO_3 caused unexpectedly higher titers of rAAV, relative to other ions (**Figure 9A**: fold-change in AAV titer in culture medium; **Figure 9B**: fold-change in Total Genomes). The concentration shown in the Figure (40, 50, 60 or 70 mM) is the concentration increase in the culturing medium provided by the addition of such KHCO_3 .

[0052] **Figure 10** shows the fold-change in the total amount of rAAV produced, and in the amount of rAAV released into the medium by cells that had been co-transfected with an Ad helper plasmid, a plasmid that provides the AAV ITRs, an enhanced green fluorescent protein-encoding transgene cassette and either an AAV2 helper plasmid or an AAV8 helper plasmid in order to provide the AAV *rep* and *cap* gene functions. At 2, 4, 6, 8, and 10 hours post-transfection, KHCO_3 was added to produce an additional concentration of 30 mM in the culturing medium and the fold-change of rAAV that had been released into the medium (AAV2-medium and AAV8-medium) and the total genomes in the cell lysis (AAV2-total and AAV8-total) were assessed at 72 hours post-transfection.

[0053] **Figures 11A-11B** show the effect of providing KHCO_3 on the enhancement of the production of rAAV of different serotypes. **Figure 11A**: shows the fold-change of rAAV released into the medium after 24 hours; **Figure 11B** shows the fold-change of total genomes of rAAV; KHCO_3 -30 denotes that KHCO_3 was added to produce an

additional concentration of 30 mM in the culturing medium; KHCO_3 -55 denotes that KHCO_3 was added to produce an additional concentration of 55 mM in the culturing medium.

[0054] **Figure 12** shows the ability of cells cultured in suspension to produce enhanced levels of rAAV in response to the provision of KHCO_3 . Provision of KHCO_3 sufficient to increase the concentration of KHCO_3 in the culturing medium by greater than about 20 mM enhanced production of rAAV5 and rAAV6 after 20 hours.

DETAILED DESCRIPTION OF THE INVENTION:

I. The Methods of the Present Invention

[0055] The present invention is directed to methods for increasing the efficiencies with which recombinant adeno-associated virus (rAAV) are packaged, so as to increase their production titers. More specifically, the invention relates to a method for increasing the production titer of rAAV by transfected cells by increasing the ionic strength of the cell culture media through the administration of additional ions.

[0056] As used herein, the term “**AAV**” is intended to denote adeno-associated virus, and may be used to refer to the virus itself or derivatives thereof. The term covers all subtypes and both naturally-occurring and recombinant forms. As used herein, the term “**rAAV**” is intended to denote a recombinantly-modified version of AAV that comprises a polynucleotide sequence not of AAV origin (*i.e.*, a polynucleotide heterologous to AAV). The rAAV may be single-stranded or double-stranded, and may be composed of deoxyribonucleotides or ribonucleotides.

[0057] As used herein, the term “**AAV helper functions**” denotes AAV proteins (*e.g.*, **Rep** and **Cap**) and/or polynucleotides of AAV that are required for the replication and packaging of an rAAV. Such AAV helper functions are provided by an “**AAV helper function-providing polynucleotide**,” which as such term is used herein is a virus, plasmid vector, a non-plasmid vector, or a polynucleotide that has been integrated into a cellular chromosome, that provides AAV helper functions. AAV helper plasmids that may be used in accordance with the present invention to provide **AAV helper functions**, such as pAAV-RC (Agilent; Addgene; Cell Biolabs), pAAV-RC2 (Cell

Biolabs), *etc.*, are commercially available. Plasmid **pAAV-RC2 (SEQ ID NO:1; Figure 3)** is an AAV helper plasmid that may be used in accordance with the present invention to provide **AAV helper functions**.

Coding Strand of Plasmid pAAV-RC2 (SEQ ID NO:1):

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ccggggccccc cctcgagggtc gacgggtatcg ggggagctcg caggggtctcc
atthttgaagc gggagggtttg aacgcgcagc cgccatgccg gggthtttacg
agattgtgat taaggthcccc agcgaccttg acgagcatct gcccggcatt
tctgacagct ttgtgaactg ggtggccgag aaggaatggg agttgccgcc
agattctgac atggatctga atctgattga gcaggcacc ctagcctggg
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gccccggagg ctctthttctt tgtgcaattt gagaaggggag agagctactt
ccacatgcac gtgctcgtgg aaaccaccgg ggtgaaatcc atggthtttg
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gaacagtatt taagcgctg thttgaatct acggagcgta aacggthgg
ggcgagcat ctgacgcagc gtgctgcagac gcaggagcag aacaaagaga
atcagaatcc caattctgat gcgcccgtga tcagatcaaa aacttcagcc
aggtacatgg agctggthcg gtggctcgtg gacaaggggg ttacctcgga
gaagcagthg atccaggagg accaggctc atacatctcc thcaatgcgg
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cgtggaggac atthtccagca atcggattta taaaattthg gaactaaacg
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aagthtcggca agaggaacac catctggctg thtgggctg caactaccgg
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gccgthgcaa gaccggatgt tcaaattthg actacccgc cgtctggatc
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[0058] In SEQ ID NO:1, residues 85-1950 of **pAAV-RC2** encode the **Rep** protein, **Rep78** (with residues 484-663 corresponding to the **P19** promoter, residues 1464-1643 corresponding to the **P40** promoter and residues 1668-1676 being a donor site); residues 1967-4174 encode the capsid protein, **VP1**; residues 1992-2016 encodes a portion of the **Rep68** protein; residues 4175-4256 encode a polyA sequence; residues 4610-4626 are *M13 Rev* sequences; residues 4634-4650 are *Lac* operator sequences; 4658-4688 are *Lac* promoter sequences; residues 4951-5675 correspond to pMB ori sequences, residues 5771-6631 encode an ampicillin resistance determinant; and residues 6632-6730 are *bla* promoter sequences (**Figure 3**).

