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(54) Title: COMPOSITION AND METHODS FOR HIGHLY EFFICIENT GENE TRANSFER USING AAV CAPSID VARIANTS

(57) Abstract: Improved compositions and methods for AAV mediated gene therapy are disclosed.

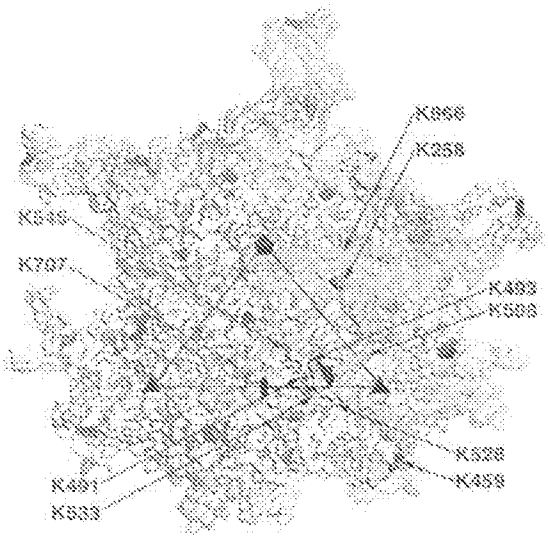


Figure 1A

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Composition and methods for highly efficient gene transfer using AAV capsid variants

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This application claims priority to US Provisional Application Nos. 61/635,273 and 61/794,995 filed April 18, 2012 and March 15, 2013 respectively, the entire contents of each being incorporated herein by reference as though set forth in full.

FIELD OF THE INVENTION

This application relates to the fields of gene therapy and molecular biology. More specifically, this invention provides adeno-associated viral vectors comprising protein capsid variants which improve the transduction efficiency of AAV vectors comprising therapeutically beneficial transgenes.

BACKGROUND OF THE INVENTION

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

Adeno-associated virus (AAV) is a small (20 nm), replication-defective, non-enveloped virus. Many distinct AAV serotypes have been characterized in human and nonhuman primates. The AAV genome is comprised of single-stranded DNA with 145 bp inverted terminal repeats (ITRs) at both ends. There are two open reading frames (ORFs), rep and cap. While the rep products are essential for AAV replication, 3 capsid proteins (VP1, VP2, and VP3) are expressed from the cap gene. VP1, VP2 and VP3 come together at 1:1:10 ratio to form an icosahedral capsid (Xie Q et al, 2002). During recombinant AAV (rAAV) vector production, an expression cassette flanked by ITRs is packaged into AAV capsid. The genes required for replication of AAV are not included in the cassette. Recombinant AAV is considered the safest and one of the most widely used viral vectors for in vivo gene transfer. The vectors can infect cells from multiple tissue types providing strong and persistent

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transgene expression. They are also non-pathogenic and have a low immunogenicity profile (High KA, 2011).

One of the immediate goals for gene therapy trials is optimizing vectors to maximize tissue transduction while minimizing the vector dose. Upon entry into the cell, AAV capsid proteins are subject to proteasome mediated degradation. Phosphorylation of surface-exposed tyrosine residues of AAV capsid represents one of the first steps that leads to degradation of the virus via the ubiquitin-proteasome pathway (Zhong L *et al*, 2007). Most of the regulated proteolysis in the cell occurs through this pathway. Ubiquitin is a small protein (~8.5 kDa) that can be found in all eukaryotic cells. Ubiquitin is attached to the side-chain of amino-acids of a substrate protein. After additional ubiquitin proteins are attached to the substrate via the initially attached ubiquitin, a polyubiquitin chain is formed and the substrate is marked for degradation (Thrower JS *et al* 2000, Peng J *et al* 2003, Bedford L *et al* 2011). It has been shown that mutation of surface-exposed tyrosine residues leads to an increase in transduction efficiency of AAV2 vectors (Zhong L *et al*, 2008). More recently, several groups have shown that the strategy is effective also with other AAV serotypes in several tissues, including AAV serotype 6 and 8.

Clearly, a need exists in the art for compositions and methods which improve the transduction of AAV carrying clinically important transgenes in patients in need thereof.

SUMMARY OF THE INVENTION

In accordance with the present invention, novel AAV variants are provided which exhibit increased transduction efficiency when compared to AAV serotypes (e.g., AAV1, AAV2, AAV8, AAV-rh74), which lack the modifications disclosed herein. Such improved vectors are useful for transduction of a variety of tissues, including liver, muscle, brain, and retina.

In one embodiment, an adeno-associated virus (AAV) vector comprising an altered VP1 capsid protein is provided, the altered capsid protein comprising lysine residue substitutions, thereby reducing ubiquination of the capsid and increasing the transduction efficiency of variant AAV into target tissues and cells. In one embodiment, the vector further comprises a heterologous nucleic acid, (e.g., a minigene comprising AAV inverted terminal repeats and a heterologous nucleic acid sequence) operably linked to regulatory sequences which direct expression of a product from the heterologous nucleic acid sequence in a host cell. In a preferred embodiment, the AAV vector comprises one or more lysine

substitutions in VP1 as provided in the tables set forth herein. In another embodiment, the AAV vector is of the AAV8 serotype and contains an alteration provided in Table 3.

In a preferred embodiment, the AAV vectors of the invention comprising the variant capsid proteins are useful for expression of therapeutic peptides or therapeutic nucleic acids. Such peptides include, without limitation, an anti-viral RNAi molecule, Factor VIII, Factor IX or a functional fragment thereof. Additional expression products include for example, IgG, IgM, IgA, IgD, IgE, chimeric immunoglobulins, humanized antibodies, or single chain antibodies. In one aspect the expression product is an RNAi that is useful for inhibiting HCV infection and replication. In another embodiment, the expression product is an antisense nucleic acid useful for down modulating a target cell of interest.

In another embodiment of the invention, a pharmaceutical composition comprising the variant AAV vectors of the invention in a biologically compatible carrier is provided. Also encompassed by the present invention are cell cultures comprising the vectors disclosed herein.

The invention also encompasses a method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with an AAV vector as disclosed herein, wherein said AAV vector comprises the transgene, wherein the presence of lysine substitution in the VP1 capsid sequence in said vector is associated with reduced ubiquitination and increased transduction efficiency.

In a final aspect, the invention provides decoy viral variants that are inefficient at infecting cells but are effective to block antibody neutralization of viral variants carrying beneficial transgenes due to the structural similarities of the two viral variants. Exemplary capsid variants for this purpose include for example, K38R, K143R, K510R and K709R.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Lysines on the surface of AAV1, AAV2 and AAV8: PDB numbers of AAV serotypes used here are as follows: AAV1: 3NG9, AAV2: 1LP3, and AAV8: 2QA0. The arrows represent the respective Lysine residues. (A) There are 11 Lysines on the surface of AAV1 VP3. Residue colors are as follows: K258 red, K459 blue, K491 yellow, K493 magenta, K508 cyan, K528 dark salmon, K533 light green, K545 light blue (slate), K567 dark salmon, K666 light cyan, K707 gray. (B) There are 10 Lysines on the surface of AAV2 VP3. Residue colors are as follows: K258 red, K490 yellow, K507 cyan, K527 dark salmon, K532 light green, K544 light blue (slate), K549 light yellow, K556 light magenta,

K665 light cyan, K706 gray (C) There are 8 Lysines on the surface of AAV8 VP3. Residue colors are as follows : K259 red, K333 green, K510 cyan, K530 dark salmon, K547 light blue (slate), K569 dark salmon, K668 light cyan, K709 gray. Note that K528 and K567 of AAV1 and K530 and K569 of AAV8 are side by side in the strructure and showed with the same color.

Figure 2: Several Lysine residues of AAV8 capsid were mutated to Arginine. Blood from the animals was collected 8 weeks after virus injection via tail vein. hF.IX levels are detected by ELISA (A) The residues that are predicted to be ubiqutinated by the software with high and medium confidence levels were mutated to arginine. 2.5×10^{10} virus particles per mouse were injected via tail vein (B,C) The residues that are predicted with low confidence to be ubiqutinated by the software were mutated to arginine. 2.5×10^{9} virus particles per mouse were injected via tail vein. (B) K569R and K668R capsid mutations (C) Affect of K38R, K143R, K259R, K510R, K547R capsid mutations are compared to affect of K530R mutation.

Figure 3: Combination of the K to R capsid mutations (A) Combination of K137R, K33R and K530R mutations are still higher than the wild type but not statistically different from the K530R. (B) K709R mutation negatively affects the AAV8 transduction, Addition of K709R mutation to K(137/333/530)R mutant also decreases the transduction of the virus. C) Combination of multiple Lysine to arginine mutations does not increase the transduction rate. (D) Combination of three lysine to arginine residues with four or six tyrosine to phenylalanine residues decreases the transduction rate.

Figure 4: AAV1 transduction (A) CTL killing of HHL5-B7 hepatocytes transduced with AAV-1 lysine mutants at three different MOIs 5K, 50K and 500K. Peptide (IPQYGYLTL for AAV1; VPQYGYLTL for AAV2) was used as a positive control. LDH release correlates with the cell killing. (B) Total number of GFP positive cells and GFP expression was compared among different constructs at 50K and 500K MOIs.

Figure 5: AAV2 transduction: A) AAV2 K137R, K527R or K532R mutants were compared to WT AAV2 in terms of HHL5 cell line transduction rate. The cells were transfected at 10K, 50K, 100K and 500K MOIs and checked 24 hours later for GFP expression. B) A graph showing cytotoxicity as measured by LDL release of variants tested.

Figure 6: CTL assay in which target hepatocyte cells were transduced with AAV-2 vector at increasing concentrations and then incubated with HLA-matched effector cells. AAV vectors encoded wild-type AAV-2 capsid or single lysine mutations as indicated. Effectors were derived from PBMC expanded in vitro against AAV-2 MHC Class I epitope VPQYGYLTL and effector-to-target ratio was 10:1. Results are expressed as percentage of CTL activity (% cytotoxicity compared to cells treated with 10% SDS as a maximum cytotoxicity control after background subtraction) with respect to the wild type vector.

Figure 7: RH74 data: Human F.IX transgene expression levels in plasma measured by an ELISA specific for human F.IX.A) Definition of TMR=K(137/333/530)R, Definition of HMF= Y(253/275/447/703/707/733)F. B) Definition of HMR: K(137/259/333/530/552/569)R, Definition of 195/202: G195A+ L199V+ S201P+ G202N. While HMR+195/202, HMR+195/202+K(38)R, HMR+195/202+K(51)R, HMR+195/202+K(61)R , and HMR+195/202+K(77)R produced higher hFIX production upon injection to mice, HMR+195/202+K(122/123)R or HMR+195/202+K(142/413)R injection did not produce any detectable hFIX at all. C): RHM13_1 mutant produced similar hFIX levels compared to Rh74 WT whereas hFIX levels derived from RHM17_1-treated mice were barely above background levels. D) RHM14_2 mutant produced similar hFIX levels compared to Rh74 WT; RHM15_1 performance was in-between that of AAV8 and AAVrh74 WT.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have found that mutating the lysine residues on AAV capsids to arginine residues increases AAV transduction efficiency. Our initial experiments showed that a single substitution of a lysine residue predicted to be a target for ubiquitination resulted in higher levels of expression of the human factor IX (FIX) transgene in mice compared to animals receiving unmodified AAV vectors. The AAV lysine mutants described herein could be used to advantage to generate vectors that target the liver, CNS, muscle, and other organs with higher efficiency compared to the wild type AAV capsids. Thus, this discovery can be used to develop therapeutics to treat hemophilia A, B, Huntington's disease, and virtually any other disease that requires increased transduction levels of desirable transgenes into a target tissue of interest.

The following definitions are provided to facilitate an understanding of the present invention.

I. DEFINITIONS:

"Gene therapy" is the insertion of genes into an individual's cells and/or tissues to treat a disease, commonly hereditary diseases wherein a defective mutant allele is replaced or supplemented with a functional one.

"Adeno-associated viruses", from the parvovirus family, are small viruses with a genome of single stranded DNA. These viruses can insert genetic material at a specific site on chromosome 19 and are preferred because they are not associated with pathogenic disease in humans.

A "therapeutic" peptide or protein is a peptide or protein that may alleviate or reduce symptoms that result from an absence or defect in a protein in a cell or subject. Alternatively, a "therapeutic" peptide or protein is one that otherwise confers a benefit to a subject, e.g., anti-cancer effects. Therapeutic peptides and proteins include, but are not limited to, CFTR (cystic fibrosis transmembrane regulator protein), dystrophin (including the protein product of dystrophin mini-genes, see, e.g, Vincent et al., (1993) Nature Genetics 5:130), utrophin (Tinsley et al., (1996) Nature 384:349), clotting factors (Factor XIII, Factor IX, Factor X, etc.), monoclonal antibodies (Lewis et al., 2002), erythropoietin, the LDL receptor, lipoprotein lipase, ornithine transcarbamylase, β-globin, α-globin, spectrin, α-antitrypsin, adenosine deaminase, hypoxanthine guanine phosphoribosyl transferase, β-glucocerebrosidase, sphingomyelinase, lysosomal hexosaminidase, branched-chain keto acid dehydrogenase, hormones, growth factors (e.g., insulin-like growth factors 1 and 2, platelet derived growth factor, epidermal growth factor, nerve growth factor, neurotrophic factor -3 and -4, brain-derived neurotrophic factor, glial derived growth factor, transforming growth factor α and β, and the like), cytokines (e.g., α-interferon, β-interferon, interferon-γ, interleukin-2, interleukin-4, interleukin 12, granulocyte-macrophage colony stimulating factor, lymphotoxin), suicide gene products (e.g., herpes simplex virus thymidine kinase, cytosine deaminase, diphtheria toxin, cytochrome P450, deoxycytidine kinase, and tumor necrosis factor), proteins conferring resistance to a drug used in cancer therapy, tumor suppressor gene products (e.g., p53, Rb, Wt-1, NF1, VHL, APC, and the like), and any other peptide or protein that has a therapeutic effect in a subject in need thereof.

Further exemplary therapeutic peptides or proteins include those that may be used in the treatment of a disease condition including, but not limited to, cystic fibrosis (and other

diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood disorders, AIDS, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (e.g., Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), and diseases of solid organs (e.g., brain, liver, kidney, heart).

The term "promoters" or "promoter" as used herein can refer to a DNA sequence that is located adjacent to a DNA sequence that encodes a recombinant product. A promoter is preferably linked operatively to an adjacent DNA sequence. A promoter typically increases an amount of recombinant product expressed from a DNA sequence as compared to an amount of the expressed recombinant product when no promoter exists. A promoter from one organism can be utilized to enhance recombinant product expression from a DNA sequence that originates from another organism. For example, a vertebrate promoter may be used for the expression of jellyfish GFP in vertebrates. In addition, one promoter element can increase an amount of recombinant products expressed for multiple DNA sequences attached in tandem. Hence, one promoter element can enhance the expression of one or more recombinant products. Multiple promoter elements are well-known to persons of ordinary skill in the art.

In one embodiment, high-level constitutive expression will be desired. Examples of such promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter/enhancer, the cytomegalovirus (CMV) immediate early promoter/enhancer (see, e.g., Boshart et al, Cell, 41:521-530 (1985)), the SV40 promoter, the dihydrofolate reductase promoter, the cytoplasmic β-actin promoter and the phosphoglycerol kinase (PGK) promoter.

In another embodiment, inducible promoters may be desired. Inducible promoters are those which are regulated by exogenously supplied compounds, either in cis or in trans, including without limitation, the zinc-inducible sheep metallothionine (MT) promoter; the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter; the T7 polymerase promoter system (WO 98/10088); the tetracycline-repressible system (Gossen et al, Proc. Natl. Acad. Sci. USA, 89:5547-5551 (1992)); the tetracycline-inducible system (Gossen et al., Science, 268:1766-1769 (1995); see also Harvey et al., Curr. Opin. Chem. Biol., 2:512-518 (1998)); the RU486-inducible system (Wang et al., Nat. Biotech., 15:239-243 (1997) and Wang et al., Gene Ther., 4:432-441 (1997)]; and the rapamycin-inducible system (Magari et al., J. Clin. Invest., 100:2865-2872 (1997); Rivera et al., Nat. Medicine.

2:1028-1032 (1996)). Other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, or in replicating cells only.

In another embodiment, the native promoter for the transgene or nucleic acid sequence of interest will be used. The native promoter may be preferred when it is desired that expression of the transgene or the nucleic acid sequence should mimic the native expression. The native promoter may be used when expression of the transgene or other nucleic acid sequence must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

In one embodiment, the recombinant viral genome comprises a transgene operably linked to a tissue-specific promoter. For instance, if expression in skeletal muscle is desired, a promoter active in muscle may be used. These include the promoters from genes encoding skeletal α-actin, myosin light chain 2A, dystrophin, muscle creatine kinase, as well as synthetic muscle promoters with activities higher than naturally-occurring promoters . See Li et al., Nat. Biotech., 17:241-245 (1999). Examples of promoters that are tissue-specific are known for liver albumin, Miyatake et al. J. Virol., 71:5124-32 (1997); hepatitis B virus core promoter, Sandig et al., Gene Ther. 3:1002-9 (1996); alpha-fetoprotein (AFP), Arbuthnot et al., Hum. Gene Ther., 7:1503-14 (1996)], bone (osteocalcin, Stein et al., Mol. Biol. Rep., 24:185-96 (1997); bone sialoprotein, Chen et al., J. Bone Miner. Res. 11 :654-64 (1996)), lymphocytes (CD2, Hansal et al., J. Immunol., 161:1063-8 (1998); immunoglobulin heavy chain; T cell receptor a chain), neuronal (neuron-specific enolase (NSE) promoter, Andersen et al. Cell. Mol. Neurobiol., 13:503-15 (1993); neurofilament light-chain gene, Piccioli et al., Proc. Natl. Acad. Sci. USA, 88:5611-5 (1991); the neuron-specific vgf gene, Piccioli et al., Neuron, 15:373-84 (1995)]; among others.

The term "enhancers" or "enhancer" as used herein can refer to a DNA sequence that is located adjacent to the DNA sequence that encodes a recombinant product. Enhancer elements are typically located upstream of a promoter element or can be located downstream of or within a DNA coding sequence (e.g., a DNA sequence transcribed or translated into a recombinant product or products). Hence, an enhancer element can be located 100 base pairs, 200 base pairs, or 300 or more base pairs upstream or downstream of a DNA sequence that encodes recombinant product. Enhancer elements can increase an amount of recombinant product expressed from a DNA sequence above increased expression afforded by a promoter

element. Multiple enhancer elements are readily available to persons of ordinary skill in the art.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the expression of a polypeptide coding sequence in a host cell or organism.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, infection, PEG-fusion and the like.

The introduced nucleic acid may or may not be integrated (covalently linked) into nucleic acid of the recipient cell or organism. In bacterial, yeast, plant and mammalian cells, for example, the introduced nucleic acid may be maintained as an episomal element or independent replicon such as a plasmid. Alternatively, the introduced nucleic acid may become integrated into the nucleic acid of the recipient cell or organism and be stably maintained in that cell or organism and further passed on to or inherited by progeny cells or organisms of the recipient cell or organism. Finally, the introduced nucleic acid may exist in the recipient cell or host organism only transiently.

The term "selectable marker gene" refers to a gene that when expressed confers a selectable phenotype, such as antibiotic resistance, on a transformed cell or plant.

The term "operably linked" means that the regulatory sequences necessary for expression of the coding sequence are placed in the DNA molecule in the appropriate positions relative to the coding sequence so as to effect expression of the coding sequence. This same definition is sometimes applied to the arrangement of transcription units and other transcription control elements (e.g. enhancers) in an expression vector.

The term "oligonucleotide" as used herein refers to sequences, primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo- or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The phrase "specifically hybridize" refers to the association between two single-stranded nucleic acid molecules of sufficiently complementary sequence to permit such hybridization under pre-determined conditions generally used in the art (sometimes termed "substantially complementary"). In particular, the term refers to hybridization of an oligonucleotide with a substantially complementary sequence contained within a single-stranded DNA or RNA molecule of the invention, to the substantial exclusion of hybridization of the oligonucleotide with single-stranded nucleic acids of non-complementary sequence.

The term "primer" as used herein refers to a DNA oligonucleotide, either single-stranded or double-stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield a primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15-25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able to anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use

in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non-complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non-complementary bases may be interspersed within the oligonucleotide primer sequence, provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template-primer complex for the synthesis of the extension product.

Polymerase chain reaction (PCR) has been described in U.S. Patents Nos. 4,683,195, 4,800,195, and 4,965,188, the entire disclosures of which are incorporated by reference herein.

The term "isolated" may refer to a compound or complex that has been sufficiently separated from other compounds with which it would naturally be associated. "Isolated" is not meant to exclude artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with fundamental activity or ensuing assays, and that may be present, for example, due to incomplete purification, or the addition of stabilizers.

The term "immune response" is meant to refer to any response to an antigen or antigenic determinant by the immune system of a vertebrate subject. Exemplary immune responses include humoral immune responses (e.g. production of antigen-specific antibodies) and cell-mediated immune responses (e.g. lymphocyte proliferation), as defined herein below.

II. METHODS OF USING AND METHODS OF ADMINISTRATION OF THE VARIANT ADENOASSOCIATED VIRAL VECTORS OF THE INVENTION

The methods of the present invention provide a means for delivering heterologous nucleic acid sequences into a broad range of host cells, including both dividing and non-dividing cells. The vectors and other reagents, methods and pharmaceutical formulations of the present invention are additionally useful in a method of administering a protein or peptide to a subject in need thereof, as a method of treatment or otherwise. In this manner, the protein or peptide may thus be produced in vivo in the subject. The subject may be in need of the protein or peptide because the subject has a deficiency of the protein or peptide, or because the production of the protein or peptide in the subject may impart some therapeutic effect, as a method of treatment or otherwise, and as explained further below.

In general, the present invention may be employed to deliver any foreign nucleic acid with a biological effect to treat or ameliorate the symptoms associated with any disorder related to gene expression. Illustrative disease states include, but are not limited to: cystic fibrosis (and other diseases of the lung), hemophilia A, hemophilia B, thalassemia, anemia and other blood coagulation disorders, AIDs, Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, epilepsy, and other neurological disorders, cancer, diabetes mellitus, muscular dystrophies (e.g., Duchenne, Becker), Gaucher's disease, Hurler's disease, adenosine deaminase deficiency, glycogen storage diseases and other metabolic defects, retinal degenerative diseases (and other diseases of the eye), diseases of solid organs (e.g., brain, liver, kidney, heart), and the like.

In addition, the present invention may be employed to deliver nucleic acids encoding monoclonal antibodies or fragments thereof that are known to provide beneficial biological effects to treat or ameliorate the symptoms associated with cancers, infectious diseases, and autoimmune diseases such as rheumatoid arthritis. Other sequences may encode for example cytokines such as interferon-alpha that may modulate the course of a disease.

Gene transfer has substantial potential use in understanding and providing therapy for disease states. There are a number of inherited diseases in which defective genes are known and have been cloned. In some cases, the function of these cloned genes is known. In general, the above disease states fall into two classes: deficiency states, usually of enzymes, which are generally inherited in a recessive manner, and unbalanced states, at least sometimes involving regulatory or structural proteins, which are inherited in a dominant manner. For deficiency state diseases, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations. For unbalanced disease states, gene transfer could be used to create a disease state in a model system, which could then be used in efforts to counteract the disease state. Thus the methods of the present invention permit the treatment of genetic diseases. As used herein, a disease state is treated by partially or wholly remedying the deficiency or imbalance that causes the disease or makes it more severe. The use of site-specific integration of nucleic sequences to cause mutations or to correct defects is also possible.

Finally, the instant invention finds further use in diagnostic and screening methods, whereby a gene of interest is transiently or stably expressed in a cell culture system, or alternatively, a transgenic animal model.

III. SUBJECTS, PHARMACEUTICAL FORMULATIONS, VACCINES, AND MODES OF ADMINISTRATION

The present invention finds use in both veterinary and medical applications. Suitable subjects include both avians and mammals, with mammals being preferred. The term "avian" as used herein includes, but is not limited to, chickens, ducks, geese, quail, turkeys and pheasants. The term "mammal" as used herein includes, but is not limited to, humans, bovines, ovines, caprines, equines, felines, canines, lagomorphs, etc. Human subjects are the most preferred. Human subjects include fetal, neonatal, infant, juvenile and adult subjects.

In particular embodiments, the present invention provides a pharmaceutical composition comprising a virus particle of the invention in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc. For injection, the carrier will typically be a liquid. For other methods of administration, the carrier may be either solid or liquid, such as sterile, pyrogen-free water or sterile pyrogen-free phosphate-buffered saline solution. For inhalation administration, the carrier will be respirable, and will preferably be in solid or liquid particulate form. As an injection medium, it is preferred to use water that contains the additives usual for injection solutions, such as stabilizing agents, salts or saline, and/or buffers.

In other embodiments, the present invention provides a pharmaceutical composition comprising a cell in which an AAV provirus is integrated into the genome in a pharmaceutically-acceptable carrier or other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

By "pharmaceutically acceptable" it is meant a material that is not biologically or otherwise undesirable, e.g., the material may be administered to a subject without causing any undesirable biological effects. Thus, such a pharmaceutical composition may be used, for example, in transfection of a cell ex vivo or in administering a viral particle or cell directly to a subject.

The present invention further provides a method of delivering a nucleic acid to a cell. For in vitro methods, the virus -may be administered to the cell by standard viral transduction methods, as are known in the art. Preferably, the virus particles are added to the cells at the appropriate multiplicity of infection according to standard transduction methods appropriate for the particular target cells. Titers of virus to administer can vary, depending upon the target cell type and the particular virus vector, and may be determined by those of skill in the art

without undue experimentation. Alternatively, administration of a parvovirus vector of the present invention can be accomplished by any other means known in the art.

Recombinant virus vectors are preferably administered to the cell in a biologically-effective amount. A "biologically-effective" amount of the virus vector is an amount that is sufficient to result in infection (or transduction) and expression of the heterologous nucleic acid sequence in the cell. If the virus is administered to a cell in vivo (e.g., the virus is administered to a subject as described below), a "biologically-effective" amount of the virus vector is an amount that is sufficient to result in transduction and expression of the heterologous nucleic acid sequence in a target cell.

The cell to be administered the inventive virus vector may be of any type, including but not limited to neural cells (including cells of the peripheral and central nervous systems, in particular, brain cells), lung cells, retinal cells, epithelial cells (e.g., gut and respiratory epithelial cells), muscle cells, pancreatic cells (including islet cells), hepatic cells, myocardial cells, bone cells (e.g., bone marrow stem cells), hematopoietic stem cells, spleen cells, keratinocytes, fibroblasts, endothelial cells, prostate cells, germ cells, and the like. Alternatively, the cell may be any progenitor cell. As a further alternative, the cell can be a stem cell (e.g., neural stem cell, liver stem cell). Moreover, the cells can be from any species of origin, as indicated above.

In particular embodiments of the invention, cells are removed from a subject, the parvovirus vector is introduced therein, and the cells are then replaced back into the subject. Methods of removing cells from subject for treatment ex vivo, followed by introduction back into the subject are known in the art. Alternatively, the rAAV vector is introduced into cells from another subject, into cultured cells, or into cells from any other suitable source, and the cells are administered to a subject in need thereof.

Suitable cells for ex vivo gene therapy include, but are not limited to, liver cells, neural cells (including cells of the central and peripheral nervous systems, in particular, brain cells), pancreas cells, spleen cells, fibroblasts (e.g., skin fibroblasts), keratinocytes, endothelial cells, epithelial cells, myoblasts, hematopoietic cells, bone marrow stromal cells, progenitor cells, and stem cells.

Dosages of the cells to administer to a subject will vary upon the age, condition and species of the subject, the type of cell, the nucleic acid being expressed by the cell, the mode of administration, and the like. Typically, at least about 10^2 to about 10^8, preferably about 10^3 to about 10^6 cells, will be administered per dose. Preferably, the cells will be administered in a "therapeutically-effective amount".

A "therapeutically-effective" amount as used herein is an amount that is sufficient to alleviate (e.g., mitigate, decrease, reduce) at least one of the symptoms associated with a disease state. Alternatively stated, a "therapeutically-effective" amount is an amount that is sufficient to provide some improvement in the condition of the subject.

A further aspect of the invention is a method of treating subjects in vivo with the inventive virus particles. Administration of the parvovirus particles of the present invention to a human subject or an animal in need thereof can be by any means known in the art for administering virus vectors.

Exemplary modes of administration include oral, rectal, transmucosal, topical, transdermal, inhalation, parenteral (e.g., intravenous, subcutaneous, intradermal, intramuscular, and intraarticular) administration, and the like, as well as direct tissue or organ injection, alternatively, intrathecal, direct intramuscular, intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspenions in liquid prior to injection, or as emulsions. Alternatively, one may administer the virus in a local rather than systemic manner, for example in a depot or sustained-release formulation.

In particularly preformed embodiments of the invention, the nucleotide sequence of interest is delivered to the liver of the subject. Administration to the liver may be achieved by any method known in art, including, but not limited to intravenous administration, intraportal administration, intrabilary administration, intra-arterial administration, and direct injection into the liver parenchyma.

Preferably, the cells (e.g., liver cells) are infected by a recombinant parvovirus vector encoding a peptide or protein, the cells express the encoded peptide or protein and secrete it into the circulatory system in a therapeutically-effective amount (as defined above). Alternatively, the vector is delivered to and expressed by another cell or tissue, including but not limited to, brain, pancreas, spleen or muscle.

In other preferred embodiments, the inventive parovirus particles are administered intramuscularly, more preferably by intramuscular injection or by local administration (as defined above). In other preferred embodiments, the parovirus particles of the present invention are administered to the lungs.

The parovirus vector disclosed herein may be administered to the lungs of a subject by any suitable means, but are preferably administered by administering an aerosol suspension of respirable particles comprised of the inventive parvovirus vectors, which the

subject inhales. The respirable particles may be liquid or solid. Aerosols of liquid particles comprising the inventive parvovirus vectors may be produced by any suitable means, such as with a pressure-driven aerosol nebulizer or an ultrasonic nebulizer, as is known to those of skill in art. See, e.g. U.S. Pat. No. 4,501,729. Aerosols of solid particles comprising the inventive virus vectors may likewise be produced with any solid particulate medicament aerosol generator, by techniques known in the pharmaceutical art.

Dosages of the inventive parvovirus particles will depend upon the mode of administration, the disease or condition to be treated, the individual subject's condition, the particular virus vector, and the gene to be delivered and can be determined in a routine manner. Exemplary doses for achieving therapeutic effects are virus titers of at least about 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, 10^{14}, 10^{15} ,10^{16} transducting units or more, preferably about 10^8 to 10^{13} transducting units, yet more preferably 10^{12} transducing units.

In summary, the parvovirus vectors, reagents, and methods of the present invention can be used to direct a nucleic acid to either dividing or non-dividing cells, and to stably express the heterologous nucleic acid therein. Using this vector system, it is now possible to introduce into cells, in vitro or in vivo, genes that encode proteins that affect cell physiology. The vectors of the present invention can thus be useful in gene therapy for disease states or for experimental modification of cell physiology.

The following example is provided to illustrate certain embodiments of the invention. It is not intended to limit the invention in anyway.

EXAMPLE I

Lysine to Arginine mutations affect AAV transduction rate and MHC delivery

Identification of lysine residues to be targeted in AAV1 and AAV8 vectors

We used the UbPred software to predict the possible ubiquitination sites on AAV1, AAV2, AAV8 and Rh74 capsid proteins (Radivojac P *et al*, 2010). UbPred software is available online at http://www.ubpred.org/index.html. The output of the analysis is the prediction of the lysine residues important for ubiquitination within the indicated AAV serotype capsid sequence. See Figure 1 and Table 1. These are the following:

AAV1 Capsid Protein VP1 Sequence:
MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGL
DKGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF

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QAKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQEPDSSSGIGKTGQQPAKKRLNFGQTGDSES
VPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRVIT
TSTRTWALPTYNNHLYKQISSASTGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINN
NWGFRPKRLNFKLFNIQVKEVTTNDGVTTIANNLTSTVQVFSDSEYQLPYVLGSAHQGCLPP
FPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEEVPFHSSYAHS
QSLDRLMNPLIDQYLYYLNRTQNQSGSAQNKDLLFSRGSPAGMSVQPKNWLPGPCYRQQRV
SKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASHKDDEDKFFPMSGVMIFGKESAGAS
NTALDNVMITDEEEIKATNPVATERFGTVAVNFQSSSTDPATGDVHAMGALPGMVWQDRD
VYLQGPIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANPPAEFSATKFASFITQYST
GQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNNGLYTEPRPIGTRYLTRP

Lysine

position	Score	Ubiquitinated	
26	0.59	No	
31	0.55	No	
33	0.47	No	
38	0.61	No	
51	0.54	No	
61	0.72	Yes	Medium confidence
77	0.63	Yes	Low confidence
84	0.72	Yes	Medium confidence
122	0.17	No	
123	0.26	No	
137	0.90	Yes	High confidence
142	0.62	Yes	Low confidence
143	0.81	Yes	Medium confidence
161	0.70	Yes	Medium confidence
168	0.25	No	
169	0.26	No	
258	0.49	No	
310	0.14	No	
313	0.15	No	
322	0.48	No	
459	0.81	Yes	Medium confidence
476	0.43	No	
491	0.20	No	
493	0.28	No	
508	0.62	Yes	Low confidence
528	0.67	Yes	Low confidence
533	0.70	Yes	Medium confidence
545	0.65	Yes	Low confidence
567	0.66	Yes	Low confidence
621	0.50	No	
641	0.50	No	
650	0.45	No	
666	0.58	No	
689	0.65	Yes	Low confidence
693	0.65	Yes	Low confidence
707	0.78	Yes	Medium confidence

Legend:

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Label	Score range	Sensitivity	Specificity
Low confidence	0.62 < s < 0.69	0.464	0.903
Medium confidence	0.69 < s < 0.84	0.346	0.950
High confidence	0.84 < s < 1.00	0.197	0.989

Desirable mutants in AAV-1

ID	*AAV-1 mutant*
MUT1-1	**K61R**
MUT1-2	**K84R**
MUT1-3	**K137R**
MUT1-4	**K143R**
MUT1-5	**K161R**
MUT1-6	**K459R**
MUT1-7	**K528R**
MUT1-8	**K533R**
MUT1-9	**K707R**

AAV8 capsid protein VP1 sequence:

MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGL
DKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF
QAKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPARKRLNFGQTGDSE
SVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVI
TTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLI
NNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSAHQGCLP
PFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFTYTFEDVPFHSSYAH
SQSLDRLMNPLIDQYLYYLSRTQTTGGTANTQTLGFSQGGPNTMANQAKNWLPGPCYRQQR
VSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSNGILIFGKQNAAR
DNADYSDVMLTSEEEIKTTNPVATEEYGIVADNLQQQNTAPQIGTVNSQGALPGMVWQNRD
VYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQSKLNSFITQYST
GQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTEGVYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
26	0.52	No	
31	0.51	No	
33	0.16	No	
38	0.62	Yes	Low confidence
51	0.56	No	
61	0.68	Yes	Low confidence
77	0.65	Yes	Low confidence
122	0.17	No	

123	0.32	No	
137	0.92	Yes	High confidence
142	0.53	No	
143	0.65	Yes	Low confidence
162	0.28	No	
163	0.29	No	
170	0.24	No	
259	0.49	No	
312	0.09	No	
317	0.12	No	
324	0.43	No	
333	0.75	Yes	Medium confidence
478	0.59	No	
510	0.50	No	
530	0.71	Yes	Medium confidence
547	0.40	No	
569	0.66	Yes	Low confidence
623	0.47	No	
643	0.44	No	
652	0.51	No	
668	0.64	Yes	Low confidence
691	0.66	Yes	Low confidence
695	0.67	Yes	Low confidence
709	0.68	Yes	Low confidence

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	0.62 < s < 0.69	0.464	0.903
Medium confidence	0.69 < s < 0.84	0.346	0.950
High confidence	0.84 < s < 1.00	0.197	0.989

Table 3 appended hereto provides the yield of vector obtained using the different capsid mutants, including AAV8 of the invention. The table also indicates whether the mutation resulted in increased, decreased or comparable transduction efficiencies when compared to wild type vectors of the same serotype.

AAV2 capsid protein VP1 sequence:

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLDK
GEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQA
KKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVP
DPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTS
TRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNW
GFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPA
DVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQS
LDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKT
SADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNV

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DIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYL
QGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQ
VSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
24	0.20	No	
26	0.49	No	
33	0.38	No	
39	0.89	Yes	High confidence
51	0.53	No	
61	0.67	Yes	Low confidence
77	0.68	Yes	Low confidence
92	0.56	No	
105	0.58	No	
122	0.14	No	
123	0.18	No	
137	0.87	Yes	High confidence
142	0.58	No	
143	0.77	Yes	Medium confidence
161	0.70	Yes	Medium confidence
169	0.31	No	
258	0.51	No	
309	0.14	No	
314	0.17	No	
321	0.46	No	
490	0.73	Yes	Medium confidence
507	0.61	No	
527	0.78	Yes	Medium confidence
532	0.75	Yes	Medium confidence
544	0.61	No	
549	0.68	Yes	Low confidence
556	0.62	Yes	Low confidence
620	0.47	No	
640	0.45	No	
649	0.42	No	
665	0.54	No	
688	0.68	Yes	Low confidence
692	0.68	Yes	Low confidence
706	0.66	Yes	Low confidence

Legend:

Label	Score range	Sensitivity	Specificity
Low confidence	$0.62 \leq s \leq 0.69$	0.464	0.903
Medium confidence	$0.69 \leq s \leq 0.84$	0.346	0.950
High confidence	$0.84 \leq s \leq 1.00$	0.197	0.989

AAV-Rh74 capsid protein VP1 sequence

MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGL

DKGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVF
QAKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDSES
VPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRVITT
STRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQRLINN
NWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSAHQGCLPPF
PADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYNFEDVPFHSSYAHS
QSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNWLPGPCYRQQRV
STTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSSGVLMFGKQGAG
KDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNSQGALPGMVWQN
RDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADPPTTFNQAKLASFITQ
YSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTEGTYSEPRPIGTRYLTRNL

Lysine position	Score	Ubiquitinated	
26	0.60	No	
31	0.53	No	
33	0.27	No	
38	0.23	No	
51	0.38	No	
61	0.68	Yes	Low confidence
77	0.65	Yes	Low confidence
122	0.17	No	
123	0.30	No	
137	0.90	Yes	High confidence
142	0.35	No	
143	0.35	No	
162	0.36	No	
163	0.33	No	
169	0.27	No	
170	0.25	No	
259	0.46	No	
312	0.13	No	
317	0.16	No	
324	0.46	No	
333	0.77	Yes	Medium confidence
478	0.51	No	
510	0.49	No	
530	0.67	Yes	Low confidence
547	0.52	No	
552	0.69	Yes	Medium confidence
569	0.67	Yes	Low confidence
623	0.49	No	
643	0.44	No	
652	0.54	No	
668	0.56	No	
691	0.68	Yes	Low confidence
695	0.67	Yes	Low confidence
709	0.69	Yes	Medium confidence

Legend:

Label	Score range	Sensitivity	Specificity

Low confidence	$0.62 < s < 0.69$	0.464	0.903
Medium confidence	$0.69 < s < 0.84$	0.346	0.950
High confidence	$0.84 < s < 1.00$	0.197	0.989

Desirable mutants in AAV-rh74 (also see Table 3)

ID	*AAV-rh74 mutant*
Mut74-1	K26R
Mut74-2	K31R
Mut74-3	K33R
Mut74-4	K38R
Mut74-5	K51R
Mut74-6	K77R
Mut74-7	K137R
Mut74-8	K163R
Mut74-9	K169R
Mut74-10	K259R
Mut74-11	K333R
Mut74-12	K530R
Mut74-13	K547R
Mut74-14	K552R
Mut74-15	K569R
Mut74-16	K668R
Mut74-17	K691R
Mut74-18	K695R
Mut74-19	K709R

The following primer sets were utilized to create the lysine containing capsid variants of the invention:

Primers used for mutagenesis:

* AAV1 primers:

AAV1 K61R
Sense: 5'- CTT CAA CGG ACT CGA CAG GGG GGA GCC -3'
Antisense: 5'- GGC TCC CCC CTG TCG AGT CCG TTG AAG -3'

AAV1 K84R
Sense: 5'- GCC TAC GAC CAG CAG CTC AGA GCG GGT GAC -3'
Antisense: 5'- GTC ACC CGC TCT GAG CTG CTG GTC GTA GGC -3'

AAV1 K137R
Sense: 5'- TGG TTG AGG AAG GCG CTA GGA CGG CTC CT -3'
Antisense: 5'- AGG AGC CGT CCT AGC GCC TTC CTC AAC CA -3'

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AAV1 K143R
Sense: 5'- CTA AGA CGG CTC CTG GAA AGA GAC GTC CGG TAG -3'
Antisense:5'- CTA CCG GAC GTC TCT TTC CAG GAG CCG TCT TAG -3'

AAV1 K161R
Sense: 5'- CGG GCA TCG GCA GGA CAG GCC AGC A -3'
Antisense:5'- TGC TGG CCT GTC CTG CCG ATG CCC G -3'

AAV1 K459R
Sense: 5'- AGT CCG GAA GTG CCC AAA ACA GGG ACT TGC TGT -3'
Antisense:5'- ACA GCA AGT CCC TGT TTT GGG CAC TTC CGG ACT -3'

AAV1 K528R
Sense: 5'- GCA CTG CTA TGG CCT CAC ACA GAG ACG ACG AAG -3'
Antisense:5'- CTT CGT CGT CTC TGT GTG AGG CCA TAG CAG TGC -3'

AAV1 K533R
Sense: 5'- CAA AGA CGA CGA AGA CAG GTT CTT TCC CAT GAG CG -3'
Antisense:5'-CGC TCA TGG GAA AGA ACC TGT CTT CGT CGT CTT TG -3'

AAV1 K707R
Sense: 5'- TGCAGTACACATCCAATTATGCAAGATCTGCCAACG TTG -3'
Antisense:5'- CAACGTTGGCAGATCTTGCATAATTGGATGTGTACTGCA -3'

- AAV8 Primers

AAV8 K137R
Sense: 5'- GGT TGA GGA AGG CGC TAG GAC GGC TCC TGG -3'
Antisense:5'- CCA GGA GCC GTC CTA GCG CCT TCC TCA ACC -3'

AAV8 K333R
Sense: 5'- GCA GAA TGA AGG CAC CAG GAC CAT CGC CAA TAA CC -3'
Antisense:5'- GGT TAT TGG CGA TGG TCC TGG TGC CTT CAT TCT GC -3'

AAV8 K530R
Sense: 5'- GCA TCG CTA TGG CAA CAC ACA GAG ACG ACG AGG -3'
Antisense:5'- CCT CGT CGT CTC TGT GTG TTG CCA TAG CGA TGC -3'

AAV8 K709R
Sense: 5'- GTACACCTCCAACTACTACAGATCTACAAGTGTGGACTTTG -3'
Antisense:5'- CAAAGTCCACACTTGTAGATCTGTAGTAGTTGGAGGTGTAC -3'

- AAV2 Primers

AAV2 K39R
Sense: 5'-GCCCGCAGAGCGGCATAGGGACGACAG-3'
Antisense: 5'-CTGTCGTCCCTATGCCGCTCTGCGGGC-3'

AAV2 K137R
Sense: 5'-CCTGGTTGAGGAACCTGTTAGGACGGCTCCGG-3'
Antisense: 5'-CCGGAGCCGTCCTAACAGGTTCCTCAACCAGG-3'

AAV2 K143R
Sense: 5'-AGACGGCTCCGGGAAAAAGGAGGCCGGTA-3'

Antisense: 5'-TACCGGCCTCCTTTTTCCCGGAGCCGTCT-3'

AAV2 K161R
Sense: 5'-CCTCGGGAACCGGAAGGGCGGGCC-3'
Antisense: 5'-GGCCCGCCCTTCCGGTTCCCGAGG-3'

AAV2 K490R
Sense: 5'-CCGCCAGCAGCGAGTATCAAGGACATCTGCGG-3'
Antisense: 5'-CCGCAGATGTCCTTGATACTCGCTGCTGGCGG-3'

AAV2 K527R
Sense: 5'-CGGCCATGGCAAGCCACAGGGACGATGAA-3'
Antisense: 5'-TTCATCGTCCCTGTGGCTTGCCATGGCCG-3'

AAV2 K532R
Sense: 5'-ACAAGGACGATGAAGAAAGGTTTTTTCCTCAGAGCGG-3'
Antisense: 5'-CCGCTCTGAGGAAAAAACCTTTCTTCATCGTCCTTGT-3'

- AAV-rh74 Primers

AAV-rh74 K137R
Sense: 5'-CTGGTTGAATCGCCGGTTAGGACGGCTCCTG-3'
Antisense: 5'-GACCAACTTAGCGGCCAATCCTGCCGAGGAC-3'

AAV-rh74 K333R
Sense: 5'-GCAGAATGAAGGCACCAGGACCATCGCCAATAACC-3'
Antisense: 5'-GGTTATTGGCGATGGTCCTGGTGCCTTCATTCTGC-3'

AAV-rh74 K530R
Sense: 5'-GTTGCCATGGCTACCCACAGGGACGACGAA-3'
Antisense: 5'-TTCGTCGTCCCTGTGGGTAGCCATGGCAAC-3'

AAV-rh74 K552R
Sense: 5'-GGAAACAGGGAGCTGGAAGAGACAACGTGGACTAT-3'
Antisense: 5'-ATAGTCCACGTTGTCTCTTCCAGCTCCCTGTTTCC-3'

AAV-rh74 K569R
Sense: 5'-CTAACCAGCGAGGAAGAAATAAGGACCACCAACCC-3'
Antisense: 5'-GGGTTGGTGGTCCTTATTTCTTCCTCGCTGGTTAG-3'

AAV-rh74 K691R
Sense: 5'-CGAGTGGGAGCTGCAGAGGGAGAACAGCAA-3'
Antisense: 5'-TTGCTGTTCTCCCTCTGCAGCTCCCACTCG-3'

AAV-rh74 K695R
Sense: 5'-GCTGCAGAAGGAGAACAGCAGACGCTGGAACC-3'
Antisense: 5'-GGTTCCAGCGTCTGCTGTTCTCCTTCTGCAGC-3'

AAV-rh74 K709R
Sense: 5'-AGTACACTTCCAACTACTACAGATCTACAAATGTGGACTTTGC-3'
Antisense: 5'-GCAAAGTCCACATTTGTAGATCTGTAGTAGTTGGAAGTGTACT-3'

Table 5 provides a series of AAV vectors derived from mutagenesis that were used to package liver-specific AAV transgene expression cassettes for FIX. Packaging efficiency was indistinguishable from that observed with wild type, unmodified AAV8 vectors.

Lot number	Lysine mutant	Preparation size (Roller Bottles)	Yield per roller bottle (vector genomes)
KA712	AAV8 K137R	10	1.53E13
KA713	AAV8 K333R	10	9.46E12
KA714	AAV8 K530R	10	1.61E13
KA535	AAV8 (wild type)	10	6.63E12

Capsid mutants which contain 2, 3, 4, 5, 6, 7 or more altered lysine residues in any of the capsid proteins described herein to further increase transduction efficiency are also within the scope of the invention. The data also reveals that certain mutations result in variants which exhibited significantly reduced transduction efficiency. Such variants could be used in combination with variants which exhibit increased transduction efficiencies, to act as decoys to neutralize or saturate an antibody directed immune response to the incoming vectors, thereby enabling vectors carrying desirable transgenes to more efficiently enter cells.

Figure 1 shows schematic diagrams of the capsid surface. The data presented in Figures 2A, 2B and 2C demonstrate that altering lysine residues in the VP1 capsid on AAV8 alters the level of transgene produced due to altered transduction levels. Figure 3A, 3B and 3C show the effects of single and multiple mutations on HF.IX production in transduced cells. Figure 3D demonstrates that a combination mutations, e.g., three lysine to arginine residues with four or six tyrosine to phenylalanine residues decreases transduction rates.

CTL Killing of HHL5-B7 Hepatocytes using AAV lysine mutants

We assessed CTL killing of hepatocytes transduced with certain of the AAV lysine mutants disclosed here. The following materials and methods were employed to assess CTL killing of transduced hepatocytes.

Vector generation

AAV vectors were produced in HEK-293 cells using a triple transfection approach as previously described (Matsushita, 1998) and purified with cesium chloride gradient

centrifugation methods (Ayuso, 2010). AAV epitope peptides were synthesized by Genemed Synthesis and resuspended at a concentration of 5mg/ml in 100% DMSO.

***In vitro* expansion of T cells**

Human PBMCs (Cellular Technology LTD) were thawed, washed, counted, and resuspended at a concentration of 2 x 10^6 cells/ml in AIM-V lymphocyte media (Gibco) containing 3% human serum (Bioreclamation), 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco). For each expansion condition, 1x10^6 (500 µl) cells were added per well in a 24 well plate (BD Falcon) in a volume of 500 µl. An additional 1x10^6 (500 µl) of autologous irradiated splenocytes (3000 rad) were also added to each well as feeder cells, along with 2.5 µg/ml of human β-2-microglobulin (Lee Biosolutions), and 10 ng/ml of human recombinant IL-7 (R&D Systems). Cells were expanded in the presence of AAV peptide at a final concentration of 10 µg/ml at 37°C in 5% CO_2. Human IL-2 (Roche) at a concentration of 10 ng/ml was added to the cell culture after the first 24 hours and replenished every 48 hours thereafter. Cells were divided into new wells as necessary and antigenic stimulation (antigen and feeder cells) was repeated every 7-10 days for up to 3 rounds of restimulation.