[0059] As used herein, the term “**non-AAV helper functions**” denotes proteins of Ad, CMV, HSV or other non-AAD viruses (*e.g.*, E1a, E1b, E2a, VA and E4) and/or polynucleotides of Ad, CMV, HSV or other non-AAD viruses that are required for the replication and packaging of an rAAV. Such non-AAV helper functions are provided by a “**non-AAV helper function-providing polynucleotide**,” which as such term is used herein is a virus, plasmid vector, a non-plasmid vector, or a polynucleotide that has been integrated into a cellular chromosome, that provides non-AAV helper functions. The vector, **pHelper** and derivatives thereof (commercially available from Cell Biolabs, Inc., Invitrogen and Stratagene) are suitable non-AAV helper function-providing polynucleotide (see, *e.g.*, Matsushita, T. *et al.* (1998) “*Adeno-Associated Virus Vectors Can Be Efficiently Produced Without Helper Virus*,” *Gene Ther.* 5:938-945; Sharma, A. *et al.* (2010) “*Transduction Efficiency Of AAV 2/6, 2/8 And 2/9 Vectors For Delivering Genes In Human Corneal Fibroblasts*,” *Brain Res. Bull.* 81(2-3):273-278). Plasmid **pHelper-Kan** (SEQ ID NO:2; **Figure 4**) is a **non-AAV helper function-providing polynucleotide** that may be used in accordance with the present invention to provide **non-AAV helper functions**.

Coding Strand of Plasmid pHelper-Kan (SEQ ID NO:2):

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gatcttcacc	tagatccttt	taaattaaaa	atgaagtttt	aatcaatct
aaagtatata	tgagtaaact	tggtctgaca	gtcagaagaa	ctcgtcaaga

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aggcgataga aggcgatgcg ctgcgaatcg ggagcggcga taccgtaaag
cacgaggaag cggtcagccc attcgccgcc aagctcttca gcaatatcac
gggtagccaa cgctatgtcc tgatagcggc cgcacacacc cagccggcca
cagtcgatga atccagaaaa gcggccattt tccaccatga tattcggcaa
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ccttgagcct ggcgaaacagt tcggctggcg cgagcccctg atgctcttcg
tccagatcat cctgatcgac aagaccggct tccatccgag tacgtgctcg
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aaggaaggga agaaagcgaa aggagcgggc gctagggcgc tggcaagtgt
agcggtcacg ctgcgcgtaa ccaccacacc cgccgcgctt aatgcgcgcg
tacagggcgc gatggatcc

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[0060] In **SEQ ID NO:2**, residues 1-5343 of **pHelper-Kan** are derived from adenovirus, and include a polynucleotide encoding the **E2A** protein (residues 258-1847); residues 5344-8535 are derived from adenovirus, and include a polynucleotide encoding the **E4orf6** protein; residues 9423-10011 correspond to ori sequences; residues 10182-10976 encode a kanamycin resistance determinant expressed by a *bla* promoter sequence (residues 10977-11081); residues 11107-11561 correspond to f1 ori sequences (**Figure 4**).

[0061] As discussed above, **AAV helper function-providing polynucleotides** and **non-AAV helper function-providing polynucleotides** are typically employed in concert with an rAAV plasmid vector to comprise a triple plasmid transfection system. Multiple commercially available rAAV plasmid vectors (*e.g.*, **pAV-CMV-EGFP**, **pGOI**, *etc.* (Cell Biolabs, Inc., Invitrogen and Stratagene)) may be used in accordance with the present invention. An illustrative rAAV plasmid vector that may be used in

accordance with the present invention is **pAV-CMV-EGFP (SEQ ID NO:3; Figure 5)** which comprises a 5' ITR, a U6 promoter, CMV enhancer and promoter sequences, a polynucleotide encoding the enhanced green fluorescent protein (**EGFP**) (Gambotto, A. *et al.* (2000) "*Immunogenicity Of Enhanced Green Fluorescent Protein (EGFP) In BALB/C Mice: Identification Of An H2-Kd-Restricted CTL Epitope*," *Gene Ther.* 7(23):2036-2040; Tsien, R.Y. (1998) "*The Green Fluorescent Protein*," *Annu. Rev. Biochem.* 67:509-544; Cinelli, R.A. *et al.* (2000) "*The Enhanced Green Fluorescent Protein As A Tool For The Analysis Of Protein Dynamics And Localization: Local Fluorescence Study At The Single-Molecule Level*," *Photochem. Photobiol.* 71(6):771-776; Chopra A. (2008) "*Recombinant Adenovirus With Enhanced Green Fluorescent Protein*," In: *MOLECULAR IMAGING AND CONTRAST AGENT DATABASE (MICAD)*, National Center for Biotechnology Information, Bethesda MD), FLAG-tag and 6xHis-tag sites for facilitating recovery or localization of expressed proteins, an SV40 poly(A) site and a 3' ITR.

Coding Strand of Plasmid pAV-CMV-EGFP (SEQ ID NO:3):

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cctgcaggca gctgcgcgct cgctcgctca ctgaggccgc ccgggcgctcg
ggcgaccttt ggtcgcccgg ccctccagtg agcgagcgcg cagagagggga
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[0062] In **SEQ ID NO:3**, residues 1-128 of **pAV-CMV-EGFP** correspond to the 5' ITR; residues 201-441 are U6 promoter sequences; residues 562-865 are human cytomegalovirus (CMV) immediate early enhancer sequences; residues 866-1068 comprise the CMV immediate early promoter; residues 1192-1911 comprise a mammalian codon-optimized polynucleotide that encodes the EGFP; residues 1918-1941 encode the FLAG-tag; residues 1951-1968 encode the 6xHis-tag; residues 2139-2260 encode the SV40 poly(A) sequence; residues 2293-2433 correspond to the 3' ITR; residues 2508-22963 correspond to F1 ori sequences; residues 3350-4210 encode an ampicillin resistance determinant and its signal sequence (residues 3350-3418) expressed by a *bla* promoter sequence (residues 3245-3349); residues 4381-4969 correspond to an ori sequence (**Figure 5**).

[0063] A second illustrative rAAV plasmid vector that may be used in accordance with the present invention is **pAV-TBG-EGFP (SEQ ID NO:4; Figure 6)** which comprises a 5' ITR, a thyroid hormone-binding globulin (TBG) promoter, a polynucleotide encoding the enhanced green fluorescent protein (EGFP), FLAG-tag

and 6xHis-tag sites for facilitating recovery or localization of expressed proteins, an SV40 poly(A) site and a 3' ITR.

Coding Strand of Plasmid pAV-TBG-EGFP (SEQ ID NO:4):

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ggcgaccttt ggtcgcccg cctcagtgag cgagcgagcg cgcagagagg
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 ttttacgggt cctggccttt tgctggcctt ttgctcacat gt

[0064] In SEQ ID NO:4, residues 1-130 of pAV-TBG-EGFP correspond to the 5' ITR; residues 150-854 are TBG promoter sequences, with residues 415-824 comprising the TBG promoter; residues 886-1608 encode the EGFP; residues 1630-1653 encode the FLAG-tag; residues 1663-1680 encode the 6xHis-tag; residues 1851-1972 encode the poly(A) sequence; residues 2005-2145 corresponds to the 3' ITR; residues 2220-2675 correspond to F1 ori sequences; residues 3062-3922 encode an ampicillin resistance determinant and its signal sequence (residues 3062-3130) expressed by a *bla* promoter sequence (residues 2957-3061); residues 4093-4681 correspond to an ori sequence (Figure 6).

[0065] As used herein, the term “**production titer**” is intended to denote the amount of concentration of infectious rAAV in a preparation. Such amounts or concentrations are preferably determined by titering the AAV or rAAV in such preparation. The production titers of the rAAV preparations of the present invention are preferably titrated after subjecting producing cells (*e.g.*, HEK293 transformed with an rAAV plasmid vector, an AAV helper vector providing **Rep** and **Cap** proteins, and an Ad helper vector providing required adenovirus transcription and translation factors) to three rounds of freeze / thawing, followed by sonication to release the rAAV particles. The preparation is then centrifuged. The employed AAV vector is localized to the supernatant. An aliquot of the preparation is treated with proteinase K, and the number of AAV genomes is determined. An aliquot of the preparation is infected into HeLa-32C2 cells (which express AAV2 **Rep** and **Cap** proteins), and infectious titer is measured using the infectious center assay (ICA) (François, A. *et al.* (2018) “*Accurate Titration of Infectious AAV Particles Requires Measurement of Biologically Active Vector Genomes and Suitable Controls*,” Molec. Ther. Meth. Clin. Develop. 10:223-236) or more preferably, as the median tissue culture infective dose (TCID₅₀) (Zen, Z. *et al.* (2004) “*Infectious Titer Assay For Adeno-Associated Virus Vectors With Sensitivity Sufficient To Detect Single Infectious Events*,” Hum. Gene Ther. 15:709-715).

[0066] As used herein, an rAAV production titer is said to be “**increased**” by the methods of the present invention if the production titer obtained from the use of the methods of the present invention is at least 10% greater, more preferably at least 20% greater, still more preferably at least 30% greater, still more preferably at least 40%

greater, still more preferably at least 50% greater, still more preferably at least 60% greater, still more preferably at least 70% greater, still more preferably at least 80% greater, still more preferably at least 90% greater, still more preferably at least 2-fold greater, still more preferably at least 110% greater, still more preferably at least 120% greater, still more preferably at least 130% greater, still more preferably at least 140% greater, still more preferably at least 2.5-fold greater, still more preferably at least 160% greater, still more preferably at least 170% greater, still more preferably at least 180% greater, still more preferably at least 190% greater, and still more preferably at least 3-fold greater than the titer obtained from a similarly conducted production in which the additionally provided ions were not provided.

[0067] The rAAV whose production titer may be increased using the methods of the present invention may comprise any transgene cassette that permits the rAAV to be packaged into an rAAV plasmid vector that may be encapsidated within an AAV capsid particle. Without limitation, such transgene cassette(s) may be of human, primate (including chimpanzee, gibbon, gorilla, orangutan, *etc.*), cercopithecine (including baboon, cynomolgus monkey, velvet monkey, *etc.*), canine, glirine (including rat, mouse, hamster, guinea pig, *etc.*), feline, ovine, caprine, or equine origin.

[0068] In preferred embodiments, such an rAAV or rAAV plasmid vector will encode a protein (*e.g.*, an enzyme, hormone, antibody, receptor, ligand, *etc.*), or comprise a transcribed nucleic acid, that is relevant to a genetic or heritable disease or condition, such that it may be used in gene therapy to treat such disease or condition.