CTL ASSAY

CTL assay was performed as previously described (Pien, 2009). Briefly, lactate dehydrogenase (LDH) release following CTL-mediated target lysis was measured with the CytoTox 96 Non Radioactive Cytotoxicity Assay (Promega). Four thousand HHL5 hepatocyte target cells were plated in each well of a Microtest Primaria flat-bottom 96-well plate (BD Falcon) in DMEM containing no serum. Target cells were transduced at an MOI of 5000, 50,000, and 500,000 of AAV capsid and incubated for 18 hours at 37°C 5% CO_2. Following treatment and incubation, plated target cells were washed once with media prior to the addition of epitope-specific cytotoxic T lymphocytes, expanded as described above. CTLs were added at an effector-target cell ratio of 10:1 for 4 hours at 37°C, 5% CO_2 and LDH was measured after a 30 minute incubation at room temperature with enzymatic substrate read at 490nm using a spectrophotometer (Spectramax).

Flow cytometry

GFP expression following AAV transduction was measured by flow cytometry. Human hepatocytes from cell lines HHL5 or Huh7 were plated in DMEM containing 10%

fetal bovine serum, 1% L-glutamine (Gibco), and 1% penicillin/streptomycin (Gibco) at a density of 250,000 cells/well in a Primaria Multiwell 24-well plate (BD Falcon). Cells were transduced with 5000, 50000, or 5000000 MOI of AAV vector and incubated for 18 hours at 37°C 5% CO_2. Following incubation, cells were trypsinized, washed twice with PBS 2% FBS, and fixed with 2% paraformaldehyde. Samples were aquired on a FACS Canto II flow cytometer using the FACSDiva® (BD Biosciences) and further analysis was performed using Flowjo® software (Treestar).

CTL ASSAY RESULTS

In order to further test effect of the lysine mutations on viral transduction, we utilized an in vitro CTL-mediated cytotoxicity assay previously developed by our lab to test the functionality of AAV vectors (Pien et al.). AAV1 and AAV2 transduction results are shown in Figures 4 and 5 and 6. In Figure 4, all lysine mutations in the AAV-1 capsid resulted in a decrease in CTL-mediated killing of target cells, suggesting that the lysine mutations led to less efficient processing and presentation of surface antigen upon transduction. Furthermore, the effect of lysine mutations appears to be additive, with the triple and quadruple lysine mutants showing the greatest decrease of CTL-mediated killing (Figure 4A, B). Mutations to the AAV-2 capsid showed a similar effect. See Figures 5 and 6. Figure 7 shows the transduction results obtained when Rh74 variants were tested.

In summary, we have found that mutating the lysine residues on AAV capsids to arginine residues increases AAV transduction efficiency. Our experiments identified several variants that upon transduction, resulted in higher levels of expression of the human factor IX (FIX) transgene in mice compared to animals receiving unmodified AAV vectors.

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Radivojac, P., Vacic, V., Haynes, C., Cocklin, R. R., Mohan, A., Heyen, J. W., Goebl, M. G., and Iakoucheva, L. M. Identification, Analysis and Prediction of Protein Ubiquitination Sites. *Proteins: Structure, Function, and Bioinformatics.* 78(2):365-380. (2010)

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

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Table 1

Comparison of AAV1, AAV2 and AAV8 lysine positions and their Ubiquitination predictions