[0069] The methods of the present invention may be used to increase the production titer of rAAV and rAAV plasmid vectors in cells that have been transfected with a desired rAAV or rAAV plasmid vector, and with such one or more viruses and/or helper plasmids that can provide proteins or RNA molecules that are not provided by such rAAV or rAAV plasmid vectors, but are required for their production. As discussed above, such proteins or RNA molecules include the genes encoding the **Rep52** and **Rep78** proteins that are required for vector transcription control and replication, and for the packaging of viral genomes into the viral capsule, and, in the case of rAAV, *cap* genes that encode VP capsid proteins required to form infectious particles. Such proteins or RNA molecules also include the viral transcription and translation factors

(E1a, E1b, E2a, VA and E4) required for AAV proliferation. In one embodiment for producing the rAAV of the present invention, all of these genes and RNA molecules are provided on the same helper virus (or more preferably, helper vector) so as to comprise, in concert with an rAAV, a double plasmid transfection system. More preferably, however, for producing the rAAV of the present invention, the required *rep* and *cap* genes are provided by one plasmid, and the genes that encode the viral transcription and translation factors are provided on a second plasmid, so that such plasmids, in concert with the rAAV, comprise a triple plasmid transfection system.

[0070] The methods of the present invention may be employed to increase the production titer of rAAV belonging to any serotype, including the AAV1, AAV2, AAV5, AAV6, AAV7, AAV8, AAV9 and AAV10 serotypes and the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9, and rAAV10 serotypes, and including hybrid serotypes (*e.g.*, AAV2/5 and rAAV2/5, which is a hybrid of serotypes 2 and 5 and thus has the tropism of both such serotypes).

[0071] The methods of the present invention may be employed to enhance the production titers of rAAV that are to be produced using “helper” RNA or proteins provided by an adenovirus, a herpes simplex virus, a cytomegalovirus, a vaccinia virus or a papillomavirus.

[0072] The methods of the present invention may be employed to enhance the production titers of rAAV produced by cells in adherent monolayer culture or in suspension culture, and may be used with any method capable of producing rAAV. Preferably, however, rAAV is produced by transfecting baby hamster kidney (BHK) cells, or more preferably, human embryonic kidney (HEK) cells grown in tissue culture with the plasmid vectors described above. The BHK cell line BHK-21 (ATCC CCL-10), which lacks endogenous retroviruses is a preferred BHK cell line. The HEK cell line HEK293 (ATCC CRL-1573) and its derivatives, such as HEK293T (ATCC CRL-3216, which is a highly transfectable derivative of the HEK293 cell line into which the temperature-sensitive gene for SV40 T-antigen was inserted) or HEK293T/17 (ATCC® CRL-11268, which was selected for its ease of transfection) are particularly preferred. The HEK293T/17 SF cell line (ATCC ACS-4500) is a derivative of the 293T/17 cell

line (ATCC CRL-11268), adapted to serum-free medium and suspension, and may be employed if desired.

[0073] The preferred base medium of the present invention for culturing such cells is Eagle's Minimum Essential Medium (ATCC Catalog No. 30-2003) or Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Manassas, VA). Fetal bovine serum (*e.g.*, FBS; HyClone Laboratories, South Logan, UT) is added to a final concentration of 10% in order to make the complete growth medium. Eagle's Minimum Essential Medium and Dulbecco's Modified Eagle's Medium are complex media that contain amino acids, vitamins, and optionally glucose, in addition to various inorganic salts. Although different sources differ slightly in the concentrations of such salts, Dulbecco's Modified Eagle's Medium (commercially available from, *e.g.*, ThermoFisher Scientific) additionally contains approximately the inorganic salts shown in **Table 1**. The media differ in that Dulbecco's modified Eagle's medium contains approximately four times as much of the vitamins and amino acids present in the original formula of Eagle's Minimum Essential Medium, and two to four times as much glucose. Additionally, it contains iron in the form of ferric sulfate and phenol red for pH indication (Yao, T *et al.* (2017) "*Animal-Cell Culture Media: History, Characteristics, And Current Issues*," *Reproduc. Med. Biol.* 16(2): 99-117).

Table 1			
Inorganic Salt	Formula	Concentration	
		mg/L	Molarity
Calcium Chloride	CaCl ₂	200	1.80 mM
Ferric Nitrate	Fe(NO ₃) ₃ ·9H ₂ O	0.1	0.25 μM
Magnesium Sulfate (Anhyd.)	MgSO ₄	97.67	0.81 mM
Potassium Chloride	KCl	400	5.37 mM
Sodium Bicarbonate	NaHCO ₃	3700	44.04 mM
Sodium Chloride	NaCl	6400	109.5 mM
Sodium Phosphate Monobasic	NaH ₂ PO ₄ ·H ₂ O	125	0.78 mM
Sodium Phosphate Dibasic	Na ₂ HPO ₄ ·H ₂ O		

[0074] Cells to be used for such transfection are preferably passaged twice weekly to maintain them in exponential growth phase. For small-scale transfections, an aliquot of, for example, 1×10^6 HEK293 or BHK cells per well on a multi-well plate, or 1.5×10^7 HEK293 cells per 15-cm dish, may be employed. For large-scale production HEK293 or BHK cells may be collected from multiple confluent 15-cm plates, and split

into two 10-layer cell stacks (Corning, Corning, NY) containing 1 liter of complete culturing medium. In one embodiment, such cells are grown for 4 days in such medium before transfection. The day before transfection, the two cell stacks may be trypsinized and the cells (*e.g.*, approximately 6×10^8 cells) may be resuspended in 200 ml of medium. Preferably, the cells are allowed to attach for 24 hours before transfection. Confluency of the cell stacks may be monitored using a Diaphot inverted microscope (Nikon, Melville, NY) from which the phase-contrast hardware had been removed in order to accommodate the cell stack on the microscope stage.

[0075] As used herein, the phrase “**ionic strength**” is intended to denote the concentration of ions in a solution. The present invention enhances rAAV production titers by increasing the ionic strength of the culture medium by providing additional ions to the medium used to culture rAAV transfected cells. In one embodiment, the provided ions are cations. Suitable cations include Na^+ , K^+ , Ca^{++} , and Mg^{++} . Such cations may be provided as an inorganic salt or as a salt of organic molecule. In another embodiment, the provided ions are anions. Suitable anions include inorganic anions such as: CO_3^- , HCO_3^- , HPO_4^- , PO_4^- , SCN^- (thiocyanate), SO_4^- , HSO_4^- , and Cl^- , and organic ions, such as: acetate (CH_3COO^-), aspartate, biphthalate, bitartrate, butoxyethoxy acetate, caprylate, citrate ($\text{C}_6\text{H}_5\text{O}_7^-$), dehydroacetate, diacetate, dihydroxy glycinate, d-saccharate, gluconate, glutamate, glycinate, glycosulfate, hydroxymethane sulfonate, lactate, methionate, oxalate, phenate, phenosulfonate, propionate, propionate, saccharin, salicylate, sarcosinate, sorbate, thioglycolate, and toluene sulfonate.

[0076] Such cations or anions may be provided at any concentration sufficient to enhance rAAV production titers over the titers produced in the same culture medium without any such additionally provided cations. Suitable concentrations of such cations or anions include concentrations sufficient to increase the initial concentration of such ion in a culturing medium by from about 30 mM to about 80 mM, by from about 40 mM to about 80 mM, by from about 50 mM to about 80 mM, by from about 60 mM to about 80 mM, by from about 70 mM to about 80 mM, or by about 80 mM, with such concentrations being in addition to any concentration of such ion present in such culture medium prior to such addition. If such culture medium did not initially contain the ions to be administered, then such added ions are preferably provided in an amount sufficient

to provide concentrations of the provided ions in such culture medium of from about 30 mM to about 80 mM, by from about 40 mM to about 80 mM, by from about 50 mM to about 80 mM, by from about 60 mM to about 80 mM, by from about 70 mM to about 80 mM or to about 80 mM.

[0077] The ions or salts that are to be added to the initial culture medium may be added at any time prior to the harvesting of produced rAAV. Preferably, such ions or salts will have been added at least about 1 hour, at least about 2 hours, at least about 3 hours, at least about 4 hours, at least about 5 hours, at least about 6 hours, at least about 7 hours, at least about 8 hours, at least about 9 hours, at least about 10 hours, at least about 12 hours, at least about 15 hours, at least about 18 hours, at least about 20 hours, at least about 22 hours, or at least about 24 hours after the initiation of the culturing.

[0078] As used herein, the term “**about**” when used with reference to a concentration, amount, or time, is intended to denote such concentration and also a range of concentrations that is within $\pm 40\%$ of such concentration, and more preferably within $\pm 30\%$ of such concentration, and still more preferably within $\pm 20\%$ of such concentration, and still more preferably within $\pm 10\%$ of such concentration, and still more preferably within $\pm 5\%$ of such concentration. Thus, for example, a recited concentration of 10.0 mM denotes a concentration of 10.0 mM, as well as a concentration between 6-14 mM, and more preferably a concentration between 7-13 mM and still more preferably a concentration between 8-12 mM, and still more preferably a concentration between 9-11 mM, and still more preferably a concentration between 9.5-10.5 mM.

[0079] Thus, for example, since Dulbecco's Modified Eagle's Medium has an initial K^+ concentration of about 5.37 mM, a provision of additional K^+ sufficient to increase the concentration of such cation by about 30 mM would cause the culture medium to have a resultant Na^+ concentration of about 35.4 mM. Likewise, since Dulbecco's Modified Eagle's Medium has an initial HCO_3^- concentration of about 44.04 mM, a provision of additional HCO_3^- sufficient to increase the concentration of such cation by about 30 mM would cause the culture medium to have a resultant HCO_3^- concentration of about 74.04 mM.

[0080] In particular, the present invention thus provides a method for increasing the production titer of recombinantly-modified AAV (rAAV) that comprises the steps:

- (A) culturing cells that have been transfected with such rAAV in a culture medium for an initial period under conditions sufficient to permit the production of rAAV;
- (B) changing the ionic strength of the culture medium after the initial period by adding one or more ions, and preferably one or more ions other than Na^+ , to the culture medium, in an amount sufficient to increase the concentration of such ion in the culture medium by from about 30 mM to about 80 mM;
- (C) continuing the culturing of the cells to thereby produce a production titer of rAAV that is greater than a titer obtained in the absence of step (B).

[0081] The invention particularly contemplates the use of KHCO_3 to enhance rAAV production titer. Such KHCO_3 is preferably provided in an amount sufficient to increase the concentrations of K^+ and HCO_3^- in the culture medium by about 20 mM, by about 30 mM, by about 40 mM, or by about 50 mM. Such addition would cause the K^+ concentration in Dulbecco's Modified Eagle's Medium to be about 25 mM, about 35 mM, about 45 mM, or about 55 mM, and would cause the HCO_3^- concentration in such medium to be about 64 mM, about 74 mM, about 84 mM or about 94 mM. If such culture medium did not contain K^+ and HCO_3^- ions, then such KHCO_3 is preferably provided in an amount sufficient to provide concentrations of K^+ and HCO_3^- in such culture medium of about 20 mM, of about 30 mM, or of about 40 mM.

II. Pharmaceutical Compositions of the Present Invention

[0082] The invention additionally includes pharmaceutical compositions that comprise a pharmaceutically acceptable preparation of rAAV produced in accordance with the methods of the present invention, and a pharmaceutically acceptable carrier. The rAAV of such pharmaceutical compositions comprises a transgene cassette that encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, and is present in such pharmaceutical composition in an amount effective to ("**effective amount**")

[0083] The term "**pharmaceutically acceptable**" means approved by a regulatory agency of the Federal or a state government or listed in the US Pharmacopeia or other

generally recognized pharmacopeia for use in animals, and more particularly in humans. The term “**carrier**” refers to a diluent, adjuvant (*e.g.*, Freund’s adjuvant complete and incomplete), excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Suitable pharmaceutical excipients are described in US Patents No. 8,852,607; 8,192,975; 6,764,845; 6,759,050; and 7,598,070.