Color Label: [illegible], Blue: Medium confidence, Red: High confidence, Black: No ubiquitination prediction

```
AAV1      MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLD 60
AAV8      MAADGYLPDWLEDNLSEGIREWWALKPGAPKPKANQQKQDDGRGLVLPGYKYLGPFNGLD 60
AAV2      MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLD 60
AAV-rh74  MAADGYLPDWLEDNLSEGIREWWDLKPGAPKPKANQQKQDNGRGLVLPGYKYLGPFNGLD 60

AAV1      KGEPVNAADAAALEHDKAYDQQLKAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120
AAV8      KGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120
AAV2      KGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQ 120
AAV-rh74  KGEPVNAADAAALEHDKAYDQQLQAGDNPYLRYNHADAEFQERLQEDTSFGGNLGRAVFQ 120

AAV1      AKKRVLEPLGLVEEGAKTAPGKKRPVEQSPQ-EPDSSSGIGKTGQQPAKKRLNFGQTGDS 179
AAV8      AKKRVLEPLGLVEEGAKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPARKRLNFGQTGDS 180
AAV2      AKKRVLEPLGLVEEPVKTAPGKKRPVEHSPV-EPDSSSGTGKAGQQPARKRLNFGQTGDA 179
AAV-rh74  AKKRVLEPLGLVESPVKTAPGKKRPVEPSPQRSPDSSTGIGKKGQQPAKKRLNFGQTGDS 180

AAV1      ESVPDPQPLGEPPATPAAVGPTTMASGGGAPMADNNEGADGVGNASGNWHCDSTWLGDRV 239
AAV8      ESVPDPQPLGEPPAAPSGVGPNTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV 240
AAV2      DSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRV 239
AAV-rh74  ESVPDPQPIGEPPAGPSGLGSGTMAAGGGAPMADNNEGADGVGSSSGNWHCDSTWLGDRV 240

AAV1      ITTSTRTWALPTYNNHLYKQISSASTG-ASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQ 298
AAV8      ITTSTRTWALPTYNNHLYKQISNGTSGGATNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ 300
AAV2      ITTSTRTWALPTYNNHLYKQIS--SQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQ 297
AAV-rh74  ITTSTRTWALPTYNNHLYKQISNGTSGGSTNDNTYFGYSTPWGYFDFNRFHCHFSPRDWQ 300

AAV1      RLINNNWGFRPKRLNFKLFNIQVKEVTTNDGVTTIANNLTSTVQVFSDSEYQLPYVLGSA 358
AAV8      RLINNNWGFRPKRLSFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA 360
AAV2      RLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSA 357
AAV-rh74  RLINNNWGFRPKRLNFKLFNIQVKEVTQNEGTKTIANNLTSTIQVFTDSEYQLPYVLGSA 360

AAV1      HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEE 418
AAV8      HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFQFTYTFED 420
AAV2      HQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFED 417
AAV-rh74  HQGCLPPFPADVFMIPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFEFSYNFED 420

AAV1      VPFHSSYAHSQSLDRLMNPLIDQYLYYLNRTQNQSGSAQNKDLLFSRGSPAGMSVQPKNW 478
AAV8      VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQTTGGTANTQTLGFSQGGPNTMANQAKNW 480
AAV2      VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNW 477
AAV-rh74  VPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTQSTGGTAGTQQLLFSQAGPNNMSAQAKNW 480
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AAV1 LPGPCYRQQRVSKTKTDNNNSNFTWTGASKYNLNGRESIINPGTAMASHKDDEDKFFPMS 538
AAV8 LPGPCYRQQRVSTTTGQNNNSNFAWTAGTKYHLNGRNSLANPGIAMATHKDDEERFFPSN 540
AAV2 LPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQS 537
AAV-rh74 LPGPCYRQQRVSTTLSQNNNSNFAWTGATKYHLNGRDSLVNPGVAMATHKDDEERFFPSS 540

AAV1 GVMIFGKESAGASNTALDNVMITDEEEKATNPVATERFGTVAVNFQSSSTDPATGDVHA 598
AAV8 GILIFGKQNAARDNADYSDVMLTSEEEKTTNPVATEEYGIVADNLQQQNTAPQIGTVNS 600
AAV2 GVLIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNT 597
AAV-rh74 GVLMFGKQGAGKDNVDYSSVMLTSEEEIKTTNPVATEQYGVVADNLQQQNAAPIVGAVNS 600

AAV1 MQALPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKNPPPQILIKNTPVPANP 658
AAV8 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP 660
AAV2 QGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANP 657
AAV-rh74 QGALPGMVWQNRDVYLQGPIWAKIPHTDGNFHPSPLMGGFGLKHPPPQILIKNTPVPADP 660

AAV1 PAEFSATKFASFITQYSTGQVSVEIEWELQKENSKRWNPEVQYTSNYAKSANVDFTVDNN 718
AAV8 PTTFNQSKLNSFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTSVDFAVNTE 720
AAV2 STTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYNKSVNVDFTVDTN 717
AAV-rh74 PTTFNQAKLASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTSNYYKSTNVDFAVNTE 720

AAV1 GLYTEPRPIGTRYLTRP- 735
AAV8 GVYSEPRPIGTRYLTRNL 738
AAV2 GVYSEPRPIGTRYLTRNL 735
AAV-rh74 GTYSEPRPIGTRYLTRNL 738

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Table 2
Ubiquitination prediction of AAV8 capsid

Lysine	surface	prediction by ubipred	VECTOR PRODUCED
26	NA(not in VP3)		
31	NA(not in VP3)		
33	NA(not in VP3)		
38	NA(not in VP3)	Low confidence	Yes
51	NA(not in VP3)		
61	NA(not in VP3)	Low confidence	
77	NA(not in VP3)	Low confidence	
122	NA(not in VP3)		
123	NA(not in VP3)		
137	NA(not in VP3)	High confidence	YES
142	NA(not in VP3)		
143	NA(not in VP3)	Low confidence	Yes
162	NA(not in VP3)		
163	NA(not in VP3)		
170	NA(not in VP3)		
259	YES		YES
312			
317			
324			
333	YES	Medium confidence	YES
478			
510	YES		YES
530		Medium Confidence	YES
547	YES		YES
569	YES	Low confidence	YES
623			
643			
652			NOT PACKED
668	YES	Low confidence	YES
691		Low confidence	
695		Low confidence	
709	YES	Low confidence	YES

UbPred software (http://www.ubpred.org/):

Radivojac P., Vacic, V., Haynes, C., Cocklin, R. R., Mohan, A., Heyen, J. W., Goebl, M. G., and Iakoucheva, L. M. Identification, Analysis and Prediction of Protein Ubiquitination Sites. *Proteins: Structure, Function, and Bioinformatics. 78(2):365-380. (2010)*

Table 3

AAV8 mutation	Vector	YIELD (vector concentration vg/RB)	Comparison to WT
WT	hAAT-hFIX16	1.24 E+13	NA
K38R	hAAT-hFIX16	2.32 E+13	decreased
K137R	hAAT-hFIX16	1.53 E+13	increased
K143R	hAAT-hFIX16	2.21 E+13	decreased
K259R	hAAT-hFIX16	1.03 E+13	slight increase
K333R	hAAT-hFIX16	9.46 E+12	increased
K510R	hAAT-hFIX16	1.04 E+13	decreased
K530R	hAAT-hFIX16	1.61 E+13	increased
K547R	hAAT-hFIX16	1.17 E+13	same
K569R	hAAT-hFIX16	1.38 E+13	increased
K668R	hAAT-hFIX16	1.41 E+13	same
K709R	hAAT-hFIX16	1.38 E+13	decreased
K530R/K569R	hAAT-hFIX16	1.34 E+13	same
K(137/333/530) R	hAAT-hFIX16	7.00 E+12	increased
K(137/333/530/709)R	hAAT-hFIX16	6.64 E+12	decreased
K(137/333/530/569)R	hAAT-hFIX16	9.03 E+12	same
K(137/259/333/530/547/569)R	hAAT-hFIX16	7.62 E+12	same
Y(253/275/447/703/707/733)Y	hAAT-hFIX16	9.87 E+12	slight increase
Y(253/275/447/703/707/733)Y + K (137/333/530) R	hAAT-hFIX16	3.24 E+12	decreased
Y (253/275/447/733) F	hAAT-hFIX16	8.58 E+12	slight increase
Y(253/275/447/733)F + K(137/333/530)R	hAAT-hFIX16	3.30 E+12	decreased
K(137/333/530/547)R	hAAT-hFIX16	5.85 E+12	Not tested

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K(137/259/333/530/547)R	hAAT-hFIX16	7.68 E+12	Not tested
Y733F	hAAT-hFIX16	8.30 E+12	increased
Y(733/447) F	hAAT-hFIX16	1.19 E+13	increased
Y(703/707)F	hAAT-hFIX16	1.05 E+13	increased
Y(733/447/253)F	hAAT-hFIX16	1.35 E+13	increased
Y(733/447/275) F	hAAT-hFIX16	7.26 E+12	increased

AAV1 mutation	Vector	YIELD (vector concentration vg/RB)
AAV1 WT	CMV-GFP	6.71E+12
AAV1 K137R/K459R/K533R	CMV-GFP	8.35E+12
AAV1 K(137/459/533/707)R	CMV-GFP	6.97E+12
AAV1 K137R	CMV-GFP	7.96E+12
AAV1 K459R	CMV-GFP	6.57E+12
AAV1 K533R	CMV-GFP	5.00E+12
AAV1 K707R	CMV-GFP	5.66E+12
AAV1 K528R	CMV-GFP	7.28E+12
AAV1 Y50A	CMV-GFP	7.93E+12

MHC delivery

AAV2 mutation	Vector	YIELD (vector concentration vg/RB)
AAV2	CMV GFP	1.15e12
AAV2 K137R	CMV-GFP	6.24E+12
AAV2 K532R	CMV-GFP	5.73E+12
AAV2 K527R	CMV-GFP	4.92E+12
AAV2 Y50A	CMV-GFP	5.59E+12

RH74 mutation	Vector	YIELD (vector concentration vg/RB)
RH74 WT	hAAT-hFIX19	1.51E+13
Rh74 TMR	hAAT-hFIX16	7.62E+12
Rh74 TMR+552R	hAAT-hFIX16	6.91E+12
Rh74 TMR+709R	hAAT-hFIX16	4.18E+12
Rh74 HMF	hAAT-hFIX16	6.83E+12
Rh74 HMR+195/202	hAAT-hFIX19	3.79E+12
Rh74 HMR+195/202+K38R	hAAT-hFIX19	3.00E+12
Rh74 HMR+195/202+K51R	hAAT-hFIX19	3.54E+12
Rh74 HMR+195/202+K61R	hAAT-hFIX19	5.14E+12
Rh74 HMR+195/202+K77R	hAAT-hFIX19	5.86E+12
Rh74 HMR+195/202+K(122/123)R	hAAT-hFIX19	5.51E+12
Rh74 HMR+195/202+K(142/143)R	hAAT-hFIX19	5.75E+12
RHM13_1	hAAT-hFIX19	7.3132E+12
RHM 17_1	hAAT-hFIX19	5.928E+12
RHM14_2	hAAT-hFIX19	6.084E+12
RHM15_1	hAAT-hFIX19	5.0396E+12

Definition of TMR= K(137/333/530)R
Definition of HMF= Y(253/275/447/703/707/733)F
Definition of HMR : K(137/259/333/530/552/569)R
Definition of 195/202 : G195A+ L199V+ S201P+ G202N

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WHAT IS CLAIMED IS:

1. An improved adeno-associated virus (AAV) vector comprising a VP1 capsid protein comprising one or more lysine substitutions, said vector further comprising a minigene comprising AAV inverted terminal repeats and a heterologous nucleic acid sequence operably linked to regulatory sequences which direct expression of a product from the heterologous nucleic acid sequence in a host cell, said lysine substitution being effective to inhibit ubiquitination of said capsid protein, thereby increasing transduction of said AAV vector into a target cell.

2. The AAV vector of claim 1 having a serotype selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV-rh74, AAV5, AAV6, AAV7, AAV8 and AAV9.

3. The AAV vector of claim 1, comprising a VP1 capsid protein having at least one lysine substitution at a lysine residue shown in the tables, wherein said at least one substitution increases transduction efficiency.

4. The AAV vector of claim 2 comprising an altered AAV8 VP1capsid protein selected from the group consisting of AAV8 K137R, AAV8 K333R and AAV8 K530R.

5. The AAV vector according to any one of the previous claims wherein the expression product of the heterologous nucleic acid sequence is a therapeutic peptide or nucleic acid.

6. The AAV vector according to claim 5, wherein the therapeutic peptide is a coagulation factor selected from the group consisting of Factor VIII, Factor IX or a functional fragment thereof.

7. The AAV8 vector according to claim 4, wherein the expression product of the heterologous nucleic acid sequence is an IgG, IgM, IgA, IgD, IgE, chimeric immunoglobulin, humanized antibody, or a single chain antibody.

8. The AAV8 vector according to claim 7, wherein the expression product of the heterologous nucleic acid sequence is a chimeric immunoglobulin.

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9. The AAV8 vector according to claim 4, wherein the expression product of the heterologous nucleic acid sequence is a single chain antibody.

10. The AAV vector of claim 5 wherein the expression product is an antiviral RNAi.

11. The AAV vector of claim 10, wherein said inhibitory RNA is effective to inhibit HCV infection and replication.

12. The AAV vector of claim 10, wherein said inhibitory RNA is effective to inhibit expression of a eukaryotic target gene.

13. The AAV vector of claim 4, wherein the transgene encodes a disease-modifying cytokine.

14. The AAV vector of claim 4, wherein the transgene encodes a pair of zinc finger nucleases.

15. The AAV vector of claim 1 comprising 2, 3, or 4 lysine substitutions.

16. The AAV vector of claim 3 further comprising a second AAV vector comprising a VP1 capsid protein having at least one lysine substitution at a lysine residue shown in the tables, wherein said substitution reduces transduction efficiency, delivery of said second AAV vector being effective to neutralize an antibody response to a first AAV vector.

17. A pharmaceutical composition comprising the AAV vector according to claim 3, and a physiological compatible carrier therefor.

18. A cell culture comprising the AAV vector according to claim 1.

19. A method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with the pharmaceutical composition of according to claim 17 wherein said AAV vector comprises the transgene, wherein the presence of said lysine substitution in said VP1 capsid protein is associated a reduction in ubiquitination of said capsid, thereby increasing transduction efficiency of target cells with said vector.

20. The method of claim 19, wherein said transgene is Factor IX.

21. A pharmaceutical composition comprising the AAV vectors according to claim 16 , and a physiological compatible carrier therefor.

22. A method of delivering a transgene to a cell in a subject, said method comprising the step of contacting the cell with the pharmaceutical composition of according to claim 21 wherein said first AAV vector comprises the transgene, wherein the presence of said lysine substitution in said VP1 capsid protein is associated a reduction in ubiquitination of said capsid, thereby increasing transduction efficiency of target cells with said vector and said second vector exhibits reduced transduction efficiency relative to wild type and is effective to neutralize an undesired antibody response to said first AAV vector.

23. The method of claim 22, wherein said transgene is Factor IX.

AAV1

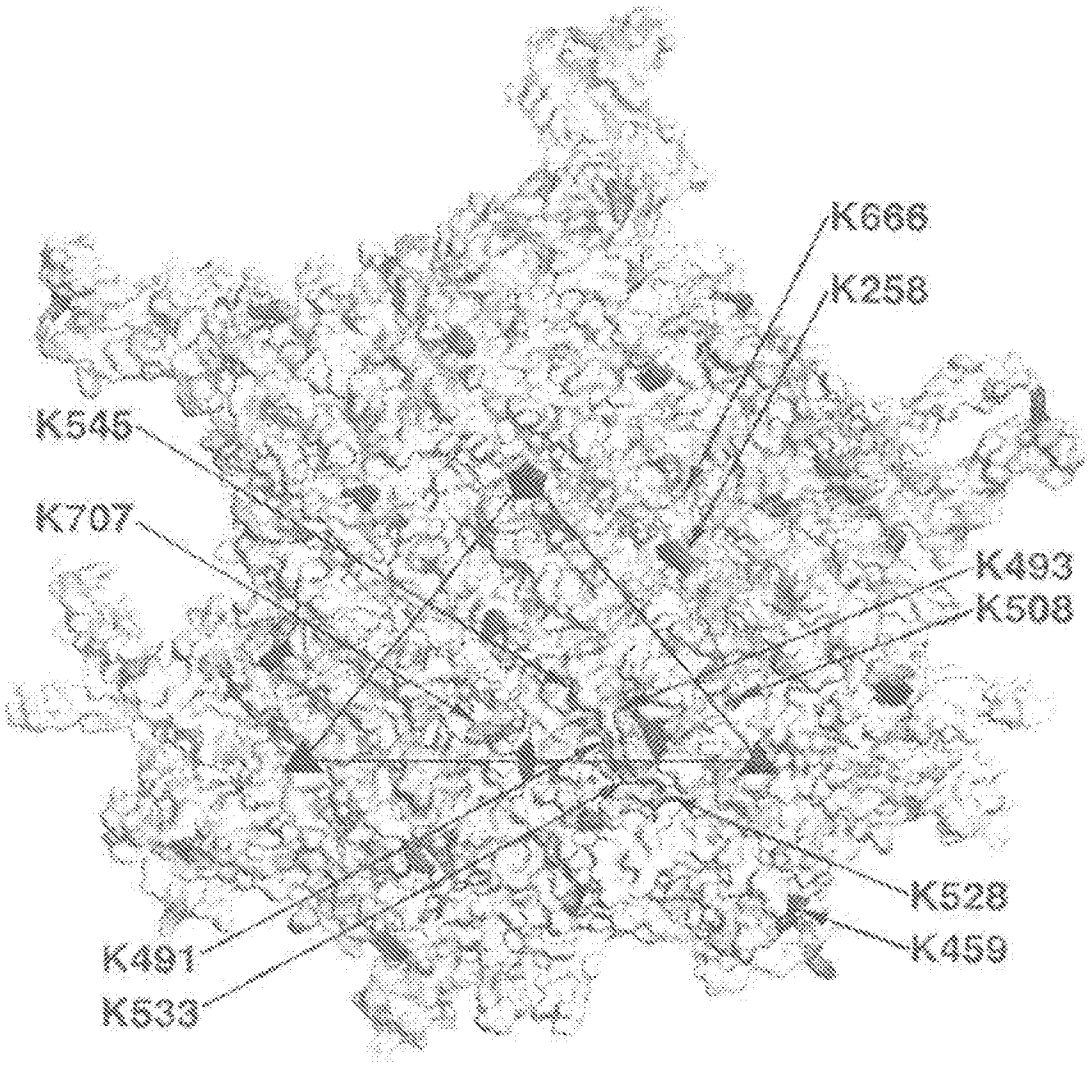


Figure 1A

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AAV2

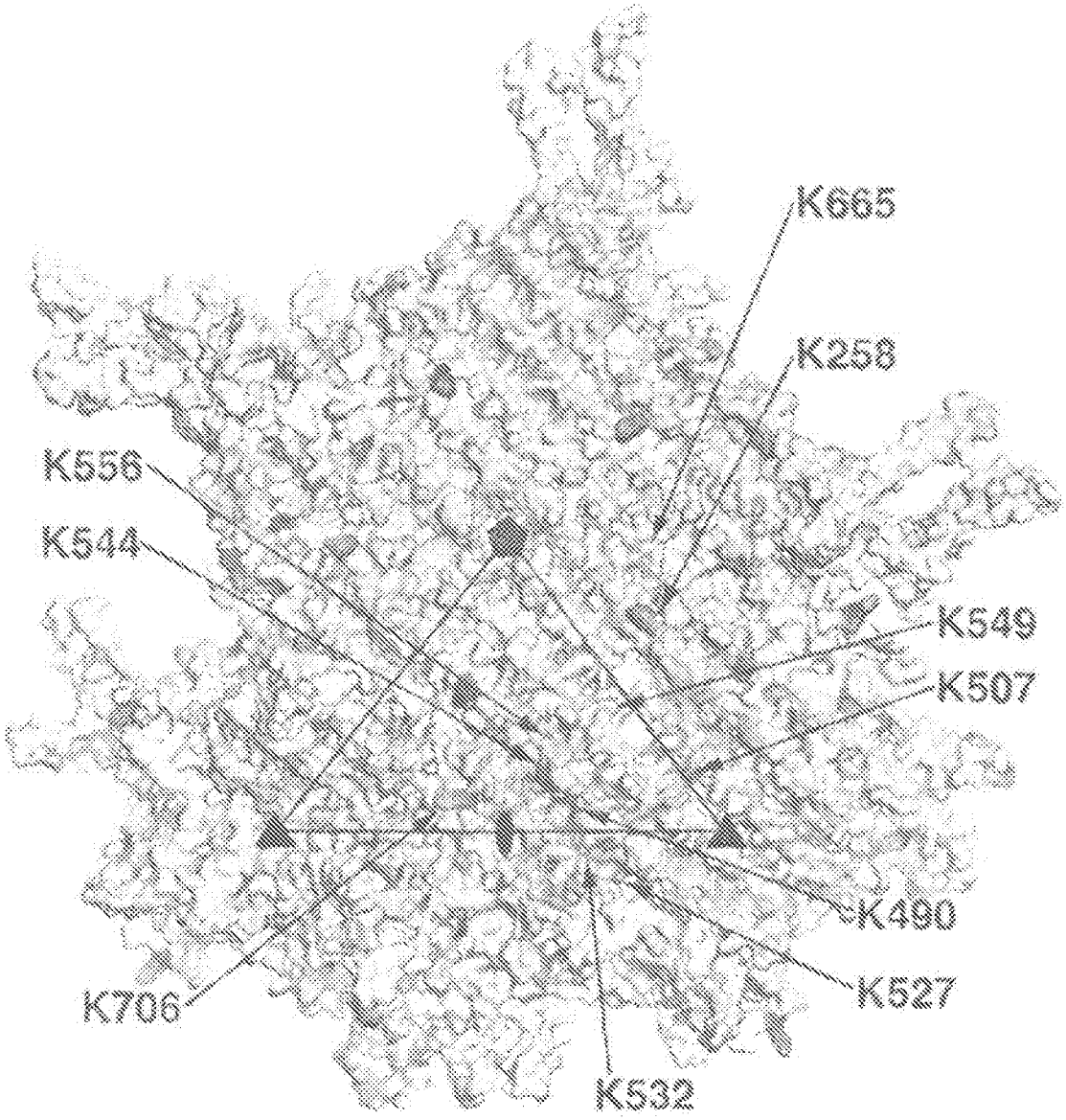


Figure 1B

AAV8

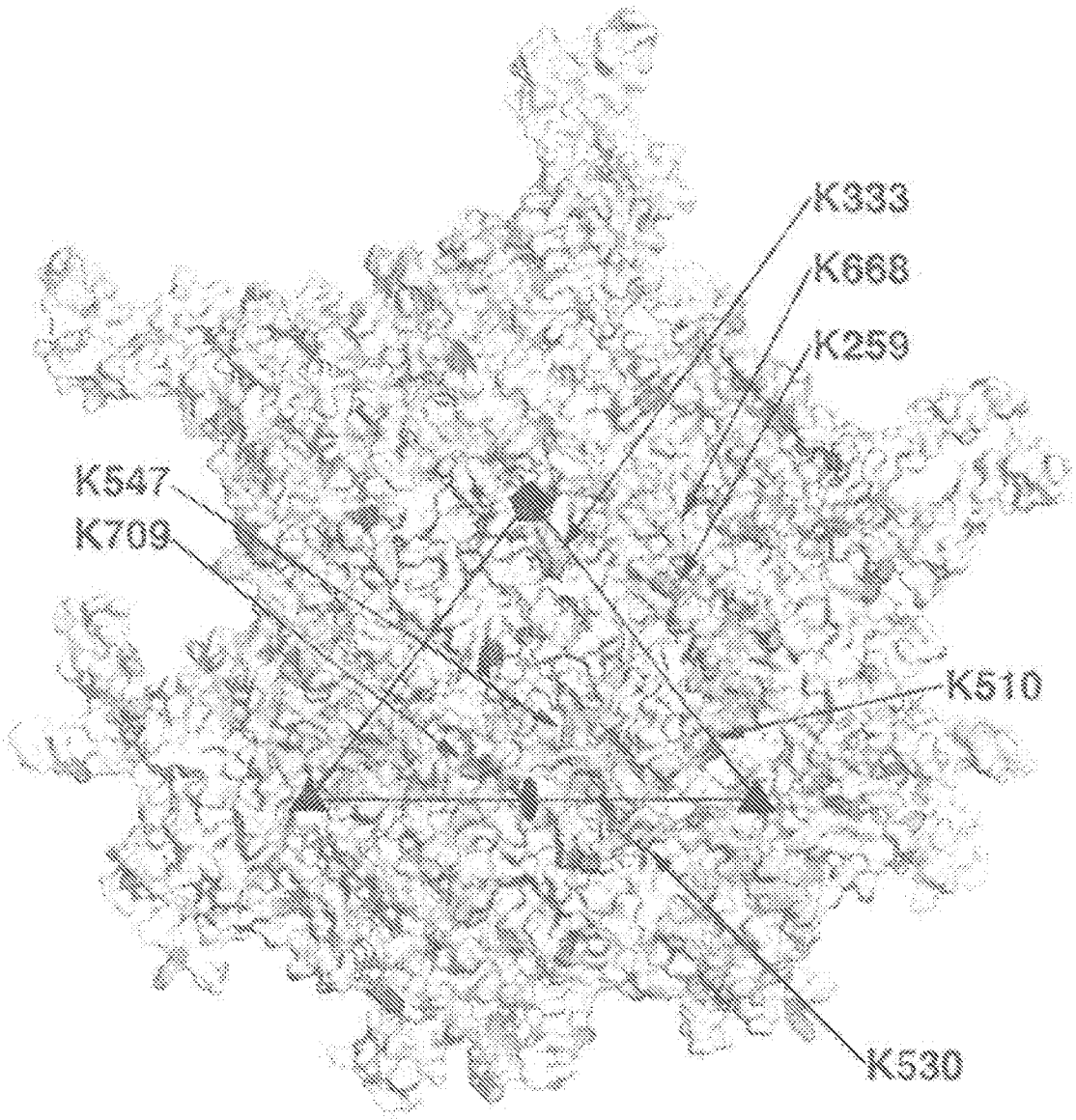


Figure 1C

Sarepta Exhibit 1002, page 1299

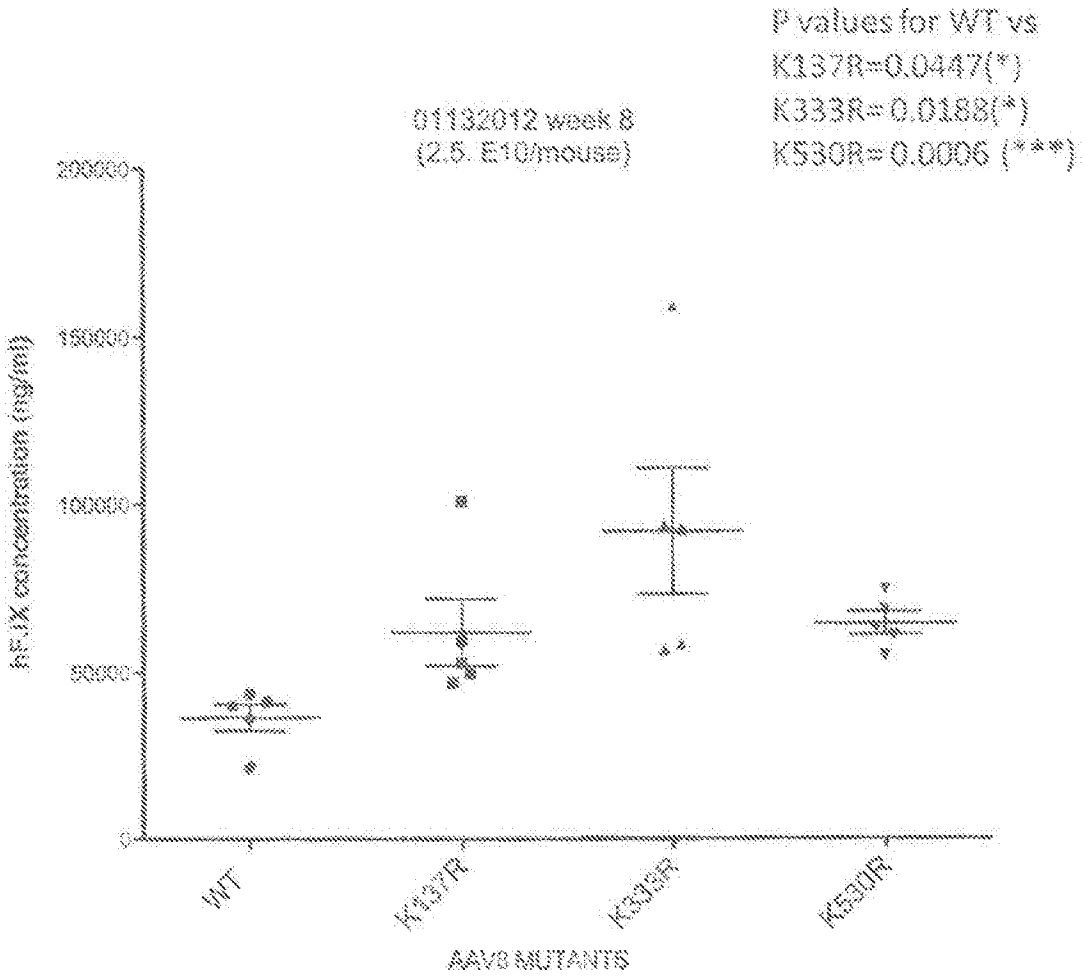


Figure 2A

Sarepta Exhibit 1002, page 1300

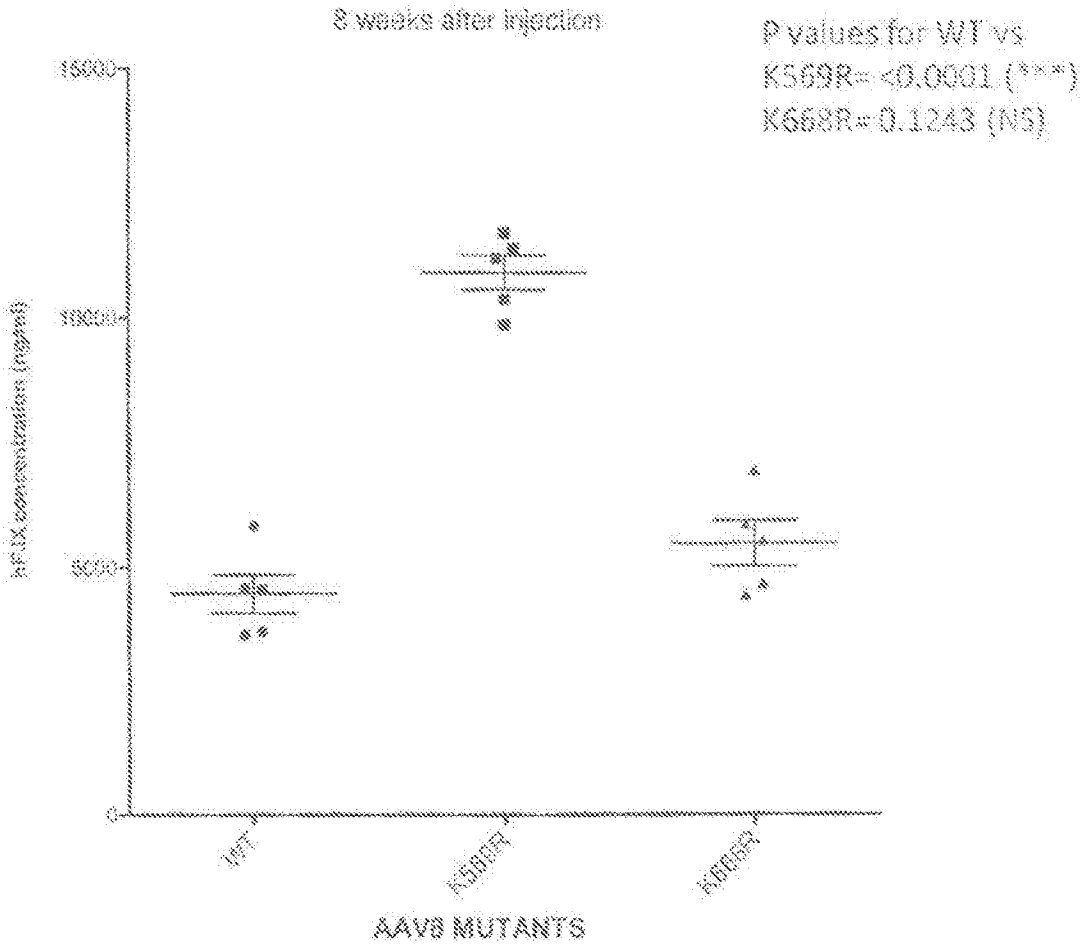


Figure 2B

Sarepta Exhibit 1002, page 1301

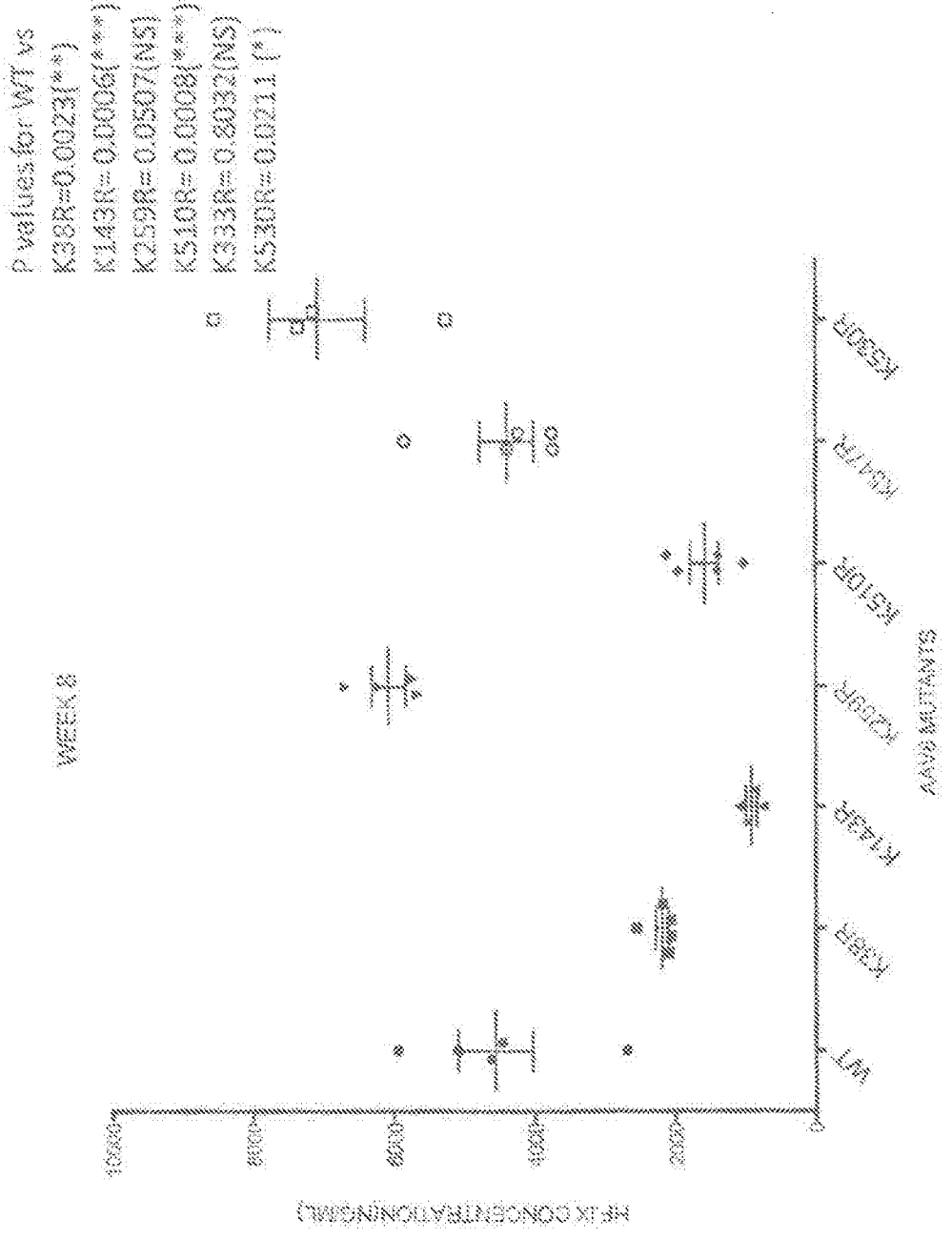

P values for WT vs
K38R=0.0023(**)
K143R= 0.0006(***)
K259R= 0.0507(NS)
K510R= 0.0008(***)
K333R= 0.6032(NS)
K530R= 0.0211 (*)
WEEK 8
AAV9 VARIANTS
WT
K530R
K333R
K259R
K143R
K38R
K510R
NF-L (CONCENTRATION/NGML)
0
2000
4000
6000
8000
10000

Figure 2C

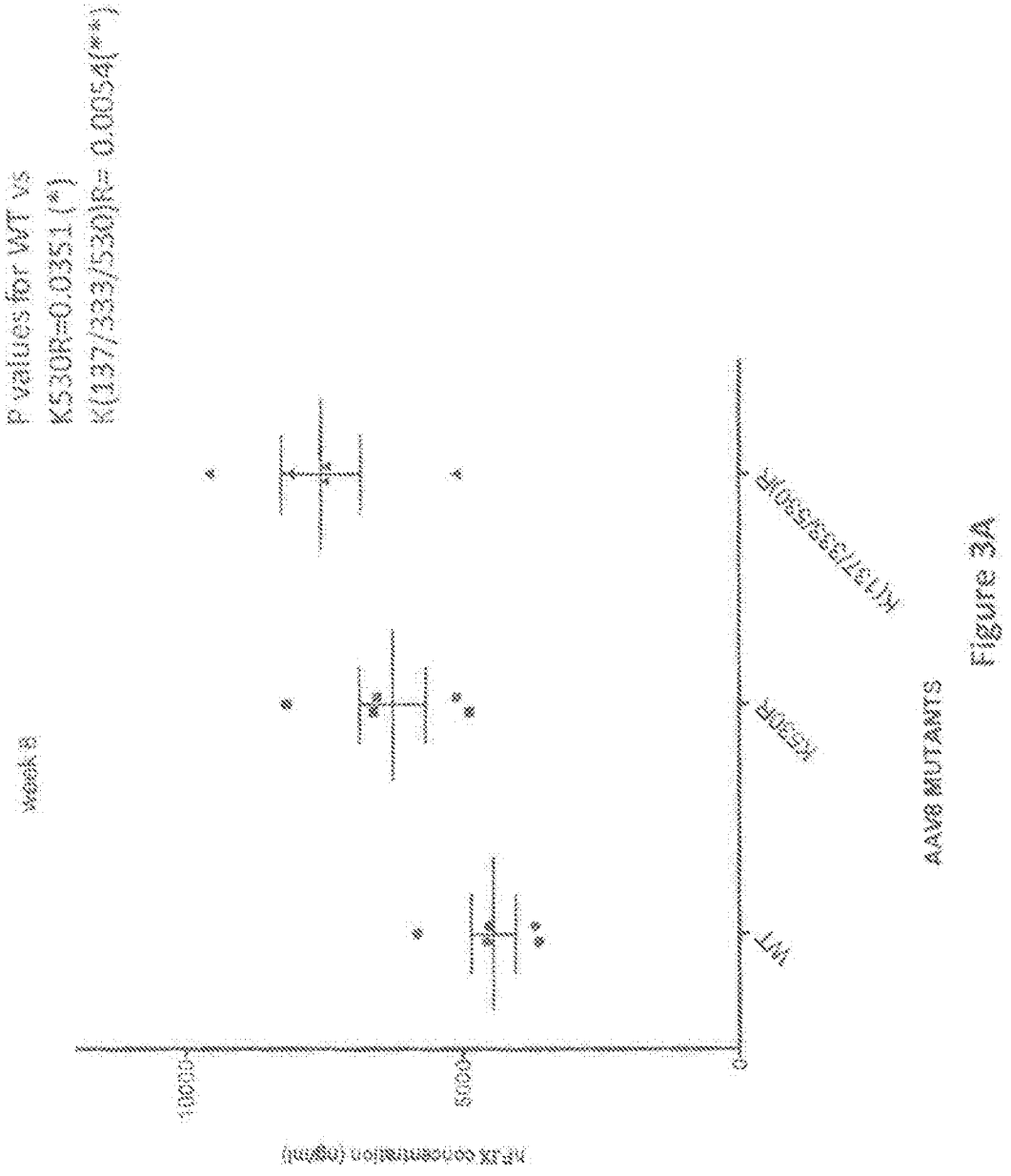

P values for WT vs
K530R=0.0351 (*)
K(137/333/530)R= 0.0054(**)
week 8
vg/kg
AAV8 MUTANTS
WT
K530R
K(137/333/530)R

Figure 3A

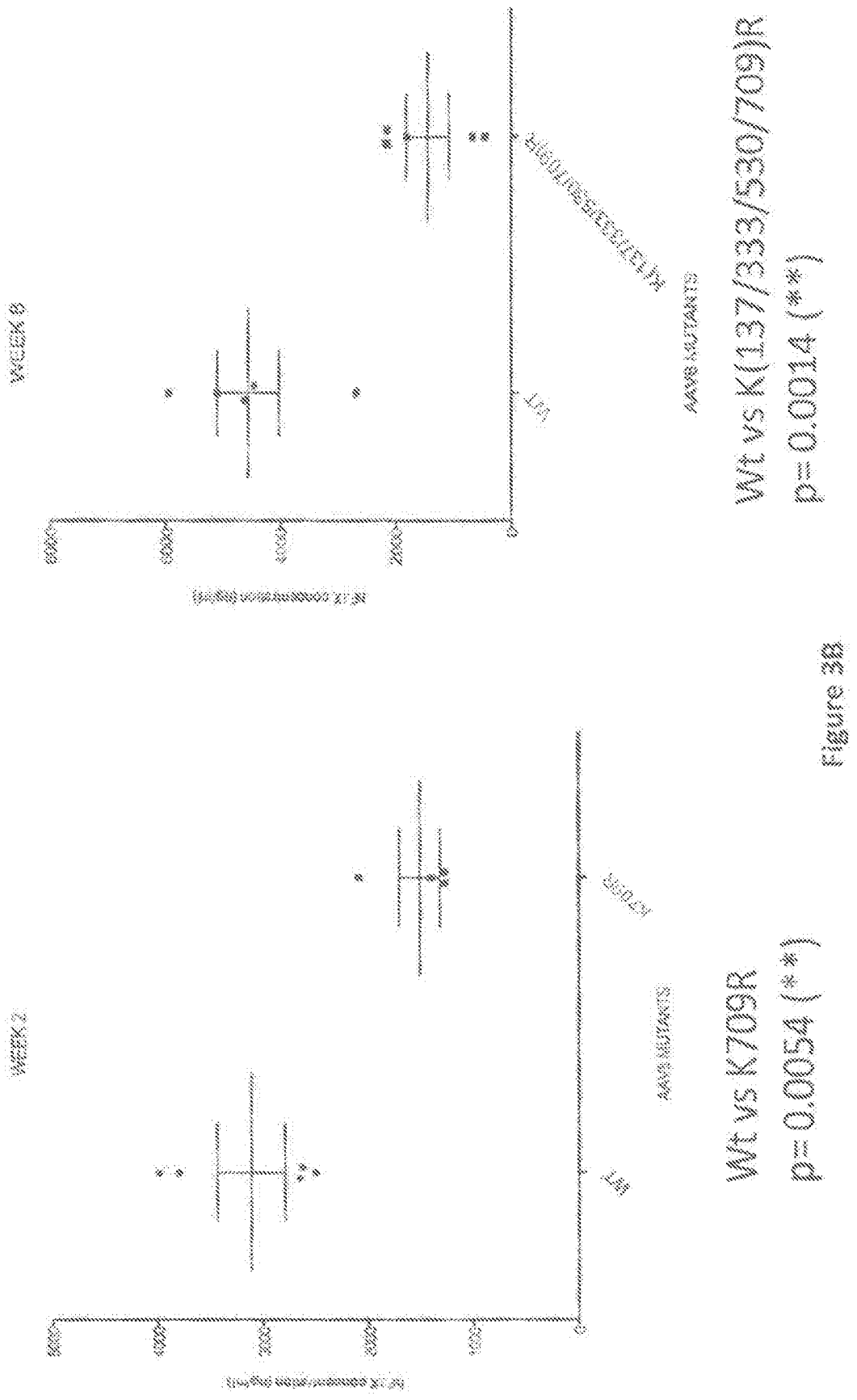


Figure 3B

Sarepta Exhibit 1002, page 1304

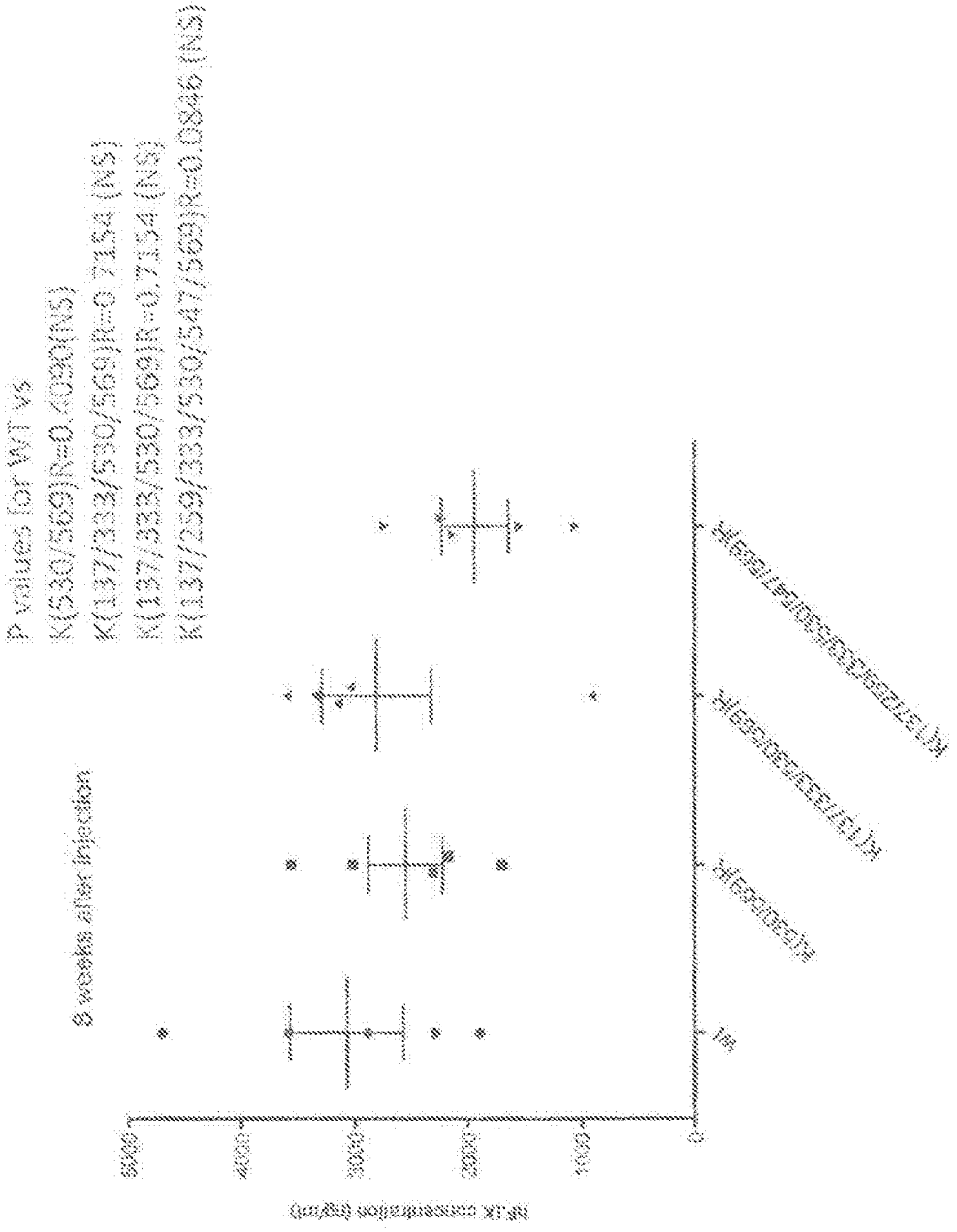

P values for WT vs
K(530/569)R=0.4030(NS)
K(137/333/530/569)R=0.7154 (NS)
K(137/333/530/560)R=0.7154 (NS)
K(137/259/333/530/547/560)R=0.0846 (NS)
8 weeks after injection
hFIX concentration (ng/ml)
6000
4000
3000
2000
1000
0
WT
K(137/259/333/530/547/560)R
K(137/333/530/560)R
K(530/569)R
AAV8 MUTANTS

Figure 3C

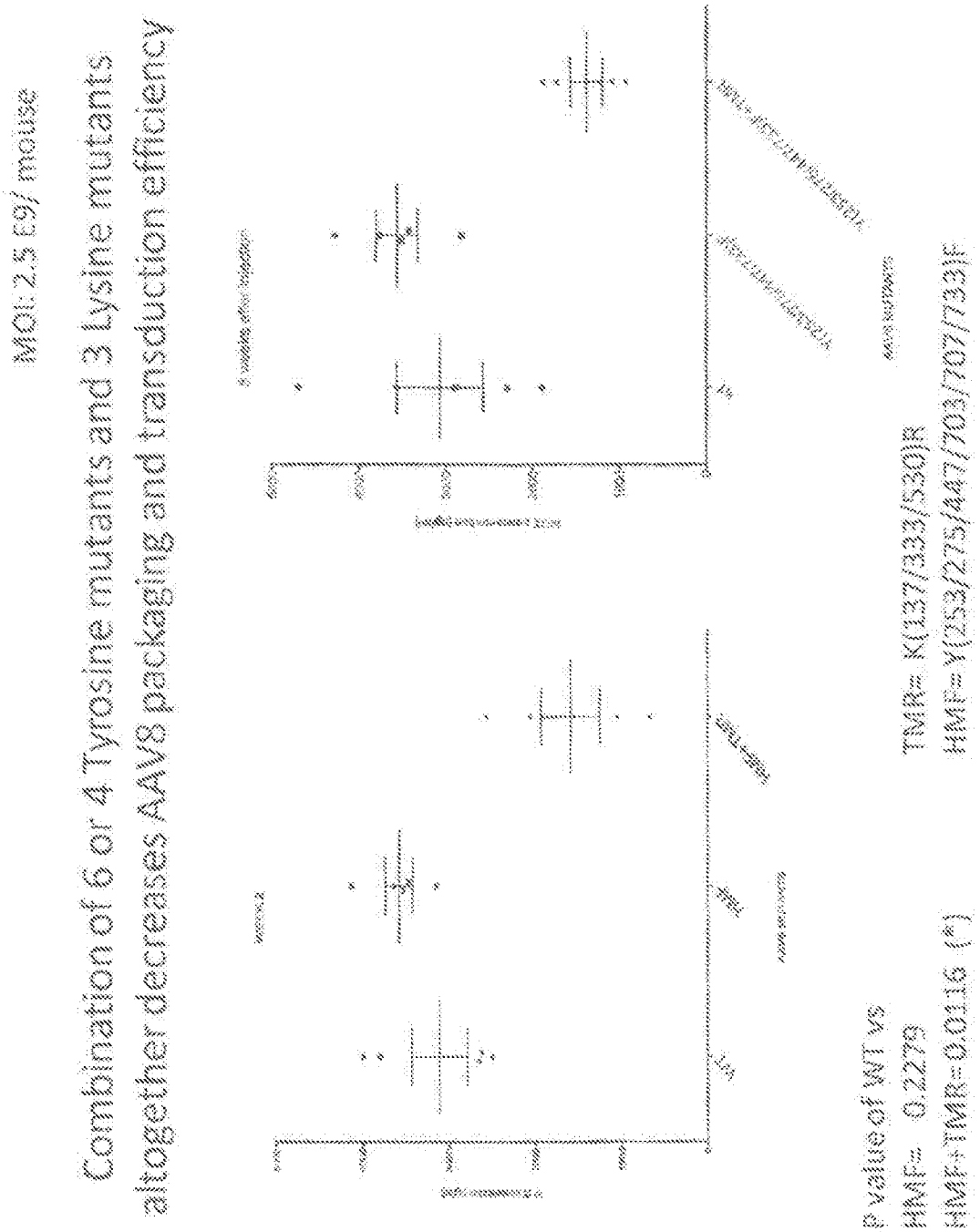

MOI: 2.5 E9/ mouse
Combination of 6 or 4 Tyrosine mutants and 3 Lysine mutants altogether decreases AAV8 packaging and transduction efficiency
p value of WT vs
HMF= 0.2279
HMF+TMR=0.0116 (*)
TMR= K(137/333/530)R
HMF= Y(253/275/447/703/707/731)F

Figure 3D

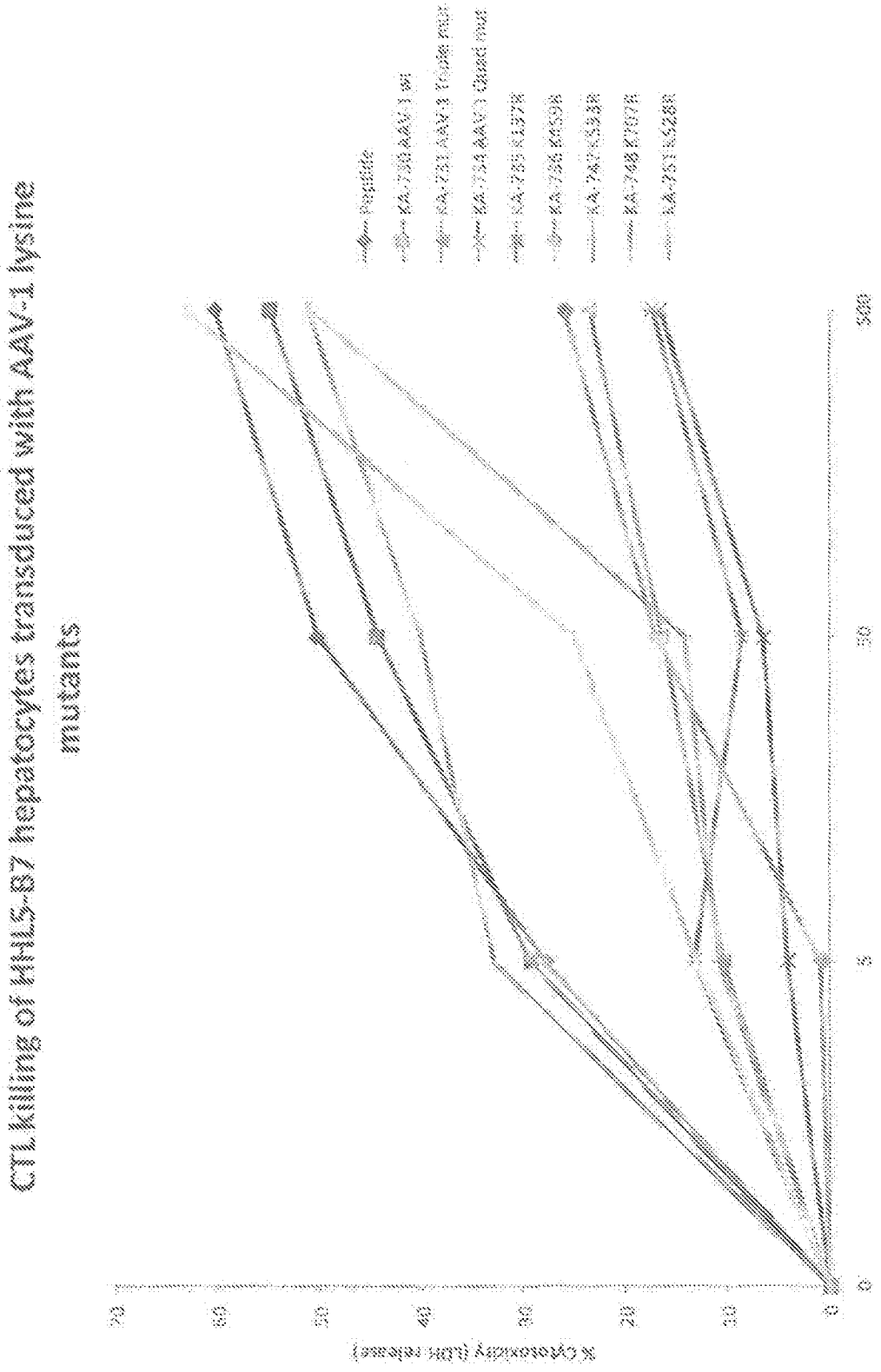

CTL killing of HHL5-87 hepatocytes transduced with AAV-1 lysine mutants
% Cytotoxicity (51Cr release)
MOI (K)
Peptide
AA-738 AAV-1 wt
AA-738 AAV-1 lysine mut
AA-734 AAV-1 Quad mut
AA-265 K137R
AA-736 K459R
AA-787 S383R
AA-748 T707R
AA-531 S528R

Figure 4A

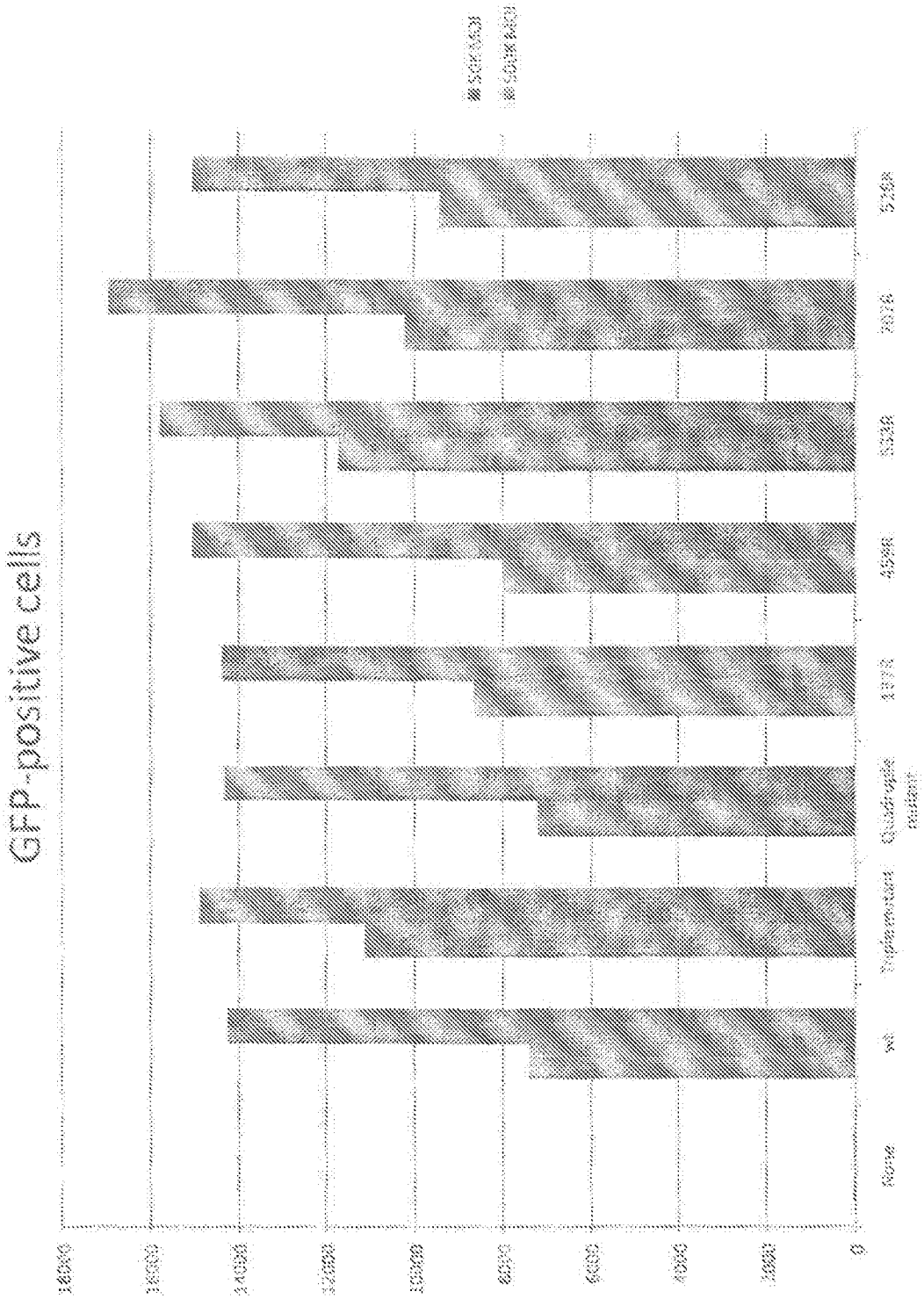

GFP-positive cells
16000
14000
12000
10000
8000
6000
4000
2000
0
None
WT
Triplet-mutant
Quadruple-mutant
4YFF
4596
5238
7076
5259

Figure 4B

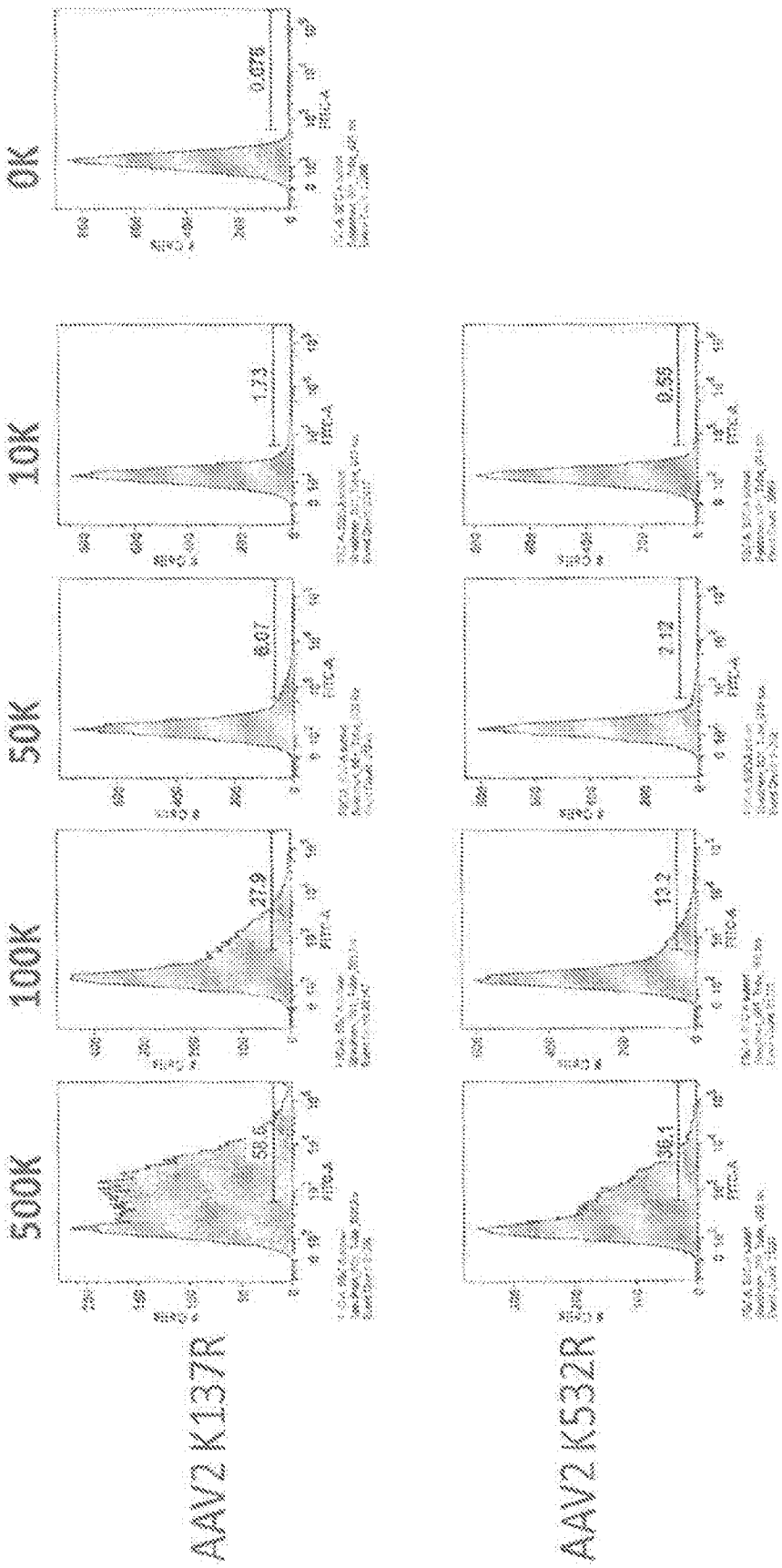


Figure 5A

Sarepta Exhibit 1002, page 1309

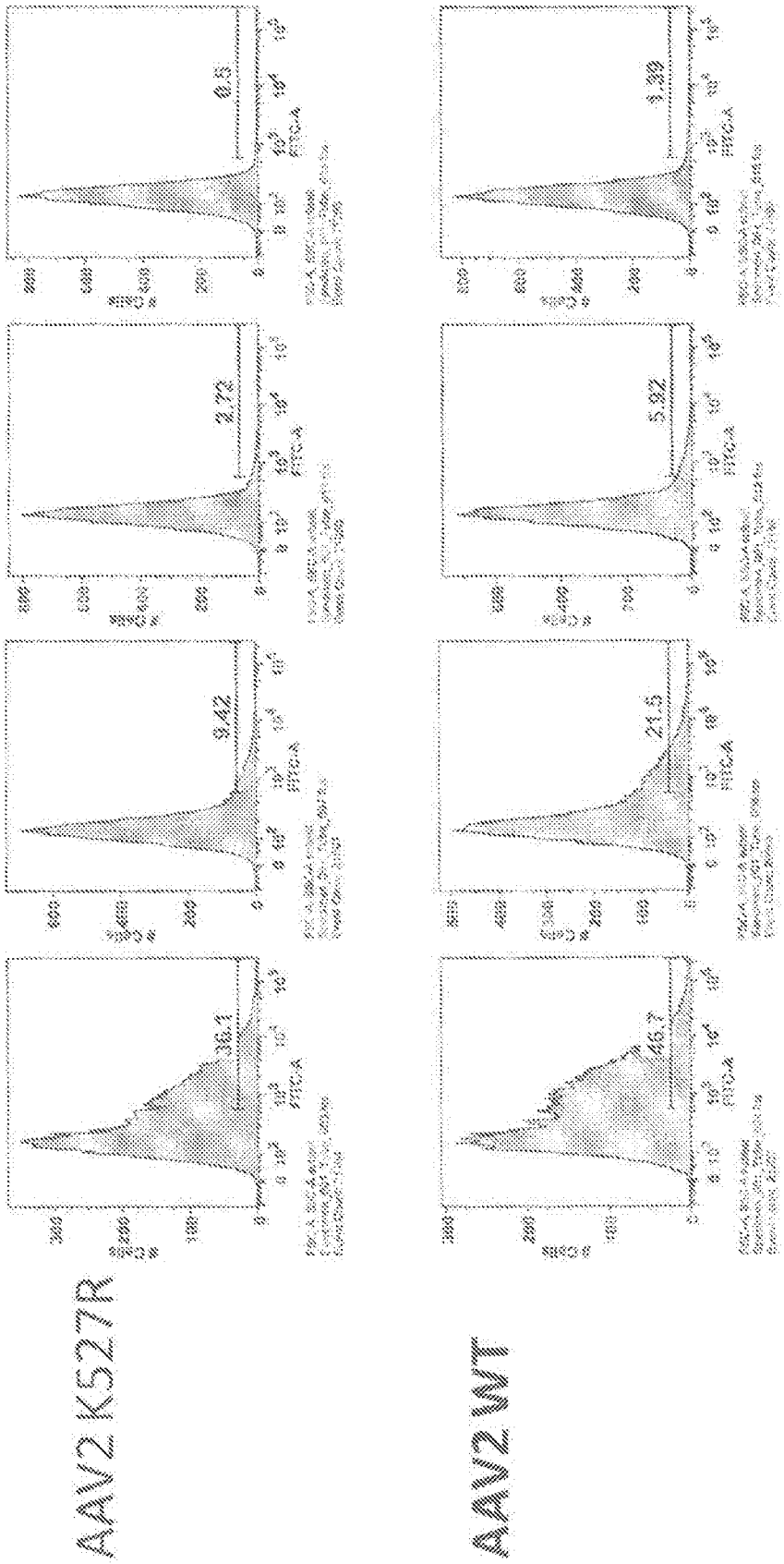


Figure 5A (cont'd)

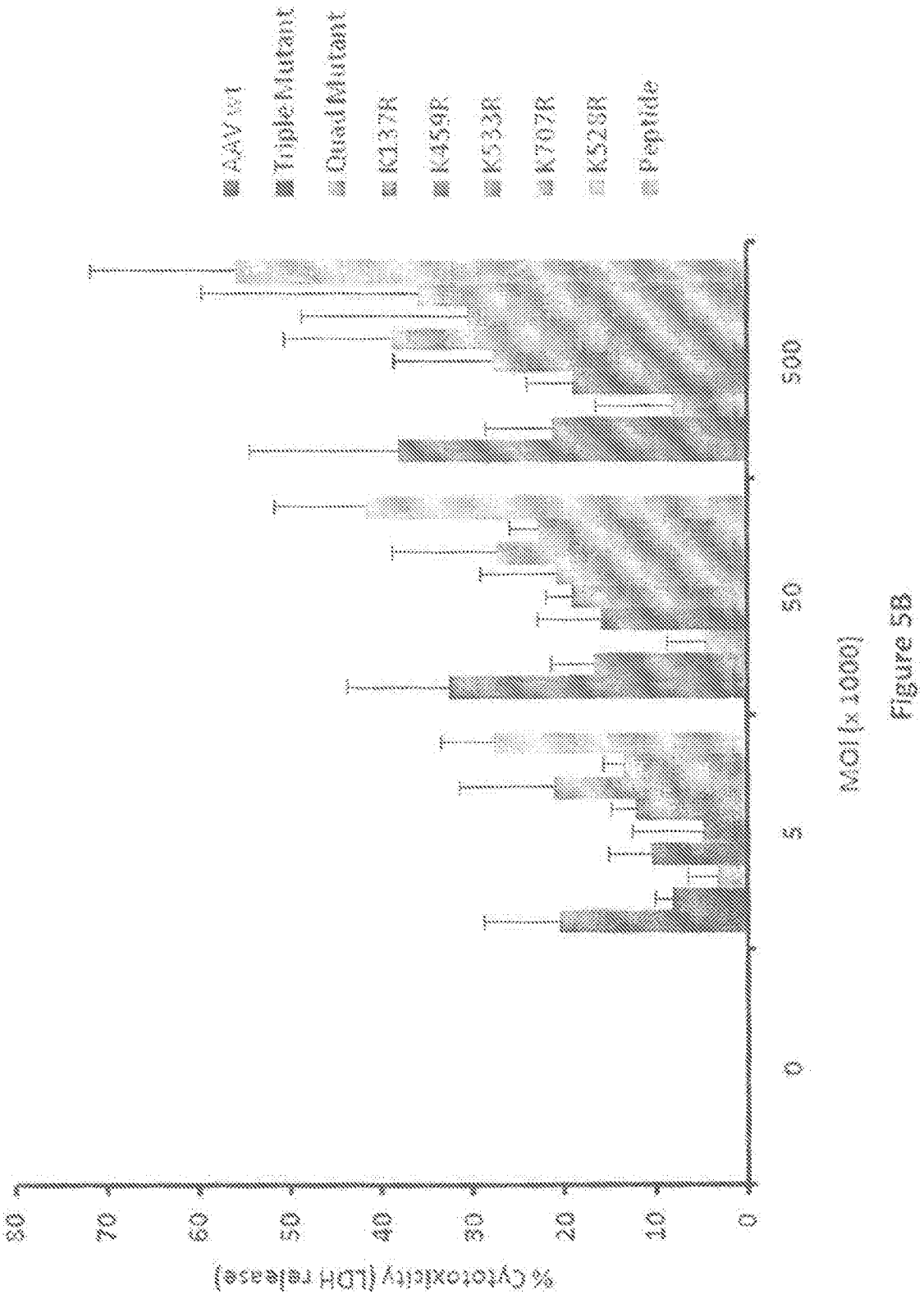


Figure 5B

Sarepta Exhibit 1002, page 1311

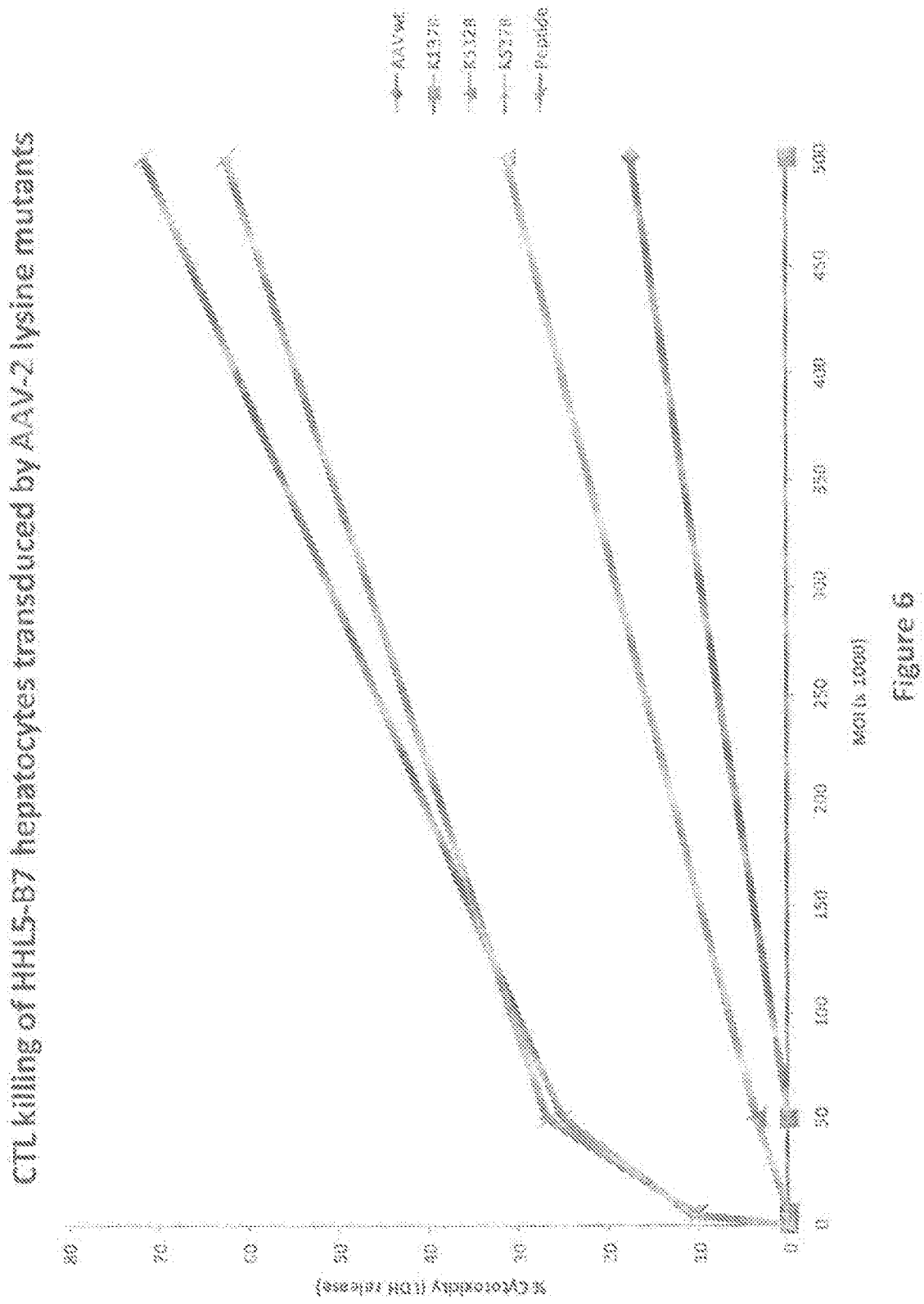


Figure 6

Sarepta Exhibit 1002, page 1312

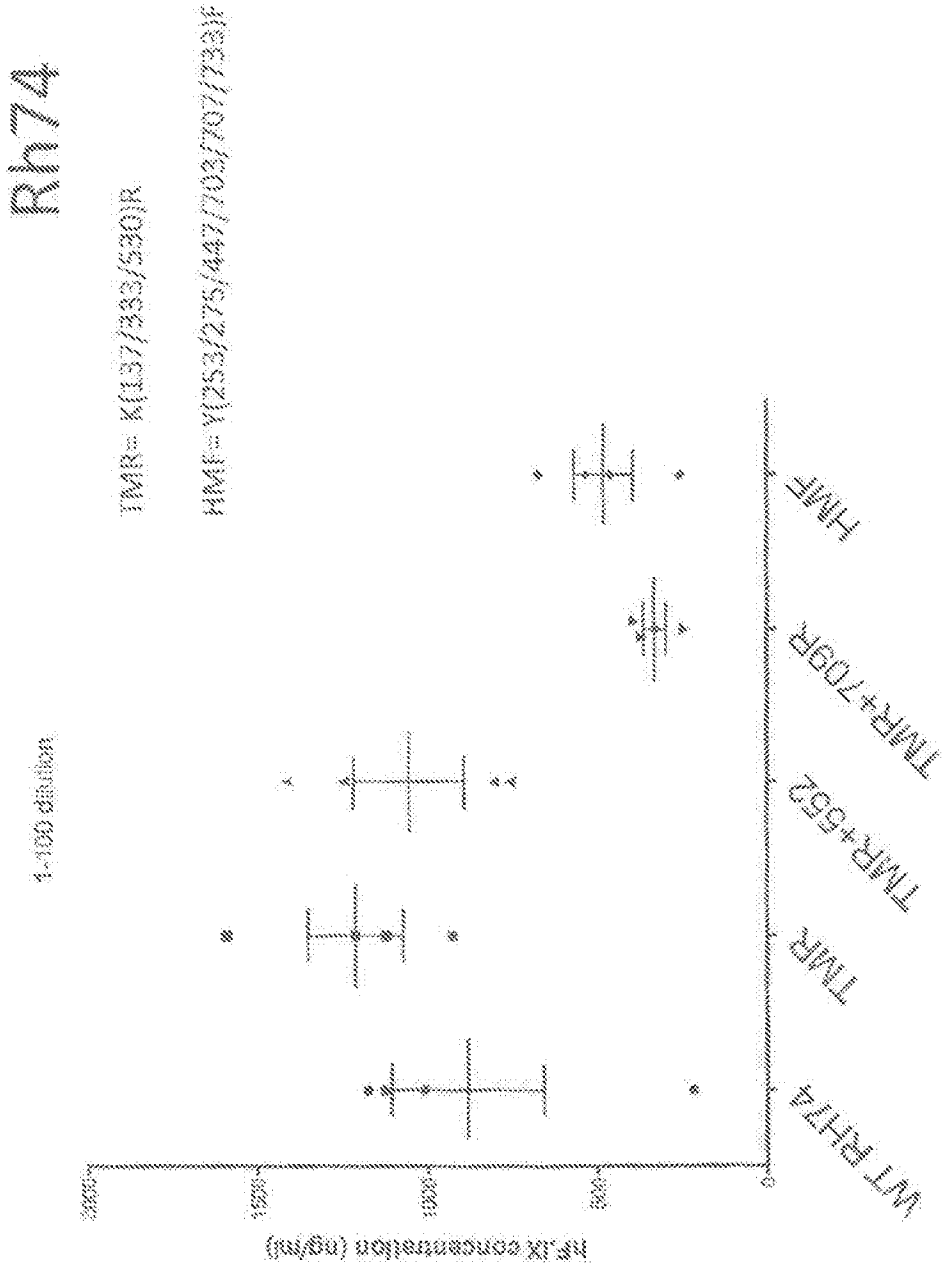
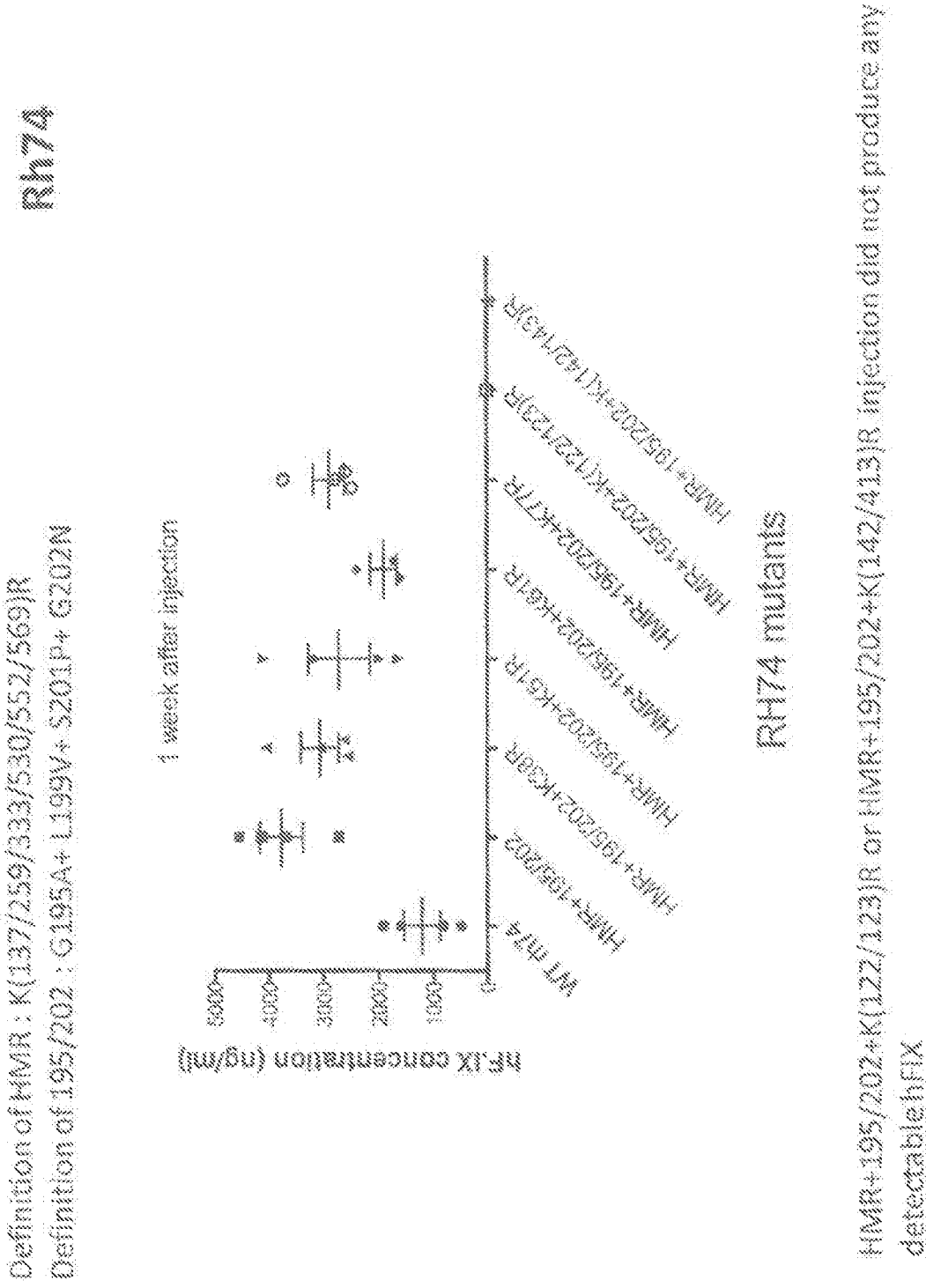


Figure 7A

Sarepta Exhibit 1002, page 1313



HMR+195/202+K(122/123)R or HMR+195/202+K(142/413)R injection did not produce any detectable hFIX

Figure 7B

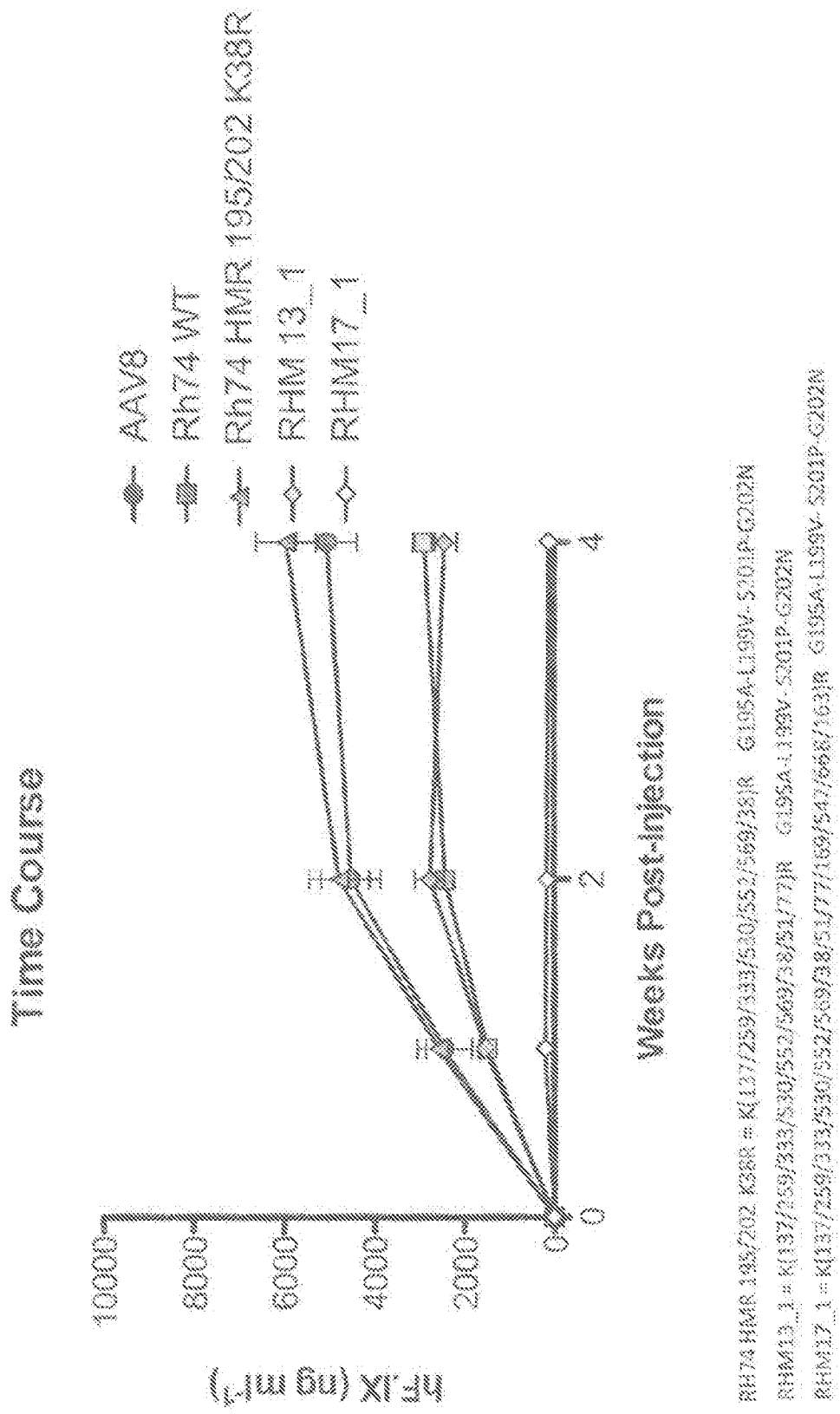


Figure 7C

Sarepta Exhibit 1002, page 1315

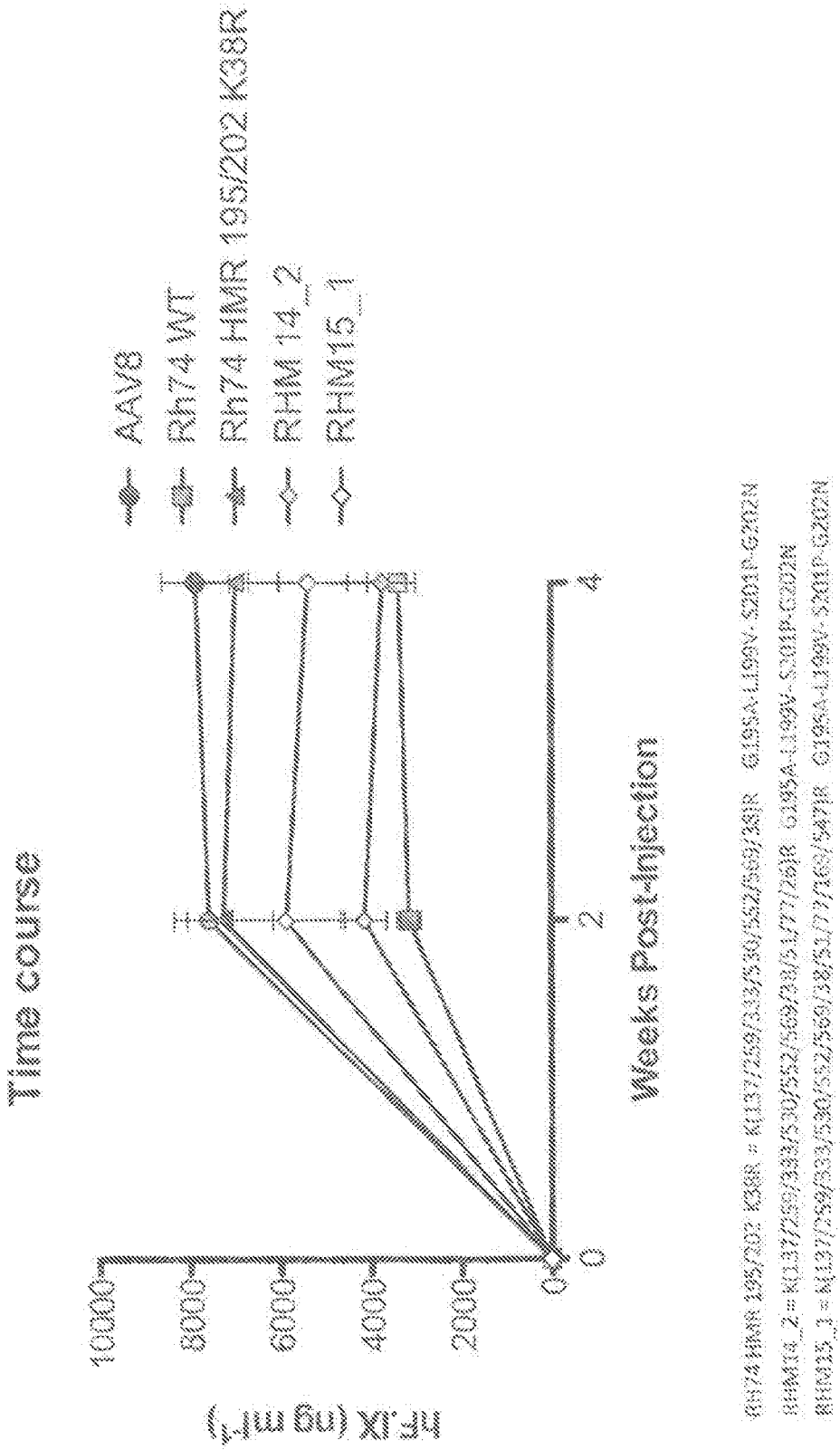

Time course
AAV8
Rh74 WT
Rh74 HMR 195/202 K38R
RHM 14_2
RHM15_1
hFIX (ng ml⁻¹)
10000
8000
6000
4000
2000
0
0
2
4
Weeks Post-Injection
Rh74 HMR 195/202 K38R = KU377259/G33S30A52T69I38R G195A-L199V- S201P-G202N
RHM14_2 = KU377259/G33S30A52S69I38R/Q61T77I260R G195A-L199V- S201P-G202N
RHM15_1 = KU377259/G33S30A52S69I38R/Q61T77I260R/Q478R G195A-L199V- S201P-G202N

Figure 7D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US13/37170

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/864, 7/01; C07K 14/015; A61K 48/00; C07H 21/04 (2013.01)
USPC - 435/320.1, 115, 424/93.2, 233.1, 199.1; 514/44R
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 15/00, 15/864, 15/861, 15/86, 15/85, 15/79, 15/63; A61K 35/76, 35/713, 48/00 (2013.01)
USPC: 435/320.1, 948, 457, 456, 455, 440, 91.1, 173.3, 235.1, 115, 71.1, 69.1; 424/93.2, 93.21, 93.1, 93.6, 204.1, 205.1, 233.1, 192.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; DialogPRO; Pubmed; Google; Google Scholar; DialogPRO; PubMed; 'AAV,' 'rAAV,' adeno*, virus, lysine, ubiquitin, proteasom*, 'factor VIII,' 'factor IX,' substitut*, 'vp1,' vector, transduc, serotype

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2009/0197338 (VANDENBERGHE, L et al.), August 6, 2009; abstract; paragraphs [0005], [0006], [0008], [0013], [0026], [0066], [0076], [0169]	1-4, 5/1-5/4, 6/5/1-6/5/4, 7-9, 10/5/1-10/5/4, 11/10/5/1-11/10/5/4, 12/10/5/1-12/10/5/4, 13, 17-20 ----- 14-16, 21-23
Y	WO 2011/126808 A1 (WILSON, JM et al.), October 13, 2011; page 2, lines 12-30; page 29, lines 12-26	14
Y	US 7629322 B2 (KLEINSCHMIDT, J et al.), December 8, 2009, column 1, lines 7-14; lines 16-30; column 5, lines 46-52	15
Y	US 6127525 A (CRYSTAL, RG et al.), November 28, 2011; column 3, lines 59-67 to column 4, lines 1-12	16, 21-23
A	SANLIOGLU, S et al. Rate Limiting Steps Of AAV Transduction And Implications For Human Gene Therapy. Current Gene Therapy. 2001, Vol. 1, No. 2, pp 137-147; page 140, column 1, paragraph 2 to page 141, column 1, paragraph 1.	1
A	CIECHANOVER, A. The Ubiquitin-Proteasome System: Death Of Proteins is Required For Life Of Cells. Celltransmissions. September 2003, Vol. 19, No. 3, pp 3-10; <URL="http://www.sigmaaldrich.com/technical-service-home/publications/cell-transmissions.html"> page 3, column 1, paragraph 3 to column 2, paragraph 1.	1

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 August 2013 (13.08.2013)	21 AUG 2013

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1615	2	100	200
INDEPENDENT CLAIMS IN EXCESS OF 3	1614	11	460	5060
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**5460**

Electronic Acknowledgement Receipt

EFS ID:	36662812
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	23-JUL-2019
Filing Date:	
Time Stamp:	16:55:34
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$5460
RAM confirmation Number	072419INTEFSW00004655031952
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Prelim_for_resp_to_Notice.pdf	103105 281db5c61983866da8228032bcc46f7caca668c3	yes	11
	Multipart Description/PDF files in .zip description				
	Document Description		**Start**	**End**	
	Preliminary Amendment		1	1	
	Specification		2	2	
	Claims		3	9	
	Applicant Arguments/Remarks Made in an Amendment		10	11	
Warnings:					
Information:					
2	Specification	Sub_Spec_-_clean_copy.pdf	524546 adb9df80739d1793308d6a5d6946cb905fbe122f	no	98
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Information:					
3	Specification	Sub_Spec_-_Marked-up_copy.pdf	537390 3754627ad5fd281c3821b9bdae3c60bbcb021bdd	no	99
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This is not an USPTO supplied IDS fillable form					

5	Foreign Reference	WO_2003_042397_A2.pdf	20678974 7e10419a60942cf89835b58169759c41af6d0ada	no	461
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6	Foreign Reference	WO_2004_027019_A2_Part1.pdf	15293089 202d4b12bdbcf014f5d6f7ea5955ac71827b0dc7	no	90
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9	Foreign Reference	WO-2013_158879_A1.pdf	7498336 0daac765baaff92aba66aa927437c29a54f8530c	no	60
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10	Non Patent Literature	AHMADIANKIA_2013_813-821.pdf	1028248 4837f5f2d0454bb20e6e021b5ae6ed489d2ad84d	no	9
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11	Non Patent Literature	ARNESEN_2009_8157-8162.pdf	659638 88271294bd6ebfb1e0f89b2e127646f06bf45116	no	6
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12	Non Patent Literature	ASOKAN_2006_8961-8969.pdf	700350 25ef54bbd84c6999f7dcc5b88a81606d7b3711e7	no	9
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13	Non Patent Literature	BARK_2001_1774-1775.pdf	352289 55416f278fb5d9f01c21829dc44b799831dd507e	no	2
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14	Non Patent Literature	BARTLETT_2000_2777-2785.pdf	970522 f7f977596b4c16e151db73a4393b9f0c210b0681	no	9
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15	Non Patent Literature	BEN-SAADON_2004_41414-41421.pdf	1310093 7f71352ca33ad647e0ce2c6c5455c3414b0647ee	no	9
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16	Non Patent Literature	BLEKER_2005_2528-2540.pdf	1198503 10d259790fa0fdec3e697f6240025543f1b01624	no	13
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17	Non Patent Literature	BOSSIS_2003_6799-6810.pdf	1132374 a45a0296b7eb5b00721bdb4bf8b9d99a19eb6760	no	12
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19	Non Patent Literature	COULTON_2010_3235-3243.pdf	2078671 52cab6c6efdf88b5c01a3cdbca8036f7f8173591	no	23
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22	Non Patent Literature	GAO_2002_11854-11856.pdf	955952 609be4c0f38e49f0e9e22b5f5b61b5fe3da8dcf6	no	6
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33	Non Patent Literature	MAJCHRZYKIEWICZ-KOEHORST_2015_75-83.pdf	674602 7fe3edf64f6338f82b3b018c0b374ca25198d4b0	no	9
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34	Non Patent Literature	MURRAY_2006_6171-6176.pdf	616323 8500cc03f857125ef0f3f5483b5a10e0ce1272a7	no	6
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Information:					
35	Non Patent Literature	NAM_2007_12260-12271.pdf	865187 cfdadf9e30fe17b8ae7835a50d3363ca4e59c869	no	12
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Information:					
36	Non Patent Literature	OPIE_2003_6995-7006.pdf	1002111 e0bf5f93002ec59d117f1b3eb9d6cb86508f6c14	no	12
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37	Non Patent Literature	PERRY_2008_661-699.pdf	4760554 a3c8753aae80d6570e96c5763ed4a11b6fab0b56	no	39
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Information:					
38	Non Patent Literature	PERSSON_1985_523-527.pdf	839226 4e75c9e12a4e793c673d9b05cbfeddc07a93b995	no	5
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40	Non Patent Literature	PLUMB_2004_2331-2337.pdf	258925 0373918c60e4ef865d05b9a8048bd5b741905ecb	no	7
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41	Non Patent Literature	POLEVODA_2003_30686-30697.pdf	1765439 765e393bfe70d3f56ab7ce64ca66436cddbe5e67	no	13
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42	Non Patent Literature	POPA-WAGNER_2012_9163-9174.pdf	1106662 e2277e9318094050782404e94fb5b0f62c7b067f	no	12
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47	Non Patent Literature	SANLIOGLU_2000_9184-9196.pdf	1133019 eeec108dfb0603f847a3cc8d0ab4fdd6b36adf2e	no	13
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53	Non Patent Literature	SUMMERFORD_1999_78-82.pdf	573695 612d13d40133eeeae33d597b493c7603bf6d55d4	no	5
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54	Non Patent Literature	VANVLIET_2009_167-177.pdf	2632722 7a1624604a39bcdd0a0f98b4a16c493a520cd3fa	no	11
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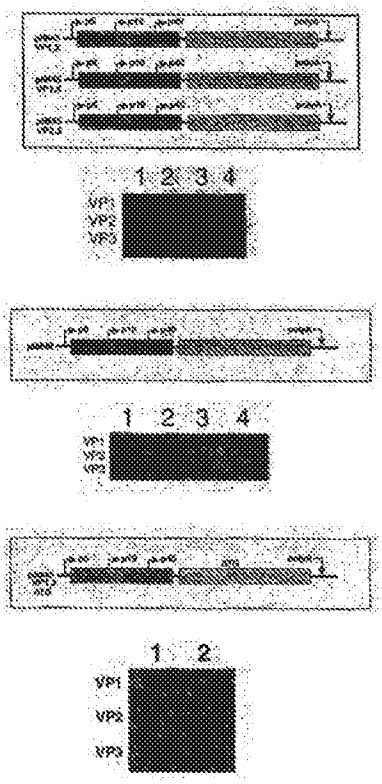
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(54) Title: IMPROVED RAAV EXPRESSION SYSTEMS FOR GENETIC MODIFICATION OF SPECIFIC CAPSID PROTEINS



(57) Abstract: Disclosed are improved recombinant adeno-associated viral (rAAV) vectors having mutations in one or more capsid proteins. Exemplary vectors are provided that have altered affinity for heparin or heparin sulfate, as well as vectors, expression systems, and rAAV virions that lack functional VP2 protein expression, but are nevertheless, fully virulent. Also provided by the invention are rAAV vector-based compositions, virus particles, host cells, and pharmaceutical formulations that comprise them useful in the expression of selected therapeutic proteins, polypeptides, peptides, antisense oligonucleotides and/or ribozymes in selected mammals, including organs, tissues, and human host cells.

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DESCRIPTION

IMPROVED RAAV EXPRESSION SYSTEMS FOR GENETIC MODIFICATION OF SPECIFIC CAPSID PROTEINS

1. BACKGROUND OF THE INVENTION

The present application claims priority to United States Provisional Application Serial No. 60/377,315, filed May 1, 2002, the entire contents of which is specifically incorporated herein by reference. The United States government has certain rights in the present invention pursuant to grant numbers HL59412 and HL51811 from the National Institutes of Health.

1.1 FIELD OF THE INVENTION

The present invention relates generally to the fields of molecular biology and virology, and in particular, to the development of gene delivery vehicles. The invention provides improved recombinant adeno-associated virus (rAAV) vectors that while deleted for VP2, are still able to form infectious virion particles, as well as other AAV vector compositions useful in expressing a variety of nucleic acid segments, including those encoding therapeutic proteins polypeptides, peptides, antisense oligonucleotides, and ribozyme constructs, in various gene therapy regimens. Methods are also provided for preparing and using these modified rAAV-based vector constructs in a variety of viral-based gene therapies, and in particular, treatment and prevention of human diseases using conventional gene therapy approaches. The invention also provides multicomponent vector systems which may be used to assess the relative efficiency and infectivity of a variety of AAV particles having mutated, or deleted capsid proteins.

1.2 DESCRIPTION OF RELATED ART

Major advances in the field of gene therapy have been achieved by using viruses to deliver therapeutic genetic material. The adeno-associated virus (AAV) has attracted considerable attention as a highly effective viral vector for gene therapy due to its low immunogenicity and ability to effectively transduce non-dividing cells. AAV has been shown to infect a variety of cell and tissue types by using heparin sulfate proteoglycan (HSPG) as its primary cellular receptor. The natural tropism of AAV for the abundantly expressed HSPG presents a challenge to specifically targeting particular cell populations.

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For safety and targeting considerations it is highly desirable to have a vector that cannot infect its natural host cell types.

2. SUMMARY OF THE INVENTION

The present invention overcomes these and other limitations inherent in the prior art by providing new rAAV-based genetic constructs that encode one or more mammalian therapeutic polypeptides for the prevention, treatment, and/or amelioration of various disorders resulting from a deficiency in one or more of such polypeptides. In particular, the invention provides AAV-based genetic constructs encoding one or more mammalian therapeutic proteins, polypeptides, peptides, antisense oligonucleotides, and ribozymes, as well as variants, and/or active fragments thereof, for use in the treatment and prophylaxis of a variety of conditions and mammalian diseases and disorders.

Current AAV2 targeting strategies involve inserting DNA sequences that code for specific receptor ligands within the capsid open reading frame of the pIM45 plasmid. While this approach has identified surface positions capable of tolerating peptide insertions, there are certain limitations. Because the three capsid proteins share the same open reading frame and stop codon, the amino acid sequence of the major capsid protein, VP3, and any peptide ligands inserted in this region of the open reading frame, are contained within the 2 larger and significantly less abundant capsid proteins, VP1 and VP2.

In order to target peptide ligands to a specific capsid protein, the inventors have investigated an alternative method for the production of recombinant AAV2 vectors. By mutating the capsid proteins' start codons the inventors have generated pIM45 plasmids that only express one capsid protein: pIM45-VP1, pIM45-VP2 (acg/atg), and pIM45-VP3. Such plasmids can be complemented with plasmids that express the remaining 2 capsid proteins (pIM45-VP2,3, pIM45-VP1,3, and pIM45-VP1,2, respectively) in order to produce viable recombinant AAV2 vectors. Interestingly, the plasmid, pIM45-VP1,3 is also capable of producing infectious virions in the absence of VP2 expression. Expression of the capsid proteins in this manner allows for the genetic modification of a specific capsid protein across its entire sequence. As a result, more control of the position and number of expressed peptide insertions is obtained in producing recombinant AAV2 vectors. This system allows for the production of novel targeted recombinant AAV2 vectors containing significantly larger peptide insertions in an individual capsid protein without disruption of the remaining capsid structure.

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In one embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP1 and VP3 capsid proteins substantially in the absence of functional VP2 protein. Surprisingly, the inventors have shown that such a vector can produce an infectious virion in the absence of exogenous VP2 protein.

The lack or substantial absence of functional VP2 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP2 start codon, or alternatively in the VP2 start codon itself. An exemplary vector described herein is pIM45-VP1,3.

In another embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP1 and VP2 capsid proteins substantially in the absence of functional VP3 protein. Although such vector cannot produce an infectious virion in the absence of exogenous VP3 protein, if a second helper vector that encodes a functional VP3 protein is employed to coinfect cells with this vector, infectious virions can be obtained.

The lack or substantial absence of functional VP3 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP3 start codon, or alternatively in the VP3 start codon itself. An exemplary vector described herein is pIM45-VP1,2.

In a third embodiment, the invention concerns rAAV vectors that comprise a nucleic acid segment modified to express functional VP2 and VP3 capsid proteins substantially in the absence of functional VP1 protein. Although such vector cannot produce an infectious virion in the absence of exogenous VP1 protein, if a second helper vector that encodes a functional VP1 protein is employed to coinfect cells with this vector, infectious virions can be obtained.

The lack or substantial absence of functional VP1 protein may be the result of at least a first mutation in the capsid gene sequence region that comprises the VP1 start codon, or alternatively in the VP1 start codon itself. An exemplary vector described herein is pIM45-VP2,3.

A yet further embodiment of the invention is an expression vector that expresses an rAAV capsid protein selected from the group consisting of VP1, VP2, and VP3, each in the absence of substantially any other rAAV protein, such as the other capsid proteins or helper functions.

This expression vector may comprise, for example, a mutation at position 1 of the cap gene, a mutation at position 138 of the cap gene, or a mutation at position 203 of the cap

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gene. Exemplary such vectors provided herein are pIM45-VP1, pIM45-VP2, or pIM45-VP3, which produce substantially a single VP1, VP2, or VP3 protein, respectively.

Another embodiment of the invention is an expression vector that expresses: (a) rAAV capsid proteins VP1 and VP2 in the absence of substantial amounts of VP3 protein; (b) rAAV capsid proteins VP1 and VP3 in the absence of substantial amounts of VP2 protein; or (c) rAAV capsid proteins VP2 and VP3 in the absence of substantial amounts of VP1 protein.

Such vector typically comprises: (a) at least one mutation in the start codon of the VP1 protein and at least one mutation in the start codon of the VP2 protein; (b) at least one mutation in the start codon of the VP1 protein and at least one mutation in the start codon of the VP3 protein; or (c) at least one mutation in the start codon of the VP2 protein and at least one mutation in the start codon of the VP3 capsid protein.

For example, the vector may comprise: (a) at least one mutation at position 1 and at least one mutation at position 138 of the cap gene, (b) at least one mutation at position 1 and at least one mutation at position 203 of the cap gene; or (c) at least one mutation at position 138 and at least one mutation at position 203 of the cap gene. Vectors pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 described herein, are representative examples of each of such vectors, respectively.

The invention also provides in an important embodiment, an rAAV expression system substantially lacking in expression of VP2 protein. This VP2-free system comprises:(a) at least a first rAAV vector comprising at least a first heterologous nucleic acid segment inserted into the capsid sequence region, with the segment encoding at least a first heterologous peptide; and (b) at least a second expression vector that expresses functional VP1 and VP3 capsid proteins in the absence of substantial quantities of VP2 protein, or at least a second and a third expression vector that separately express functional VP1 and VP3 capsid proteins, each of these second and third plasmids expressing a single VP1 or VP3 protein, both in the absence of substantial amounts of VP2 protein.

For example, the system will preferably comprise at least a first rAAV vector that substantially lacks VP2 expression. Such expression systems will give rise to infectious virions, so long as the helper plasmids provide sufficient exogenous VP1 and VP3 protein to permit the rAAV vector to form the capsid.

In one embodiment, when it is desirable to "target" particular cells, cell surfaces, or cell surface ligands or receptors, it may be desirable to alter the sequence of the capsid gene through the addition of one or more relatively short nucleic acid segments that encode at least

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1 or more targeting peptides that, when these heterologous peptides are expressed on the surface of an rAAV virion comprising the vector, the peptide sequence contained within the altered capsid protein will permit the selective targeting of the rAAV virions comprising them to one or more specific types of cells, cell surfaces, or cell surface receptors when the particles are used to transfect a plurality or population of such host cells. The inventors contemplate that the exploitation of such targeting peptide sequences, when expressed on the surface of the rAAV virions as contained within the capsid proteins, may be critical in localizing, enhancing, improving, or increasing the specificity of the rAAV virions for a particular cell type, or may even be useful in permitting transduction of cells or cell types that previously were not appropriate host cells for AAV infection. Such methods could be particularly desirable in altering the native affinity of one or more of the various known serotypes of AAV to one or more host cells not previously capable of efficient transfection by one or more particular serotypes. For example, by appropriate insertion of one or more peptide epitopes, ligands, or recognition sequences, an rAAV serotype 1 vector may be able to efficiently transfect a cell line not readily transfected by wild-type rAAV1 vectors. Likewise, an rAAV serotype 2 vector may be sufficiently modified by addition of appropriate targeting ligands to effectively transfect one or more cell lines, cells types, tissues, or organs, not previously capable of efficient transfection using the unmodified wild-type rAAV2 vector.

As such, preferred embodiments include those VP2-free rAAV expression systems, wherein at least a first peptide inserted into one or more of the capsid protein sequences, permits the rAAV virion to transfect a specific organ tissue, or host cell, with a higher efficiency than an unmodified rAAV vector.

The VP2-free rAAV expression systems of the invention may utilize any rAAV vector, including those of serotypes 1, 2, 3, 4, 5, or 6, and may employ at least two helper plasmids such as pIM45-VP1, pIM45-VP2, or pIM45-VP3, as the second and third expression vectors required in the system to provide exogenous VP1, VP2, and/or VP3 as may be required for efficient virion formation by the rAAV vectors. When only a second helper plasmid is desired, a single vector may be employed such as, for example, pIM45-VP1,3. Alternatively, so long as at least VP1 and VP3 are provided to the system, either on a single plasmid, each on separate plasmids, or by exogenous supplementation of one or both of the purified protein(s) themselves, a fully functional, fully virulent rAAV virion may be reconstituted from the disclosed expression system, either in the presence of

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functional VP2 protein, or alternatively, substantially in the absence of any endogenously- or exogenously-provided VP2 protein.

When the use of such vectors is contemplated for introduction of one or more exogenous proteins, polypeptides, peptides, ribozymes, and/or antisense oligonucleotides, to a particular cell transfected with the vector, one may employ the rAAV vectors or the VP2-free rAAV expression systems disclosed herein by genetically modifying the vectors to further comprise at least a first exogenous polynucleotide operably positioned downstream and under the control of at least a first heterologous promoter that expresses the polynucleotide in a cell comprising the vector to produce the encoded peptide, protein, polypeptide, ribozyme, or antisense oligonucleotide. Such constructs may employ heterologous promoters that are constitutive, inducible, or even cell-specific promoters. Exemplary such promoters include, but are not limited to, a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

The vectors or expression systems may also further comprise one or more enhancers, regulatory elements, transcriptional elements, to alter or effect transcription of the heterologous gene cloned in the rAAV vectors. For example, the rAAV vectors of the present invention may further comprise at least a first CMV enhancer, a synthetic enhancer, or a cell- or tissue-specific enhancer. The exogenous polynucleotide may also further comprise one or more intron sequences.

In other aspects, the invention concerns methods for altering, reducing, or eliminating, the binding of particular rAAV vectors for particular ligands. In an illustrative embodiment, the invention provides rAAV vectors that have altered affinity for heparin, heparin sulfate, and heparin sulfate proteoglycan. This vector comprises at least a first mutation in the capsid gene, wherein the mutation substantially reduces or eliminates the affinity of a viral particle comprising the vector for binding to heparin, heparin sulfate, or heparin sulfate proteoglycan. Preferably, these rAAV vectors comprise one or more Arginine to Alanine mutations, and particularly one or more Arginine to Alanine mutations at position 585 or position 588 of the capsid polypeptide sequence. In rAAV vectors comprising either a single R585A or R588A mutation, or a double mutant comprising both the R585A and the R588A mutations, affinity for heparin sulfate binding by the vector was eliminated. Such vectors are therefore important when one wishes to design improved rAAV vectors that comprise particular capsid protein mutations that either have increased or reduced affinity for one or more particular ligands.

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In all aspects of the invention, the exogenous polynucleotides that are comprised within one or more of the improved rAAV vectors disclosed herein will be of mammalian origin, with human, murine, porcine, bovine, ovine, feline, canine, equine, epine, caprine, and lupine polynucleotides being particularly preferred.

As described above, the exogenous polynucleotide will preferably encode one or more proteins, polypeptides, peptides, ribozymes, or antisense oligonucleotides, or a combination of these. In fact, the exogenous polynucleotide may encode two or more such molecules, or a plurality of such molecules as may be desired. When combinational gene therapies are desired, two or more different molecules may be produced from a single rAAV expression system, or alternatively, a selected host cell may be transfected with two or more unique rAAV expression systems, each of which will provide unique heterologous polynucleotides encoding at least two different such molecules.

In other embodiment, the invention also concerns the disclosed rAAV vectors comprised within an infectious adeno-associated viral particle, comprised within one or more pharmaceutical vehicles, and may be formulated for administration to a mammal such as a human for therapeutic, and/or prophylactic gene therapy regimens. Such vectors may also be provided in pharmaceutical formulations that are acceptable for veterinary administration to selected livestock, domesticated animals, pets, and the like.

The invention also concerns host cells that comprise the disclosed rAAV vectors and expression systems, particularly mammalian host cells, with human host cells being particularly preferred.

Compositions comprising one or more of the disclosed rAAV vectors, expression systems, infectious AAV particles, host cells also form part of the present invention, and particularly those compositions that further comprise at least a first pharmaceutically-acceptable excipient for use in the manufacture of medicaments and methods involving therapeutic administration of such rAAV vectors. Such pharmaceutical compositions may optionally further comprise liposomes, a lipid, a lipid complex; or the rAAV vectors may be comprised within a microsphere or a nanoparticle. Pharmaceutical formulations suitable for intramuscular, intravenous, or direct injection into an organ or tissue of a human are particularly preferred.

Other aspects of the invention concern recombinant adeno-associated virus virion particles, compositions, and host cells that comprise one or more of the AAV vectors disclosed herein, such as for example pharmaceutical formulations of the vectors intended for administration to a mammal through suitable means, such as, by intramuscular, intravenous,

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or direct injection to cells, tissues, or organs of a selected mammal. Typically, such compositions may be formulated with pharmaceutically-acceptable excipients as described hereinbelow, and may comprise one or more liposomes, lipids, lipid complexes, microspheres or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired.

Kits comprising one or more of the disclosed vectors, virions, host cells, viral particles or compositions; and (ii) instructions for using the kit in therapeutic, diagnostic, or clinical embodiments also represent preferred aspects of the present disclosure. Such kits may further comprise one or more reagents, restriction enzymes, peptides, therapeutics, pharmaceutical compounds, or means for delivery of the compositions to host cells, or to an animal, such as syringes, injectables, and the like. Such kits may be therapeutic kits for treating or ameliorating the symptoms of particular diseases, and will typically comprise one or more of the modified AAV vector constructs, expression systems, virion particles, or therapeutic compositions described herein, and instructions for using the kit.

Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, expression systems, compositions, and host cells described herein in the preparation of medicaments for treating or ameliorating the symptoms of various polypeptide deficiencies in a mammal. Such methods generally involve administration to a mammal, or human in need thereof, one or more of the disclosed vectors, virions, host cells, or compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a deficiency in the affected mammal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms.

3. Brief Description of the Drawings

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

FIG. 1A, FIG. 1B and **FIG. 1C** show generation of plasmids that express two capsid proteins through missense mutation of individual capsid protein start codons. FIG. 1A shows mutations required to eliminate VP1 and VP2 expression. Immunoblot of whole cell

lysates using B1 antibody that recognizes all three capsids following transfection of plasmids, pIM45(lane1); pIM45-VP2,3 (lane2); pIM45-VP1,3 (lane3); and pIM45-M203L (lane 4). Note, lane 4 is the initial attempt to produce plasmid that expresses only VP1 and VP2. Further mutations are required. FIG. 1B shows mutations required to eliminate VP3 expression. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid following transfection of pIM45 (lane1); pIM45-M203L (lane2); pIM45-M203,211L (lane3); pIM45-M203,211,235L (lane4). Note, pIM45-M203,211,235L is designated pIM45-VP1,2. FIG. 1C shows alternative mutation used to eliminate VP3 expression while maximizing expression of VP2 protein. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid proteins following transfection of pIM45 (lane1) and pIM45-VP1,2A (lane3) in which the start codon for VP2 protein is changed from ACG to ATG.

FIG. 2 shows generation of plasmids that express a single capsid protein. Immunoblot of whole cell lysates using B1 antibody that recognizes all three capsid proteins following transfection of pIM45 (lane1); pIM45-VP1 (lane2); pIM45-VP2 (lane3) pIM45-VP2A (lane4); pIM45-VP3 (lane5).

FIG. 3A and **FIG. 3B** show production and purification of rAAV2-like particles that lack expression of specific capsid proteins. FIG. 3A shows analysis of effects of missense mutations required to eliminate VP3 expression. Left panel shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from pIM45 (lane1); pIM45-M203L (lane2); pIM45-M211L (lane3); pIM45-M235L (lane4), pIM45-M203,211,235 (lane 5). Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe. FIG. 3B shows analysis of effects of eliminating a single capsid on the production and purification of virus particles. Left panel shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from pIM45 (lane1); pIM45-VP1,2 (lane2); pIM45-VP1,3 (lane3); and pIM45-VP2,3 (lane4). Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe.

FIG. 4 shows complementation capsid plasmid groups employed to produce viable rAAV2 particle preparations. Group VP0 is a control group consisting of pIM45 and pIM45-

VP0 (all capsid expression eliminated). Group VP1 is group consisting of pIM45-VP1 and pIM45-VP2,3 in which expression of VP1 is isolated. Group VP2/VP2A is group consisting of pIM45-VP2 or pIM45-VP2A and pIM45-VP1,3 in which expression of VP2 is isolated, and in case of pIM45-VP2A, VP2 expression is maximized. Group VP3 is group consisting of pIM45-VP3 and pIM45-VP1,2 in which expression of VP3 is isolated. Isolation of specific capsid proteins allows genetic modification of the isolated capsid without further modifying remaining capsids. Alternatively, genetic modification of two capsids can be accomplish without further modification of remaining capsid. These groups are cotransfected with pXX6 (Ad helper functions) and pTR-UF5 (terminal repeats flanking expression cassette with CMV promoter driving expression of GFP) to produce rAAV vectors.