[0084] Generally, the ingredients of compositions of the invention are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate, or as an aqueous solution in a hermetically sealed container such as a vial, an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline, or other diluent can be provided so that the ingredients may be mixed prior to administration.

[0085] The invention also provides a pharmaceutical pack or kit comprising one or more containers such pharmaceutical composition. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0086] The rAAV of such pharmaceutical compositions is preferably packaged in a hermetically sealed container, such as a vial, an ampoule or sachette indicating the quantity of the molecule, and optionally including instructions for use. In one embodiment, the rAAV of such kit is supplied as a dry sterilized lyophilized powder or water-free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water, saline, or other diluent to the appropriate concentration for administration to a subject. The lyophilized material should be stored at between 2°C and 8°C in their original container and the material should be administered within 12 hours, preferably within 6 hours, within 5 hours, within 3 hours, or within 1 hour after being reconstituted. In another embodiment, the rAAV of such kit is supplied as an aqueous solution in a hermetically sealed container and can be diluted, *e.g.*, with water, saline, or other diluent, to the appropriate concentration for administration to a subject. The kit can further comprise one or more other prophylactic and/or therapeutic agents useful for the treatment of the disease or condition, in one or more containers; and/or the kit can further comprise one or more cytotoxic antibodies that bind one or more cancer antigens associated with cancer. In certain embodiments, the other prophylactic or therapeutic agent is a chemotherapeutic. In other embodiments, the prophylactic or therapeutic agent is a biological or hormonal therapeutic.

III. Uses of the Invention

[0087] The methods of the present invention may be used to facilitate the production of rAAV, and may particularly be used to facilitate the production of rAAV that comprise transgene cassettes that encode a protein (*e.g.*, an enzyme, hormone, antibody, receptor, ligand, *etc.*), or of rAAV that comprise a transcribed nucleic acid, that is relevant to a genetic or heritable disease or condition, such that it may be used in gene therapy to treat such disease or condition. Examples of such diseases and conditions include: achromatopsia (**ACHM**); alpha-1 antitrypsin (**AAT**) deficiency; Alzheimer's Disease; aromatic L-amino acid decarboxylase (**AADC**) deficiency; choroideremia (**CHM**); cancer; Duchenne muscular dystrophy; dysferlin deficiency; follistatin gene deficiency (**BMD5IBM**); hemophilia A; hemophilia B; hepatitis A; hepatitis B; hepatitis C; Huntington's disease; idiopathic Parkinson's disease; late-infantile neuronal ceroid lipofuscinosis (**LINCL**, an infantile form of Batten disease); Leber congenital amaurosis (**LCA**); Leber's hereditary optic neuropathy (**LHON**); limb

girdle muscular dystrophy 1B (**LGMD1B**); limb girdle muscular dystrophy 1C (**LGMD1C**); limb girdle muscular dystrophy 2A (**LGMD2A**); limb girdle muscular dystrophy 2B (**LGMD2B**); limb girdle muscular dystrophy 2I (**LGMD2I**); limb girdle muscular dystrophy 2L (**LGMD2L**); lipoprotein lipase (**LPL**) deficiency; metachromatic leukodystrophy; neurological disability; neuromotor deficit; neuroskeletal impairment; Parkinson's disease; rheumatoid arthritis; Sanfilippo A syndrome; spinal muscular atrophy (**SMA**); X-linked retinoschisis (**XLRS**); α -sarcoglycan deficiency (**LGMD2D**); β -sarcoglycan deficiency (**LGMD2E**); γ -sarcoglycan deficiency (**LGMD2C**) and δ -sarcoglycan deficiency (**LGMD2F**).

IV. Embodiments of the Invention

[0088] The invention concerns a method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), the recombinantly-modified adeno-associated virus (AAV) helper vector produced from such method, and uses and compositions thereof. It is particularly directed to the following embodiments **E1-E19**:

E1. A method for increasing the production titer of recombinantly-modified adeno-associated virus (rAAV), wherein the method comprises the steps:

- (A) culturing cells that have been transfected with the rAAV in an initial culture medium for an initial period under conditions sufficient to permit the production of rAAV, wherein the cells additionally contain an AAV helper function-providing polynucleotide and a non-AAV helper function-providing polynucleotide;
- (B) changing the ionic strength of the culture medium after the initial period by adding one or more ions other than Na^+ to the culture medium; and
- (C) continuing the culturing of the cells to thereby produce a production titer of with the rAAV that is greater than a titer obtained in the absence of step (B).

E2. The method of **E1**, wherein each of the added ion(s) is provided in an amount sufficient to increase the concentration of such ion in the initial culture medium by from about 10 mM to about 80 mM.