FIG. 5A and **FIG. 5B** show production and purification of rAAV2-like particles from complementation groups described in FIG. 4. FIG. 5A, right panel, shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from Group VP0(lane1); Group VP1 (lane2); Group VP2 (lane3); Group VP2A (lane4); and Group VP3 (lane5). Note, lane 4 shows production of particle stock with increased level of VP2 protein in resultant particles composed of all three capsid proteins. FIG. 5A, Right panel shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe. FIG. 5B, left panel, shows immunoblot using B1 antibody that recognizes all three capsid proteins of purified particle stocks from transfection of pIM45-VP2A and pIM45-VP3 showing production of rAAV2-like particles composed of VP2 and VP3 with increased VP2 levels relative to VP3. FIG. 5B, right panel, shows dot blot autoradiograph of DNA extracted from same particle stocks. Aliquots from an iodixinal step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabelled GFP probe.

FIG. 6A, FIG. 6B and **FIG. 6C** depict production of rAAV2-like particles with large peptide insertions in VP1 and VP2 capsid proteins. FIG. 6A shows production scheme for insertion of large peptides in VP1 and VP2 (top) involves insertion of peptide immediately after amino acid 138 in a plasmid that expresses only VP1 and VP2 (pIM45-VP1,2A) and complementing this plasmid with plasmid, pIM45-VP3, to produce particles. Production

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scheme for insertion of large peptides only in VP2 (bottom) involves insertion of peptide immediately after amino acid 138 in a plasmid that expresses only VP2 (pIM45-VP2A) and complementing this plasmid with plasmid, pIM45-VP1,3 to produce particles. FIG. 6B shows immunoblot of purified rAAV2-like particles produced by above production schemes with protein, leptin, inserted in VP1 and VP2 or only in VP2. FIG. 6B, left panel, shows immunoblot probed with antibody recognizing all three capsids proteins. FIG. 6B, right panel, shows immunoblot probed with antibody recognizing inserted peptide, leptin. Both panels: Lane 1: pIM45; Lane 2: pIM45-VP1,2A-Leptin/pIM45-VP3; Lane 3: pIM45-VP2A-Leptin/pIM45-VP1,3; Lane 4: pIM45-VP3 only; pIM45-VP1,3 only. FIG. 6C shows immunoblot of purified rAAV2-like particles produced by above production schemes with protein, GFP, inserted in VP1 and VP2 or only in VP2. FIG. 6C, left panel, shows immunoblot probed with antibody recognizing all three capsids proteins. FIG. 6C, right panel, shows immunoblot probed with antibody recognizing inserted peptide, GFP. Both panels: Lane 1: pIM45; Lane 2: pIM45-VP1,2A-GFP/pIM45-VP3; Lane 3: pIM45-VP2A-GFP/pIM45-VP1,3; Lane 4: pIM45-VP3 only; pIM45-VP1,3 only.

FIG. 7 shows Western blot of iodixanol virus stocks. Equal volumes of virus stock were separated by 10% SDS-PAGE and analyzed by Western blot using the B1 antibody.

FIG. 8 shows heparin-agarose binding profiles of mutant capsids. Approximately 5×10^{10} particles were applied to 500 µl of heparin-agarose affinity matrix at a 100 mM NaCl concentration, washed extensively with the loading buffer, and bound capsids were eluted with 2 M NaCl. Pooled fractions were denatured and slot blotted onto nitrocellulose for immunodetection with mAb B1. For each mutant, L is the total amount of iodixanol purified virus that was loaded onto the heparin agarose column; FT is the total virus that flowed through the column, W is the wash; E, eluate.

FIG. 9A and **FIG. 9B** show production and purification of AAV serotypes. FIG. 9A shows equivalent amounts of iodixanol purified AAV1, AAV2 and AAV5 were separated by 10% PAGE and analyzed by Western blot using the B1 antibody. FIG. 9B shows heparin-agarose binding properties of AAV2, AAV1 and AAV5. Abbreviations are the same as FIG. 8.

FIG. 10 shows particle-to-infectivity ratios of mutants relative to wild type. The particle-to-infectivity ratio for each mutant was calculated by dividing the average genomic titer by the average green cell assay titer (Table 2). The P/I ratio of each mutant was then normalized to wild type by dividing the P/I of each mutant by the P/I of wild type rAAV2, and the $\log_{10}$ value of the ratio was plotted. Wild type, therefore, equals one and is indicated

by the dashed line. Grey bars, mutant viruses with infectivity comparable to wild type; Black bars, mutant viruses that are heparin binding deficient; White bars, mutant viruses with an undetermined block to infectivity; Asterisks indicate those mutants for which no green cells were scored. For these mutants the green cell assay titer used was the limit of detection in the assay. Thus, the log difference is a minimum estimate.

FIG. 11 shows GFP transduction ability of mutants in HeLa C12 cells. Cells were infected with wild type rAAV or mutant virus at an MOI=500 genomic particles and an Ad5 MOI=10 pfu per cell. Twenty-four hours post infection cells were fixed with 2% paraformaldehyde and the number of GFP positive cells was determined by FACS analysis.

FIG. 12A and **FIG. 12B** show binding and uptake of rAAV2 and R585A/R588A genomes in Hela C12 cells. FIG. 12A shows 10^6 cells were infected with rAAV2 or R585/R588A at an MOI=100 or 1000 genome containing particles per cell, respectively. At the indicated times, infection media was removed and saved. The cells were washed and harvested, and Hirt DNA was extracted from both the infection media and the cell pellet. Southern analysis was performed using an [α-^{32}P]-dATP labeled GFP probe. FIG. 12b shows the percent bound/internalized DNA was calculated by dividing the total DNA present in both the media and the cell pellet by the amount bound/internalized for each time point. The average of three determinations is shown. Error bars indicate a standard deviation.

FIG. 13A, FIG. 13B, FIG. 13C and **FIG. 13D** show modifying the heparin binding properties of AAV5. FIG. 13A shows alignment of AAV2 amino acid residues 585 through 590 to residues predicted by amino acid alignment to be structurally equivalent in AAV5. FIG. 13B shows Western blot of iodixanol virus stocks. Equal volumes of virus were separated by 10% SDS-PAGE and analyzed by Western blot using the B1 antibody. FIG. 13C shows novel heparin binding properties of AAV5-HS. Heparin-agarose binding was performed as described in FIG. 8. See FIG. 8 for abbreviations. FIG. 17D shows. the log of the particle-to-infectivity ratio of the rAAV5 variants normalized to wild type rAAV2 as described in FIG. 10.

FIG. 14 shows an immunoslotblot of total capsid protein from novel production system following standard purification procedures. Immunoslotblot was probed with anti-VP1,2,3 monoclonal antibody. 1. pIM45/pIM45-VP0; 2. pIM45-VP1/pIM45-VP2,3; 3. pIM45-VP2acg/pIM45-VP1,3; 4. pIM45-VP2atg/pIM45-VP1,3; 5. pIM45-VP3/pIM45-VP1,2.

FIG. 15 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and system plasmid combinations. Numbering scheme is the same as described in FIG. 14.

Equal volume aliquots from an iodixinol step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabeled GFP probe.

FIG. 16 shows the *in vivo* transduction ability of recombinant AAV vectors produced using various system components. GFP fluorescence microscopy was performed on Hela C12 infected at an MOI of 1000 genomes/cell 24 hours post infection.

FIG. 17 shows the Immunoblot and dot blot autoradiograph of virions produced from pTR-UF5; pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 plasmids following standard purification protocols. The capsid proteins VP1, VP2, and VP3 are indicated. No virions were obtained in 40% iodixanol fraction from plasmid pIM45-VP1,2.

FIG. 18 shows the *in vivo* transduction ability of recombinant AAV vectors containing only two capsid proteins. GFP fluorescence microscopy was performed on Hela C12/24 hours post infection.

FIG. 19 depicts an immunoblot of protein fractions collected from iodixinol purified passed over a heparin-agarose column. Immunoblot was probed with anti-VP1,2,3 monoclonal antibody. C, 5E+10 virus particles loaded directly onto blot, FT, flowthrough fraction, W, wash fraction, E, 2M NaCl fraction

FIG. 20 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and rAAV R585A, R588A. Equal volume aliquots from an iodixinal step gradient were with incubated with DNAseI, inactivated with EDTA, digested with proteinase K, phenol:chloroform extracted, and precipitated with ethanol. DNA was transferred to nitrocellulose and probed with radiolabeled GFP probe.

FIG. 21 shows the *in vivo* transduction ability of pTR-UF5 and R585A, R588A. GFP fluorescence microscopy was performed on Hela C12 and HEK 293 cells infected at an MOI of 1000 genomes/cell 24 hours post infection.

FIG. 22 shows a slot blot autoradiograph of an *in vivo* DNA tracking time course experiment of pTR-UF5, rAAV R585A, R588A. Media and cells infected with pTR-UF5 and rAAV R585A, R588A were collected at 1,4, and 20 hours post infection. Hirt DNA was extracted, transferred to nitrocellulose and probed with a radiolabeled GFP probe.

FIG. 23 shows a schematic diagram of the pIM45 vector showing the rep and cap sequences.

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4. DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would nevertheless be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

4.1 rAAV TYPE 2

The adeno-associated virus type 2 (AAV2) is a small, non-enveloped parvovirus that has received considerable attention as a gene therapy vector (see, *e.g.*, Muzyczka and Berns, 2001). The capsid has a diameter of approximately 20 nm formed by an icosahedral lattice with T=1 symmetry (60 structurally equivalent subunits). In purified virions, three structural proteins, VP1, VP2, and VP3 with molecular masses of 87, 73, and 62 Kda, respectively, are present in a molar ratio of 1:1:18 (Buller and Rose, 1978). mRNAs encoding capsid proteins are synthesized from a single open reading frame and use alternative splicing and start codons to produce three VP proteins that share an identical 532 carboxyl-terminal amino acid domain (Becerra *et al.*, 1988; Becerra *et al.*, 1985), with VP2 and VP3 containing successive amino terminal truncations of VP1.

The atomic structure of the AAV2 capsid has been determined to a resolution of 3.5 angstroms (Xie *et al.*, 2002). In this model, sixty copies of VP3 minus 14 amino terminal residues are present in an icosahedral arrangement. The VP3 protein contains 8 anti-parallel β-strands that adopt a barreloid structure similar to capsid proteins of other non-enveloped viruses. Loops of variable length connect the interior β-barrel scaffold and extend outwards to form the capsid surface. Cryo-electron microscopy of empty AAV2 particles generated a surface density map that described holes, spikes and canyon features similar to those found in other parvoviruses (Kronenberg *et al.*, 2001). Before the crystal structure was available, several alternative methods were investigated in an attempt to localize specific regions of the capsid. Neutralizing antibody screening of peptide sequences derived from VP1 found multiple antigenic determinants distributed on the capsid exterior in both linear and conformation dependent forms (Moskalenko *et al.*, 2000). Computer modeling of AAV structure based on the known atomic structure of the related canine parvovirus coupled with

genetic modification of the capsid identified several positions that were on the surface of the capsid and could tolerate insertions and substitutions (Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Rabinowitz *et al.*, 1999; Ried *et al.*, 2002; Shi *et al.*, 2001; Wu *et al.*, 2000; Yang *et al.*, 1998).

Cell membrane binding and entry initiates all viral infections. Non-enveloped viruses rely on membrane bound extracellular receptors for attachment to the cell membrane. AAV2 has evolved a dynamic and multistep infectious entry pathway that utilizes the abundantly expressed heparan sulfate proteoglycan (HSPG) as its primary target (Summerford and Samulski, 1998). Two co-receptors, αVβ5 integrin and basic fibroblast growth factor receptor (bFGFR) have been identified, which act as secondary receptors that may stabilize virus attachment or participate during internalization (Duan *et al.*, 1999; Qing *et al.*, 1999; Summerford *et al.*, 1999). HSPG is a macromolecule expressed by many cell types and is a component of the extracellular matrix of most tissues (see, *e.g.*, Hileman *et al.*, 1998; Mull9oy and Linhardt, 2001). Attached to the core protein are glycosaminoglycan side chains heparin and heparin sulfate (HS). These carbohydrate polymers are formed by disaccharide repeats consisting of alternating N-acetylglucosamine and iduronic acid residues in a α1,4 linkage. The saccharides can be modified by N-sulfation as well as 2-O and 6-O-sulfation to impart a dense overall negative charge at physiological pH. As a result, HS interacts with an extensive range of proteins primarily by electrostatic attraction between the electron dense sulfate groups and a cluster of positively charged amino acids. Two linear consensus-binding sequences, XBBXBX (SEQ ID NO:1) and XBBBXXBX (SEQ ID NO:2), and a conformation dependent sequence, TXXBXXTBXXXTBB (SEQ ID NO:3), (where B is any basic amino acids including His, Lys or Arg and X is any hydropathic amino acid and T is a turn) have been reported (Hileman *et al.*, 1998). Although HSPG is thought to participate in attachment during the infectious process of numerous human viruses (Liu and Thorp, 2002), information about the molecular mechanisms of these interactions is limited. A report describing the atomic structure of the foot and mouth disease virus co-crystallized with a HS pentasaccharide is available and serves as the only model defined at the atomic level that describes the molecular interaction between a non-enveloped icosahedral virus and HS (Fry *et al.*, 1999).

Several laboratories have attempted to retarget AAV vectors to non-permissive cell types by inserting sequences coding for short foreign peptides into VP3. Interestingly, insertions at position 587, including an L14 integrin binding peptide, a myc tag, an IgG binding domain truncation of protein A and an endothelial cell targeting peptide, abolished

the natural heparin binding ability of virus capsids with these alterations (Girod *et al.*, 1999; Grifman *et al.*, 2001; Nicklin *et al.*, 2001; Ried *et al.*, 2002; Shi *et al.*, 2001). Similarly, an alanine repeat insertion at position 509, an L14 peptide insertion at position 520, a hemagluttinin tag insertion at positions 522 and 591, and peptides derived from the human luteinizng hormone receptor and the bovine papilloma virus at inserted positions 520 and 584, respectively, have been reported to disrupt heparin binding (Shi *et al.*, 2001; Wu *et al.*, 2000). Curiously, alanine substitutions of acidic residues between 561 and 565 also reduced heparin binding, suggesting that nearby basic residues were affected (Wu *et al.*, 2000). Finally, a substitution mutation of two arginines and a glutamine at positions 585, 588, and 587, respectively, binds poorly to heparin-agarose (Wu *et al.*, 2000). Taken together, these genetic modifications suggested two potential heparin-binding loci that cluster between positions 509-522 and 561-591.

4.2 RAAV CAPSID PROTEINS

Supramolecular assembly of 60 individual capsid protein subunits into a non-enveloped, T-1 icosahedral lattice capable of protecting a 4.7 kb single-stranded DNA genome is a critical step in the life-cycle of the helper-dependent human parvovirus, adeno-associated virus2 (AAV2). The mature 20 nm diameter AAV2 particle is composed of three structural proteins designated VP1, VP2, and VP3 (molecular masses of 87, 73, and 62 kDa respectively) in a ratio of 1:1:18. Based on its symmetry and these molecular weight estimates, of the 60 capsid proteins comprising the particle, three are VP1 proteins, three are VP2 proteins, and fifty-four are VP3 proteins. The employment of three structural proteins makes AAV serotypes unique among parvoviruses, as all others known package their genomes within icosahedral particles composed of only two capsid proteins. The anti-parallel β-strand barreloid arrangement of these 60 capsid proteins results in a particle with a defined tropism that is highly resistant to degradation.

The AAV2 genome contains two large open reading frames (ORF), rep and cap, flanked by inverted terminal repeats. The AAV2 capsid proteins are produced in an overlapping fashion from the cap ORF; arising through alternative mRNA splicing of the transcript (initiated at the p40 promoter), with subsequent alternative translational start codon usage. A common stop codon is employed for all three capsid proteins. Correct capsid protein stoichiometry is maintained by translating VP1 from the 2.4 KB mRNA, while VP2 and VP3 arise from the 2.3-kB mRNA using a weaker ACG start codon for VP2 protein production with resultant read-through translation for the production of the VP3 protein.

Differing only in the length of their N-terminus, these proteins are produced such that the amino acid sequence of VP3 is contained within the significantly less abundant and longer VP1 and VP2 proteins. As such, VP1's unique 137 amino acid N-terminal extension of VP2 contains a phospholipase enzymatic activity important for viral infectivity. Similarly, VP2 extends the N-terminus of VP3 by 64 amino acids with this VP1/VP2 overlap region possessing a putative nuclear localization signal (NLS) involved in the nuclear translocation of VP2. The VP3 region common to all three capsid proteins contains the critical β-barrel structural motifs characteristic of all parvoviruses and particle surface loops involved in determining viral tropism.

While specific activities have been attributed to regions of an individual AAV2 capsid protein, the role of each capsid protein in the structural formation of the particle is less clear. Early studies in which all AAV2 capsid expression was eliminated revealed that capsid protein expression is required for the accumulation of single stranded genomes. It follows that AAV2 particle assembly occurs within the nucleus, and a putative NLS for VP2 has been localized to the VP1/VP2 overlap region in transfected COS cells.

In the absence of VP1 expression, this study suggested a major role of VP2 is the nuclear localization of VP3. However, since VP1 was deleted in this study, one cannot rule out that VP1 has the ability to nuclear localize VP3. Site-directed missense mutagenesis of the individual capsid proteins' start codons suggested that infectious particles are obtained only when all three capsid proteins are present. In contrast, later genetic analysis demonstrated that in the absence of VP1, VP2 and VP3 are able to encapsidate progeny genomes. Similarly, *in vitro* assembly of purified individual AAV capsid proteins demonstrated that VP2 and VP3 could form an AAV2-like particle. Baculovirus expression of the AAV2 capsid proteins within SF9 cells suggests an absolute requirement for VP2, although this study failed to eliminate VP3-like fragments produced by the VP2-baculovirus. However, it is feasible that studies of AAV2 assembly in baculovirus have subtle differences with particles assembled in mammalian cells.

An examination of the assembly process of the related autonomous canine parvovirus, CPV, in baculovirus observed significantly more aggregation of capsid proteins in insect cells. In addition, the results of the baculovirus and NLS studies have the caveat that they were performed in the absence of AAV2 Rep proteins, Ad helper gene functions, and a replicating AAV2 genome. Furthermore, the p40 promoter in these studies does not control AAV2 capsid protein expression, resulting in altered stoichiometry of the available capsid protein pool. Indeed, the above concerns seem warranted, as a recent insertional mutagenesis

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study of the AAV2 cap ORF, using standard AAV2 production protocols, reported the purification of an AAV2-like particle composed of only the VP3 protein. Therefore, despite the uncertainty of the precise role of VP1 and VP2 in particle formation, the evidence thus far suggests that the VP3 protein is absolutely required for the formation of an AAV2 particle. Finally, co localization studies of AAV2 assembly in 293 cells demonstrated an interaction of AAV2 Rep and capsid proteins with Ad proteins and the replicating genome in the nucleus, thus, supporting a current model of AAV2 assembly which proposes nucleoplasmic formation of empty particles with subsequent maturation of the particle as a result of Rep 52/40 mediated translocation of capsid protein associated single stranded genomes into the preformed particles.

4.3 Genetic Modification of rAAV Capsid Proteins

Great interest in the assembly, structure, and mutability of the AAV2 particle results from its promise as a recombinant gene delivery vehicle (rAAV2) *in vivo*. Essential to the clinical development of rAAV2 vectors for gene therapy is the ability to target specific tissue types. Manipulation of the rAAV2 particle in order to control its cellular receptor interactions is essential for vector targeting. The feasibility of various targeting strategies based on AAV cap ORF mutagenesis is currently an area of active investigation. A better understanding of the AAV2 particle surface architecture through systematic scanning-alanine and insertional mutagenesis of the AAV cap ORF and recent publication of the AAV2 crystal structure has identified several amino acid regions on the surface of the particle that tolerate sequence alteration without loss of capsid stability or integrity.

However, small changes in charge, sequence, and/or position of the mutation can result in dramatic changes in the mutant particle phenotype. One limitation in sequence mutation of the overlapping cap ORF is that mutation of only one capsid protein across its entire sequence is currently not possible. The full potential in manipulation of the particle is not reached with direct alteration of regions of capsid overlap. Predicted surface regions of capsid overlap leading to defective phenotypes upon mutagenesis may allow production of viable particles if such mutations were only in one or two of the capsid proteins. An additional degree of flexibility in modifying the rAAV2 particle would result from the ability to mutate the entire coding region of a specific capsid protein without altering the remaining two capsid proteins. Indeed, while mutations in the C-terminus of the VP3 region have been reported to be completely defective in particle formation following insertion of HA and 6xHis tags into the overlapping cap ORF, a recent report focusing on the purification of

rAAV2 particles demonstrated that the C-terminus of VP3 is capable of accepting a 6xHis tag if VP1 and VP2 are not altered. This rAAV2 production strategy involved expressing VP1 and VP2 from one construct, and expressing the VP3-6xHis fusion protein from a CMV promoter in a second plasmid. In the absence of the isolation of a specific capsid protein's expression, the N-terminal 137 amino acids of VP1 are the only region of the cap ORF where mutations are restricted to a single capsid protein. Successful insertions within this region have included HA and serpin. The VP1/VP2 overlap region (amino acid 138-202) also has been receptive to sequence modification. Insertions in this region have included HA, serpin and luetinizing hormone receptor ligand sequences immediately following amino acid 138 in the cap ORF.

The success of inserting sequences to the VP1 and VP1/VP2 regions may be due in part to less disruption of the integrity of the particle compared to insertion in the VP3 region of capsid overlap (amino acid 203-735). It is important to note that these mutant particles would require further mutation of the putative heparin-binding motif to restrict infection to the target cell. Not surprisingly, since it is the longest region of capsid protein overlap, contains many critical structural motifs, and targeting sequences in this region have 60 representatives in the rAAV particle, mutations in the VP3 region of the AAV2 cap ORF have resulted in the highest number of defective phenotypes. Yet, one location within the VP3 region has received much attention for the successful insertion of small targeting sequences in all three capsid proteins (amino acid 587). One major advantage of targeting insertions to this position is that the resultant mutant particle also has lost the ability to bind its native receptor. Viable mutations in the VP3 region of the cap ORF have been restricted in size (<30 amino acids).

One caveat of creating genetically-targeted rAAV2 particles, is the consideration that many cell surface receptors have ligands whose coding sequence are much larger than those successfully inserted directly into the overlapping cap ORF. Due to the modest size of this ORF (~2kB), the insertion of larger peptide sequences into the capsid coding sequences may result in serious disruption of splicing, read-through translation, capsid structure and/or stability. The insertion of large sequences into the rAAV2 particle have been limited to a study involving the fusion of the CD34 single chain antibody coding sequence with the N-termini of the individual capsid proteins following isolation of their expression to separate CMV promoters. Viable CD34-retargeted rAAV particles of extremely low titer were produced only when this fusion was to VP2 protein, and co-expression of wild-type VP2 protein was required. Nonetheless, the fusion of large peptide sequences to the N-terminus

of VP2 does not interfere with the incorporation of this capsid protein into the rAAV2 particle.

4.4 Wild-Type AAV2 Binds to Heparan Sulfate Proteoglycan

The adeno-associated virus type-2 (AAV2) uses heparan sulfate proteoglycan (HSPG) as its primary cellular receptor. In order to identify amino acids within the capsid of AAV2 that contribute to HSPG association, biochemical information about heparin/heparin sulfate (HS), AAV serotype protein sequence alignments, and data from previous capsid studies was used to select residues for mutagenesis. In the present invention, charged-to-alanine substitution mutagenesis was performed on individual and combinations of basic residues for the production and purification of recombinant viruses that contained a GFP reporter gene cassette. Intact capsids were assayed for their ability to bind to heparin-agarose *in vitro* and virions that packaged DNA were assayed for their ability to transduce normally permissive cell lines. It was found that mutation of arginine residues at position 585 or 588 eliminated binding to heparin-agarose. Mutation of residues R484, R487, and K532 showed partial binding to heparin-agarose. A general correlation between heparin-agarose binding and infectivity was observed as measured by GFP transduction; however, a subset of mutants that partially bound heparin-agarose (R484A and K532A) were completely non-infectious, suggesting that they had additional blocks to infectivity that were unrelated to heparin binding. Conservative mutation of positions R585 and R588 to lysine slightly reduced heparin-agarose binding, and had comparable effects on infectivity. Substitution of AAV2 residues 585 through 590 into a location predicted to be structurally equivalent in AAV5 generated a hybrid virus that bound to heparin-agarose efficiently, was able to package DNA, but was non-infectious. Taken together, these suggest that residues R585 and R588 are primarily responsible for heparin sulfate binding and mutation of these residues has little effect on other aspects of the viral life cycle.

Computer modeling using the AAV2 VP3 atomic coordinates revealed that residues which contribute to heparin binding form a cluster of five basic amino acids on the surface of each three-fold axis of symmetry related spike. Three other kinds of mutants were found as well. Mutants, R459A, H509A and H526A/K527A bound heparin as well as wild type but were defective for transduction. Another mutant, H358A, was defective for capsid assembly. Finally, a mutant R459A produced significantly lower levels of full capsids, suggesting a packaging defect.

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4.5 Pharmaceutical Compositions

The genetic constructs of the present invention may be prepared in a variety of compositions, and may also be formulated in appropriate pharmaceutical vehicles for administration to human or animal subjects. The AAV molecules of the present invention and compositions comprising them provide new and useful therapeutics for the treatment, control, and amelioration of symptoms of a variety of disorders. Moreover, pharmaceutical compositions comprising one or more of the nucleic acid compounds disclosed herein, provide significant advantages over existing conventional therapies -- namely, (1) their reduced side effects, (2) their increased efficacy for prolonged periods of time, (3) their ability to increase patient compliance due to their ability to provide therapeutic effects following as little as a single administration of the selected therapeutic AAV composition to affected individuals. Exemplary pharmaceutical compositions and methods for their administration are discussed in significant detail hereinbelow.

The invention also provides compositions comprising one or more of the disclosed vectors, expression systems, virions, viral particles; or mammalian cells. As described hereinbelow, such compositions may further comprise a pharmaceutical excipient, buffer, or diluent, and may be formulated for administration to an animal, and particularly a human being. Such compositions may further optionally comprise a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle, or may be otherwise formulated for administration to the cells, tissues, organs, or body of a mammal in need thereof. Such compositions may be formulated for use in therapy, such as for example, in the amelioration, prevention, or treatment of conditions such as peptide deficiency, polypeptide deficiency, tumor, cancer or other malignant growth, neurological dysfunction, autoimmune diseases, lupus, cardiovascular disease, pulmonary disease, ischemia, stroke, cerebrovascular accidents, diabetes and diseases of the pancreas, neural diseases, including Alzheimer's, Huntington's, Tay-Sach's, and Parkinson's diseases, memory loss, trauma, motor impairment, and the like, as well as biliary, renal or hepatic disease or dysfunction, as well as musculoskeletal diseases including, for example, arthritis, cystic fibrosis (CF), amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), muscular dystrophy (MD), and such like, to name only a few.

In certain embodiments, the present invention concerns formulation of one or more of the rAAV compositions disclosed herein in pharmaceutically acceptable solutions for administration to a cell or an animal, either alone or in combination with one or more other

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modalities of therapy, and in particular, for therapy of human cells, tissues, and diseases affecting man.

It will also be understood that, if desired, nucleic acid segments, RNA, DNA or PNA compositions that express one or more of therapeutic gene products may be administered in combination with other agents as well, such as, *e.g.*, proteins or polypeptides or various pharmaceutically-active agents, including one or more systemic or topical administrations of therapeutic polypeptides, biologically active fragments, or variants thereof. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The rAAV compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA, DNA, or PNA compositions.

Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation.

Typically, these formulations may contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be varied and may conveniently be between about 1 or 2% and about 70% or 80% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically-useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In certain circumstances it will be desirable to deliver the AAV vector-based therapeutic constructs in suitably formulated pharmaceutical compositions disclosed herein either subcutaneously, intraocularly, intravitreally, parenterally, intravenously, intracerebroventricularly, intramuscularly, intrathecally, orally, intraperitoneally, by oral or nasal inhalation, or by direct injection to one or more neural cells, nervous tissues, or even by direct injection or administration to the brain, CNS or to the peripheral nervous system. The

methods of administration may also include those modalities as described in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as freebase or pharmacologically acceptable salts may be prepared in sterile water and may also suitably mixed with one or more surfactants, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms of the AAV-based viral compositions suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U. S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the

condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active AAV vector-delivered therapeutic polypeptide-encoding DNA fragments in the required amount in the appropriate solvent with several of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The AAV vector compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug-release capsules, and the like.

As used herein, "carrier" includes any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human, and in particular, when administered to the human eye. The preparation of an aqueous composition

that contains a protein as an active ingredient is well understood in the art. Typically, such compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified.

The amount of AAV compositions and time of administration of such compositions will be within the purview of the skilled artisan having benefit of the present teachings. It is likely, however, that the administration of therapeutically-effective amounts of the disclosed compositions may be achieved by a single administration, such as for example, a single injection of sufficient numbers of infectious particles to provide therapeutic benefit to the patient undergoing such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple, or successive administrations of the AAV vector compositions, either over a relatively short, or a relatively prolonged period of time, as may be determined by the medical practitioner overseeing the administration of such compositions. For example, the number of infectious particles administered to a mammal may be on the order of about 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, or even higher, infectious particles/ml given either as a single dose, or divided into two or more administrations as may be required to achieve therapy of the particular disease or disorder being treated. In fact, in certain embodiments, it may be desirable to administer two or more different AAV vector compositions, either alone, or in combination with one or more other therapeutic drugs to achieve the desired effects of a particular therapy regimen.

4.6 Liposome-, Nanocapsule-, and Microparticle-Mediated Delivery

In certain embodiments, the inventors contemplate the use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the compositions of the present invention into suitable host cells. In particular, the rAAV vector delivered gene therapy compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like.

Such formulations may be preferred for the introduction of pharmaceutically acceptable formulations of the nucleic acids or the rAAV constructs disclosed herein. The formation and use of liposomes is generally known to those of skill in the art (see for example, Couvreur *et al.*, 1977; Couvreur, 1988; Lasic, 1998; which describes the use of liposomes and nanocapsules in the targeted antibiotic therapy for intracellular bacterial infections and diseases). Recently, liposomes were developed with improved serum stability and circulation half-times (Gabizon and Papahadjopoulos, 1988; Allen and Choun, 1987; U. S. Patent 5,741,516, specifically

incorporated herein by reference in its entirety). Further, various methods of liposome and liposome like preparations as potential drug carriers have been reviewed (Takakura, 1998; Chandran *et al.*, 1997; Margalit, 1995; U. S. Patent 5,567,434; U. S. Patent 5,552,157; U. S. Patent 5,565,213; U. S. Patent 5,738,868 and U. S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally resistant to transfection by other procedures including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, 1990; Muller *et al.*, 1990). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, drugs (Heath and Martin, 1986; Heath *et al.*, 1986; Balazsovits *et al.*, 1989; Fresta and Puglisi, 1996), radiotherapeutic agents (Pikul *et al.*, 1987), enzymes (Imaizumi *et al.*, 1990a; Imaizumi *et al.*, 1990b), viruses (Faller and Baltimore, 1984), transcription factors and allosteric effectors (Nicolau and Gersonde, 1979) into a variety of cultured cell lines and animals. In addition, several successful clinical trails examining the effectiveness of liposome-mediated drug delivery have been completed (Lopez-Berestein *et al.*, 1985a; 1985b; Coune, 1988; Sculier *et al.*, 1988). Furthermore, several studies suggest that the use of liposomes is not associated with autoimmune responses, toxicity or gonadal localization after systemic delivery (Mori and Fukatsu, 1992).

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm to 4 μm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

Liposomes bear resemblance to cellular membranes and are contemplated for use in connection with the present invention as carriers for the peptide compositions. They are widely suitable as both water- and lipid-soluble substances can be entrapped, *i.e.* in the aqueous spaces and within the bilayer itself, respectively. It is possible that the drug-bearing liposomes may even be employed for site-specific delivery of active agents by selectively modifying the liposomal formulation.

In addition to the teachings of Couvreur *et al.* (1977; 1988), the following information may be utilized in generating liposomal formulations. Phospholipids can form a variety of structures other than liposomes when dispersed in water, depending on the molar ratio of lipid to water. At low ratios the liposome is the preferred structure. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations. Liposomes can

show low permeability to ionic and polar substances, but at elevated temperatures undergo a phase transition which markedly alters their permeability. The phase transition involves a change from a closely packed, ordered structure, known as the gel state, to a loosely packed, less-ordered structure, known as the fluid state. This occurs at a characteristic phase-transition temperature and results in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can alter the permeability of liposomes. Certain soluble proteins, such as cytochrome c, bind, deform and penetrate the bilayer, thereby causing changes in permeability. Cholesterol inhibits this penetration of proteins, apparently by packing the phospholipids more tightly. It is contemplated that the most useful liposome formations for antibiotic and inhibitor delivery will contain cholesterol.

The ability to trap solutes varies between different types of liposomes. For example, MLVs are moderately efficient at trapping solutes, but SUVs are extremely inefficient. SUVs offer the advantage of homogeneity and reproducibility in size distribution, however, and a compromise between size and trapping efficiency is offered by large unilamellar vesicles (LUVs). These are prepared by ether evaporation and are three to four times more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an important determinant in entrapping compounds is the physicochemical properties of the compound itself. Polar compounds are trapped in the aqueous spaces and nonpolar compounds bind to the lipid bilayer of the vesicle. Polar compounds are released through permeation or when the bilayer is broken, but nonpolar compounds remain affiliated with the bilayer unless it is disrupted by temperature or exposure to lipoproteins. Both types show maximum efflux rates at the phase transition temperature.

Liposomes interact with cells *via* four different mechanisms: Endocytosis by phagocytic cells of the reticuloendothelial system such as macrophages and neutrophils; adsorption to the cell surface, either by nonspecific weak hydrophobic or electrostatic forces, or by specific interactions with cell-surface components; fusion with the plasma cell membrane by insertion of the lipid bilayer of the liposome into the plasma membrane, with simultaneous release of liposomal contents into the cytoplasm; and by transfer of liposomal lipids to cellular or subcellular membranes, or vice versa, without any association of the liposome contents. It often is difficult to determine which mechanism is operative and more than one may operate at the same time.

The fate and disposition of intravenously injected liposomes depend on their physical properties, such as size, fluidity, and surface charge. They may persist in tissues for h or days, depending on their composition, and half lives in the blood range from min to several h. Larger

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liposomes, such as MLVs and LUVs, are taken up rapidly by phagocytic cells of the reticuloendothelial system, but physiology of the circulatory system restrains the exit of such large species at most sites. They can exit only in places where large openings or pores exist in the capillary endothelium, such as the sinusoids of the liver or spleen. Thus, these organs are the predominate site of uptake. On the other hand, SUVs show a broader tissue distribution but still are sequestered highly in the liver and spleen. In general, this *in vivo* behavior limits the potential targeting of liposomes to only those organs and tissues accessible to their large size. These include the blood, liver, spleen, bone marrow, and lymphoid organs.

Targeting is generally not a limitation in terms of the present invention. However, should specific targeting be desired, methods are available for this to be accomplished. Antibodies may be used to bind to the liposome surface and to direct the antibody and its drug contents to specific antigenic receptors located on a particular cell-type surface. Carbohydrate determinants (glycoprotein or glycolipid cell-surface components that play a role in cell-cell recognition, interaction and adhesion) may also be used as recognition sites as they have potential in directing liposomes to particular cell types. Mostly, it is contemplated that intravenous injection of liposomal preparations would be used, but other routes of administration are also conceivable.

Alternatively, the invention provides for pharmaceutically acceptable nanocapsule formulations of the AAV vector-based polynucleotide compositions of the present invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (Henry-Michelland *et al.*, 1987; Quintanar-Guerrero *et al.*, 1998; Douglas *et al.*, 1987). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 μm) should be designed using polymers able to be degraded *in vivo*. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention. Such particles may be are easily made, as described (Couvreur *et al.*, 1980; Couvreur, 1988; zur Muhlen *et al.*, 1998; Zambaux *et al.* 1998; Pinto-Alphandry *et al.*, 1995 and U. S. Patent 5,145,684, specifically incorporated herein by reference in its entirety).

4.7 Additional Modes of Delivery

In addition to the methods of delivery described above, the following techniques are also contemplated as alternative methods of delivering the disclosed rAAV vector based polynucleotide compositions to a target cell or animal. Sonophoresis (*i.e.*, ultrasound) has been used and described in U. S. Patent 5,656,016 (specifically incorporated herein by reference in its entirety) as a device for enhancing the rate and efficacy of drug permeation into and through the

circulatory system. Other drug delivery alternatives contemplated are intraosseous injection (U. S. Patent 5,779,708), microchip devices (U. S. Patent 5,797,898), ophthalmic formulations (Bourlais *et al.*, 1998), transdermal matrices (U. S. Patent 5,770,219 and U. S. Patent 5,783,208) and feedback-controlled delivery (U. S. Patent 5,697,899), each specifically incorporated herein by reference in its entirety.

4.8 Promoters and Enhancers

Recombinant AAV vectors form important aspects of the present invention. The term "expression vector or construct" means any type of genetic construct containing a nucleic acid in which part or all of the nucleic acid encoding sequence is capable of being transcribed. In preferred embodiments, expression only includes transcription of the nucleic acid, for example, to generate a biologically-active therapeutic peptides, polypeptides, proteins, antisense molecules, or catalytic RNA ribozymes from a transcribed gene.

Particularly useful vectors are contemplated to be those vectors in which the nucleic acid segment to be transcribed is positioned under the transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrases "operatively positioned," "under control" or "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

In preferred embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an cytokine or serpin-encoding gene in its natural environment. Such promoters may include promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell.

Naturally, it will be important to employ a promoter that effectively directs the expression of the serpin or cytokine-encoding DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook *et al.* (1989), incorporated herein by reference. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high-level expression of the introduced DNA segment, or the promoters may direct tissue- or cell-specific

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expression of the therapeutic constructs, such as, for example, an islet cell- or pancreas-specific promoter such as the insulin promoter.

At least one module in a promoter functions to position the start site for RNA synthesis. The best-known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid is not believed to be critical, so long as it is capable of expressing the serpin or cytokine-polypeptide encoding nucleic acid segment in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter, such as a CMV or an HSV promoter. In certain aspects of the invention, β-actin, and in particular, chicken β-actin promoters have been shown to be particularly preferred for certain embodiments of the invention.

In various other embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of transgenes. The use of other viral or mammalian cellular or bacterial phage promoters that are well known in the art to achieve expression of a transgene is contemplated as well, provided that the levels of expression are sufficient for a given purpose. A variety of promoter elements have been described in Tables 1 and 2 that may be employed, in the context of the present invention, to regulate the expression of the present serpin or cytokine-encoding nucleic acid segments comprised within the recombinant AAV vectors of the present invention.

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Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

ILLUSTRATIVE PROMOTER AND ENHANCER ELEMENTS

PROMOTER/ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Banerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1984; Kiledjian *et al.*, 1988; Porton *et al.*; 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987; Winoto and Baltimore, 1989; Redondo *et al.*; 1990
HLA DQ a and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1988
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990

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PROMOTER/ENHANCER	REFERENCES
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-Dra	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*; 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase I	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987; Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
β-Globin	Trudel and Constantini, 1987
c-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsh *et al.*, 1990
α_{1}-Antitrypsin	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987; Schaffner *et al.*, 1988

PROMOTER/ENHANCER	REFERENCES
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; de Villiers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a, b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987; Stephens and Hentschel, 1987
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rosen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

INDUCIBLE ELEMENTS

ELEMENT	INDUCER	REFERENCES
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987, Karin *et al.*, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983;

ELEMENT	INDUCER	REFERENCES
		Chandler *et al.*, 1983; Lee *et al.*, 1984; Ponta *et al.*, 1985; Sakai *et al.*, 1988
β-Interferon	poly(rI)x poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angel *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angel *et al.*, 1987b
SV40	Phorbol Ester (TPA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2κb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a, b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous DNA segment, such as DNA segment that leads to the transcription of a biologically-active serpin or cytokine polypeptide or a ribozyme specific for such a biologically-active serpin or cytokine polypeptide product, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are thus cells having DNA segment introduced through the hand of man.

To express a biologically-active serpin or cytokine encoding gene in accordance with the present invention one would prepare an rAAV expression vector that comprises a biologically-active serpin or cytokine polypeptide-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between

about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context. Particularly preferred recombinant vector constructs are those that comprise an rAAV vector. Such vectors are described in detail herein.

4.9 Mutagenesis and Preparation of Modified Nucleotide Compositions

In certain embodiments, it may be desirable to prepared modified nucleotide compositions, such as, for example, in the generation of the nucleic acid segments that encode either parts of the AAV vector itself, or the promoter, or even the therapeutic gene delivered by such rAAV vectors. Various means exist in the art, and are routinely employed by the artisan to generate modified nucleotide compositions.

Site-specific mutagenesis is a technique useful in the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art. As will be appreciated, the technique typically employs a bacteriophage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector, or melting of two strands of a double stranded vector that includes within its sequence a DNA sequence encoding the desired ribozyme or other nucleic acid construct. An oligonucleotide primer bearing the desired mutated sequence is synthetically prepared. This primer is then annealed with the single-stranded DNA preparation, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand

encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected nucleic acid sequences using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting, as there are other ways in which sequence variants may be obtained. For example, recombinant vectors encoding the desired gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

4.10 NUCLEIC ACID AMPLIFICATION

In certain embodiments, it may be necessary to employ one or more nucleic acid amplification techniques to produce the nucleic acid segments of the present invention. Various methods are well-known to artisans in the field, including for example, those techniques described herein:

Nucleic acid, used as a template for amplification, may be isolated from cells contained in the biological sample according to standard methodologies (Sambrook *et al.*, 1989). The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to convert the RNA to a complementary DNA. In one embodiment, the RNA is whole cell RNA and is used directly as the template for amplification.

Pairs of primers that selectively hybridize to nucleic acids corresponding to the ribozymes or conserved flanking regions are contacted with the isolated nucleic acid under conditions that permit selective hybridization. The term "primer", as defined herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty base pairs in length, but longer sequences can be employed. Primers may be provided in double-stranded or single-stranded form, although the single-stranded form is preferred.

Once hybridized, the nucleic acid:primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

Next, the amplification product is detected. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or

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fluorescent label or even *via* a system using electrical or thermal impulse signals (*e.g.*, Affymax technology).

A number of template dependent processes are available to amplify the marker sequences present in a given template sample. One of the best-known amplification methods is the polymerase chain reaction (referred to as PCR™), which is described in detail in U. S. Patent No. 4,683,195, U. S. Patent No. 4,683,202 and U. S. Patent No. 4,800,159 (each of which is incorporated herein by reference in its entirety).

Briefly, in PCR™, two primer sequences are prepared that are complementary to regions on opposite complementary strands of the marker sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase, *e.g.*, *Taq* polymerase. If the marker sequence is present in a sample, the primers will bind to the marker and the polymerase will cause the primers to be extended along the marker sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the marker to form reaction products, excess primers will bind to the marker and to the reaction products and the process is repeated.

A reverse transcriptase PCR™ amplification procedure may be performed in order to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.* (1989). Alternative methods for reverse transcription utilize thermostable, RNA-dependent DNA polymerases. These methods are described in Int. Pat. Appl. Publ. No. WO 90/07641 (specifically incorporated herein by reference). Polymerase chain reaction methodologies are well known in the art.

Another method for amplification is the ligase chain reaction ("LCR"), disclosed in EPA No. 320 308, and incorporated herein by reference in its entirety. In LCR, two complementary probe pairs are prepared, and in the presence of the target sequence, each pair will bind to opposite complementary strands of the target such that they abut. In the presence of a ligase, the two probe pairs will link to form a single unit. By temperature cycling, as in PCR™, bound ligated units dissociate from the target and then serve as "target sequences" for ligation of excess probe pairs. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence.

Qβ Replicase (QβR), described in Int. Pat. Appl. No. PCT/US87/00880, incorporated herein by reference, may also be used as still another amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to

that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence that can then be detected.

An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[α-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention.

Strand Displacement Amplification (SDA), described in U. S. Patent Nos. 5,455,166, 5,648,211, 5,712,124 and 5,744,311, each incorporated herein by reference, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation. A similar method, called Repair Chain Reaction (RCR), involves annealing several probes throughout a region targeted for amplification, followed by a repair reaction in which only two of the four bases are present. The other two bases can be added as biotinylated derivatives for easy detection. A similar approach is used in SDA. Target specific sequences can also be detected using a cyclic probe reaction (CPR). In CPR, a probe having 3' and 5' sequences of non-specific DNA and a middle sequence of specific RNA is hybridized to DNA that is present in a sample. Upon hybridization, the reaction is treated with RNase H, and the products of the probe identified as distinctive products that are released after digestion. The original template is annealed to another cycling probe and the reaction is repeated.

Still another amplification methods described in GB Application No. 2 202 328, and in Int. Pat. Appl. No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety, may be used in accordance with the present invention. In the former application, "modified" primers are used in a PCR™-like, template- and enzyme-dependent synthesis. The primers may be modified by labeling with a capture moiety (*e.g.*, biotin) and/or a detector moiety (*e.g.*, enzyme). In the latter application, an excess of labeled probes is added to a sample. In the presence of the target sequence, the probe binds and is cleaved catalytically. After cleavage, the target sequence is released intact to be bound by excess probe. Cleavage of the labeled probe signals the presence of the target sequence.

Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR Gingeras *et al.*, Int. Pat. Appl. Publ. No. WO 88/10315, incorporated herein by reference. In NASBA, the nucleic acids can be prepared for amplification by standard phenol/chloroform extraction, heat denaturation of a clinical sample, treatment with lysis buffer and minispin columns for isolation of DNA and RNA or guanidinium chloride extraction of RNA. These

amplification techniques involve annealing a primer that has target specific sequences. Following polymerization, DNA/RNA hybrids are digested with RNase H while double stranded DNA molecules are heat denatured again. In either case the single stranded DNA is made fully double stranded by addition of second target specific primer, followed by polymerization. The double-stranded DNA molecules are then multiply transcribed by an RNA polymerase such as T7 or SP6. In an isothermal cyclic reaction, the RNAs are reverse transcribed into single stranded DNA, which is then converted to double stranded DNA, and then transcribed once again with an RNA polymerase such as T7 or SP6. The resulting products, whether truncated or complete, indicate target specific sequences.

Davey *et al.*, EPA No. 329 822 (incorporated herein by reference in its entirety) disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention. The ssRNA is a template for a first primer oligonucleotide, which is elongated by reverse transcriptase (RNA-dependent DNA polymerase). The RNA is then removed from the resulting DNA:RNA duplex by the action of ribonuclease H (RNase H, an RNase specific for RNA in duplex with either DNA or RNA). The resultant ssDNA is a template for a second primer, which also includes the sequences of an RNA polymerase promoter (exemplified by T7 RNA polymerase) 5' to its homology to the template. This primer is then extended by DNA polymerase (exemplified by the large "Klenow" fragment of *E. coli* DNA polymerase I), resulting in a double-stranded DNA ("dsDNA") molecule, having a sequence identical to that of the original RNA between the primers and having additionally, at one end, a promoter sequence. This promoter sequence can be used by the appropriate RNA polymerase to make many RNA copies of the DNA. These copies can then re-enter the cycle leading to very swift amplification. With proper choice of enzymes, this amplification can be done isothermally without addition of enzymes at each cycle. Because of the cyclical nature of this process, the starting sequence can be chosen to be in the form of either DNA or RNA.

Miller *et al.*, Int. Pat. Appl. Publ. No. WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "RACE" and "one-sided PCR™" (Frohman, 1990, specifically incorporated herein by reference).

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Methods based on ligation of two (or more) oligonucleotides in the presence of nucleic acid having the sequence of the resulting "di-oligonucleotide," thereby amplifying the di-oligonucleotide, may also be used in the amplification step of the present invention.

Following any amplification, it may be desirable to separate the amplification product from the template and the excess primer for the purpose of determining whether specific amplification has occurred. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (see *e.g.*, Sambrook *et al.*, 1989).

Alternatively, chromatographic techniques may be employed to effect separation. There are many kinds of chromatography which may be used in the present invention: adsorption, partition, ion-exchange and molecular sieve, and many specialized techniques for using them including column, paper, thin-layer and gas chromatography.

Amplification products must be visualized in order to confirm amplification of the marker sequences. One typical visualization method involves staining of a gel with ethidium bromide and visualization under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the amplification products can then be exposed to x-ray film or visualized under the appropriate stimulating spectra, following separation.

In one embodiment, visualization is achieved indirectly. Following separation of amplification products, a labeled, nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, and the other member of the binding pair carries a detectable moiety.

In one embodiment, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art and can be found in many standard books on molecular protocols. See Sambrook *et al.*, 1989. Briefly, amplification products are separated by gel electrophoresis. The gel is then contacted with a membrane, such as nitrocellulose, permitting transfer of the nucleic acid and non-covalent binding. Subsequently, the membrane is incubated with a chromophore-conjugated probe that is capable of hybridizing with a target amplification product. Detection is by exposure of the membrane to x-ray film or ion-emitting detection devices.

One example of the foregoing is described in U. S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and

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blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

4.11 Methods of Nucleic Acid Delivery and DNA Transfection

In certain embodiments, it is contemplated that one or more RNA, DNA, PNAs and/or substituted polynucleotide compositions disclosed herein will be used to transfect an appropriate host cell. Technology for introduction of PNAs, RNAs, and DNAs into cells is well known to those of skill in the art.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Wong and Neumann, 1982; Fromm *et al.*, 1985; Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984; Suzuki et al., 1998; Vanbever *et al.*, 1998), direct microinjection (Capecchi, 1980; Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979; Takakura, 1998) and lipofectamine-DNA complexes, cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990; Klein *et al.*, 1992), and receptor-mediated transfection (Curiel *et al.*, 1991; Wagner *et al.*, 1992; Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

4.12 Expression Vectors

The present invention contemplates a variety of AAV-based expression systems, and vectors. In one embodiment the preferred AAV expression vectors comprise at least a first nucleic acid segment that encodes a therapeutic peptide, protein, or polypeptide. In another embodiment, the preferred AAV expression vectors disclosed herein comprise at least a first nucleic acid segment that encodes an antisense molecule. In another embodiment, a promoter is operatively linked to a sequence region that encodes a functional mRNA, a tRNA, a ribozyme or an antisense RNA.

As used herein, the term "operatively linked" means that a promoter is connected to a functional RNA in such a way that the transcription of that functional RNA is controlled and regulated by that promoter. Means for operatively linking a promoter to a functional RNA are well known in the art.

The choice of which expression vector and ultimately to which promoter a polypeptide coding region is operatively linked depend directly on the functional properties desired, e.g., the

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location and timing of protein expression, and the host cell to be transformed. These are well known limitations inherent in the art of constructing recombinant DNA molecules. However, a vector useful in practicing the present invention is capable of directing the expression of the functional RNA to which it is operatively linked.

RNA polymerase transcribes a coding DNA sequence through a site where polyadenylation occurs. Typically, DNA sequences located a few hundred base pairs downstream of the polyadenylation site serve to terminate transcription. Those DNA sequences are referred to herein as transcription-termination regions. Those regions are required for efficient polyadenylation of transcribed messenger RNA (mRNA).

A variety of methods have been developed to operatively link DNA to vectors *via* complementary cohesive termini or blunt ends. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted and to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

4.13 Biological Functional Equivalents

Modification and changes to the structure of the polynucleotides and polypeptides of wild-type rAAV vectors to provide the improved rAAV virions as described in the present invention to obtain functional viral vectors that possess desirable characteristics, particularly with respect to improved delivery of therapeutic gene constructs to selected mammalian cell, tissues, and organs for the treatment, prevention, and prophylaxis of various diseases and disorders, as well as means for the amelioration of symptoms of such diseases, and to facilitate the expression of exogenous therapeutic and/or prophylactic polypeptides of interest *via* rAAV vector-mediated gene therapy. As mentioned above, one of the key aspects of the present invention is the creation of one or more mutations into specific polynucleotide sequences that encode one or more of the therapeutic agents encoded by the disclosed rAAV constructs. In certain circumstances, the resulting polypeptide sequence is altered by these mutations, or in other cases, the sequence of the polypeptide is unchanged by one or more mutations in the encoding polynucleotide to produce modified vectors with improved properties for effecting gene therapy in mammalian systems.

When it is desirable to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, second-generation molecule, the amino acid changes may be achieved by changing one or more of the codons of the encoding DNA sequence, according to Table 3.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the polynucleotide sequences disclosed herein, without appreciable loss of their biological utility or activity.

TABLE 3

Amino Acids		Codons						
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (-0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (-3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (-1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (-2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those that are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity,

hydrophilicity, charge, size, and the like. Exemplary substitutions which take several of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

4.14 Therapeutic and Diagnostic Kits

The invention also encompasses one or more of the modified rAAV vector compositions described herein together with one or more pharmaceutically-acceptable excipients, carriers, diluents, adjuvants, and/or other components, as may be employed in the formulation of particular rAAV-polynucleotide delivery formulations, and in the preparation of therapeutic agents for administration to a mammal, and in particularly, to a human. In particular, such kits may comprise one or more of the disclosed rAAV compositions in combination with instructions for using the viral vector in the treatment of such disorders in a mammal, and may typically further include containers prepared for convenient commercial packaging.

As such, preferred animals for administration of the pharmaceutical compositions disclosed herein include mammals, and particularly humans. Other preferred animals include murines, bovines, equines, porcines, canines, and felines. The composition may include partially or significantly purified rAAV compositions, either alone, or in combination with one or more additional active ingredients, which may be obtained from natural or recombinant sources, or which may be obtainable naturally or either chemically synthesized, or alternatively produced *in vitro* from recombinant host cells expressing DNA segments encoding such additional active ingredients.

Therapeutic kits may also be prepared that comprise at least one of the compositions disclosed herein and instructions for using the composition as a therapeutic agent. The container means for such kits may typically comprise at least one vial, test tube, flask, bottle, syringe or other container means, into which the disclosed rAAV composition(s) may be placed, and preferably suitably aliquoted. Where a second therapeutic polypeptide composition is also provided, the kit may also contain a second distinct container means into which this second composition may be placed. Alternatively, the plurality of therapeutic biologically active compositions may be prepared in a single pharmaceutical composition, and may be packaged in a single container means, such as a vial, flask, syringe, bottle, or other suitable single container means. The kits of the present invention will also typically include a means for containing the vial(s) in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vial(s) are retained.

4.15 Ribozymes

As mentioned above, one aspect of the invention concerns the use of the modified capsid vectors to deliver catalytic RNA molecules (ribozymes) to selected mammalian cells and tissues to effect a reduction or elimination of expression of one or more native DNA or mRNA molecules, so as to prevent or reduce the amount of the translation product of such mRNAs. Ribozymes are biological catalysts consisting of only RNA. They promote a variety of reactions involving RNA and DNA molecules including site-specific cleavage, ligation, polymerization, and phosphoryl exchange (Cech, 1989; Cech, 1990). Ribozymes fall into three broad classes: (1) RNAse P, (2) self-splicing introns, and (3) self-cleaving viral agents. Self-cleaving agents include hepatitis delta virus and components of plant virus satellite RNAs that sever the RNA genome as part of a rolling-circle mode of replication. Because of their small size and great specificity, ribozymes have the greatest potential for biotechnical applications. The ability of ribozymes to cleave other RNA molecules at specific sites in a catalytic manner has brought them into consideration as inhibitors of viral replication or of cell proliferation and gives them potential advantage over antisense RNA. Indeed, ribozymes have already been used to cleave viral targets and oncogene products in living cells (Koizumi *et al.*, 1992; Kashani-Sabet *et al.*, 1992; Taylor and Rossi, 1991; von-Weizsacker *et al.*, 1992; Ojwang *et al.*, 1992; Stephenson and Gibson, 1991; Yu *et al.*, 1993; Xing and Whitton, 1993; Yu *et al.*, 1995; Little and Lee, 1995).

Two kinds of ribozymes have been employed widely, hairpins and hammerheads. Both catalyze sequence-specific cleavage resulting in products with a 5N hydroxyl and a 2N,3N-cyclic phosphate. Hammerhead ribozymes have been used more commonly, because they impose few restrictions on the target site. Hairpin ribozymes are more stable and, consequently, function better than hammerheads at physiologic temperature and magnesium concentrations.

A number of patents have issued describing various ribozymes and methods for designing ribozymes. See, for example, U.S. Patent Nos. 5,646,031; 5,646,020; 5,639,655; 5,093,246; 4,987,071; 5,116,742; and 5,037,746, each specifically incorporated herein by reference in its entirety. However, the ability of ribozymes to provide therapeutic benefit *in vivo* has not yet been demonstrated.

Although proteins traditionally have been used for catalysis of nucleic acids, another class of macromolecules has emerged as useful in this endeavor. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, 1987; Gerlach

et al., 1987; Forster and Symons, 1987). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, 1981; Michel and Westhof, 1990; Reinhold-Hurek and Shub, 1992). This specificity has been attributed to the requirement that the substrate bind *via* specific base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Ribozyme catalysis has primarily been observed as part of sequence-specific cleavage/ligation reactions involving nucleic acids (Joyce, 1989; Cech *et al.*, 1981). For example, U. S. Patent No. 5,354,855 (specifically incorporated herein by reference) reports that certain ribozymes can act as endonucleases with a sequence-specificity greater than that of known ribonucleases and approaching that of the DNA restriction enzymes. Thus, sequence-specific ribozyme-mediated inhibition of gene expression may be particularly suited to therapeutic applications (Scanlon *et al.*, 1991; Sarver *et al.*, 1990). Recently, it was reported that ribozymes elicited genetic changes in some cells lines to which they were applied; the altered genes included the oncogenes H-*ras*, c-*fos* and genes of HIV. Most of this work involved the modification of a target mRNA, based on a specific mutant codon that is cleaved by a specific ribozyme.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the

specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, 1992). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* (1992). Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz (1989), Hampel *et al.* (1990) and U. S. Patent 5,631,359 (specifically incorporated herein by reference). An example of the hepatitis δ virus motif is described by Perrotta and Been (1992); an example of the RNaseP motif is described by Guerrier-Takada *et al.* (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990; Saville and Collins, 1991; Collins and Olive, 1993); and an example of the Group I intron is described in U. S. Patent 4,987,071 (specifically incorporated herein by reference). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

In certain embodiments, it may be important to produce enzymatic cleaving agents that exhibit a high degree of specificity for the RNA of a desired target, such as one of the sequences disclosed herein. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of a target mRNA. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required, although in preferred embodiments the ribozymes are expressed from DNA or RNA vectors that are delivered to specific cells.

Small enzymatic nucleic acid motifs (*e.g.*, of the hammerhead or the hairpin structure) may also be used for exogenous delivery. The simple structure of these molecules increases the ability of the enzymatic nucleic acid to invade targeted regions of the mRNA structure. Alternatively, catalytic RNA molecules can be expressed within cells from eukaryotic promoters (*e.g.*, Scanlon *et al.*, 1991; Kashani-Sabet *et al.*, 1992; Dropulic *et al.*, 1992; Weerasinghe *et al.*, 1991; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Sarver *et al.*, 1990). Those skilled in the art realize that any ribozyme can be expressed in eukaryotic cells from the

appropriate DNA vector. The activity of such ribozymes can be augmented by their release from the primary transcript by a second ribozyme (Int. Pat. Appl. Publ. No. WO 93/23569, and Int. Pat. Appl. Publ. No. WO 94/02595, both hereby incorporated by reference; Ohkawa *et al.*, 1992; Taira *et al.*, 1991; and Ventura *et al.*, 1993).

Ribozymes may be added directly, or can be complexed with cationic lipids, lipid complexes, packaged within liposomes, or otherwise delivered to target cells. The RNA or RNA complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, aerosol inhalation, infusion pump or stent, with or without their incorporation in biopolymers.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595 (each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Hammerhead or hairpin ribozymes may be individually analyzed by computer folding (Jaeger *et al.*, 1989) to assess whether the ribozyme sequences fold into the appropriate secondary structure, as described herein. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 or so bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif may be designed to anneal to various sites in the mRNA message, and can be chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman *et al.* (1987) and in Scaringe *et al.* (1990) and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5′-end, and phosphoramidites at the 3′-end. Average stepwise coupling yields are typically >98%. Hairpin ribozymes may be synthesized in two parts and annealed to reconstruct an active ribozyme (Chowrira and Burke, 1992). Ribozymes may be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2′-amino, 2′-C-allyl, 2′-flouro, 2′-o-methyl, 2′-H (for a review see e.g., Usman and Cedergren, 1992). Ribozymes may be purified by gel electrophoresis using general methods or by high-pressure liquid chromatography and resuspended in water.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see e.g., Int. Pat. Appl. Publ. No. WO 92/07065; Perrault *et al*, 1990;

Pieken *et al.*, 1991; Usman and Cedergren, 1992; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U.S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

A preferred means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990; Gao and Huang, 1993; Lieber *et al.*, 1993; Zhou *et al.*, 1990). Ribozymes expressed from such promoters can function in mammalian cells (Kashani-Sabet *et al.*, 1992; Ojwang *et al.*, 1992; Chen *et al.*, 1992; Yu *et al.*, 1993; L'Huillier *et al.*, 1992; Lisziewicz *et al.*, 1993). Although incorporation of the present ribozyme constructs into adeno-associated viral vectors is preferred, such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, other viral DNA vectors (such as adenovirus vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraocular, retinal, subretinal, intraperitoneal, intracerebroventricular, intrathecal delivery, and/or direct injection to one or more tissues of the brain. More detailed descriptions of ribozyme and rAAV

vector delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Ribozymes and the AAV vectored-constructs of the present invention may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of one or more neural diseases, dysfunctions, cancers,, and/or disorders. In this manner, other genetic targets may be defined as important mediators of the disease. These studies lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules).

4.16 Antisense Oligonucleotides

In certain embodiments, the AAV constructs of the invention will find utility in the delivery of antisense oligonucleotides and polynucleotides for inhibiting the expression of a selected mammalian mRNA in neural cells.

In the art the letters, A, G, C, T, and U respectively indicate nucleotides in which the nucleoside is Adenosine (Ade), Guanosine (Gua), Cytidine (Cyt), Thymidine (Thy), and Uridine (Ura). As used in the specification and claims, compounds that are "antisense" to a particular PNA, DNA or mRNA "sense" strand are nucleotide compounds that have a nucleoside sequence that is complementary to the sense strand. It will be understood by those skilled in the art that the present invention broadly includes oligonucleotide compounds that are capable of binding to the selected DNA or mRNA sense strand. It will also be understood that mRNA includes not only the ribonucleotide sequences encoding a protein, but also regions including the 5'-untranslated region, the 3'-untranslated region, the 5'-cap region and the intron/exon junction regions.

The invention includes compounds which are not strictly antisense; the compounds of the invention also include those oligonucleotides that may have some bases that are not complementary to bases in the sense strand provided such compounds have sufficient binding affinity for the particular DNA or mRNA for which an inhibition of expression is desired. In addition, base modifications or the use of universal bases such as inosine in the oligonucleotides of the invention are contemplated within the scope of the subject invention.

The antisense compounds may have some or all of the phosphates in the nucleotides replaced by phosphorothioates (X=S) or methylphosphonates ($X=CH_3$) or other C_{1-4} alkylphosphonates. The antisense compounds optionally may be further differentiated from

native DNA by replacing one or both of the free hydroxy groups of the antisense molecule with C_{1-4} alkoxy groups (R=C_{1-4} alkoxy). As used herein, C_{1-4} alkyl means a branched or unbranched hydrocarbon having 1 to 4 carbon-atoms.

The disclosed antisense compounds also may be substituted at the 3′ and/or 5′ ends by a substituted acridine derivative. As used herein, "substituted acridine," means any acridine derivative capable of intercalating nucleotide strands such as DNA. Preferred substituted acridines are 2-methoxy-6-chloro-9-pentylaminoacridine, *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-3-aminopropanol, and *N*-(6-chloro-2-methoxyacridinyl)-O-methoxydiisopropylaminophosphinyl-5-aminopentanol. Other suitable acridine derivatives are readily apparent to persons skilled in the art. Additionally, as used herein "P(O)(O) -substituted acridine" means a phosphate covalently linked to a substitute acridine.

As used herein, the term "nucleotides" includes nucleotides in which the phosphate moiety is replaced by phosphorothioate or alkylphosphonate and the nucleotides may be substituted by substituted acridines.

In one embodiment, the antisense compounds of the invention differ from native DNA by the modification of the phosphodiester backbone to extend the life of the antisense molecule. For example, the phosphates can be replaced by phosphorothioates. The ends of the molecule may also be optimally substituted by an acridine derivative that intercalates nucleotide strands of DNA. Intl. Pat. Appl. Publ. No. WO 98/13526 and U. S. Patent 5,849,902 (each specifically incorporated herein by reference in its entirety) describe a method of preparing three component chimeric antisense compositions, and discuss many of the currently available methodologies for synthesis of substituted oligonucleotides having improved antisense characteristics and/or half-life.

The reaction scheme involves ^{1}H-tetrazole-catalyzed coupling of phosphoramidites to give phosphate intermediates that are subsequently reacted with sulfur in 2,6-lutidine to generate phosphate compounds. Oligonucleotide compounds are prepared by treating the phosphate compounds with thiophenoxide (1:2:2 thiophenol/triethylamine/tetrahydrofuran, room temperature, 1 hr). The reaction sequence is repeated until an oligonucleotide compound of the desired length has been prepared. The compounds are cleaved from the support by treating with ammonium hydroxide at room temperature for 1 hr and then are further deprotected by heating at about 50°C overnight to yield preferred antisense compounds.

Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure, T_m, binding

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energy, relative stability, and antisense compositions were selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those that are at or near the AUG translation initiation codon, and those sequences that were substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations were performed using v.4 of the OLIGO primer analysis software (Rychlik, 1997) and the BLASTN 2.0.5 algorithm software (Altschul *et al.*, 1997).

4.17 Exemplary Definitions

In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from native sources, chemically synthesized, modified, or otherwise prepared in whole or in part by the hand of man.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

A, an: In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denotes "one or more".

Expression: The combination of intracellular processes, including transcription and translation undergone by a polynucleotide such as a structural gene to synthesize the encoded peptide or polypeptide.

Promoter: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Regulatory Element: a term used to generally describe the region or regions of a nucleic acid sequence that regulates transcription.

Structural gene: A gene or sequence region that is expressed to produce an encoded peptide or polypeptide.

Transformation: A process of introducing an exogenous polynucleotide sequence (*e.g.*, a vector, a recombinant DNA or RNA molecule) into a host cell or protoplast in which that exogenous nucleic acid segment is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell. Transfection, electroporation, and naked nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

Transformed cell: A host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

Transgenic cell: Any cell derived or regenerated from a transformed cell or derived from a transgenic cell, or from the progeny or offspring of any generation of such a transformed host cell.

Vector: A nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

The terms "substantially corresponds to", "substantially homologous", or "substantial identity" as used herein denotes a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared. The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides. Desirably, which highly homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at

least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as e.g., the FASTA program analysis described by Pearson and Lipman (1988).

The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

"Transcriptional regulatory element" refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

As used herein, a "transcription factor recognition site" and a "transcription factor binding site" refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

As used herein, the term "operably linked" refers to a linkage of two or more polynucleotides or two or more nucleic acid sequences in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally

function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

"Transcriptional unit" refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first *cis*-acting promoter sequence and optionally linked operably to one or more other *cis*-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional *cis* sequences that are necessary for efficient transcription and translation (*e.g.*, polyadenylation site(s), mRNA stability controlling sequence(s), *etc.*

The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular subject sequence, for example, an oligonucleotide sequence, is substantially complementary to all or a portion of the selected sequence, and thus will specifically bind to a portion of an mRNA encoding the selected sequence. As such, typically the sequences will be highly complementary to the mRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the complementary portion of the sequence. In many instances, it may be desirable for the sequences to be exact matches, *i.e.* be completely complementary to the sequence to which the oligonucleotide specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region of the mRNA and will therefore be highly efficient in reducing, and/or even inhibiting the translation of the target mRNA sequence into polypeptide product.

Substantially complementary oligonucleotide sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary oligonucleotide sequences for use in the practice of the invention, and in such instances, the oligonucleotide sequences will be greater than about 90 percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and may in certain embodiments be greater than about 95

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percent complementary to the corresponding mRNA target sequence to which the oligonucleotide specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to all or a portion of the target mRNA to which the designed oligonucleotide specifically binds.

Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

5. EXAMPLES

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

5.1 EXAMPLE 1 – IMPROVED RAAV VECTORS HAVING GENETIC MODIFICATIONS IN SPECIFIC CAPSID PROTEINS

Given advances in purification methods for rAAV2, the requirements of the individual capsid protein species in rAAV2 particle formation were reexamined in the context of designing a novel rAAV2 production system that would allow for the modification of a specific capsid protein in regions of capsid sequence overlap. Currently, highly purified and concentrated preparations of rAAV2 particles are possible from two plasmid-based production systems. These systems differ in that one system supplies the necessary

adenovirus helper functions and AAV rep and cap genes from one plasmid (pDG), while the other uses two plasmids to supply these proteins (pIM45 and pXX6). These constructs are transfected into an appropriate cell type along with a construct containing a transgene expression cassette flanked by the AAV terminal repeats (*e.g.*, pTR-UF5). This example describes an rAAV2 production system based on modifications of the triple plasmid transfection method. In this system, the expression of a specific capsid protein is restricted to one pIM45 plasmid and complemented in trans with the remaining two capsid proteins expressed from a second pIM45 plasmid. This approach maintains expression of the capsid proteins in their genomic context while providing a platform for the genetic modification of a specific capsid protein or two of the capsid proteins across their entire coding sequence. Missense mutation of the capsid proteins' start codons generated pIM45 plasmids that express a single capsid protein: pIM45-VP1, pIM45-VP2 (ACG or ATG start codon), and pIM45-VP3. Such plasmids can be complemented with plasmids expressing the remaining 2 capsid proteins (pIM45-VP2,3, pIM45-VP1,3, and pIM45-VP1,2 (ACG or ATG start codon), respectively) in order to produce viable rAAV2 vectors. Using the system's plasmid components individually, a reevaluation of capsid protein requirements for the production of rAAV2 particles revealed that viable rAAV2-like particles are produced as long as the VP3 protein is present (VP1+2+3, VP1+3, VP2+3, and VP3 only). Focusing on large peptide insertions in the VP1 and VP2 proteins without altering the critical VP3 protein, the utility of this system is demonstrated through the production of viable rAAV2 particles containing 8-, 15-, and 29-kDa proteins inserted immediately following amino acid 138 in both VP1 and VP2 proteins or in VP2 protein alone. Finally, rAAV2-like particles can be produced with altered capsid protein stoichiometry if VP2 is significantly over expressed.

5.1.1 Construction of rAAV2 Capsid Mutant Plasmids That Express Two Capsid Proteins

To isolate the expression of a specific capsid protein to one pIM45 plasmid and the remaining two capsid proteins to a second pIM45 plasmid, missense mutation of the AAV2 cap ORF start codons was employed as previously described. Using site-directed mutagenesis of a pIM45 template, the VP1 start codon was mutated to leucine to generate the construct, pIM45-VP2,3, the VP2 start codon to alanine to generate the construct, pIM45-VP1,3, and the VP3 start codon to leucine to generate the construct, pIM45-VP1,2 (FIG. 1A). Western blotting analysis of capsid protein expression in whole cell lysates 48 hours post transfection of 293 cells with these plasmids in the presence of Ad5 (MOI=10) was carried

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out using the B1 antibody which recognizes all three capsid proteins (FIG. 1A). As previously reported, the expression of VP1 and VP2 could be eliminated by missense mutation of their start codons (FIG. 1A, lanes 2 and 3), and, in contrast, mutation of the VP3 start codon resulted in expression of a smaller VP3-like fragment (VP3a) (FIG. 1A, lane 4). Since this construct did not eliminate all VP3-like proteins it was renamed, pIM45-M203L. In the baculovirus study of AAV particle assembly, it was suggested that mutation of the VP3 start codon allows translational initiation to occur downstream at the next available ATG codon with correct Kozak sequences. While no additional ATG codons are found between the VP1 start codon and the start of VP3, an examination of the VP3 capsid revealed that nine additional ATG codons are present (amino acid positions 211, 235, 371, 402, 434, 523, 558, 604, and 634). Of these methionines, only those at amino acid position 211, 235, 523, 558, and 604 are in a context that is predicted favorable by Kozak. Since the VP3a fragment is slightly smaller than wildtype VP3, the contribution of continued read through translational initiation to the appearance of the VP3a fragment was examined by mutating the next two available ATG codons (M211 and M235) on a pIM45-M203L template yielding the plasmids, pIM45-M203L, pIM45-M203,211L and pIM45-M203,211,235L (FIG. 2A). Western blotting analysis of capsid protein expression in whole cell lysates 48 hours post transfection of 293 cells in the presence of Ad5 (MOI=10) revealed that translational initiation could occur at both these ATG codons. FIG. 1B (lane 2) again demonstrates the formation of VP3a following the mutation M203L. Combined mutation of M203 and M211 allowed less robust expression of a second still shorter VP3-like fragment (VP3b, FIG. 1B, lane 3). Subsequent mutation of M235 in the pIM45-M203,211L background led to disappearance of this VP3b fragment generating pIM45-VP1,2 (FIG. 1B, lane 4). Collectively, while missense mutagenesis of the VP1 start codon does not alter the sequence of the VP2 and VP3 protein expressed (pIM45-VP2,3, M1L), mutation of the VP2 start codon results in one point mutation in the expressed VP1 protein (pIM45-VP1,3, T138A), and elimination of all VP3-like proteins results in three mutations in the remaining VP1 and VP2 proteins (pIM45-VP1,2, M203,211,235L).