- E3.** The method of any one of **E1** or **E2**, wherein the production titer is at least 50% greater than the titer obtained from a similarly conducted cell culturing in the absence of the step (B).
- E4.** The method of any one of **E1-E3**, wherein the rAAV comprises a transgene cassette that encodes a protein, or comprises a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition.
- E5.** The method of any one of **E1-E4**, wherein the rAAV belongs to the rAAV1, rAAV2, rAAV5, rAAV6, rAAV7, rAAV8, rAAV9 or rAAV10 serotype, or to a hybrid of the serotypes.
- E6.** The method of **E5**, wherein the rAAV belongs to the rAAV2, rAAV5, or rAAV9 serotype, or to a hybrid of the serotypes.
- E7.** The method of any one of **E1-E6**, wherein the added ions comprise one or more of K^+ , Ca^{++} , or Mg^{++} .
- E8.** The method of any one of **E1-E7**, wherein the added ions comprise one or more of CO_3^- , HCO_3^- , HPO_4^- , PO_4^- , SCN^- , SO_4^- , HSO_4^- , and Cl^- .
- E9.** The method of any one of **E1-E7**, wherein the added ions comprise one or more of acetate, aspartate, biphthalate, bitartrate, butoxyethoxy acetate, caprylate, citrate, dehydroacetate, diacetate, dihydroxy glycinate, d-saccharate, gluconate, glutamate, glycinate, glycosulfate, hydroxymethane sulfonate, lactate, methionate, oxalate, phenate, phenosulfonate, propionate, propionate, saccharin, salicylate, sarcosinate, sorbate, thioglycolate, and toluene sulfonate.
- E10.** The method of any one of **E1-E8**, wherein the added ions comprise K^+ and CO_3^- .
- E11.** The method of any one of **E1-E10**, wherein the cells are human embryonic kidney cells.
- E12.** The method of **E11**, wherein the cells are HEK293 cells.
- E13.** The method of any one of **E1-E10**, wherein the cells are baby hamster kidney cells.
- E14.** The method of **E13**, wherein the cells are BHK21 cells.
- E15.** The method of any one of **E1-E10**, wherein the cells are sf9 insect cells.

- E16.** The method of any one of **E1-E15**, wherein the initial culture medium is Dulbecco's Modified Eagle's Medium.
- E17.** The method of **E16**, wherein the initial culture medium is supplemented with serum.
- E18.** A pharmaceutical composition that comprises:
- (A) a preparation of recombinantly-modified adeno-associated virus (rAAV) produced by the method of any one of **E1-E17**, wherein the rAAV comprises a transgene cassette that encodes a protein, or a transcribed nucleic acid, that is therapeutic for a genetic or heritable disease or condition, and wherein the pharmaceutical composition contains an effective amount of the rAAV preparation; and
 - (B) a pharmaceutically acceptable carrier.
- E19.** The preparation of recombinantly-modified adeno-associated virus (rAAV) produced by the method of any one of **E1-E17**, wherein the rAAV comprises a transgene cassette that encodes a protein, or a transcribed nucleic acid, or the pharmaceutical composition of **E18**, for use in the treatment of a genetic or heritable disease or condition.

EXAMPLES

[0089] Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention unless specified.

Example 1

Effect Of Cation and Cation Concentration on rAAV Production

[0090] The effect of cation and cation concentration on AAV production was demonstrated using cultured HEK293 cells. The culture medium was changed, and then, one hour later, the cells were transfected with:

- (1) the plasmid vector **pAAV-RC2**, which is capable of expressing the AAV *rep* and *cap* gene functions that are required for the replication and packaging of an rAAV;

- (2) the plasmid vector **pHelper**, which is capable of providing the viral transcription and translation factors (E1a, E1b, E2a, VA and E4) required for AAV proliferation; and
- (3) the rAAV plasmid vector **pAV-CMV-EGFP**, which comprises the transgene cassette encoding the enhanced green fluorescent protein (**EGFP**) and the AAV ITRs.

[0091] Five hours after such transfection, salt (either NaCl, KCl, CaCl₂ or MgCl₂) was provided to a final concentration of 0, 20, 40, 60 80 or 100 mM. **Figure 7A** shows the extent of expression of EGFP in the transfected cells and the titering of the rAAV stocks using the infectious center assay. **Figure 7B** is a graph of the fold-change in infectious centers as a function of such cation and cation concentrations. **Figure 7C** is a graph of the fold-change in Total Genomes (**TG**) of AAV as a function of such cation and cation concentrations. The results show that the provision of cations affected the total genomes (TG) produced and that the provision of NaCl, KCl and MgCl₂ increased AAV genome replication and AAV production. Provision of NaCl and KCl was found to cause the highest titers of total genomes and the greatest increase in AAV production, with the greatest increase seen at NaCl and KCl concentrations that are sufficient to increase the concentrations of such ions in the culture medium by between about 40 mM to about 80 mM. The provision of higher concentrations of cations was found to inhibit EGFP expression (NaCl $\geq$ 180mM; KCl $\geq$ 100mM; MgCl₂ $\geq$ 60mM).

Example 2

Effect Of Anion and Anion Concentration on rAAV Production

[0092] The effect of anion and anion concentration on AAV production was also demonstrated using cultured HEK293 cells. As in **Example 1**, the culture medium was changed, and one hour later, the cells were transfected with the Ad helper plasmid, the AAV helper plasmid, and the pAAV-ITR plasmid vector that provides the AAV ITRs and transgene cassette encoding the enhanced green fluorescent protein. Five hours after such transfection, salt (either K₂CO₃, KHCO₃, KH₂PO₄, KCH₃COO (potassium acetate), KCNS, K₂SO₄, KNO₃, K₃C₆H₅O₇ (potassium citrate) or KCL) was provided in an amount sufficient to increase the concentrations of such ions in the culture medium by 40, 50, 60, or 70 mM. The fold-change in rAAV infectious centers was determined after 72 hours. Provision of KHCO₃ was found to cause the greatest increase in rAAV

production, with the greatest increase seen at concentrations sufficient to increase the concentrations of such ions by between about 40 mM to about 50 mM (**Figure 8A**). **Figure 8B** is a graph of the fold-change in the titer of rAAV vector as a function of such anion and anion concentrations. The results show that the provision of anions affected the total genomes (TG) produced. The provision of high concentrations of ions (> 60 mM) was found to attenuate rAAV production. The results demonstrate that the provision of KHCO_3 in an amount sufficient to increase the concentrations of such ions in the culture medium by between about 30 mM and about 50 mM provided unexpectedly better results than those obtained with other salts (**Figures 9A-9B**). An increase in concentration by about 30 mM was considered optimum.