An alternative method has been reported for eliminating VP3 expression that limits mutation of remaining capsid sequences to one point mutation in the VP2 start codon. Changing the VP2 start codon from ACG to ATG results in loss of VP3 expression (pIM45-VP1,2A) with one point mutation in both the VP1 and VP2 proteins (T138M). Presumably, this stronger VP2 start codon prevents efficient translational initiation at the downstream VP3 start codon. The VP2 start codon was mutated to ATG on a pIM45 template (pIM45-

VP1,2A (FIG. 1C)) as an alternative means of eliminating VP3 protein (while maximizing VP2 expression). As expected, Western blotting analysis of capsid protein expression in whole cell lysates 48 hr post transfection of 293 cells in the presence of Ad5 (MOI=10) with pIM45-VP1,2A showed normal levels of VP1 protein produced, with significantly increased expression of VP2 protein (FIG. 1C, lane 2).

5.1.2 Construction of rAAV2 Capsid Plasmid Mutants That Express A Single Capsid Protein

To complete the complementary pIM45 capsid groups, pIM45 plasmids that express a single capsid protein were generated next. Employing the same missense mutations described above on templates that now only express two capsid proteins, the plasmids, pIM45-VP1, pIM45-VP2, pIM45-VP2A, and pIM45-VP3 (FIG. 2) were also generated. pIM45-VP1, has the VP2 start codon mutated to alanine and M203, M211, and M235 mutated to L in the expressed VP1 protein . pIM45-VP2 has the VP1 start codon mutated to leucine and M203, M211, and M235 mutated to L. The expressed VP2 protein contains only M203, M211, and M235 mutations. pIM45-VP3 has the VP1 start codon mutated to leucine and the VP2 start codon mutated to alanine. Like all VP3 protein in these complementary groups, the VP3 coding sequence is not mutated. Finally, pIM45-VP2A has the VP1 start codon mutated to leucine and the VP2 start codon mutated to methionine resulting in the single T138M modification of the VP2 protein being expressed. Western blotting analysis of capsid protein expression in whole cell lysates 48 hr post transfection of 293 cells with pIM45-VP1, pIM45-VP2, pIM45-VP2A, and pIM45-VP3 in the presence of Ad5 (MOI=10) demonstrated that a single capsid protein could be expressed from the pIM45 cap ORF (FIG. 2) and completed the catalogue of plasmids required of a system for further genetic manipulation of a specific capsid protein across its entire coding sequence.

5.1.3 The VP3 N-Terminal M203 and M211 are Critical for AAV Particle Formation

As control experiments for the production of AAV particles from the complementary groups of single and double capsid expressing pIM45 plasmids, particle production was examined from the individual plasmids described. Since VP3 protein makes up the bulk of the particle, and mutagenesis studies have indicated that the N-terminal region of VP3 is important for AAV particle formation, the effects of the three mutations required to eliminate VP3 expression (M203,211,235L) were investigated on the recovery of rAAV particles

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following standard production and purification protocols. The plasmids pIM45-M203L, pIM45-M211L, pIM45-M235L, and pIM45M-203,211,235L were cotransfected separately with pTR-UF5 and pXX6 in a 1:1:8 molar ratio in 293 cells and 72 hrs later the cells were harvested and particles were purified as previously reported. Western blotting of capsid protein expression and dot blot analysis of genome containing particles was carried out on the mutant virus preparations (FIG. 3A). No particles were recovered from pIM45-M203L (lane 2) indicating that the combination of VP1, VP2, and VP3a does not able form a stable AAV particle. Equally important in the formation of the particle is M211 (lane 3), as this mutation also prevented particle recovery. Whether it is the M211L in VP1, VP2, or VP3 that leads to this defective phenotype is unclear. This issue is addressed *infra* when pIM45-VP1,2 is complemented with pIM45-VP3 to produce AAV particles (Fig 4 #5). Finally, particles were obtained from pIM45-M235L (Fig 3A, lane 4) that package DNA efficiently.

5.1.4 AAV-Like Particles Can Be Produced That Lack VP1 or VP2 Protein

While the effect of mutating the individual capsid start codons on the formation of infectious AAV particles has been reported, given the improvements in AAV2 production and purification methods, control experiments were performed to reexamine the role of each capsid protein in the formation of the AAV2 particle capable of binding heparin. First examined were the effects of the elimination of one capsid protein on AAV2 particle recovery. pIM45-VP2,3, pIM45-VP1,3, pIM45-VP1,2, and pIM45-VP1,2A were transfected separately into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio and 72 hrs later the cells were harvested and particles were purified as previously reported. Western blotting, A20 ELISA, and dot blot analysis of these virus preparations were carried out (FIG. 3B) and, in agreement with previous reports, the elimination of the VP1 protein (pIM45-VP2,3) resulted in the production of an AAV-like particle that packaged genomes efficiently (lane 4). Surprisingly, in contrast with the initial report mapping the capsid start codons, transfection of the pIM45-VP1,3 plasmid resulted in the purification of an AAV-like particle capable of packaging genomes efficiently composed of only VP1 and VP3 (lane 3) that had only a modest decrease in infectivity compared to particles containing all three capsid proteins (two-fold decrease). Finally, regardless if VP2 is overexpressed, particles composed of only VP1 and VP2 were not recovered (lane 2).

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5.1.5 AAV-Like Particles Can Be Produced Composed Only of VP3 Capsid Proteins

As with the pIM45 plasmids that express two capsid proteins, the ability of a single capsid protein to form an AAV-like particle was tested. pIM45-VP1, pIM45-VP2, pIM45-VP2A, and pIM45-VP3 were transfected separately into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio and harvested cells 72 hrs later and purified particles as previously described. Western blotting of capsid proteins, A20 ELISA, and dot blot analysis of virus preparations were carried out with no detectable AAV-like particles obtained from pIM45-VP1, pIM45-VP2, or pIM45-VP2A (FIG. 3C, lanes 2 and 3). Interestingly, like a recent insertional mutagenesis study of the cap ORF, an AAV-like particle composed exclusively of VP3 protein was purified (lane 3). Like the VP2,3 AAV-like particle, this particle had a significantly lower infectious phenotype.

5.1.6 rAAV Particles With All Three Capsid Proteins Can Be Produced From Capsid Complementation Groups

Given the results of the control experiments, the ability to recover rAAV2 particles containing all three capsid proteins following transfection of two complementary pIM45 plasmids was tested (FIG. 4). To control for twice the Rep expression resulting from two pIM45 plasmids, an additional plasmid was constructed, pIM45-VP0, that expresses no capsid proteins as a result of 5 point mutations (M1L, T138A, M203,211,235L). Complementary group VP0 (FIG. 4, #1) includes pIM45 and pIM45-VP0, group VP1 includes pIM45-VP1 and pIM45-VP2,3 (FIG. 4, #2), group VP2 includes pIM45-VP2 and pIM45-VP1,3 (FIG. 4, #3), group VP2A includes pIM45-VP2A and pIM45-VP1,3 (FIG. 4, #4), and group VP3 includes pIM45-VP3 and pIM45-VP1,2 (FIG. 4, #5). Western blotting of capsid proteins, A20 ELISA, and dot blot analysis of virus preparations were carried out following transfection of the individual groups into 293 cells with pTR-UF5 and pXX6 in a 1:1:8 molar ratio. 72 hrs post transfection the cells were harvested and particles were purified as previously described. Infectious rAAV particles containing all three capsid proteins with similar yields were recovered (FIG. 5A). Interestingly, the VP2A group resulted in the formation of particles with an apparent alteration of capsid protein stoichiometry and lower infectivity compared to other groups (lane 4). The characteristics of this group suggest that this preparation may contain a single unique particle that is defective *per se* or alternatively two particles may be assembled containing all three capsid proteins at normal levels and a defective interfering particle composed of VP2 and VP3 proteins with

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altered stoichiometry. Cotransfection of the pIM45-VP2A and pIM45-VP3 plasmid should yield a particle with an altered VP2:VP3 ratio if such a defective interfering particle contributes to the low titer of this group. Indeed, an AAV-like particle with an overrepresentation of VP2 protein was purified that resembled the VP2,3 and VP3 only particles with respect to infectivity (FIG. 5B, lane 4).

5.1.7 Production of AAV Particles With Insertions in the VP1/VP2 Overlap Region

Since the VP1/VP2 overlap region has been shown to be on the surface of the particle and flexible in the acceptance of targeting epitopes, the ability of this region to accept larger insertions was examined. Presumably, large insertions in the VP3 protein would decrease ones success in obtaining a particle due to steric hindrances in assembling the 60 modified capsid subunits. Sixty ligands were considered excessive when inserting large molecules into the AAV particle, so the strategy employed was to focus larger insertions to VP1 and/or VP2 proteins. Large insertions in both VP1 and VP2 protein immediately after amino acid 138 may have less steric constraints but may produce particles with defective trafficking due to the juxtaposition of a large insertion to the putative phospholipase motif in VP1 protein. Also, since VP1, essentially an N-terminal fusion of 137 amino acids to VP2, and a CD 34 sc antibody VP2 protein fusion are readily incorporated into an AAV particle, insertion of large epitopes only at the N-terminus of VP2 may have advantages. Notably, genetic modification of the VP2 protein exclusively has not been accomplished from within a pIM45 based AAV production scheme. To address the ability to insert large peptide sequences in the VP1/VP2 overlap region of the cap ORF, directional cloning sites were generated immediately after amino acid 138 in plasmids that express VP1 and VP2 or VP2 only (FIG. 6A). The choice of *EagI*/*MluI* restriction sites resulted in two amino acids insertions on either side of further inserted sequence (RP/RT). pIM45-VP1,2A and pIM45-VP2A were chosen as templates for engineering *EagI*/*MluI* restriction sites immediately after amino acid 138 in VP1 and VP2 or in VP2 only (pIM45VP1,2AEM138, pIM45-VP2AEM138, FIG. 6A). Templates with VP2 protein over expression were used to help ensure that genetically-modified VP2 would be present at sufficient levels for assembly. The pIM45-VP1,2AEM138 plasmid was complemented with pIM45-VP3, while the pIM45-VP2AEM138 plasmid was complemented with pIM45-VP1,3 for the production of rAAV particles carrying insertion sequences. Insertion of the coding sequence for leptin and GFP was in these plasmids generated pIM45-VP1,2Alep, pIM45-VP2Alep, pIM45-VP1,2AGFP, and pIM45-VP2AGFP. These plasmids

and their complements were transfected with in 293 cells in the presence of pTR-UF5 (leptin-insertions) or pTR-dsRFP (GFP) and pXX6 in a 1:1:8 molar ratio. 72 hrs post transfection the cells were harvested and virus particles were purified as previously described. Western blotting, A20 ELISA, and dot blot analysis were carried out on the virus preparations (FIG. 6B and FIG. 6C). For the leptin-inserted virus preparations successful insertion in both VP1 and VP2 or VP2 only was possible in the purified particle, but GFP-insertion in the purified particle was only possible in the VP1 protein (VP2-GFP was excluded in both cases).

5.1.8 Discussion

This example increases the flexibility in probing the surface of the particle by isolating the expression of a given capsid protein to a separate plasmid. Such an approach allows for manipulation of this capsid protein only within the produced particle and allows for retesting regions of capsid overlap for the acceptance of sequence modification. Alternatively, the system also allows for the modification of only two of the capsid proteins while leaving the third protein unmodified. Using the missense mutation of capsid start codons to generate all required plasmids, characterization of the catalogue of plasmids required for this system yielded interesting results concerning the role of each capsid protein in the assembly of AAV-like particles.

Elimination of VP3-like fragments illustrates importance of VP3 N terminus, as particles with these mutations in the VP1 and VP2 proteins were recovered following complementation of pIM45-VP1,2 with pIM45-VP3. Evidence that ability to modify individual capsid proteins in regions of overlap may allow production of particles that were defective for production when mutations are in all three proteins. Increases the flexibility in manipulation of the particle for targeting purposes. Recently, isolation of the expression of a C-terminal modified VP3 separately allowed for modification of the c-terminus of VP3 with his tag and production of viable recombinant virus follow nickel chromatography. Like the study involving the VP3-6xHis tag where the modified capsid protein was isolated and VP1 and VP2 did not carry the insertion, lethal mutations in the overlapping N-terminus region of VP3 (M203,211) resulted in particles from complementation group 3 when these mutations were only in VP1 and VP2 with normal VP3.

The present system allows for complementation and recovery of rAAV2 particles with all capsid proteins present. Since it allows for the genetic modification of only one or two of the capsid proteins, it can also be used for studies of previously reported lethal mutations in overlapping capsid sequences to see if mutations at the same positions in fewer

capsid proteins rescue the position for particle manipulation. Important genetic modification would include insertion of genetic sequence for retargeting the virus, purification of the virus, monitoring of the virus particle following infection, or presentation of immunogenic epitopes on the surface of the virus particle.

Insertions of large peptides (leptin and GFP) into the overlapping region of VP1 and VP2 resulted in the purification of virus like particles carrying these insertions. This required preliminary isolation of the expression of VP1 and VP2 (pIM45-VP1,2A) or VP2 only (pIM45-VP2A) to a separate plasmid followed by insertion of peptide sequences after amino acid 138 allowed for the production of peptide inserted AAV-like particles following complementation with pIM45-VP3 or pIM45-VP1,3. This example is the first report of the purification of an AAV-like particle containing a mutation in the VP2 protein exclusively. Estimated similar stoichiometry of capsid proteins in particle. Retain ability to package genomes, bind A20, and are infectious as they retain native tropism due to intact heparin binding motif. VP2 overexpression may have ensured the inclusion of modified VP2 protein large insertions with VP2 acg start codon produced significantly less modified VP2 proteins.

5.2 Example 2 – Heparin Sulfate Binding Motif in AAV2 Capsid Proteins Required for Native Tropism

In this example, charged-to-alanine substitution mutants were made to analyze the effects of single and combinatorial mutations in the capsid gene. New point mutants that result in assembly, packaging, and receptor binding deficiencies have been discovered. Importantly, five amino acids, arginines 484, 487, 585, and 588, and one lysine at position 532 have been identified that appear to mediate the natural affinity of AAV for HSPG. Those observations contribute to the current map of the AAV capsid and provide a reagent for the discovery of novel, heparin independent targeting ligands.

5.2.1 Materials and Methods

5.2.1.1 Plasmids

Plasmid pIM45 (previously called pIM29-45) contains the Rep and Cap coding sequences from AAV with expression controlled by their natural promoters (McCarty *et al.*, 1991). It was used as the parent template for construction of all the AAV2 mutant vectors.

Plasmid pXX6 supplies the adenovirus helper gene products in *trans* to allow rAAV production in an adenovirus free environment (Xiao *et al.*, 1998).

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Plasmid pTR2-UF5 supplies the recombinant AAV DNA to be packaged. It contains a cytomegalovirus promoter driving expression of a green fluorescent protein (GFP) reporter gene flanked by AAV2 terminal repeats (Klein *et al.*, 1998). Plasmid pTR5-UF11 was constructed using an expression cassette consisting of a strong constitutive CBA promoter (Xu *et al.*, 2001), GFP reporter gene (Zolotukhin *et al.*, 1996), woodchuck hepatitis virus posttranscriptional regulatory element WPRE (Donello *et al.*, 1998) and bovine growth hormone gene polyadenylation signal. The cassette was assembled using standard molecular biology techniques and substituted for the *lacZ* cassette in the plasmid backbone pAAV5Rn*lacZ* containing AAV5 terminal repeats (Chiorini *et al.*, 1999).

Plasmids pXYZ1, pXYZ5 contain the AAV1 and AAV5 Cap coding sequences, respectively, in addition to AAV2 Rep coding sequence with an ACG start codon under control of the AAV2 p5 promoter (Zolotukhin *et al.*, 2002). Plasmid pAAV5-2 contains the AAV5 nucleotides 260 to 4448 without terminal repeats (Chiorini *et al.*, 1999).

5.2.1.2 Construction of Mutant Capsid Plasmids

Quickchange site directed mutagenesis (Stratagene) was performed on plasmid pIM45 as per the manufacturer's instructions. For each AAV2 mutant, two complementary PCR primers that contained alanine or lysine substitutions in addition to a silent change for restriction endonuclease screening purposes were used to introduce changes into pIM45. For construction of AAV5-HS, pAAV5-2 was used as the parental template. Sequences for the oligonucleotides used are available upon request. PCR products were digested with DpnI to remove methylated template DNA, phenol:cholorform:isoamyl (25:24:1) extracted, ethanol precipitated, and transformed into electrocompetent JM109 cells. Miniprep DNA was extracted from overnight LB/amp cultures and screened with the appropriate restriction enzyme. All mutants were sequenced prior to use. Transfection quality plasmid DNA was produced by standard alkaline lysis method of a 1-liter TB culture followed by PEG precipitation and cesium chloride gradient purification.

5.2.1.3 Cell Culture

Human embryonic kidney 293's and cervical carcinoma HeLa C12's, a gift from Dr. Phil Johnson (Clark *et al.*, 1996) were grown in Dulbecco Modified Eagle Medium (Gibco-BRL) supplemented with 100U/ml penicillin, 100U/ml streptomycin, 10% bovine calf serum, sodium pyruvate and L-glutamine. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

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5.2.1.4 PRODUCTION OF RAAV2 PARTICLES

To produce AAV2 virions, low passage 293's were seeded so that they were approximately 75% confluent at transfection time. A triple plasmid transfection protocol (Xiao *et al.*, 1998) was followed that included pIM45 to supply Rep and mutated capsid genes, pTR2-UF5 (Klein *et al.*, 1998) to supply recombinant DNA with AAV2 terminal repeats and a CMV driven GFP reporter gene, and pXX6 (Xiao *et al.*, 1998) to supply the adenovirus helper functions in *trans*. A total of 60 μg of plasmid DNA in a 1:1:1 molar ratio was transfected by lipofectamine (Invitrogen).

To produce pseudotyped rAAV1 and rAAV5 particles, a total of 60 μg of pXYZ1 or pXYZ5 (Zolotukhin *et al.*, 2002) was co-transfected with pTR2-UF5 plasmid DNA in a 1:1 molar ratio as above. To produce rAAV5 and rAAV5-HS virions a total of 60 μg of pAAV5 or pAAV5-HS was co-transfected with pTR5-UF11.

Purification of rAAV has been described previously (Zolotukhin *et al.*, 1999; Zolotukhin *et al.*, 2002). Briefly, 72 hr after transfection, cells were harvested and the pellets were resuspended in lysis buffer (.15M NaCl, 50 mM Tris-Cl pH=8.5). Virus was released by three cycles of freezing and thawing. Benzonase (Sigma) was added to the cell lysate to a final concentration of 140 U/ml and incubated at 37°C for 30 min. Cell debris was pelleted by centrifugation at 3,700× g for 30 min and the supernatant was loaded onto a 15%-25%-40%-60% iodixanol (5,5'[2-hydroxy-1,3-propanediyl)bis(acetyl-amino)] bis[N,N'-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide] step gradient (Nycomed). The 40% fraction was collected after centrifugation at 69,000× g for 1 hr and stored at -80°C until further use.

5.2.1.5 VIRUS TITER DETERMINATION

To determine the concentration of intact capsid particles, the A20 ELIZA (American Research Bioproducts) was used. The A20 antibody detects intact, fully assembled particles, both full and empty (Wistuba *et al.*, 1995). Iodixinal purified stocks were serially diluted and processed by the manufacturer's recommended protocol. Only readings within the linear range of the kit standard were used.

To determine the concentration of DNA-containing particles, real-time (RT)-PCR™ was performed using a Perkin Elmer-Applied Biosystems (Foster City, CA) Prism 7700 sequence detector system. Equal volumes of iodixanol purified virus stocks were treated with 600 U/ml benzonase in 50 mM Tris-CL pH=7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$ at 37°C

for 30 min. 280 U/ml proteinase-K was added to reactions adjusted to 10 mM EDTA and 5% SDS, and then incubated at 37°C for 30 min. Reactions were extracted with phenol/chloroform/isoamyl-alcohol (25:24:1) and undigested DNA was precipitated overnight with ethanol and glycogen carrier. Precipitated DNA pellets were resuspended in 100 µl of water. Five µl was used for RT-PCR™ analysis in a reaction mixture that included 900 nM each of GFP forward (5'-TTCAAAGATGACGGGAACTACAA-3') (SEQ ID NO:4) and reverse (5'-TCAATGCCCTTCAGCTCGAT-3') (SEQ ID NO:5) primers, 250 nM Taqman probe (5'-6FAM-CCCGCGCTGAAGTCAAGTTCGAAG-TAMRA-3') (SEQ ID NO:6), 1X Taqman universal PCR master mix in a total volume of 50 µl. Cycling parameters were 1 cycle each of 50°C, 5 mins, and 95°C, 10 mins, followed by 40 cycles of 95°C, 15 sec and 60°C, 1 min. Only values within the linear portion of a standard curve having a coefficient of linearity greater than .98 were accepted. The average RT-PCR™ titer was calculated from virus preparations assayed three times.

To determine the infectious titer of the wt and mutant virus stocks, a green cell assay (GCA) was performed essentially as previously described (Zolotukhin *et al.*, 1999). Briefly, HeLa C12 cells were seeded in a 96 well plate so that they were approximately 75% confluent at infection time. Cells were infected with 10-fold serial dilutions of iodixanol purified mutant viruses and Ad5 at a constant multiplicity of infection (MOI) = 10. Cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 hrs and examined by fluorescence microscopy. The average GCA titer was calculated by averaging the number of green cells counted in individual wells from two or three virus preparations assayed three times. Particle to infectivity ratios were calculated by dividing the average RT-PCR™ titer by the average GCA titer. In some figures, this number was expressed as a $\log_{10}$ value with rAAV2 arbitrarily set to one.

5.2.1.6 *IN VITRO* HEPARIN BINDING ASSAY

Bio-Rad microspin columns were treated with silicon dioxide to minimize non-specific binding of the virus to the column wall. A 500 µp heparin-agarose (Sigma H-6508) gravity column was prepared by washing with 3 column volumes each of 1X TD (137 nM NaCl, 15 mM KCl, 10 mM Na_2PO_4, 5 mM $MgCl_2$, 2 mM KH_2PO_4, pH=7.4), 1X TD + 2M NaCl and 1X TD. Approximately equal numbers of virus particles were added to 1X TD to a final volume of 600 µl and loaded onto the column. The column was washed with 7 column volumes of 1X TD. Bound virus was eluted with 1X TD + 2M NaCl. The entire volume of

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the flow through, wash, and eluate fractions were pooled separately, denatured by boiling in SDS, and slot blotted onto nitrocellulose for immunoblot analysis. The membrane (Osmonics) was blocked in PBS/0.05% Tween-20 + 5% dry milk, and incubated with B1 antibody (Wistuba *et al.*, 1997) at a 1:3000 dilution for 18 hrs at 4°C. Anti-mouse IgG-horse radish peroxidase was used to detect bands by enhanced chemiluminescence (Amersham-Pharmacia).

5.2.1.7 Fluorescence Activated Cell Sorting (FACS)

HeLa C12 cells were seeded in 6 well plates so that they were approximately 75% confluent at infection time. Cells were infected with an rAAV MOI=500 based on the genomic titer as determined by DNA dot blot assay (Zolotukhin *et al.*, 1999). Adenovirus type-5 was used at an MOI=10 plaque forming units (pfu). Twenty-four hours postinfection, cells were washed, trypsinized, and fixed in 2% paraformaldyhede. FACS analysis for GFP expression was done in the ICBR Flow Cytometry lab of the University of Florida on a Becton-Dickinson FACScan.

5.2.1.8 Cell Attachment Assay

10^5 Hela C12 cells were infected with rAAV2 at a genome containing particle MOI = 100 or R585A/R588A at an MOI = 1000 as determined by RT-PCR™. Cells were incubated at 37°C in a 5% CO_2 atmosphere until harvesting. At indicated time points, the infection media was removed and saved and the cells were washed four times with PBS before being scraped. Low molecular weight DNA from the infection media and the cell pellet was extracted by the Hirt procedure (Hirt, 1967). DNA pellets were resuspended in 0.2M NaOH, incubated at 37°C for 20 mins, and slot blotted onto nitrocellulose. DNA was UV cross-linked to the nitrocellulose and probed at 65°C for 18 hrs with [α-^{32}P]-dATP labeled GFP probe in hybridization buffer (7% SDS, 10 mM EDTA and .5M Na_2HPO_4). Membranes were washed twice in 2X SSC/0.1%SDS, 0.2XSSC/0.1%SDS, 0.1X SSC/0.1%SDS, and rinsed with water. The membranes were then exposed to film and quantitated using a BAS-1000 phosphor imager (Fuji).

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5.2.2 Results

5.2.2.1 Selection and Generation of AAV Mutants

A considerable body of information regarding the determinants of HS-protein interactions suggests that their association is driven mainly by electrostatic attraction between acidic sulfate groups on the polysaccharide and basic R-groups on amino acids in the target protein (Hermens *et al.*, 1999; Hileman *et al.*, 1998). It was hypothesized that similar electrostatic interactions would govern HSPG-AAV2 association. In order to evaluate the role of particular amino acids in receptor binding, a panel of mutants was generated by site directed mutagenesis of selected residues. The selection was confined primarily to basic amino acids (His, Lys, Arg) in VP3 as AAV-like particles composed only of VP3 proteins have been purified by heparin affinity chromatography. Any basic amino acid substitution mutant that previously had demonstrated capsid instability or efficient purification by heparin affinity chromatography (Wu *et al.*, 2000) was excluded from the pool of mutants.

Seven AAV serotypes have been reported (Bantel Schaal and zur Hausen, 1984; Gao *et al.*, 2002; Hoggan *et al.*, 1996; Parks *et al.*, 1967; Rutledge *et al.*, 1998). Several groups have shown that rAAV2 and rAAV3 bind efficiently to heparin sulfate (Rabinowitz *et al.*, 2002; Shi *et al.*, 2001; Wu *et al.*, 2000). A single report concerning rAAV1 suggests that it binds with low affinity, if at all, to heparin (Rabinowitz *et al.*, 2002). In contrast, rAAV4 and rAAV5 do not bind heparin and instead recognize 2,3 O-linked and 2,6 N-linked sialic acid moieties (Kaludov *et al.*, 2001). Indeed, this may account for their different cellular tropisms. It was reasoned that residues conserved among all five serotypes were probably not participating directly in receptor discrimination and binding and were excluded from further consideration. Additionally, a number of charge to alanine substitution mutants in the AAV capsid had been identified, and these had been characterized for their ability to bind heparin sulfate columns (Wu *et al.*, 2000) and amino acid positions that did not affect heparin binding or had been shown to be assembly mutants were excluded from further study. Using a Clustal W algorithm, a sequence alignment of capsid proteins from serotypes 1-5 was generated, and 9 basic residues in AAV2 that were conserved in AAV3 and/or AAV1 but were uncharged or acidic in AAV4 and AAV5 were identified that had not previously been tested for heparin-agarose binding (Table 4). In addition to these 9 amino acids, Wu *et al.* (2000) described a virus deficient for heparin binding with alanine substitution mutations at positions 585, 587, and 588. Finally, during the course of these studies, the atomic structure of AAV2 was solved (Xie *et al.*, 2002) and suggested that residues 484, 513, and 532 might

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participate in a heparin-binding pocket as they were located close to residues 585, 587, and 588. These six extra residues were also included to complete the mutant panel (Table 4).

TABLE 4

RESIDUES CHOSEN FOR MUTAGENESIS

VP Residue[a]	AAV Serotype[b] 2	3	1	4	5
358	H	H	H	Q	T
447	R	R	R	S	S
459	R	R	D	T	G
484	R	R	R	K	R
487	R	R	R	G	G
509	H	H	H	T	E
513	R	R	R	R	A
526	H	H	H	A	N
527	K	K	K	G	N
532	K	K	K	K	N
544	K	K	K	P	S
566	R	R	K	A	Q
585	R	S	S	S	S
587	N	N	S	S	T
588	R	T	T	N	T

[a] Residues selected for mutagenesis were generated by a sequence alignment of the VP1 capsid protein from each serotype using the Clustal W algorithm (Vector NTI 5.2, Informax).

[b] Amino acids are represented by their one letter abbreviation. Blue letters represent positively charged, basic amino acids. Red letters represent any other amino acid.

5.2.2.2 MUTANT VIRUS PRODUCTION AND PHYSICAL CHARACTERIZATION

A series of single and combinatorial capsid mutants were generated from the pool of candidate residues in the AAV2 capsid gene (Table 4). To designate the mutant viruses, the number of the mutated amino acid based on its position in VP1 was used. Iodixanol purified virus stocks were checked by western blot using the monoclonal antibody B1. The B1 antibody recognizes a linear epitope in the extreme carboxyl terminus of all three VP proteins from AAV serotypes 1, 2, 3 and 5 (Rabinowitz *et al.*, 2002; Wobus *et al.*, 2000). With the

exception of H358A, capsid proteins were detected in all virus stocks (FIG. 7). To confirm that assembled capsids, rather than subunits or assembly intermediates, had been purified, the particle concentration was measured with an A20 antibody ELISA (Table 5). The A20 antibody recognizes a structural epitope that is found only on assembled capsids with or without packaged DNA (Grimm *et al.*, 1998). Although there was some variability between stocks due to different transfection efficiencies and purification recoveries, only the H358A mutant was negative by A20 ELISA assay. Excluding H358A, a particle concentration range was determined that spanned 1.5 logs and correlated reasonably well with the B1 antibody results (FIG. 7; Table 5). Several possibilities may account for this range of particle titers, including that capsid subunits containing these mutations (i) form intact particles inefficiently, (ii) are unstable during purification or (iii) formed a particle with a partially disrupted A20 epitope. Since none of these mutations fell within the antigenic regions that have been mapped for A20 (Wobus *et al.*, 2000), these results suggested that the A20 epitope had probably not been modified but rather the stability or assembly of some of the mutants was altered so that fewer particles were recovered after iodixanol centrifugation (FIG. 7; Table 5).

TABLE 5

TITERS AND HEPARIN BINDING PROPERTIES OF MUTANTS

Mutant virus[a]	Particle titer[b]		Infectious titer[c]	Particle to infectivity	Heparin binding[e]	Empty/ Full[g]
	A20/ml	Genome/ml	(IU/ml)	d		
rAAV2 (WT)	1.5×10^{12}	4.6×10^{11}	1.8×10^{10}	25	+	3.4
H3558A	$<1.0 \times 10^{8}$	$<1.0 \times 10^{6}$	$<1.0 \times 10^{4}$	N/D[f]	N/D	N/D
R447A	1.2×10^{12}	3.4×10^{10}	1.3×10^{9}	25	+	35.9
R459A	9.1×10^{10}	7.2×10^{8}	$<1.0 \times 10^{4}$	>72500	+	126.3
R484A	1.5×10^{11}	3.0×10^{10}	$<1.0 \times 10^{4}$	>2976667	+/-	5.1
R487A	5.4×10^{11}	2.2×10^{11}	2.3×10^{8}	954	+/-	2.5
H509A	4.6×10^{10}	2.3×10^{9}	6.9×10^{5}	3285	+	20.3
R513A	2.9×10^{11}	1.7×10^{10}	1.6×10^{8}	106	+	17.9
K532A	1.1×10^{11}	3.6×10^{10}	$<1.0 \times 10^{4}$	>3633333	+/-	3.0
K544A	2.0×10^{11}	1.7×10^{10}	8.3×10^{8}	20	+	11.9
R566A	5.1×10^{11}	1.6×10^{10}	7.4×10^{8}	21	+	32.6
R585A	5.0×10^{11}	4.8×10^{10}	1.7×10^{7}	2812	-	1.4

Mutant virus[a]	Particle titer[b] A20/ml	Particle titer[b] Genome/ml	Infectious titer[c] (IU/ml)	Particle to infectivity[d]	Heparin binding[e]	Empty/ Full[g]
R587A	4.4×10^{11}	1.3×10^{10}	1.7×10^{7}	165	+	34.7
R588A	2.4×10^{11}	5.6×10^{10}	3.0×10^{6}	18521	-	4.2
H526A, K527A	1.4×10^{11}	8.2×10^{10}	5.5×10^{7}	1489	+	1.8
R585A, R588A	1.2×10^{12}	9.2×10^{11}	1.9×10^{7}	48421	-	1.2
R585K	1.3×10^{12}	3.7×10^{10}	4.0×10^{8}	92	+	35.4
R585K, R588K	1.4×10^{12}	3.9×10^{10}	8.9×10^{7}	436	+	34.9
AAV1	N/D	3.7×10^{10}	1.1×10^{9}	37	+/-	N/D
AAV5	N/D	3.4×10^{10}	3.2×10^{6}	10692	-	N/D
AAV5-HS	N/D	8.0×10^{8}	$<1.0 \times 10^{4}$	>80000	+	N/D

[a] Two letters flanking a number designate each mutant. The first letter is the one letter abbreviation for the wild type amino acid followed by its numerical position in VP1 followed by the one letter abbreviation for the amino acid to which it was mutated.

[b] A20 particle titers were determined as described using the A20 ELISA assay. Genomic titers were determined by RT-PCR™.

[c] Infectious titers were determined by green cell assay as described by counting GFP fluorescent cells.

[d] Particle-to-infectivity ratio was calculated by dividing the average genomic titer as determined by RT-PCR™ by the average green cell assay titer.

[e] Determined by heparin-agarose binding assay. +, >95% virus recovered in the eluate; +/-, >50 recovered in the eluate; -, <5% of virus recovered in the eluate.

[f] N/D, not determined.

[g] Empty-to-full ratio was determined by dividing the A20 particle titer by the average genomic titer.

To determine whether any mutations affected DNA packaging, the titer of DNA containing virions was determined by real-time (RT) PCR™ (Clark *et al.*, 1999; Veldwijk *et al.*, 2002) (Table 5) and confirmed by DNA dot blot hybridization. Although there was variation between preparations, the majority of the capsid mutants were able to package detectable DNA (Table 5). As expected, H358A was negative for DNA packaging, as it did not produce virus particles. It was concluded that none of the capsids in the mutant panel that made A20 positive particles were completely defective for DNA packaging. However, by comparing the A20 ELISA and PCR titers, it was noted that stocks of mutant R459A contained 40-fold more empty particles than wild type rAAV2. Thus, R459 could have a role in DNA packaging. Although less dramatic, mutants R447A, R566A, R587A, R585K, and R585K/R588K had approximately 10-fold more empty particles than rAAV2. The remainder of the virus preparations packaged DNA at levels comparable to wild type AAV2 (Table 5).

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5.2.2.3 *IN VITRO* HEPARIN BINDING OF CAPSID MUTANTS

To assess the ability of mutant capsids to bind heparin sulfate, a modification of an assay previously described by Wu *et al.* (2002) was used. Virus preparations that had been purified by iodixanol step gradients were loaded on heparin agarose columns and the entire volume of the flow through, wash, and eluate fractions were pooled separately, denatured, and slot blotted onto nitrocellulose for immunoblot analysis with B1 antibody. A representative Western analysis for each mutant is shown in FIG. 8. As expected, wild type AAV2 was not observed in the flow through or wash fractions and most of the virus bound to the column was recovered at the elution step. Eight other mutants, R447A, R459A, H509A, R513A, K544A, K566A, N587A, and H526A/K527A, had a heparin-agarose binding phenotype indistinguishable from wild type. The results with R513A confirmed a previous report (Wu *et al.*, 2000) in which a double mutant at positions 513 and 514 was positive for heparin binding. In marked contrast, it was observed that any capsid harboring a non-conservative mutation at position 585 or 588 was detected only in the flow through and wash. Intermediate heparin-agarose binding phenotypes in mutants R484A, R487A and K532A were also detected with approximately equal levels of signal detected in the flow through, wash, and eluate. The results with K532A were inconsistent with previous results in which a mutant containing alanine substitutions at positions 527 to 532 was found to be positive for heparin binding (Wu *et al.*, 2000). These data suggested that at least five amino acids had the potential to contribute to the electrostatic attraction between AAV and heparin sulfate. These included predominantly R585 and R588, and to a lesser but detectable extent, R484, R487, K532.

To confirm that the charge at R585 and R588 was primarily responsible for heparin interaction, two viruses were generated with conservative mutations, R585K and R585K/R588K, and tested them in the *in vitro* heparin binding assay. Both lysine and arginine residues are positively charged, however, lysine is slightly larger due to an additional methyl residue in the side group. Both of these capsids bound to heparin-agarose almost as well as wild type virus (FIG. 8). In each case, most of the virus was recovered in the eluate; however, the flow through and wash fractions also contained minor amounts of virus. This result suggested that both localized negative surface charge, and the relative position of the changes in this region of the capsid, are responsible for mediating the interaction with heparin-agarose.

Finally, as a control and to validate the heparin binding assay, the ability of wild type rAAV2, rAAV1, and rAAV5 to bind to heparin-agarose was compared. For this purpose, recombinant viruses were produced and purified by using a pseudotyping protocol developed to package AAV2 terminal repeat containing genomes into alternative serotype capsids (FIG. 9A) (Rabinowitz *et al.*, 2002; Zolotukhin *et al.*, 2002). Approximately equal amounts of input virus as determined by Western blot signal intensity were applied to a heparin-agarose column, and fractions from the column were slot blotted onto nitrocellulose for immunodetection using the B1 antibody (FIG. 9B). As expected, rAAV2 was efficiently retained by heparin-agarose under low ionic conditions but the majority of rAAV1 and all of rAAV5 was seen in the flow through and wash. A low amount of AAV1 was detected in the eluate. These data were consistent with previous reports (Rabinowitz *et al.*, 2002).

5.2.2.4 Multiple Mutations in the AAV2 Capsid Effect Viral Transduction

To determine how the heparin-agarose binding phenotypes correlated to infectivity, iodixanol stocks were tested for their ability to transduce HeLa C12 cells by performing a green cell assay (GCA). Cells in a 96 well plate were co-infected with Ad5 at a constant MOI=10 pfu/cell and mutant AAV virus stocks in a 10-fold dilution series. Twenty-four hours post-infection (hpi), the number of GFP expressing cells in individual wells were counted and a GCA titer was calculated (Table 5). The detection limit of this assay was approximately 10^4 transducing units/ml. The GCA titers were then normalized to genome containing physical particles by calculating a particle to infectivity (P/I) ratio. This ratio is equivalent to the number of genomes required to transduce one cell (Table 5). To get a measure of the relative impact of a particular mutation on viral infectivity, the P/I ratio of each mutant was divided by the wild type capsid P/I ratio and the $\log_{10}$ of this value was plotted in FIG. 10. This provided a simple comparison of how many genome-containing particles of each mutant were required to achieve the same number of transduced cells as the wild type virus.

Several phenotypes emerged from this analysis. Mutants R477A, K544A, and K566A were virtually identical to wild type, and mutants R513A, N587A, R585K, and R585K/R588K were only slightly defective (approximately 1 log). These seven mutants were found previously to bind heparin sulfate to the same extent as wild type rAAV2 (FIG. 8).

Three of the mutants R459A, R484A, and K532A produced virus that was essentially non-infectious with P/I ratio between 7.2×10^4 and 3.6×10^6 compared to the wild type ratio

of 25 (Table 5, FIG. 10). The P/I ratios for these mutants were minimum estimates based on the GCA assay sensitivity of 1×10^4 IU/ml. In fact, no transduction events were seen with any of these mutants.

R459A was the most severe example of three mutants (R459A, H509A, and H526A/K527A) that were essentially wild type for heparin binding but defective for transduction (FIG. 10). These mutants were presumably defective in some late stage of viral infection.

Finally, all five of the mutants that were defective or partially defective for heparin binding (R484A, R487A, K532A, R585A, and R588A) were defective for transduction. However, the loss of infectivity did not correlate completely with the loss of heparin binding (compare FIG. 8 and FIG. 10). Two of these mutants (R484A and K532A) were only partially defective for heparin binding but severely defective (>5 logs) for transduction, suggesting that some other step in viral infection was defective in these mutants in addition to heparin binding. The remaining heparin binding mutants (R487A, R585A, and R588A) had defects in transduction that approximately correlated with their ability to bind heparin.

5.2.2.5 Evaluation of R585A/R588A Cell Attachment *In Vivo*

As mentioned earlier, alanine substitutions at either position 585 or 588 were the only mutations that completely abolished binding to HS (FIG. 8), suggesting that these two arginines were primarily responsible for heparin binding. Moreover, the extent to which mutation of either or both of these residues inhibited transduction (FIG. 10, 1.5-3 logs) was approximately the same when soluble heparin sulfate is used to inhibit wild type rAAV2 infection (Handa *et al*., 2000). Those mutants were, therefore, examined in more detail.

To see if the defect in transduction of R585 and R588 mutants could be overcome by using higher input MOI's, cells were co-infected with rAAV2 or the mutant viruses at an MOI=500 genome containing particles/cell. Twenty-four hours post-infection cells were examined by fluorescence microscopy and counted by FACS. The data from three independent experiments and representative histograms are shown in FIG. 11. As expected, the defects in transduction of the single mutants, R585A and R588A, could be overcome by higher MOI's (56% and 25% transduction for R585A, and R588A, respectively). Predictably, the level of recovery of the double mutant, R585A/R588A, was lower (10% transduction). However, it was clear that the fluorescence intensity profile for the heparin binding mutants was quite different from wild type, suggesting a significant delay in the onset of GFP expression by 24 hours. In contrast, the level of transduction of the

conservative double mutant, R585K/R588K, and the heparin positive mutant, N587A, was indistinguishable from wild type.

As a more direct assay for cell attachment, Hela C12 cells were transfected and the location of viral DNA tracked. Cells were infected with rAAV2 at an MOI=100 or R585A/R588A at an MOI=1000 genome containing particles as determined by RT-PCR™. At 1, 4, and 20 hpi, the infection media was removed and saved, and the cells were washed extensively to remove any residual unbound virus. The cells were then harvested and low molecular weight DNA was extracted from both the infection media (unbound) and the cell pellet (bound or internalized) by the Hirt procedure and transferred to nitrocellulose for Southern hybridization with an [α-^{32}P]-dATP labeled GFP probe (FIG. 12A and FIG. 12B).

At all time points rAAV2 DNA was detectable both bound/internalized and in the infection media. In contrast, cells infected with 10-fold more genomic copies of R585A/R588A showed the vast majority of the signal only in the unbound fraction (FIG. 12A). Phosphor imager analysis determined that at each time point approximately one third of the total rAAV2 DNA was attached or internalized compared to only 1% of R585A/R588A (FIG. 12B). As these infections were performed at 37°C, the process of internalization should not have been prevented. This result demonstrated that the block in infection for mutant R585A/R588A occurred at the cell attachment stage or internalization stage, and correlated to heparin sulfate binding *in vitro*.

5.2.2.6 Loop Swapping Confers Heparin Binding to AAV5

Although the primary amino acid sequences are moderately divergent, the architectural position of β-sheets and loops is predicted to be very similar among AAV serotypes (Rabinowitz and Samulski, 2000). It was hypothesized that if R585 and R588 were the critical residues involved in HSPG binding, then it should be possible to substitute that region of AAV2 into AAV5 to create a hybrid virus capable of interacting with heparin-agarose. To achieve this, a recombinant virus, designated rAAV5-HS, was generated by replacing a short loop containing residues 585 through 590 from AAV2 into a region predicted to be structurally equivalent in AAV5 (FIG. 13A). Loop substitution rather than point mutagenesis was done to account for the possibility of additional Van der Waals interactions or hydrophobic contributions from nearby amino acids.

Production and purification of rAAV5-HS was unaffected by the six amino acid substitution (FIG. 13B; Table 5). When rAAV5-HS was tested in the *in vitro* heparin-

agarose binding assay, it was indistinguishable from wild type rAAV2 (FIG. 7C). These data suggested that this region of AAV5 was surface accessible, and that heparin-agarose binding could be artificially conferred by the six amino acids containing R585 and R588.

To compare the infectivity of rAAV5 and rAAV5-HS, packaged viruses were generated that contained a recombinant AAV5 genome in which the GFP reporter gene was flanked by AAV5 terminal repeats. The infectivity of these viruses was compared to rAAV2 in a GCA assay and particle-to-infectivity ratios were calculated as before (FIG. 13D). rAAV5 was able to transduce Hela C12 cells at a low efficiency, approximately 2.5 logs lower than AAV2. However, no transduction was seen with AAV5-HS ($<1 \times 10^4$ IU/ml) (Table 5; FIG. 13D). Given the minimum sensitivity of the GCA assay this meant that the P/I ratio of AAV5-HS was at least 3.5 logs higher than rAAV2 and at least 1 log higher than wild type rAAV5. It was concluded that, although substitution of these five heterologous amino acids into the AAV5 capsid restored heparin binding to the level of AAV2 capsids, it was not sufficient to produce AAV2 levels of infectivity in a cell line normally permissive for AAV2.

5.2.3 Discussion

This example describes the identification of amino acids in the capsid of AAV2 that mediate binding to heparin sulfate proteoglycans. Several lines of evidence suggest that HSPG serves as the primary receptor for AAV2. Inhibition of AAV2 infection can be demonstrated by competition with soluble analogs, GAG desulfation by sodium chlorate treatment, antibody competition, enzymatic removal of heparin, and use of mutant cell lines that express varying levels of HSPG (Handa *et al.*, 2000; Qiu *et al.*, 2000; Summerford and Samulski, 1998; Wu *et al.*, 2000). Binding to heparin sulfate is usually the result of electrostatic charge interactions between basic amino acids (R, K, or H) and negatively charged sulfate residues (Hileman *et al.*, 1998; Mulloy and Linhardt, 2001). During the course of previous mutagenesis studies, many of the basic amino acids in the AAV2 capsid that could potentially contribute to heparin sulfate binding were eliminated (Wu *et al.*, 2000). In this example, the remaining basic residues were examined by looking at their conservation in AAV serotypes 1-5. Those that were present in all five serotypes were not likely to contribute significantly to heparin binding. Those that were conserved in the heparin binding serotypes, AAV1-3, but not in the remaining serotypes were targeted for mutagenesis. Finally, by taking advantage of the fact that R585 and R588 had been previously identified as potential heparin binding amino acids (Wu *et al.*, 2000) and that these amino acids were

located in a cluster of basic residues at the three fold axis of symmetry (Xie *et al.*, 2002), all of the basic amino acids in this cluster were also targeted for mutagenesis. This approach yielded a total of 15 amino acids that could have been involved in heparin binding and alanine mutations were characterized at all of these positions. This approach, of course, does not necessarily identify *all* possible heparin binding amino acids. For example, R484, which is basic in all five serotypes was tested because of its proximity to R585 and R588 and subsequently proved to be involved in heparin binding.

5.2.3.1 HEPARIN BINDING AND INFECTIVITY

These studies indicated that capsids with mutations at residue 484, 487, 532, 585 or 588, were partially or completely defective for heparin-agarose binding. The most severe defect was found with mutations in R585 and R588. No binding to heparin sulfate columns could be detected with either mutant (FIG. 8), and both mutations reduced the particle-to-infectivity ratio by two to three logs (Table 5). Mutants that contained substitutions at both positions had even lower infectivity.

The phenotypes of R487A, R585A, and R588A, were probably due largely to defective heparin binding. For example, the double mutant R585A/R588A was approximately 500 fold more defective in cell binding and internalization than wild type (FIG. 12B) when corrected for the MOI, and approximately 2000 fold less infectious (Table 5), as judged by the change in particle-to-infectivity ratio. Another indication that heparin binding was primarily responsible for the defects in R585 and R588 was the fact that conservative mutations at these two positions (R585K and R585K/R588K) produced virus particles with properties similar to wild type (FIG. 8; FIG. 10; FIG. 11; Table 5). Results from the conservative lysine substitutions at R585 and R588 are reasonably consistent with electrostatic attraction being the primary mediator for AAV-heparin interaction. R585K, the least defective heparin binding mutant (FIG. 8), had transduction levels nearly equal to rAAV2 (FIG. 10), and R585K/R588K was only slightly more defective for heparin binding (FIG. 8) and transduction (FIG. 10), and within one log of wild type. Furthermore, when cells were infected at a high MOI, robust transduction was observed for both mutants (FIG. 11). Finally, substitution of a six amino acid sequence containing R585 and R588 imparted heparin binding to AAV5 that was comparable to that seen with AAV2 (FIG. 13). Although similar studies were not performed with the R487 position, it was clear that mutation of R487 produced virus with a more modest defect in heparin binding (FIG. 8) and in infectivity (FIG. 10).

In addition to R487A, R585, and R588, two other mutants were found that were defective for heparin binding, R484A and K532A. R484A and K532A, like R487A, had a more modest effect on binding to heparin sulfate, but unlike the other heparin binding mutants, these two mutations had a dramatic effect on transduction efficiency. Both R484A and R532A were more than 5 logs less infectious than wild type capsids (Table 5; FIG. 10). This severe defect is presumably due to a different block in the infection process that is unrelated to heparin binding, but as yet it has not been identified. The result from K532A is consistent with earlier studies that identified a mutant (mut 37) that contained six amino acid substitutions that included K532A (Wu *et al.*, 2000). Mut 37 had a phenotype identical to K532A in that it produced full virus particles that were non-infectious and more recently has been shown to have a modest defect (approximately 5 fold) in heparin binding and internalization. This potentially maps this defect to a single amino acid.

5.2.3.2 COMPUTER MODELING

Using the recently published atomic structure of AAV2 (PDB ID code: 1LP3) (Xie *et al.*, 2002), the positions of the heparin binding mutations were examined. Symmetry transformation operations from the original PDB file were applied to generate a VP3 trimer arrangement in the context of an icosahedron. When viewed in ribbon format looking directly down a three-fold axis, residues R484, R487, R532, R585 and R588, represented as balls-and-sticks, are located in a linear formation lining one side of each three-fold related spike. When viewed across the top surface of the trimer, residues R585 and R588, which are contributed by one of the peptides in the trimer, are positioned above a linear arrangement of R484, R487 and K532, which are contributed by a second peptide in the trimer. Thus, it appears that a heparin binding motif is formed from some combination of these five amino acids using amino acids from two different polypeptides. An electrostatic potential surface map of a VP3 trimer was also generated, in which areas of positive and negative charge are represented. When viewed perpendicular to the three fold-axis, the five amino acids mapped by this example appear to contribute collectively to a basic patch on one side of each three-fold related spike. The charge, clustering, and surface presentation of these residues are all consistent with a model of electrostatic attraction. Two other basic residues, H526 and K527, contribute to the basic cluster at the three fold spike but these residues do not appear to be involved in heparin binding (FIG. 8).

The five mutations that affected heparin binding were located in the large loop IV region, which among AAV serotypes has low overall sequence conservation and includes all

of the previously identified insertion and substitution mutations that affect heparin binding. Interestingly, with the exception of N587, the stretch of amino acids encompassing 585 to 590 is unique to AAV2 and is not present in AAV3, which is the other AAV serotype that has been shown to bind efficiently to heparin sulfate. Mutation of N587 had no effect on heparin-agarose binding and only minor effects on transduction. Conceivably, residues R484, R487 and K532 could be the dominant residues involved in heparin sulfate binding for AAV3.

The apparent dissociation constant (K_d) of AAV2 and heparin sulfate was determined by competition analysis to be 2×10^{9} M (Qiu *et al.*, 2000). Although this is higher than some heparin-protein interactions, it is sufficiently strong to suggest cooperative binding by one HS glycosaminoglycan chain to multiple attachment points. This example does not address whether heparin sulfate could form a bridge between basic residues in one of the threefold spikes to those in another. However, as the average chain length of heparin glycosaminoglycans varies between 50-200 disaccharide repeats that adopt a helical conformation 40-160 nm in length, it is conceivable that a heparin sulfate chain could wrap around the exterior of the capsid through cooperative binding of multiple spikes at the threefold axis of symmetry. Although a rigorous computational docking analysis was not undertaken, a heparin molecule (PDB ID code 1NTP) was manually superimposed in several orientations that placed multiple reactive sulfate and amine groups within accepted electrostatic attraction distances on pairs of residues spanning the spikes.

5.2.3.3 Mutants That Bind Heparin But Are Still Defective

Several new mutants were found that bound heparin sulfate as well as wild type but still produced defective particles. H538A was defective for particle assembly. There are a number of reported examples of mutations that disrupt AAV2 particle formation, several of which are located in the conserved β-strand regions (Rabinowitz *et al.*, 1999; Shi *et al.*, 2001; Wu *et al.*, 2000). Since H358 is neither surface accessible nor in a conserved β-strand, it is possible that it acts internally to stabilize the monomer subunit structure.

Mutants R459A, H509A, and H526A/K527 bound heparin-agarose efficiently but had particle-to-infectivity ratios that were two to more than three logs higher than wild type. Like K532A and R484A, these mutants are presumably defective in some stage of the infectious entry pathway between secondary receptor binding and uncoating. Ongoing studies in the lab are examining the block in infectivity for these mutants.

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5.2.3.4 DNA PACKAGING

The process of DNA packaging is thought to occur by an active process requiring NTP consumption coupled to the helicase activity of the small Rep proteins (King *et al.*, 2001). Although none of the mutations that assembled an A20 positive particle were completely deficient for DNA packaging, mutant R459A produced a 40-fold excess of empty capsid particles compared to rAAV2. Other studies have reported that short insertions at positions 323, 339, 466, 520, 540, 595, 597 that did not interfere with capsid formation still reduced DNA packaging to levels detectable only by PCR™ amplification (Shi *et al.*, 2001). In addition, a point mutant R432A prevents DNA packaging (Wu *et al.*, 2000). Although the relationship between these mutations and their mechanism of action is unclear, it is possible that they disrupt protein-capsid or DNA-capsid interactions.

5.2.3.5 SUMMARY OF EXEMPLARY PRODUCTION SYSTEM

An exemplary rAAV production system has been described to produce modified rAAV vectors that comprise one or more altered capsid proteins. FIG. 14 shows the results of an immunoslotblot of total capsid protein following standard purification procedures of a representative expression system of the invention. FIG. 15 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and the system plasmid combinations. FIG. 16 shows the *in vivo* transduction ability of recombinant AAV vectors produced using various system components. FIG. 17 shows an Immunoblot and dot blot autoradiograph of virions produced from pTR-UF5; pIM45-VP1,2; pIM45-VP1,3; and pIM45-VP2,3 plasmids following standard purification protocols. FIG. 18 shows the *in vivo* transduction ability of recombinant AAV vectors containing only two capsid proteins, while FIG. 19 depicts an immunoblot of protein fractions collected from iodixinol purified passed over a heparin-agarose column. Using an anti-VP1,2,3 monoclonal antibody. FIG. 20 shows a dot blot autoradiograph of DNA extracted from pTR-UF5 and rAAV R585A, R588A, while FIG. 21 summarizes an exemplary system that demonstrates the *in vivo* transduction ability of pTR-UF5 and R585A, R588A. FIG. 22 shows a slot blot autoradiograph of an *in vivo* DNA tracking time course experiment of pTR-UF5, rAAV R585A, R588A, while FIG. 23 shows a schematic diagram of the pIM45 vector showing the rep and cap sequences.

6.0 REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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CLAIMS:

1. A recombinant adeno-associated viral expression system that expresses at least a first, second, and third distinct capsid proteins, comprising:

(a) a first expression vector that encodes said first distinct capsid protein; and

(b) a second expression vector that encodes said second and said third distinct capsid proteins.

2. The recombinant adeno-associated viral expression system of claim 1, wherein said first distinct capsid protein is Vp1, and said second and said third distinct capsid proteins are Vp2 and Vp3.

3. The recombinant adeno-associated viral expression system of claim 1, wherein said first distinct capsid protein is Vp2, and said second and said third distinct capsid proteins are Vp1 and Vp3.

4. The recombinant adeno-associated viral expression system of claim 1, wherein said first distinct capsid protein is Vp3, and said second and said third distinct capsid proteins are Vp1 and Vp2.

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5. The recombinant adeno-associated viral expression system of claim 1, wherein at least one of said first, second, and third distinct capsid proteins comprises at least a first mutation.

6. The recombinant adeno-associated viral expression system of claim 5, wherein said first mutation is an insertion mutation, a point mutation, a frame-shift mutation, or a deletion mutation.

7. The recombinant adeno-associated viral expression system of claim 6, wherein said insertion mutation comprises at least a first peptide targeting ligand.

8. The recombinant adeno-associated viral expression system of claim 5, wherein said mutation alters, impairs, or prevents the binding of said capsid protein to a mammalian cell surface receptor or binding site.

9. The recombinant adeno-associated viral expression system of claim 5, wherein said mutation is a deletion or a frame-shift that alters or eliminates at least a first amino acid residue required for binding of said capsid protein to a mammalian cell surface receptor or binding site.

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10. The recombinant adeno-associated viral expression system of claim 9, wherein said mutation is a deletion or a frame-shift that alters or eliminates at least a first amino acid residue required for binding of said capsid protein to heparan sulfate proteoglycan.

11. The recombinant adeno-associated viral expression system of claim 10, wherein said mutation is at amino acid residue R484, R487, K532, R585, or R588 of said capsid protein.

12. The recombinant adeno-associated viral expression system of claim 11, wherein said mutation is an arginine to alanine mutation or an arginine to lysine mutation at amino acid residue R484, R487, R585, or R588 of said capsid protein.

13. The recombinant adeno-associated viral expression system of claim 11, wherein said mutation is a lysine to alanine mutation at amino acid residue K532 of said capsid protein.

14. The recombinant adeno-associated viral expression system of claim 10, wherein said deletion mutation further comprises a deletion of at least a second amino acid residue

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required for binding of said capsid protein to said mammalian cell surface receptor or binding site.

15. The recombinant adeno-associated viral expression system of claim 5, further comprising at least a second mutation in a second distinct capsid protein.

16. The recombinant adeno-associated viral expression system of claim 5, further comprising at least a second mutation in the same capsid protein as the first mutation.

17. The recombinant adeno-associated viral expression system of claim 16, wherein said first mutation comprises an insertion mutation that encodes at least a first peptide targeting ligand, and said second mutation is a deletion or frame-shift of at least a first amino acid residue required for binding of said capsid protein to a mammalian cell surface receptor or binding site.

18. The recombinant adeno-associated viral expression system of claim 17, wherein said first mutation comprises an insertion mutation that encodes at least a first peptide targeting ligand, and said second mutation is a deletion or frame-shift of at least a first amino acid residue required for binding of said capsid protein to heparan sulfate proteoglycan.

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19. The recombinant adeno-associated viral expression system of claim 18, wherein said first peptide targeting ligand comprises an ApoE peptide, or a targeting sequence that binds to a mammalian low-density lipoprotein (LDL) or very low density lipoprotein (VLDL) receptor.

20. The recombinant adeno-associated viral expression system of claim 1, further comprising a third expression vector that encodes the adenoviral helper gene products to permit production of said expression system in an adenovirus-free environment.

21. The recombinant adeno-associated viral expression system of claim 20, wherein said third expression vector is pXX6.

22. The recombinant adeno-associated viral expression system of claim 20, further comprising a fourth expression vector that comprises an expression cassette flanked by AAV2 terminal repeat sequences.

23. The recombinant adeno-associated viral expression system of claim 22, wherein said expression cassette comprises a first polynucleotide that comprises a first nucleic acid segment that encodes a therapeutic agent.

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24. The recombinant adeno-associated viral expression system of claim 23, wherein said therapeutic agent is a peptide, polypeptide, protein, catalytic RNA molecule, ribozyme, or an antisense oligonucleotide or antisense polynucleotide.

25. The recombinant adeno-associated viral expression system of claim 23, wherein said first polynucleotide further comprises a promoter operably linked to said first nucleic acid segment, wherein said promoter expresses said therapeutic agent.

26. The recombinant adeno-associated viral expression system of claim 25, wherein said promoter is a heterologous, tissue-specific, constitutive or inducible promoter.

27. The recombinant adeno-associated viral expression system of claim 26, wherein said promoter is selected from the group consisting of a CMV promoter, a β-actin promoter, an insulin promoter, an enolase promoter, a BDNF promoter, an NGF promoter, an EGF promoter, a growth factor promoter, an axon-specific promoter, a dendrite-specific promoter, a brain-specific promoter, a hippocampal-specific promoter, a kidney-specific promoter, an elafin promoter, a cytokine promoter, an interferon promoter, a growth factor promoter, an alpha-1 antitrypsin promoter, a brain-specific promoter, a neural cell-specific promoter, a central nervous system cell-specific promoter, a peripheral nervous system cell-specific promoter, an interleukin promoter, a serpin promoter, a hybrid CMV promoter, a hybrid β-actin

promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

28. The recombinant adeno-associated viral expression system of claim 27, wherein said promoter is a mammalian β-actin promoter.

29. The recombinant adeno-associated viral expression system of claim 23, wherein said first polynucleotide further comprises an enhancer sequence operably linked to said first nucleic acid segment.

30. The recombinant adeno-associated viral expression system of claim 29, wherein said enhancer sequence comprises a CMV enhancer, a synthetic enhancer, a liver-specific enhancer, an vascular-specific enhancer, a brain-specific enhancer, a neural cell-specific enhancer, a lung-specific enhancer, a muscle-specific enhancer, a kidney-specific enhancer, a pancreas-specific enhancer, or an islet cell-specific enhancer.

31. The recombinant adeno-associated viral expression system of claim 30, wherein said enhancer sequence comprises a CMV enhancer.

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32. The recombinant adeno-associated viral expression system of claim 23, wherein said first nucleic acid segment further comprises a post-transcriptional regulatory sequence or a polyadenylation signal.

33. The recombinant adeno-associated viral expression system of claim 32, wherein said regulatory sequence comprises a woodchuck hepatitis virus post-transcription regulatory element, or said polyadenylation signal comprises a bovine growth hormone gene polyadenylation signal.

34. The recombinant adeno-associated viral expression system of claim 22, wherein said fourth expression vector is pTR2-UF5.

35. The recombinant adeno-associated viral expression system of claim 23, wherein said therapeutic agent is a protein or polypeptide selected from the group consisting of an adrenergic agonist, an anti-apoptosis factor, an apoptosis inhibitor, a cytokine receptor, a cytokine, a cytotoxin, an erythropoietic agent, a glutamic acid decarboxylase, a glycoprotein, a growth factor, a growth factor receptor, a hormone, a hormone receptor, an interferon, an interleukin, an interleukin receptor, a kinase, a kinase inhibitor, a nerve growth factor, a netrin, a neuroactive peptide, a neuroactive peptide receptor, a neurogenic factor, a neurogenic factor receptor, a neuropilin, a neurotrophic factor, a neurotrophin, a neurotrophin receptor, an N-methyl-D-

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aspartate antagonist, a plexin, a protease, a protease inhibitor, a protein decarboxylase, a protein kinase, a protein kinsase inhibitor, a proteolytic protein, a proteolytic protein inhibitor, a semaphorin,, a semaphorin receptor, a serotonin transport protein, a serotonin uptake inhibitor, a serotonin receptor, a serpin, a serpin receptor, and a tumor suppressor.

36. The recombinant adeno-associated viral expression system of claim 35, wherein said polypeptide is selected from the group consisting of BDNF, CNTF, CSF, EGF, FGF, G-SCF, GM-CSF, gonadotropin, IFN, IFG-1, M-CSF, NGF, PDGF, PEDF, TGF, TGF-B2, TNF, VEGF, prolactin, somatotropin, XIAP1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-10(I87A), viral IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, and IL-18.

37. A recombinant adeno-associated virus virion comprising the recombinant adeno-associated viral expression system of claim 1.

38. The recombinant adeno-associated virus virion of claim 37, wherein said virion is selected from the group consisting of AAV serotype 1, AAV serotype 2, AAV serotype 3, AAV serotype 4, AAV serotype 5, and AAV serotype 6.

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39. A plurality of adeno-associated viral particles comprising the recombinant adeno-associated viral expression system of claim 1 or the recombinant adeno-associated virus virion of claim 37.

40. A mammalian cell comprising the recombinant adeno-associated viral expression system of claim 1 or the recombinant adeno-associated virus virion of claim 37.

41. The mammalian cell of claim 40, wherein said mammalian cell is a human, primate, murine, feline, canine, porcine, ovine, bovine, equine, epine, caprine, or lupine cell.

42. The mammalian cell of claim 41, wherein said human cell is a human endothelial, vascular, epithelial, liver, lung, heart, pancreas, kidney, muscle, bone, neural, or brain cell.

43. A composition comprising the recombinant adeno-associated viral expression system of claim 1, the recombinant adeno-associated virus virion of claim 37, the plurality of adeno-associated viral particles of claim 39; or the mammalian host cell of claim 40.

44. The composition of claim 43, further comprising a pharmaceutical excipient, buffer, or diluent.

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45. The composition of claim 44, formulated for administration to a human.

46. The composition of claim 43, further comprising a liposome, a lipid, a lipid complex, a microsphere, a microparticle, a nanosphere, or a nanoparticle.

47. The composition of claim 43, for use in therapy.

48. The composition of claim 47, for use in cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, liver disease, neurological disease, neurosensory dysfunction, stroke, ischemia, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Parkinson's disease, or pulmonary disease.

49. Use of a composition according to claim 43, in the manufacture of a medicament for treating cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, liver disease, neurological disease, neurosensory dysfunction, stroke, ischemia, neuromuscular disorders, eating disorders, neurological diseases, neuroskeletal impairment or disability, Alzheimer's disease, Huntington's disease, Parkinson's disease, or pulmonary disease.

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50. A kit comprising:

(a) the recombinant adeno-associated viral expression system of claim 1, the recombinant adeno-associated virus virion of claim 37, the plurality of adeno-associated viral particles of claim 39, the mammalian host cell of claim 40, or the composition of claim 43; and

(b) instructions for using said kit.

51. A method for targeting an AAV virion or viral particle to a mammalian cell that comprises a cell-surface receptor, said method comprising the step of: providing to a population of cells an AAV virion or viral particle that comprises the recombinant adeno-associated viral expression system of claim 1, in an amount and for a time effective to target said virion or said viral particle to cells of said population that express said cell-surface receptor.

52. A method for targeting an expressed therapeutic agent to a mammalian cell that comprises a cell-surface receptor, said method comprising the step of providing to a mammal that comprises a population of said cells an amount of the recombinant adeno-associated viral expression system of claim 1.

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53. The method of claim 52, wherein said expressed therapeutic agent is a peptide, polypeptide, protein, antibody, antigen binding fragment, ribozyme, or antisense molecule.

54. A method for preventing, treating or ameliorating the symptoms of a disease, dysfunction, or deficiency in a mammal, said method comprising administering to said mammal the virion of claim 37, or the viral particles of claim 39 in an amount and for a time sufficient to treat or ameliorate the symptoms of said disease, dysfunction, or deficiency in said mammal.

55. The method of claim 54, wherein said mammal is a human.

56. The method of claim 54, wherein said virion or said plurality of viral particles is administered to said mammal intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, or by direct injection into an organ or a tissue of said mammal.

57. The method of claim 56, wherein said organ or tissue is selected from the group consisting of pancreas, liver, heart, lung, brain, kidney, joint, and muscle.

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58. An rAAV vector comprising a nucleic acid segment modified to express functional VP1 and VP3 capsid proteins substantially in the absence of functional VP2 protein.

59. The rAAV vector of claim 58, wherein said vector produces an infectious virion in the absence of exogenous VP2 protein.

60. The rAAV vector of claim 58, wherein the substantial absence of functional VP2 protein is the result of at least a first mutation in the capsid gene sequence region that comprises the VP2 start codon.

61. The rAAV vector of claim 58, wherein the absence of functional VP2 protein is the result of at least a first mutation in said VP2 start codon.

62. The rAAV vector of claim 58, defined as plM45-VP1,3.

63. An rAAV vector comprising: a nucleic acid segment that has been modified to express functional VP1 and VP2 capsid proteins substantially in the absence of functional VP3 protein.

64. The rAAV vector of claim 63, wherein said vector produces an infectious virion in the presence of exogenous VP3 protein.

65. The rAAV vector of claim 64, defined as plM45-VP1,2.

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66. An rAAV vector comprising a nucleic acid segment that has been modified to express functional VP2 and VP3 capsid proteins substantially in the absence of functional VP1 protein.

67. The rAAV vector of claim 66, wherein said vector produces an infectious virion in the presence of exogenous VP1 protein.

68. The rAAV vector of claim 67, defined as pIM45-VP2,3.

69. An expression vector that expresses an rAAV capsid protein selected from the group consisting of VP1, VP2, and VP3, each substantially in the absence of any other rAAV protein.

70. The expression vector of claim 69, comprising a mutation at position 1 of the cap gene, a mutation at position 138 of the cap gene, or a mutation at position 203 of the cap gene.

71. The expression vector of claim 70, defined as pIM45-VP1, pIM45-VP2, or pIM45-VP3.

72. An expression vector that expresses: (a) rAAV capsid proteins VP1 and VP2 substantially in the absence of VP3 protein; (b) rAAV capsid proteins VP1 and VP3 substantially in the absence of VP2 protein; or (c) rAAV capsid proteins VP2 and VP3 substantially in the absence of VP1 protein.

73. The expression vector of claim 72, comprising: (a) at least one mutation in the start codon of said VP1 protein and at least one mutation in the start codon of said VP2

protein; (b) at least one mutation in the start codon of said VP1 protein and at least one mutation in the start codon of said VP3 protein; or (c) at least one mutation in the start codon of said VP2 protein and at least one mutation in the start codon of said VP3 capsid protein.

74. The expression vector of claim 73, wherein said vector comprises: (a) at least one mutation at position 1 and at least one mutation at position 138 of the cap gene, (b) at least one mutation at position 1 and at least one mutation at position 203 of the cap gene; or (c) at least one mutation at position 138 and at least one mutation at position 203 of the cap gene.

75. The expression vector of claim 74, defined as pIM45-VP1,2; pIM45-VP1,3; or pIM45-VP2,3.

76. A VP2-free rAAV expression system comprising:

(a) at least a first rAAV vector that comprises at least a first heterologous nucleic acid segment inserted into the capsid sequence region, said segment encoding at least a first heterologous peptide; and

(b) at least a second expression vector that expresses functional VP1 and VP3 capsid proteins substantially in the absence of VP2 protein, or at least a second and a third expression vector that separately express functional VP1 and VP3 capsid proteins, each substantially in the absence of VP2 protein.

77. The VP2-free rAAV expression system of claim 76, wherein said at least a first rAAV vector substantially lacks VP2 expression.

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78. The VP2-free rAAV expression system of claim 77, wherein said at least a first heterologous peptide is expressed on the surface of an rAAV virion comprising said vector.

79. The VP2-free rAAV expression system of claim 78, wherein said at least a first peptide selectively targets said rAAV virion to at least a first host cell.

80. The VP2-free rAAV expression system of claim 79, wherein said at least a first peptide selectively targets said rAAV virion to an organ- or tissue-specific host cell.

81. The VP2-free rAAV expression system of claim 76, comprising at least a second expression vector that expresses functional VP1 and VP3 capsid proteins substantially in the absence of VP2 protein.

82. The VP2-free rAAV expression system of claim 76, comprising at least a second expression vector and a third expression vector that separately express functional VP1 and VP3 capsid proteins, each substantially in the absence of VP2 protein.

83. The VP2-free rAAV expression system of claim 76, wherein said at least a second expression vector or said at least a third expression vector is pIM45-VP1, pIM45-VP2, or pIM45-VP3.

84. The VP2-free rAAV expression system of claim 83, wherein said at least a first rAAV expression vector is pIM45-VP1,3.

85. The rAAV vector of claim 58, or the VP2-free rAAV expression system of claim 76, wherein said rAAV vector further comprises at least a first exogenous polynucleotide

operably positioned downstream and under the control of at least a first heterologous promoter that expresses said polynucleotide in a cell comprising said vector.

86. The vector or expression system of claim 85, wherein said at least a first heterologous promoter is a constitutive, inducible, or cell-specific promoter.

87. The vector or expression system of claim 86, wherein said at least a first heterologous promoter is selected from the group consisting of a CMV promoter, a β-actin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter and a VP16-LexA promoter.

88. The vector or expression system of claim 87, wherein said at least a first heterologous promoter is a β-actin promoter.

89. The vector or expression system of claim 85, wherein said rAAV vector further comprises at least a first enhancer.

90. The vector or expression system of claim 89, wherein said rAAV vector further comprises at least a first CMV enhancer, a synthetic enhancer, or a cell- or tissue-specific enhancer.

91. The vector or expression system of claim 85, wherein said at least a first exogenous polynucleotide further comprises at least a first intron sequence.

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92. The vector or expression system of claim 85, wherein said at least a first exogenous polynucleotide is of human, murine, porcine, bovine, ovine, feline, canine, equine, epine, caprine, or lupine origin.

93. The vector or expression system of claim 85, wherein said at least a first exogenous polynucleotide encodes a protein, polypeptide, peptide, ribozyme, or antisense oligonucleotide.

94. The rAAV vector of claim 58, comprised within an infectious adeno-associated viral particle.

95. The rAAV vector of claim 58, comprised within a pharmaceutical vehicle.

96. The rAAV vector of claim 95, formulated for administration to a human.

97. An infectious AAV particle comprising the rAAV vector of claim 58.

98. The infectious AAV particle of claim 97, comprised within a pharmaceutical vehicle.

99. A host cell comprising the rAAV vector of claim 58, or the infectious AAV particle of claim 97.

100. The host cell of claim 99, wherein said host cell is a mammalian host cell.

101. The host cell of claim 100, wherein said host cell is a human host cell.

102. A composition comprising: (i) the rAAV vector of claim 58, the infectious AAV particle of claim 97, or the host cell of claim 99, and (ii) a pharmaceutically-acceptable excipient.

103. An rAAV vector comprising at least a first mutation in the capsid gene, wherein said mutation substantially reduces or eliminates the affinity of a viral particle comprising said vector for binding to heparin, heparan sulfate, or heparan sulfate proteoglycan.

104. The rAAV vector of claim 103, comprising at least a first mutation at Arginine position 585 or at least a first mutation at Arginine position 588 of said capsid gene.

105. The rAAV vector of claim 104, comprising at least a first Arginine to Alanine mutation at position 585 or at least a first Arginine to Alanine mutation at position 588 of said capsid gene.

106. The rAAV vector of claim 105, comprising at least a first Arginine to Alanine mutation at position 585 and at least a second Arginine to Alanine mutation at position 588 of said capsid gene.

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FIG. 1A

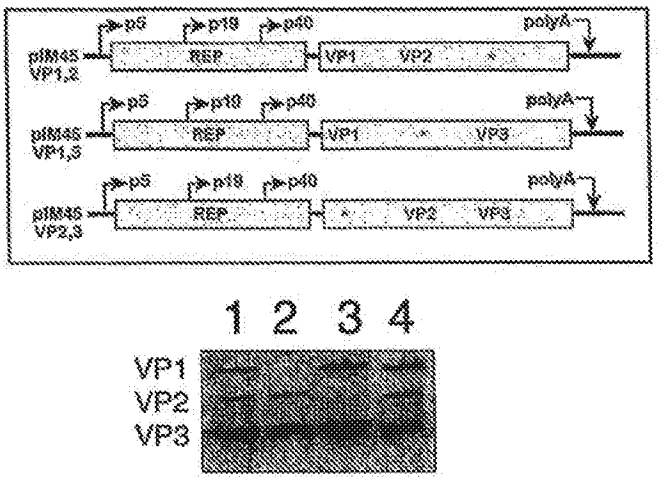


FIG. 1B

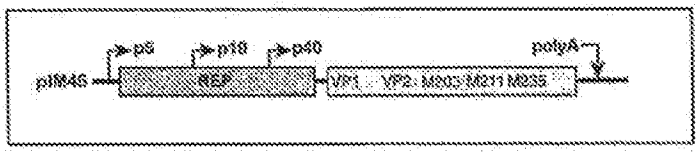
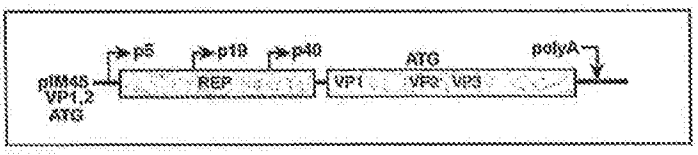


FIG. 1C



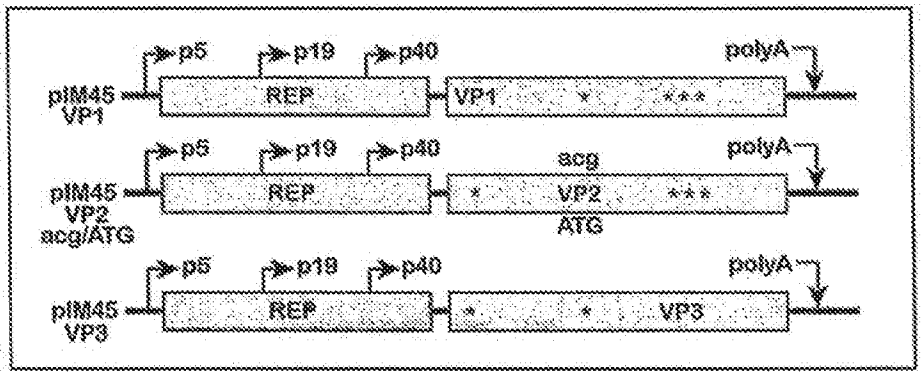

p5
p19
p40
polyA
pIM45 VP1
REP
VP1
pIM45 VP2 acg/ATG
acg
VP2
ATG
pIM45 VP3
VP3

FIG. 2A

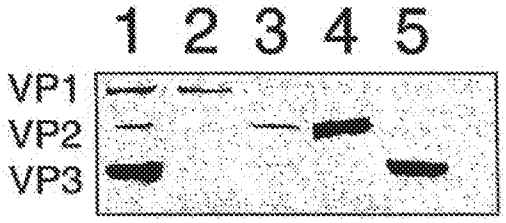

1 2 3 4 5
VP1
VP2
VP3

FIG. 2B

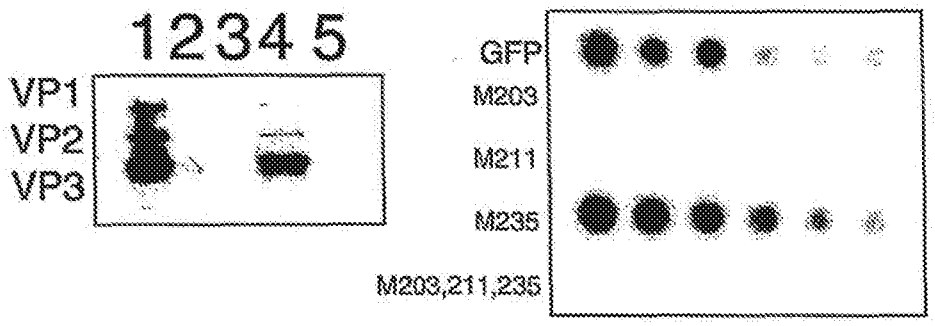


FIG. 3A

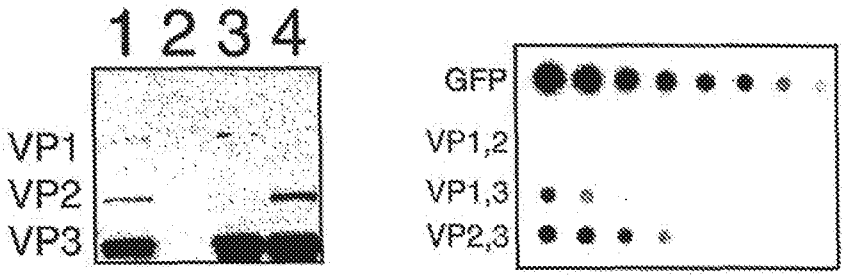


FIG. 3B

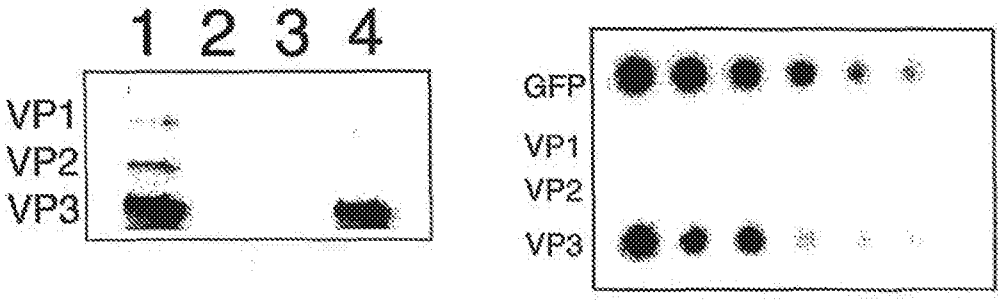
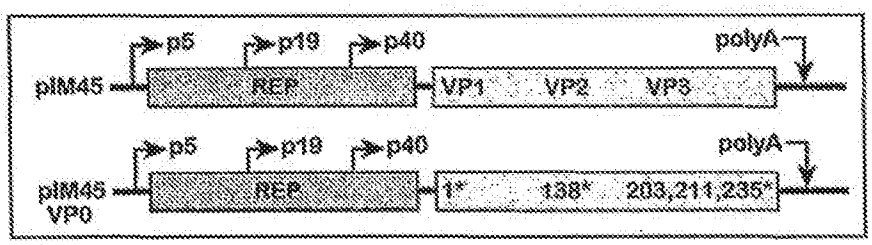
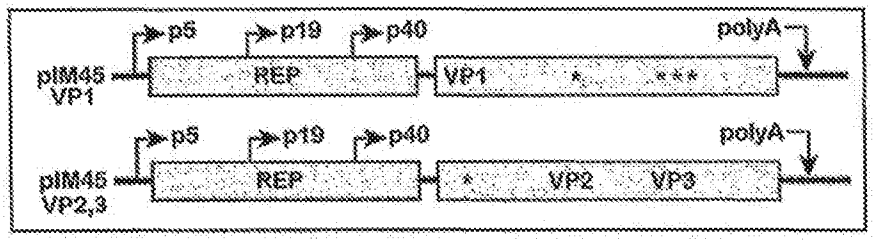


FIG. 3C

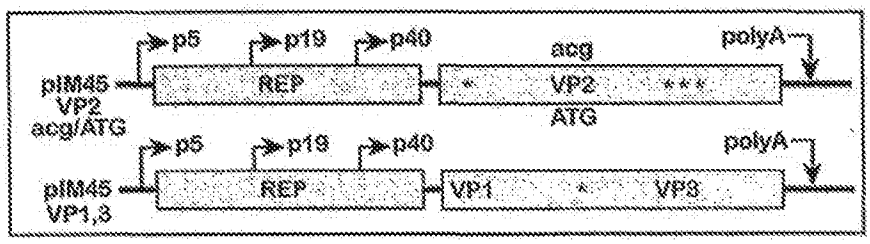
VP0



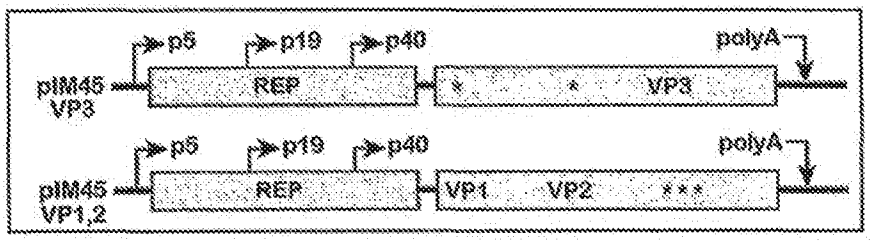
VP1



VP2/VP2A



VP3



+

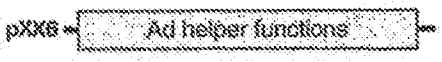


FIG. 4

+



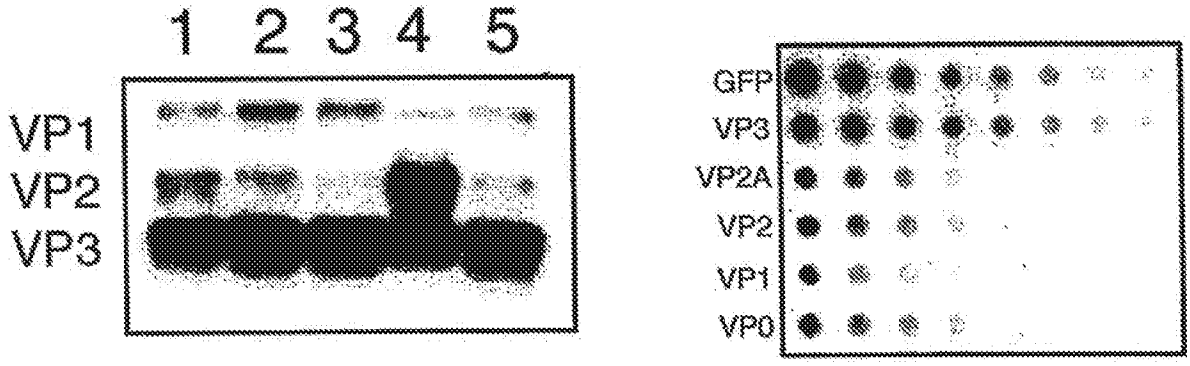

1 2 3 4 5
VP1
VP2
VP3
GFP
VP3
VP2A
VP2
VP1
VP0

FIG. 5A

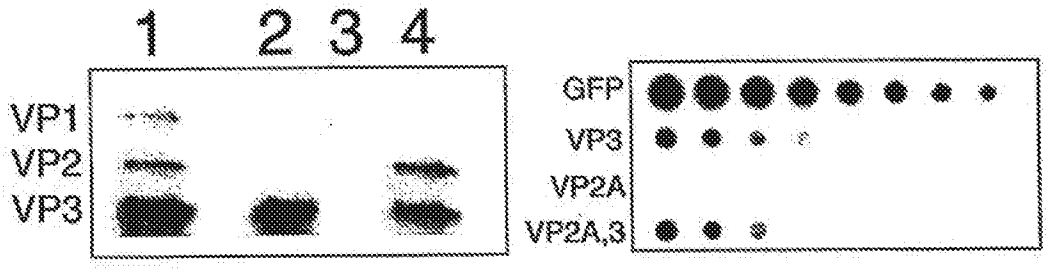

1 2 3 4
VP1
VP2
VP3
GFP
VP3
VP2A
VP2A,3

FIG. 5B

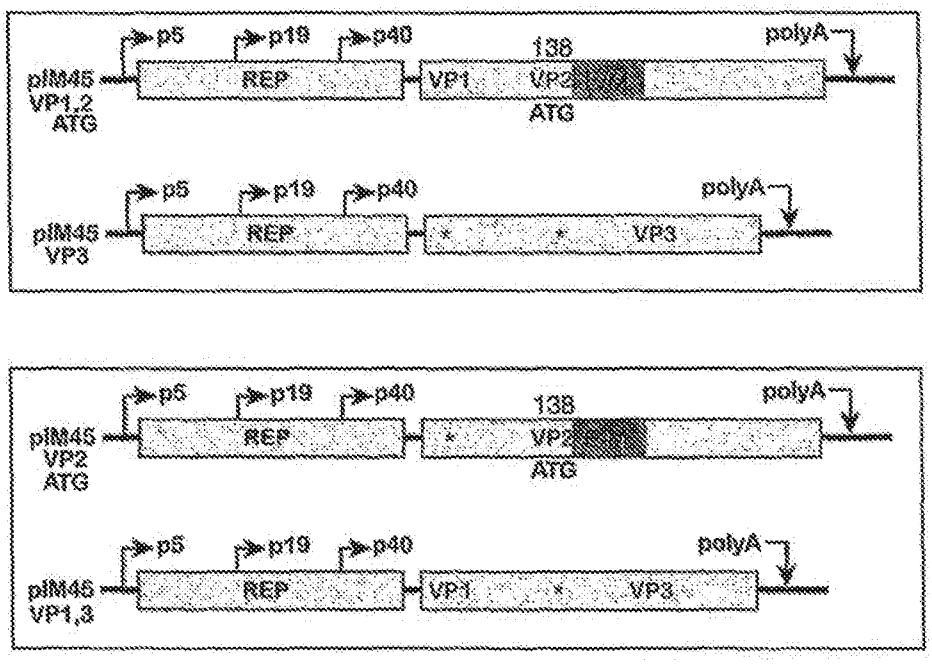


FIG. 6A

FIG. 6B

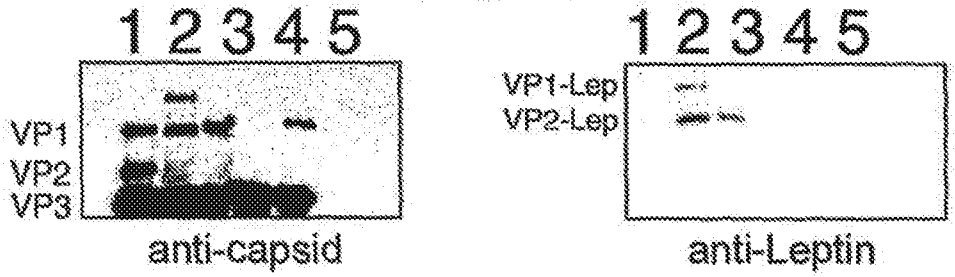
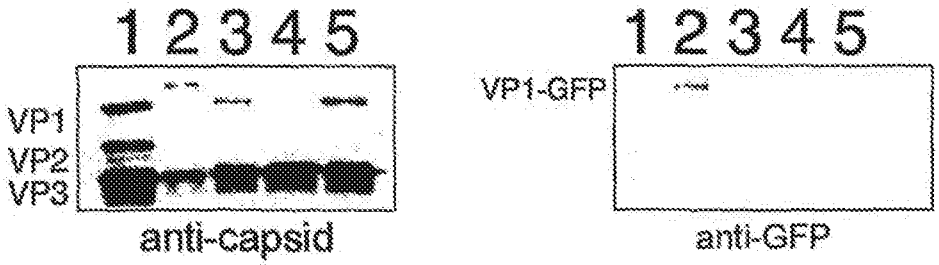


FIG. 6C



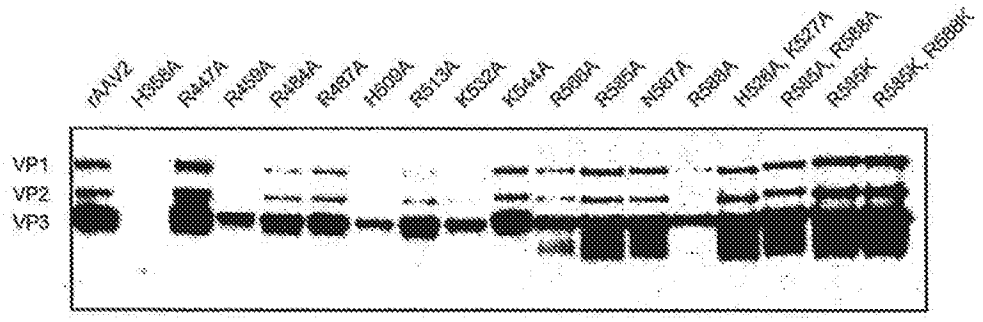

rAAV2
H558A
R447A
R459A
R484A
R487A
H509A
R513A
K532A
K564A
R569A
R585A
R587A
R588A
H638A, K527A
R585A, R588A
R585K
R585K, R588K
VP1
VP2
VP3

FIG. 7

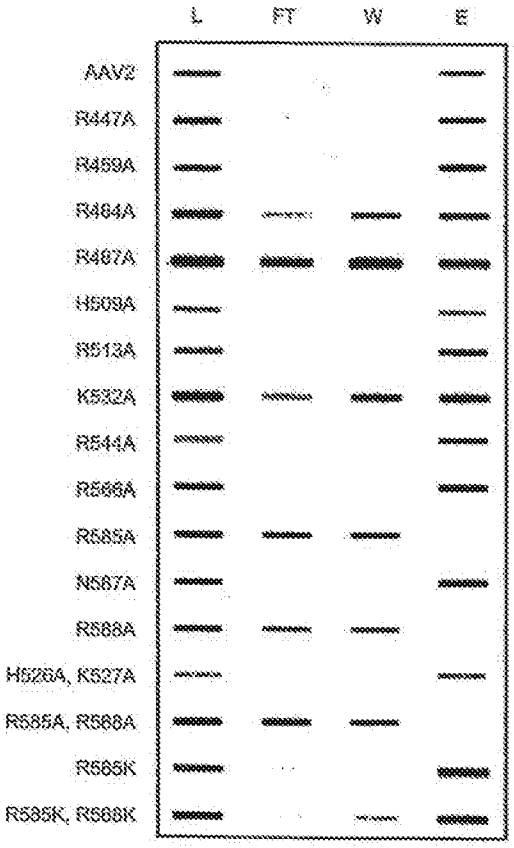
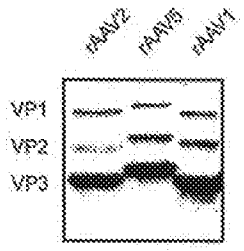

L
FT
W
E
AAV2
R447A
R459A
R464A
R487A
H509A
R513A
K532A
R544A
R566A
R585A
N587A
R588A
H526A, K527A
R585A, R588A
R585K
R585K, R588K

FIG. 8

FIG. 9A



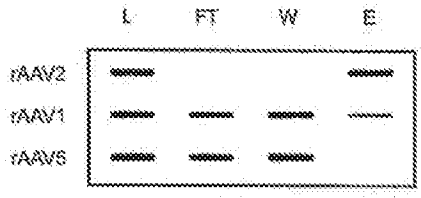


FIG. 9B

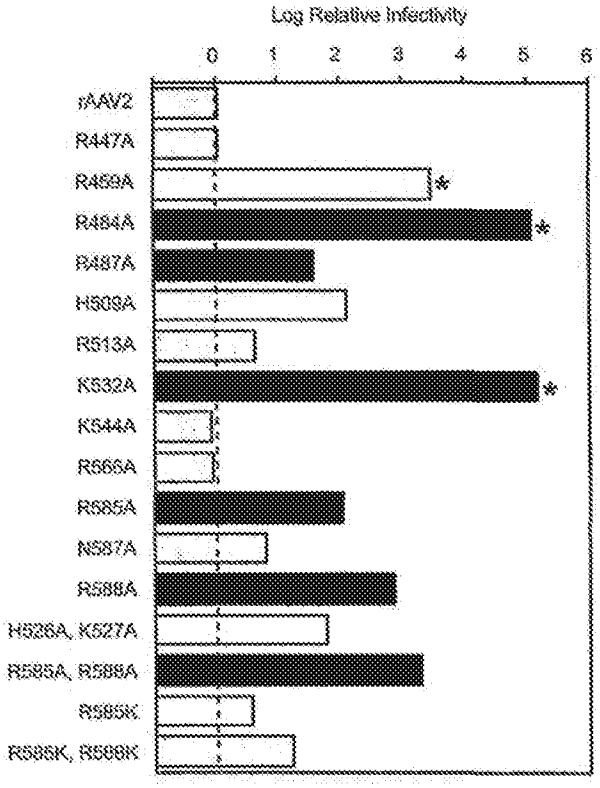

Log Relative Infectivity
0
1
2
3
4
5
6
rAAV2
R447A
R459A
R484A
R487A
H509A
R513A
K532A
K544A
R666A
R585A
N587A
R588A
H526A, K527A
R585A, R588A
R585K
R588K, R588K
*
*
*

FIG. 10

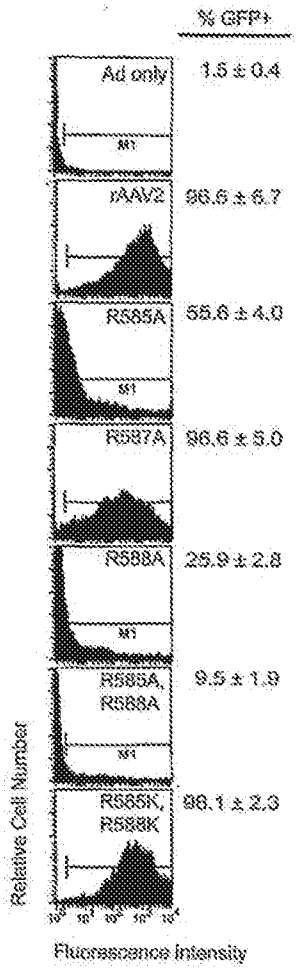
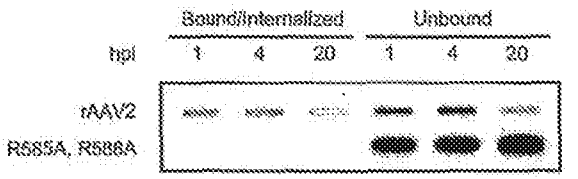

% GFP+
Ad only
1.5 ± 0.4
M1
rAAV2
96.6 ± 6.7
R585A
55.6 ± 4.0
R587A
96.6 ± 5.0
R588A
25.9 ± 2.8
R585A, R588A
9.5 ± 1.9
R585K, R588K
98.1 ± 2.3
Relative Cell Number
Fluorescence Intensity

FIG. 11

FIG. 12A



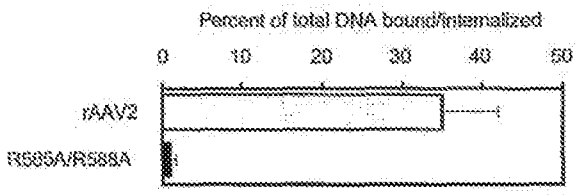
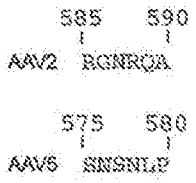


FIG. 12B

FIG. 13A



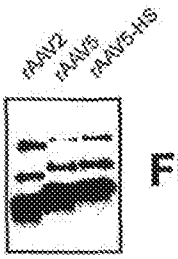
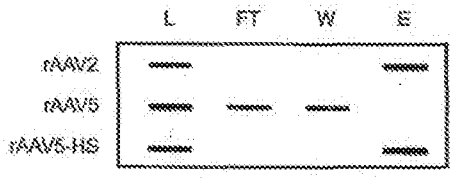


FIG. 13B

FIG. 13C



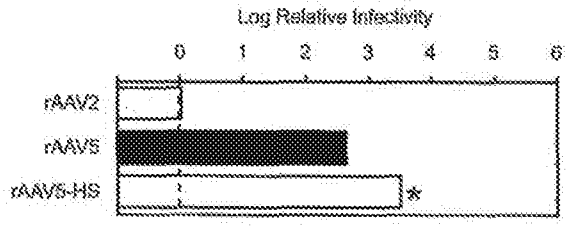


FIG. 13D

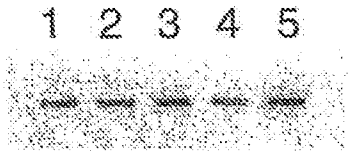


FIG. 14

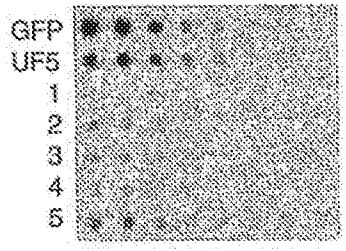


FIG. 15

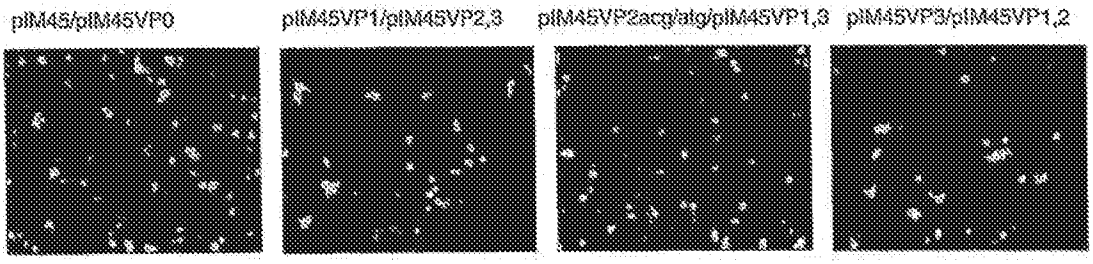


FIG. 16

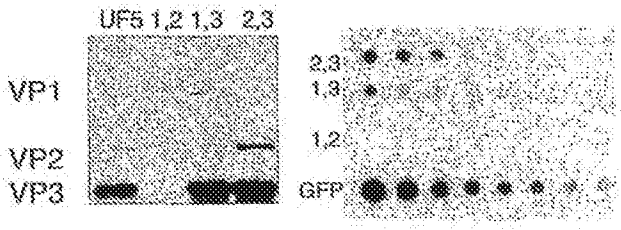
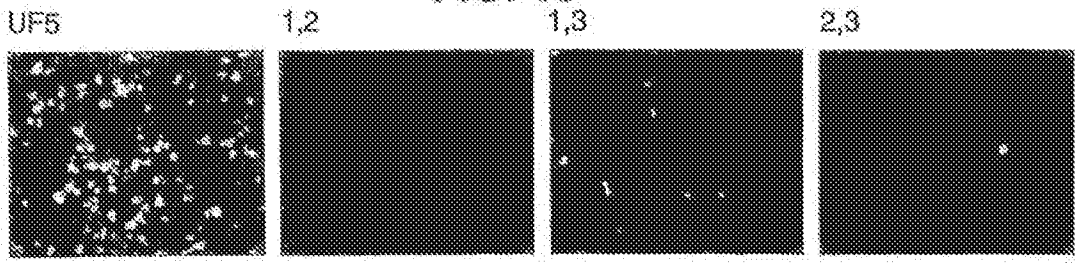


FIG. 17

FIG. 18



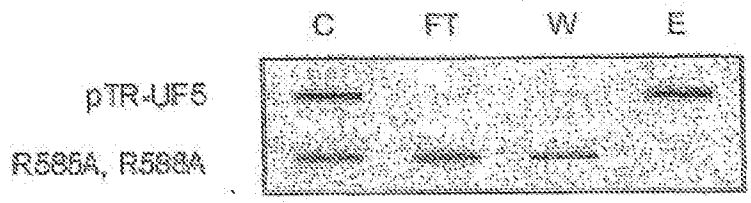


FIG. 19

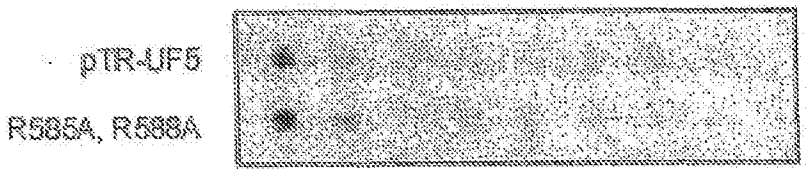
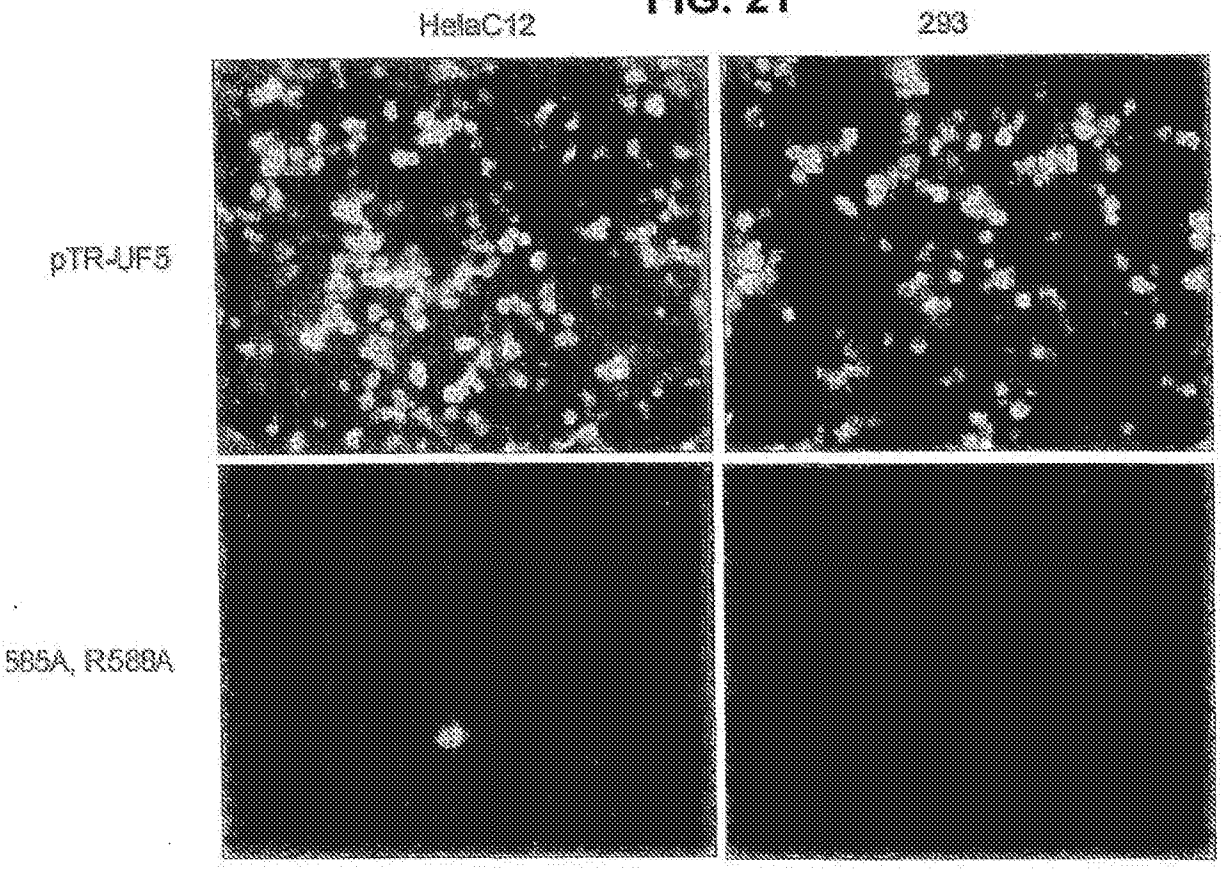


FIG. 20

FIG. 21



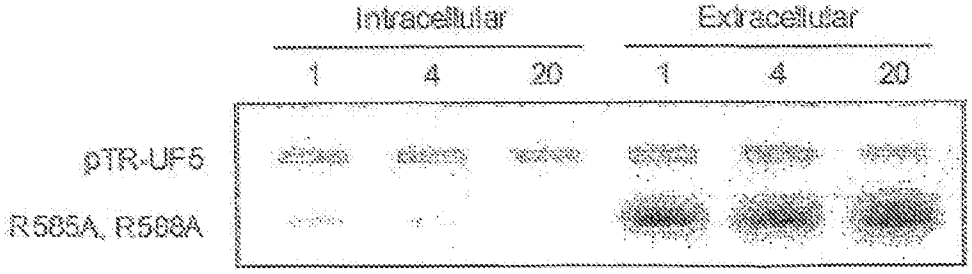


FIG. 22

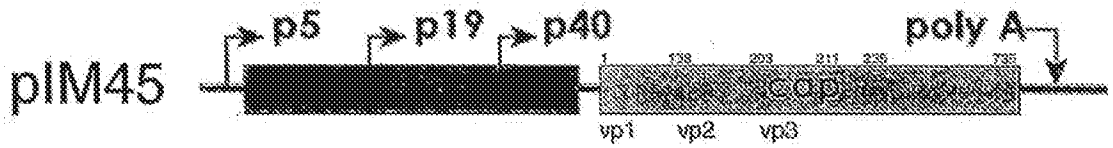


FIG. 23

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

U.S. APPLICATION NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
16/325,653	Xiaoying JIN	15979-20141.00

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

INTERNATIONAL APPLICATION NO.	
PCT/US2017/046814	
I.A. FILING DATE	PRIORITY DATE
08/14/2017	08/15/2016

CONFIRMATION NO. 1002

371
ABANDONMENT/TERMINATION
LETTER

OC000000114737409

Date Mailed: 02/19/2020

NOTIFICATION OF ABANDONMENT

The United States Patent and Trademark Office in its capacity as a Designated / Elected Office (37 CFR 1.495) has made the following determination:

- Applicant has failed to provide a proper reply to the notification mailed on 04/23/2019 within the time period set therein. Specifically, the status of each claim must be provided after the specific claim numbering by the proper status identifiers. Claims 32-60 (original)

Therefore, the above identified application is ABANDONED AS TO THE UNITED STATES OF AMERICA.

MARILYN J YOUNGER

Telephone: (571) 272-8183

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

PETITION TO WITHDRAW ABANDONMENT UNDER CFR § 1.181

MS Petition
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

This Petition under 37 C.F.R. § 1.181 is in response to the Notice of Abandonment issued on February 19, 2020. The Notice of Abandonment states that the present application is abandoned because the Response to the Notification to File Corrected Papers filed on July 23, 2019 did not indicate a proper status identifier for Claims 32-60. Applicants indicate that Claims 32-60 were properly indicated as abandoned in the Remarks section of the reply to the Notification to File Corrected Papers, and respectfully request that the Notice of Abandonment be withdrawn.

In support of this petition, Applicant provides a Third Preliminary Amendment to correct the typographical error in the Listing of Claims.

Statement of Facts:

On April 23, 2019, a Notification to File Corrected Papers was issued requiring the following:

- Independent Claim fees
- Total Claim fees
- Multiple dependent Claim fees
- Substitute specification (clean copy)
- Substitute specification (marked-up copy)
- A statement that the substitute specification contains no new matter.

On July 23, 2019, Applicants' representative filed via EFS (copy attached, excluding the references submitted with Information Disclosure Statement) a Petition for Extension of Time for one (1) month along with the following documents:

- Electronic Fees in the amount of $5,460.00
- Second Preliminary Amendment and Response to Missing Parts, including the statement that the substitute specification contains no new matter
- Copy of Substitute Specification, excluding claims and abstract (clean copy)
- Copy of Substitute Specification, excluding claims and abstract (marked-up copy)
- Information Disclosure Statement, including references

On February 19, 2020, a Notice of Abandonment was issued indicating that the USPTO received the reply to the Notification to File Corrected Papers, and alleging that the proper status identifier was not used for Claims 32-60.

Applicants respectfully submit that the submission on July 23, 2019 was timely and a Third Preliminary Amendment, correcting the status identifier in the Listing of Claims is submitted herewith.

Accordingly, Applicants respectfully request that this Petition be granted and the Notice of Abandonment be withdrawn.

Applicant believes that this communication does **not** qualify as a "supplemental reply or other paper" under the meaning of 37 C.F.R. § 1.704(c)(8) and should therefore **not** adversely impact patent term adjustment for the present application.

CONCLUSION

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account

Dated: February 21, 2020

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-5632

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

THIRD PRELIMINARY AMENDMENT UNDER 37 C.F.R. 1.115

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

Prior to examination on the merits, Applicant respectfully requests entry of this Preliminary Amendment for the above-captioned patent application. Accordingly, this response is timely filed.

Amendments to the Claims are reflected in the listing of claims which begins on page 2 of this paper.

Remarks/Arguments begin on page 9 of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (original): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

Claim 2 (canceled).

Claim 3 (original): A method of determining the heterogeneity of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS),

c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and

d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

Claims 4-28 (canceled).

Claim 29 (original): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

Claim 30 (canceled).

Claim 31 (original): A method of determining the heterogeneity of a serotype of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

Claims 32-60 (canceled).

Claim 61 (currently amended): A method of determining the serotype of an AAV particle comprising the method of claim 1 ~~any one of claims 1, 2 or 4-28~~ combined with a method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype ~~the method of any one of claims 29, 30 or 32-59~~.

Claim 62 (currently amended): A method of determining heterogeneity of an AAV particle comprising the method of claim 3 ~~any one of claims 3-28~~ combined with a method of determining the heterogeneity of a serotype of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity ~~the method of any one of claims 31-60~~.

Claim 63 (original): A recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3 or a parent polypeptide; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

Claims 64-88 (canceled).

Claim 89 (currently amended): A pharmaceutical composition comprising the rAAV particle of claim 63 ~~any one of claims 63-88~~.

Claim 90 (currently amended): A kit comprising the ~~rAAV particle of any one of claims 63-87 or the~~ pharmaceutical composition of claim 89.

Claim 91 (currently amended): An article of manufacture comprising ~~the rAAV particle of any one of claims 63-87 or~~ the pharmaceutical composition of claim 89.

Claim 92 (original): An AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein.

Claims 93-99 (canceled).

Claim 100 (currently amended): A method of improving the stability, assembly, or transduction of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting the amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

Claims 101-127 (canceled).

Claim 128 (original): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype.

Claim 129 (canceled).

Claim 130 (original): A method of determining the heterogeneity of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS),

c) determining the masses of one or more capsid proteins of the viral particle, and

d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype;

wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity.

Claims 131-133 (canceled).

Claim 134 (original): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle;

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype.

Claim 135 (canceled).

Claim 136 (original): A method of determining the heterogeneity of a serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype;

wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity.

Claims 137-141 (canceled).

Claim 142 (original): A recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent AAV particle, wherein residue numbering is based on VP1 of AAV2 and the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