Example 3

Effect of Time of Provision of KHCO_3 on rAAV Production

[0093] The effect caused by providing KHCO_3 at differing times post-transfection was also investigated. HEK293 cells were cultured and co-transfected with: (1) the above-described Ad helper plasmid, (2) the pAAV-ITR plasmid vector that provides the AAV ITRs and transgene cassette encoding the enhanced green fluorescent protein and (3) an AAV2 helper plasmid or an AAV8 helper plasmid in order to provide the AAV *rep* and *cap* gene functions. Culture medium had been changed one hour before the co-transfections. At 2, 4, 6, 8, and 10 hours post-transfection, KHCO_3 was added in an amount sufficient to increase the concentrations of such ions in the culture medium by a concentration of 30 mM and the fold-change of rAAV that had been released into the medium was assessed at 72 hours. The fold-change in the total amount of rAAV produced was also assessed (**Figure 10**). The results indicate that the greatest enhancement was seen when salts were added 4-8 hours post-transfection.

Example 4

Effect of Serotype on rAAV Production

[0094] As discussed above, prior methods for enhancing the production of rAAV were not successful for rAAV having the AAV2 serotype (Lock, M. *et al.* (2010) “*Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale*,” Hum. Gene Ther. 21:1259-1271). In order to assess the ability of KHCO_3 addition to enhance the production of rAAV of different serotypes, AAV2 helper plasmid encoding **Cap** proteins of serotypes 1, 2, 5, 6, 7, 8, 9 or 10 were

transfected into HEK293 cells along with the above-described Ad helper plasmid and a pAAV-ITR plasmid vector (pAV-TBG-EGFP) that provides the AAV ITRs and a transgene cassette encoding the enhanced green fluorescent protein. Four hours post-transfection, KHCO_3 was added to a final concentration of 30 mM and the fold-change of rAAV released into the medium was assessed at 72 hours. The results of this study are shown in **Figures 11A-11B**, and indicate that the addition of ions, and specifically the addition of KHCO_3 , significantly increased the production titer of rAAV of all serotypes tested, including the rAAV2 serotype.

Example 5

Effect of Ion Provision on Large-Scale rAAV Production

[0095] In order to demonstrate that the provision of ions enhanced production of rAAV in large-scale preparations, rAAV of serotypes 1, 5, 6 and 9 with transgene cassettes encoding the green fluorescent protein or other exogenous molecules were produced in large-scale in the presence or absence of a total concentration of 30 mM KHCO_3 in five 15 cm dishes. AAV titers were obtained after purification. The results of this demonstration are shown in **Table 2** (pDNA_001 donor construct, PiBFXNco3 and PiBFXNco11 are control vectors).

Table 2				
Effect of KHCO_3 Provision on Large-Scale AAV Production				
AAV	Transgene	Yield (per mL)	Fold-Change	KHCO_3 Addition
AAV1				
A5514-1	pAV-CMV-EGFP	1.17×10^{13}	--	None
A5514-2	pAV-CMV-EGFP	3.8×10^{13}	3.2	30 mM, 4 hours post-transfection
AAV5				
A5658	pAV-CMV-EGFP	1.14×10^{13}	--	None
A5659	pAV-CMV-GFP	3.03×10^{13}	2.7	30 mM, 4 hours post-transfection
AAV6				
A5516-1	pAV-CMV-EGFP	1.25×10^{13}	--	None
A5516-2	pAV-CMV-EGFP	2.69×10^{13}	2.2	30 mM, 4 hours post-transfection
A5555	pDNA_001 donor construct	8.99×10^{12}		None
A5556	pDNA_001 donor construct	2.64×10^{13}	2.9	30 mM, 4 hours post-transfection
AAV9				
A5474-1	PiBFXNco3	1.34×10^{13}	--	None
A5474-2	PiBFXNco3	1.61×10^{13}	1.2	30 mM, overnight post-transfection
A5475-1	PiBFXNco11	3.14×10^{12}	--	None
A5475-2	PiBFXNco11	1.46×10^{13}	4.6	30 mM, overnight post-transfection

[0096] As indicated in **Table 2**, the provision of ions, and particularly the provision of KHCO_3 , resulted in an increase in rAAV production of 1.2 to 4.6 fold, with an average fold-increase of about 3-fold.

Example 6

Effect of the Provision of Ions on the Production of rAAV by Cells Grown in Suspension

[0097] In order to demonstrate that the provision of ions enhanced production of rAAV by cells that were grown in suspension, HEK293 cells were co-transfected with: (1) the above-described Ad helper plasmid, (2) the pAAV-ITR plasmid vector that provides the AAV ITRs and transgene cassette encoding the enhanced green fluorescent protein and (3) an AAV5 helper plasmid or an AAV6 helper plasmid in order to provide the AAV *rep* and *cap* gene functions. KHCO_3 was added in an amount sufficient to increase the concentrations of such ions in the culture medium by 10, 20, 30, 40, 50 or 60 mM at 5 hours or 20 hours post-transfection. Total Genomes of produced rAAV was determined at 72 hours post-transfection. Suspension cells were cultured at 37°C, 5% CO_2 with an agitation speed of 120 rpm. The ability of cells cultured in suspension to produce enhanced levels of rAAV in response to the provision of ions is shown in **Figure 8**. As shown in **Figure 12**, provision of ions at a final concentration of greater than about 20 mM enhanced production of rAAV5 and rAAV6.

[0098] All publications and patents mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference in its entirety. While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.