Claims 143-149 (canceled).

Claim 150 (currently amended): A pharmaceutical composition comprising the rAAV particle of claim 142 ~~any one of claims 142-149~~.

Claim 151 (currently amended): A kit comprising ~~the rAAV particle of any one of claims 142-149 or~~ the pharmaceutical composition of claim 150.

Claim 152 (currently amended): An article of manufacture comprising ~~the rAAV particle of any one of claims 142-149 or~~ the pharmaceutical composition of claim 150.

Claim 153 (original): An AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

Claim 154 (currently amended): A method of improving the stability, assembly, or transduction of a rAAV particle comprising substituting one or more amino acid residues of VP1 and/or VP3 of a parent VP1 and/or VP3, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to amino acid residue 2 of the parent VP1 and/or VP3.

Claims 155-180 (canceled).

REMARKS

By virtue of this amendment, claims 61-62, 89-91, 100, 150-152, and 154 have been amended, and claims 2, 4-28, 30, 32-60, 64-88, 93-99, 101-127, 129, 131-133, 135, 137-141, 143-149, and 155-180 been canceled. Accordingly, claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142, and 150-154 are currently under consideration.

Support for the claim amendments may be found throughout the specification. For example, support may be found in original claims 29, 31, 61-62, 100-103, and 154-156. No new matter has been added.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Applicant notes that a substitute specification in response to the Notification to File Corrected Papers was filed July 23, 2019.

CONCLUSION

If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: February 21, 2020

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
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Palo Alto, CA 94304-1018
+1 (650) 813-5632

Electronic Acknowledgement Receipt

EFS ID:	36664294
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	23-JUL-2019
Filing Date:	
Time Stamp:	16:56:54
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Non Patent Literature	XIAO_1998_2224-2232.pdf	730782 c5e9b3aca28c319bf62c1afd7f67db905c4fe4ee	no	9

Warnings:

Information:					
2	Non Patent Literature	XIAOYING_2017_255-267.pdf	1179175 7aa613a98f0fbb978fdc544eadbd2f8722c31008	no	13
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Information:					
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4	Non Patent Literature	YAMASHITA_1984_4451-4459.pdf	6598702 8ac076a78f0280b2d0c4bb87a6d2b391570eb1ec	no	9
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5	Non Patent Literature	YAN_2002_2043-2053.pdf	806952 bbbce76e612d20e1cbfff7b4ff083d4d30d9c639	no	11
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6	Non Patent Literature	ZHAO_2006_283-293.pdf	1347796 fe67d699520d2b4eac648e052eebad4cbe5660af	no	19
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Information:					
7	Non Patent Literature	ZHONG_2008_7827-7832.pdf	548566 9473182b2f4bd674f6638eaa88634592862d6f4e	no	6
Warnings:					
Information:					
			Total Files Size (in bytes):	12167585	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

SECOND PRELIMINARY AMENDMENT UNDER 37 C.F.R. 1.115

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

Prior to examination on the merits, Applicant respectfully requests entry of this Preliminary Amendment for the above-captioned patent application. This Amendment is filed as part of the response to the Notification to File Corrected Papers issued April 23, 2019. A response to the Notice was due on June 23, 2019. We hereby petition for an extension of time to extend the deadline for responding from June 23, 2019 to July 23, 2019. Accordingly, this response is timely filed.

Amendments to the Specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 3 of this paper.

Remarks/Arguments begin on page 10 of this paper.

AMENDMENTS TO THE SPECIFICATION

Submitted herewith is a substitute specification as well as the "marked-up" copy of the specification (excluding claims and abstract). The "marked up" copy of the specification shows the amendments made to the specification as identified in the 1st Preliminary Amendment submitted at the time of filing of the present application on August 9, 2017 as well as removal of shading to Tables 3-6 on pages 81 and 82. The substitute specification does not include new matter.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (original): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

Claim 2 (canceled).

Claim 3 (original): A method of determining the heterogeneity of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS),

c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and

d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

Claims 4-28 (canceled).

Claim 29 (original): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

Claim 30 (canceled).

Claim 31 (original): A method of determining the heterogeneity of a serotype of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

Claims 32-60 (original).

Claim 61 (currently amended): A method of determining the serotype of an AAV particle comprising the method of claim 1 ~~any one of claims 1, 2 or 4-28~~ combined with a method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype ~~the method of any one of claims 29, 30 or 32-59~~.

Claim 62 (currently amended): A method of determining heterogeneity of an AAV particle comprising the method of claim 3 ~~any one of claims 3-28~~ combined with a method of determining the heterogeneity of a serotype of an AAV particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype;

wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity ~~the method of any one of claims 31-60~~.

Claim 63 (original): A recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3 or a parent polypeptide; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

Claims 64-88 (canceled).

Claim 89 (currently amended): A pharmaceutical composition comprising the rAAV particle of claim 63 ~~any one of claims 63-88~~.

Claim 90 (currently amended): A kit comprising the ~~rAAV particle of any one of claims 63-87 or the~~ pharmaceutical composition of claim 89.

Claim 91 (currently amended): An article of manufacture comprising ~~the rAAV particle of any one of claims 63-87 or~~ the pharmaceutical composition of claim 89.

Claim 92 (original): An AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein.

Claims 93-99 (canceled).

Claim 100 (currently amended): A method of improving the stability, assembly, or transduction of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting the amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

Claims 101-127 (canceled).

Claim 128 (original): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype.

Claim 129 (canceled).

Claim 130 (original): A method of determining the heterogeneity of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS),

c) determining the masses of one or more capsid proteins of the viral particle, and

d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype;

wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity.

Claims 131-133 (canceled).

Claim 134 (original): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle;

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype.

Claim 135 (canceled).

Claim 136 (original): A method of determining the heterogeneity of a serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS),

e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and

f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype;

wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity.

Claims 137-141 (canceled).

Claim 142 (original): A recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent AAV particle, wherein residue numbering is based on VP1 of AAV2 and the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

Claims 143-149 (canceled).

Claim 150 (currently amended): A pharmaceutical composition comprising the rAAV particle of claim 142 ~~any one of claims 142-149~~.

Claim 151 (currently amended): A kit comprising ~~the rAAV particle of any one of claims 142-149 or~~ the pharmaceutical composition of claim 150.

Claim 152 (currently amended): An article of manufacture comprising ~~the rAAV particle of any one of claims 142-149 or~~ the pharmaceutical composition of claim 150.

Claim 153 (original): An AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

Claim 154 (currently amended): A method of improving the stability, assembly, or transduction of a rAAV particle comprising substituting one or more amino acid residues of VP1 and/or VP3 of a parent VP1 and/or VP3, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to amino acid residue 2 of the parent VP1 and/or VP3.

Claims 155-180 (canceled).

REMARKS

By virtue of this amendment, claims 61-62, 89-91, 100, 150-152, and 154 have been amended, and claims 2, 4-28, 30, 32-60, 64-88, 93-99, 101-127, 129, 131-133, 135, 137-141, 143-149, and 155-180 been canceled. Accordingly, claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142, and 150-154 are currently under consideration.

Support for the claim amendments may be found throughout the specification. For example, support may be found in original claims 29, 31, 61-62, 100-103, and 154-156. No new matter has been added.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Regarding the Specification Amendment

The Notification to File Corrected Papers requests a substitute specification that does not contain shading in the tables on pages 81 and 82. In response, the specification is amended to remove the shading in Tables 3-6. No new matter is added. Substitute specifications, including both a clean version and a marked-up version, are submitted herewith. Entry of the amendments to the specification is respectfully requested.

CONCLUSION

If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: July 23, 2019

Respectfully submitted,

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METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed August 14, 2017, which claims the priority benefit of U.S. Provisional Application No. 62/375,314, filed August 15, 2016, the disclosure of each of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 159792014100SEQLIST.TXT, date recorded: February 13, 2019, size: 52 KB).

FIELD OF THE INVENTION

[0003] The present invention relates to methods for serotyping and/or determining the heterogeneity of a viral particle (*e.g.*, an adeno-associated virus (AAV) particle) using mass determination, *e.g.,* by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles.

BACKGROUND OF THE INVENTION

[0004] Complete characterization of the viral capsid proteins of viral vectors (*e.g.*, AAV vectors), including their sequence and post-translation modifications, is desired in gene therapy research and development since viral capsid proteins (VPs) are critical for viral infectivity.

[0005] Viral vector products such as recombinant Adeno-Associated Virus (rAAV) products are typically identified using molecular tools targeting the nucleic acid transgene. These methods may include polymerase chain reaction (PCR) targeting transgene-specific sequences

and Restriction Fragment Length Polymorphism (RFLP) techniques. As rAAV technologies evolve, many facilities are beginning to investigate multiple AAV capsid serotypes encoding their therapeutic transgene in an effort to improve targeted tissue tropism.

[0006] Traditional molecular identification methods identify products containing unique transgenes but are unable to discern those that have differing AAV capsid serotypes. Currently, most AAV serotype identity tests are based on SDS-PAGE banding patterns, an antibody-based ELISA, or a Western blot assay. However, the banding patterns and antibodies are not specific enough to differentiate different AAV serotypes. Gel-LC/MS/MS has been reported as a capsid serotype identification method. However, this method involves multiple steps including SDS-PAGE, in-gel digestion, and LC/MS/MS and thus requires multiple days for the analysis while providing limited sequence coverage. Methods for identifying vectors such as rAAV vectors are of interest to gene therapy vectors (see, *e.g.,* U.S. PG Pub. No. US20110275529). Thus, it would be useful to have improved methods of characterizing viral particles.

[0007] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0008] Using rAAV as an example, described herein is the use of LC/MS as an analytical tool to specifically identify different viral capsid serotypes (*e.g.,* rAAV capsid serotypes). As part of viral characterization, LC/MS can be used to augment the molecular identification methods. This analytical combination can satisfy regulatory requirements by discerning both the identity of the product's therapeutic transgene and the identity of the capsid serotype. This method can be used *e.g.,* as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in recombinant AAV gene therapy development. It can also be used to confirm VP sequences in capsid engineering research. In addition, this technique can be used to study the impact of post translation modifications, such as N terminal acetylation of viral capsid proteins, on transfection potency and intracellular protein trafficking.

[0009] The methods described herein can also be used to design AAV particles for greater stability and/or improved transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is acetylated to a higher extent compared to a wild type AAV capsid. In some embodiments, the methods can be used to design AAV particles with reduced transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is deacetylated to a higher or lower extent compared to a wild type AAV capsid.

[0010] In some aspects, the invention provides a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle; wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype. In some embodiments, the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

[0011] In some aspects, the invention provides a method of determining the heterogeneity of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of one or more capsid proteins of the viral particle, and d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0012] In some embodiments of the above aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the viral particle comprises a viral vector encoding a heterologous transgene.

[0013] In some aspects, the invention provide a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to

reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

[0014] In some aspects, the invention provides a method of determining the heterogeneity of a serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

[0015] As shown herein the methods can be performed in the absence of a gel separation step (*e.g.,* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)).

[0016] In some embodiments of the above aspects and embodiments, the viral particle comprises a viral vector encoding a heterologous transgene. In some embodiments, the viral particle belongs to a viral family selected from the group consisting of *Adenoviridae*, *Parvoviridae*, *Retroviridae*, *Baculoviridae*, and *Herpesviridae*. In some embodiments, the viral particle belongs to a viral genus selected from the group consisting of *Atadenovirus,*

Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus, Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, Penstyldensovirus, Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus, Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, Lentivirus, Spumavirus, Alphabaculovirus, Betabaculovirus, Deltabaculovirus, Gammabaculovirus, Iltovirus, Mardivirus, Simplexvirus, Varicellovirus, Cytomegalovirus, Muromegalovirus, Proboscivirus, Roseolovirus, Lymphocryptovirus, *Macavirus*, *Percavirus*, and *Rhadinovirus*.

[0017] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

[0018] In some aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0019] In some embodiments of the above aspects and embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C4 or C8

reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, the proportion of mobile phase B in the chromatography increases in a stepwise manner. In some embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In some embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In some embodiments, the liquid chromatography is ultra-performance liquid chromatography (UPLC).

[0020] In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

[0021] In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an

AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

[0022] In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

[0023] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

[0024] In some aspects, the invention provides a method of determining the heterogeneity of a serotype of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or

more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). In some embodiments, the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine. In some embodiments, the digestion is an enzymatic digestion or a chemical digestion. In some embodiments, the enzymatic digestion is an endopeptidase digestion. In some embodiments, the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion. In some embodiments, the chemical digestion is cyanogen bromide digestion or an acid digestion. In some embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

[0025] In some embodiments of the above aspects and embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C18 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, mobile phase B increases from about 2% to about 60%. In some embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC).

[0026] In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

[0027] In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1).

[0028] In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

[0029] In some embodiments, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some

embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; wherein the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; wherein the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu.

[0030] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO,, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0031] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked

by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0032] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0033] In some aspects, the invention provides a pharmaceutical composition comprising the rAAV particle as described herein. In some aspects, the invention provides a kit comprising the rAAV particle or the pharmaceutical composition as described herein. In some aspects, the invention provides an article of manufacture comprising the rAAV particle or the pharmaceutical composition as described herein.

[0034] In some aspects, the invention provides as AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of

N-terminal acetylation. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Ser, Asp or Glu. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0035] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

[0036] In some aspects, the invention provides a method of improving stability of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some embodiments, the substituted amino acid results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 is substituted. In some embodiments, the amino acid substitution at amino acid residue 2 of VP3 is substituted. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu. In some aspects, the invention provides a method of reducing the transduction of

rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

[0037] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0038] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0039] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the

host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0040] In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent particle, residue numbering based on VP1 of AAV2; wherein the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution is at amino acid residue A35, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprises a substitution with Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3; and results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprise a N57K or a N57Q substitution and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution comprise a substitution with Asp at A35 of VP1and results in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle. In some embodiments, the one or more amino acid

substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the G58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

[0041] In some aspects, the invention provides pharmaceutical compositions comprising AAV particles comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides kits comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides articles of manufacture comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

[0042] In some aspects, the invention provides a method of improving the stability of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to

deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle**.** In some embodiments, the parental Ala residue at position 35 of VP1 is substituted with Asn. In some embodiments, the parental Gly residue at position 58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

[0043] In some embodiments, the invention provides a method of improving the stability, aseembly and/or transduction efficiency of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle as described above, wherein the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from

different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0044] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0045] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution modulated deamidation of the capsid compared to the parent AAV particle.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] **FIGS. 1A-D** provide total ion Chromatograms of LC/MS of AAV2 VPs. **FIG. 1A:** 10cm long BEH C4 column with 1.7%/min gradient, **FIG. 1B:** 10cm long BEH C4 column

with 0.5%/min gradient; **FIG. 1C:** 15cm long BEH C4 column with 0.5%/min gradient, **FIG. 1D:** 15cm long BEH C8 column with 0.5%/min gradient.

[0047] **FIGS. 2A&B** provide deconvoluted mass spectra from **FIG. 1D** peak 1 (**FIG. 2A)** and **FIG. 1D** peak 2 (**FIG. 2B)**.

[0048] **FIG. 3** provides the sequence coverage of AAV2 VP1 (SEQ ID NO:3): green, tryptic peptides, blue, Lys-C peptides, pink, Asp-N peptides.

[0049] **FIGS. 4A-4C** provide MS/MS spectra of AAV2 VP N-terminal peptides. **FIG. 4A:** VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO: 4), **FIG. 4B** VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP (SEQ ID NO: 15). **FIG. 4C**: VP-3 N-terminal Asp-N derived peptide A(Ac)TGSGAPM (SEQ ID NO: 5).

[0050] **FIG. 5** provides the sequence alignment of 13 AAV serotypes black letter/white background: non-similar; blue letter/blue background: conservative; black letter/green background: block of similar; red letter/yellow background: identical; green letter/white background: weakly similar. AAVRh10 (SEQ ID NO: 17); AAV10 (SEQ ID NO: 18); AAV8 (SEQ ID NO: 19); AAV7 (SEQ ID NO: 20); AAV1 (SEQ ID NO: 21); AAV6 (SEQ ID NO: 22); AAV2 (SEQ ID NO: 23); AAV3 (SEQ ID NO: 24); AAV11 (SEQ ID NO: 25); AAV12 (SEQ ID NO: 26); AAV4 (SEQ ID NO: 27); AAV5 (SEQ ID NO: 28); AAV9 (SEQ ID NO: 29); Consensus (SEQ ID NO: 30).

[0051] **FIGS. 6A & 6B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T9 peptide YLGPF**NG**LDK (SEQ ID NO: 9) was used to monitor potential deamidation site N57 in both AAV1 and AAV2.

[0052] **FIGS. 7A & 7B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T49 peptides YNL**NG**R (SEQ ID NO: 11) and YHL**NG**R (SEQ ID NO: 12) were used to monitor potential deamidation site N511 in AAV1 and AAV2, respectively.

[0053] **FIGS. 8A & 8B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T67 peptides SANVDFTVDN**NG**LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT**NG**VYSEPR

(SEQ ID NO: 14) were used to monitor potential deamidation site N715 in AAV1 and AAV2, respectively.

[0054] **FIG. 9** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV5 deacetylated mutant variants.

[0055] **FIG. 10** illustrates an *in vitro* transduction assay for testing transduction efficiency of AAV5 deacetylated variants.

[0056] **FIG. 11** shows the efficiency of cell entry by the indicated AAV5 deacetylated variants or parental unmodified AAV5, as measured by vector genome copies/µg protein. Three cell lines were used: 293, HeLa, and HuH7.

[0057] **FIG. 12** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV5 deacetylated variants as compared to transduction with parental unmodified AAV5. Three cell lines were used: 293, HeLa, and HuH7.

[0058] **FIG. 13** provides the sequence alignment of 13 AAV serotypes, highlighting the conserved N57G58 deamidation site and the A35 residue in AAV2. AAVRh10 (SEQ ID NO: 31); AAV10 (SEQ ID NO: 31); AAV8 (SEQ ID NO: 32); AAV7 (SEQ ID NO: 33); AAV1 (SEQ ID NO: 31); AAV6 (SEQ ID NO: 31); AAV2 (SEQ ID NO: 34); AAV3 (SEQ ID NO: 35); AAV11 (SEQ ID NO: 31); AAV12 (SEQ ID NO: 36); AAV4 (SEQ ID NO: 37); AAV5 (SEQ ID NO: 38); AAV9 (SEQ ID NO: 39); Consensus (SEQ ID NO: 40).

[0059] **FIG. 14** shows a protein gel of VP1, VP2, and VP3 capsid proteins from AAV1 or AAV2 particles produced by the PCL or TTx method. *highlights the truncated VP1 (tVP1) protein.

[0060] **FIG. 15** shows the results of LC/MS analysis of deamidation of the indicated AAV2 mutants, as compared to control AAV2 capsids.

[0061] **FIG. 16** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV2 deamidation mutant variants.

[0062] **FIG. 17** illustrates an *in vitro* transduction assay for testing transduction efficiency of AAV2 deamidation variants.

[0063] **FIG. 18** shows the efficiency of cell entry by the indicated AAV2 deamidation variants or parental unmodified AAV2, as measured by vector genome copies/µg protein. Three cell lines were used: 293, HeLa, and HuH7.

[0064] **FIG. 19** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV2 deamidation variants as compared to transduction with parental unmodified AAV2. Three cell lines were used: 293, HeLa, and HuH7.

DETAILED DESCRIPTION

[0065] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle(s) comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

[0066] In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

[0067] In other aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

[0068] In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle of a serotype comprising: a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle

to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

[0069] In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0070] In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetlylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3.

I. General Techniques

[0071] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual (*Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998);

Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. Definitions

[0072] A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0073] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0074] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0075] A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, *e.g.,* two, inverted terminal repeat sequences (ITRs).

[0076] A "recombinant AAV vector (rAAV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one, *e.g.,* two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (rAAV particle)".

[0077] An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

[0078] A "parent AAV particle" and "parent AAV capsid protein" as used herein in the context of comparing N-acetylation and/or deamidation refers to an AAV particle or capsid protein into which amino acid modifications are introduced to modulate N-acetlylation and/or deamidation (*e.g.*, an AAV particle/capsid protein that is the same as or similar to the AAV particle/capsid of the subject invention but does not comprise the mutations that modulate/alter N-aceytlation and/or deamidation as described herein). In some embodiments, the parent AAV particle is a recombinant AAV particle comprising a recombinant AAV genome. In some embodiments, the parent AAV capsid particle or parent AAV capsid protein comprises amino acid substitutions that affect other aspects of the AAV particle. For example, the parent AAV particle may comprise amino acid substitutions that affect the binding of AAV to its receptor, such as affecting binding of AAV2 to heparin sulfate proteoglycan (e.g. an AAV2 HBKO particle). An AAV2 HBKO particle can be mutated to introduce amino acid substitutions that modulate N-acetylation and/or deamidation. Such a mutated AAV particle may then be compared to the parent AAV2 HBKO particle in aspects of the invention as described herein. A parent AAV capsid protein may include a parent VP1 capsid protein, a parent VP2 capsid protein, or a VP3 capsid protein.

[0079] As used herein, the term "modulate" or "alter" in reference to a parent molecule means to change a feature of the parent molecule. For example, an AAV particle with altered N-acetlylation may show increased or decreased N-acetylation compared to the parent AAV particle and an AAV particle with altered deamidation may show increased or decreased deamidation compared to the parent AAV particle.

[0080] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0081] The term "transgene" refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0082] The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0083] The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0084] The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0085] An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0086] An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also

contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0087] A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0088] "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0089] A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0090] "Percent (%) sequence identity" with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various

ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, those described in Current Protocols in Molecular Biology (Ausubel *et al.*, eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z, where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

[0091] An "isolated" molecule (*e.g.*, nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment.

[0092] "Mass spectrometry" refers to the analytical chemistry technique of identifying an amount and/or type of a compound (*e.g.,* a polypeptide) by measuring the mass-to-charge ratio and abundance of gas-phase ions. The term "mass spectrometry" may be used interchangeably herein.

[0093] "Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, *e.g.,* of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

[0094] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0095] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise.

[0096] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Methods

[0097] Certain aspects of the present disclosure relate to methods of determining the serotype of a viral particle. Other aspects of the present disclosure relate to methods of determining the heterogeneity of a viral particle. As described below, the accurate masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. These methods are based in part on the discovery described herein that direct LC/MS of different types of AAVs after denaturation may be used to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. Further, acetylations of N-termini of VP1 and VP3 may also be identified and/or monitored in different AAV serotypes. Based on these AAV results and the guidance provided herein, it is contemplated that such methods may readily be applied to profile a variety of viruses, *e.g.*, the viral families, subfamilies, and genera of the present disclosure. The methods of the present disclosure may find use, *e.g.,* in profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP

production, to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications, and/or to monitor or detect heterogeneity of a viral particle or preparation.

[0098] In some embodiments, the methods include denaturing a viral particle. In some embodiments, a viral particle such as an AAV particle may be denatured using detergent, heat, high salt, or buffering with a low or high pH. In certain embodiments, an AAV particle may be denatured using acetic acid or guanidine hydrochloride. The skilled artisan will recognize that a variety of methods useful for promoting and/or monitoring protein denaturation are available in the art and may suitably select a denaturation method compatible with liquid chromatography/mass spectrometry. For example, if heat denaturation is used, care may be applied to avoid protein precipitation and reverse phase column clogging. Similarly, high salt denaturation may be coupled with a desalting step prior to LC/MS or LC/MS/MS. In other embodiments, high pH denaturation, low pH denaturation, or denaturation using organic solvents is used.

[0099] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to liquid chromatography/mass spectrometry (LC/MS). As is known in the art, LC/MS utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions. Such mass spectral data may be used to determine, *e.g.,* molecular weight or structure, identification of particles by mass, quantity, purity, and so forth. These data may represent properties of the detected ions such as signal strength (*e.g.,* abundance) over time (*e.g.,* retention time), or relative abundance over mass-to-charge ratio.

[0100] In some embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC; the term "ultra high performance liquid chromatography" or UHPLC may be used interchangeably herein). UPLC is known in the art as an LC technique that relies upon a column with reduced particle size (*e.g.,* less than 2μm) and increased flow velocity to improve chromatographic resolution, efficiency, peak capacity, and sensitivity (see, *e.g.,* Plumb, R. *et al.* (2004) *Rapid Commun. Mass Spectrom.* 18:2331-2337). In some embodiments, UPLC refers to the use of a column with a particle size less than 2μm in liquid chromatography. In some embodiments, UPLC refers to the use of a high linear solvent

velocity (*e.g.,* as observed when operating at 6000 psi or higher) in liquid chromatography. Exemplary UPLC machines are commercially available (*e.g.,* the ACQUITY UPLC® from Waters; Milford, MA).

[0101] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) may refer to electrospray ionization mass spectrometry (ESI-MS). ESI-MS is known in the art as a technique that uses electrical energy to analyze ions derived from a solution using mass spectrometry (see, *e.g.,* Yamashita, M. and Fenn, J.B. (1984) *J. Phys. Chem.* 88:4451-4459). Ionic species (or neutral species that are ionized in solution or in gaseous phase) are transferred from a solution to a gaseous phase by dispersal in an aerosol of charged droplets, followed by solvent evaporation that reduces the size of the charged droplets and sample ion ejection from the charge droplets as the solution is passed through a small capillary with a voltage relative to ground (*e.g.,* the wall of the surrounding chamber). In some embodiments, the capillary voltage is from about 2 kV to about 10 kV, or about 2.5 kV to about 6.0 kV. In certain embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) uses a capillary voltage of about 3.5 kV. In some embodiments the capillary voltage ranges from about 1 kV to about 10 kV. In other embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) may refer to matrix-assisted laser desorption/ionization (MALDI).

[0102] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) uses a sampling cone and/or skimmer, through which ions may pass before entering the analyzer. In some embodiments, *e.g.,* when applying voltage to the capillary as described above, the sample cone is held at a lower voltage than the capillary voltage. In certain embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) uses a sampling cone voltage of about 45 V. In some embodiments the sampling cone voltage ranges from about 0 V to about 200 V.

[0103] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) uses assisted calibration. Calibration, when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (*e.g.,* a standard) for the purpose of calibrating the instrument with respect to mass detection (*e.g.,* m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak and/or position of a known standard (*e.g.,* a calibrant) to a specific mass-to-charge (m/z) ratio. Once calibrated,

the user may then perform mass spectrometry on a sample having one or more unknown compounds, or compounds present at an unknown concentration, within a certain degree of accuracy or error, and/or a desired level of reproducibility, *e.g.,* as compared to a previous or known experimental condition. Various calibrants are known in the art, including without limitation sodium iodide, sodium cesium iodide, polyethylene glycol, and perfluorotributylamine. In certain embodiments, sodium iodide is used as a calibrant. In some embodiments the calibrants are Glu-1-fibrinopeptide B and leucine encephalin peptide to lock mass during LC/MS operation.

[0104] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure, or subjecting digested fragments of a denatured viral particle of the present disclosure, to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). As is known in the art, LC/MS/MS (the term "liquid chromatography-tandem mass spectrometry" may be used interchangeably herein) utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions, where the mass spectrometry uses multiple stages of mass (*e.g.,* m/z) separation, typically separated by a fragmentation step. For example, ions of interest within a range of m/z may be separated out in a first round of MS, fragmented, and then further separated based on individual m/z in a second round of MS. Ion fragmentation may include without limitation a technique such as collision-induced dissociation (CID), higher energy collision dissociation (HCD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD).

[0105] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to reduction and/or alkylation. Means to reduce the viral particle include but are not limited to treatment with dithiothreitol, β-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). Means to alkylate the viral particle include but are not limited to treating the AAV particle with iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

[0106] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to digestion, *e.g.,* to generate fragments of VP1, VP2 and/or VP3 of an AAV particle. For example, a denatured AAV particle may be subjected to digestion to generate peptide fragments that may be analyzed, *e.g.,* using LC for separation and MS/MS for analysis

(see below for greater description). In some embodiments, the digestion is an enzymatic digestion. In some embodiments, the digestion uses chemical digestion such as CNBr treatment of instrument fragmentation (e.g., top down). In some embodiments, the digestion uses chemical digestion such as acid digestion.

[0107] In some embodiments, the enzymatic digestion is an endopeptidase digestion. An endopeptidase may include any peptidase that catalyzes the proteolysis of peptide bonds of non-terminal amino acids of a polypeptide. Known endopeptidases may include, without limitation, trypsin, chymotrypsin, AspN, Glu-C, LysC, pepsin, thermolysin, glutamyl endopeptidase, elastase, and neprilysin. In certain embodiments, the enzymatic digestion is a trypsin digestion or a LysC digestion.

[0108] In some embodiments, the liquid chromatography (*e.g.,* used in LC/MS or LC/MS/MS as described herein) is reverse phase liquid chromatography (the terms "reversed phase liquid chromatography" or RPLC may be used interchangeably herein with reverse phase liquid chromatography). As is known in the art, reverse phase liquid chromatography may refer to a chromatographic separation using a hydrophobic stationary phase (*e.g.,* a support or substrate such as a column) to adsorb hydrophobic molecules in a polar mobile phase. By decreasing the polarity of the mobile phase (*e.g.,* by adding an organic solvent), one may achieve gradient separation of molecules by hydrophobicity, since more hydrophobic molecules will stay on the column in higher concentrations of organic solvent due to stronger hydrophobic interactions with the column. In some embodiment, separation is by capillary electrophoresis (CE), size exclusion chromatography (SEC), ion exchange chromatography (IEC) such as cation exchange chromatography, hydrophobic interaction chromatography (HIC), hydrophilic interaction liquid chromatography (HILIC), but not limited to on-line LC/MS such as offline separation before MS; e.g., tips, columns; plates or cartridges.

[0109] Generally, a stationary phase suitable for reverse phase liquid chromatography (*e.g.,* a hydrophobic moiety) may be coupled to a support including without limitation a column or resin packed with particles or beads (*e.g.,* porous silica particles or polystyrene). A variety of hydrophobic stationary phases are known in the art, including without limitation hydrophobic alkyl chains, octyl or octadecyl silyl moieties, cyano moieties, and amino moieties. In some

embodiments, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In certain embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography (*e.g.,* reverse phase chromatography utilizing a C4 or C8 stationary phase). One of skill in the art may suitably select a stationary phase based on the molecule of interest (*e.g.,* a denatured AAV particle or fragment thereof).

[0110] A variety of mobile phases suitable for reverse phase liquid chromatography are known in the art. As described above, a reverse phase liquid chromatography mobile phase may include a mixture of organic (*e.g.,* hydrophobic) and aqueous (*e.g.,* polar) solvents. Increasing the proportion of organic solvent increases its power to elute hydrophobic compounds from the stationary phase. Compound retention and/or selectivity may be altered, *e.g.,* by changing the type or exposure of the stationary phase, adding polar reagents such as end capping reagents, altering the temperature, and/or altering mobile phase characteristics such as the proportion of organic solvent, pH, buffers, and the type of organic solvent used. In some embodiments, the polar component of the mobile phase may include without limitation water or an aqueous buffer. In some embodiments, the polar component of the mobile phase may include without limitation acetonitrile, methanol, ethanol, isopropyl alcohol, tetrahydrofuran (THF), and formic acid.

[0111] In some embodiments, two or more mobile phases may be used (*e.g.,* mobile phase A, mobile phase B, etc.) in a gradient or proportion of interest. In certain embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In certain embodiments, the mobile phase A comprises about 0.1% formic acid. In certain embodiments, the mobile phase A comprises about 0.1% to about 5% formic acid. In certain embodiments, the chromatography uses a mobile phase B comprising formic acid in acetonitrile. In certain embodiments, the mobile phase B comprises about 0.1% formic acid.

[0112] In some embodiments, the proportion of mobile phase B in the chromatography increases over time. For example, the proportion of mobile phase B in the chromatography may be increased in a stepwise manner. In certain embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In other embodiments, mobile phase B increases from about 2% to about 60%. In other embodiments, mobile phase B increases from about 2% to about 100% from about 1 min to about 200 min In

some embodiments, the remainder of the mobile phase is a second mobile phase of the present disclosure, *e.g.,* mobile phase A. In certain embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In other embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. One of skill in the art may suitably adjust the mobile phase of interest and the gradient timing used based on the desired chromatographic separation and/or analyte of interest.

[0113] In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC). HPLC is known in the art as a form of liquid chromatography in which a liquid solvent containing a sample is pressurized as it passes through a column containing solid phase. While traditional or low pressure LC may use gravity to pass a mobile phase through the solid phase, HPLC uses pumps to apply a pressure to the mobile phase and typically uses a solid phase with smaller particles to increase resolution. In some embodiments, the HPLC uses a pressure of between about 50 bar and about 350 bar. In some embodiments, reversed phase HPLC may be used to concentrate and/or desalt proteins (*e.g.,* AAV capsid proteins) for MS analysis.

[0114] In some embodiments, one or more parameters including without limitation source voltage, capillary temperature, ESI voltage (if using ESI-MS), CID energy, and the number of MS/MS events may be adjusted, *e.g.,* in LC/MS/MS as used herein, based on the findings described herein. In some embodiments, mass spectrometry (*e.g.,* used in LC/MS/MS as described herein) uses a source voltage (*e.g.*, capillary voltage) of about 2.5 kV. In some embodiments, mass spectrometry (*e.g.,* used in LC/MS/MS as described herein) uses a capillary temperature of about 275°C. In some embodiments, the capillary temperature ranges from about 20°C to about 400°C.

[0115] A variety of mass analyzers suitable for LC/MS and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole TOF (QTOF), and ion traps (*e.g.,* a Fourier transform-based mass spectrometer or an Orbitrap). In Orbitrap, a barrel-like outer electrode at ground potential and a spindle-like central electrode are used to trap ions in trajectories rotating elliptically around the central electrode with

oscillations along the central axis, confined by the balance of centrifugal and electrostatic forces. The use of such instruments employs a Fourier transform operation to convert a time domain signal (*e.g.,* frequency) from detection of image current into a high resolution mass measurement (*e.g.,* nano LC/MS/MS). Further descriptions and details may be found, *e.g.,* in Scheltema, R.A. *et al.* (2014) *Mol. Cell Proteomics* 13:3698-3708; Perry, R.H. *et al.* (2008) *Mass. Spectrom. Rev.* 27:661-699; and Scigelova, M. *et al.* (2011) *Mol. Cell Proteomics* 10:M111.009431.

[0116] As described above, in some embodiments, the MS includes nano LC/MS/MS, *e.g.,* using an Orbitrap mass analyzer. In some embodiments, the ion source may include a stacked-ring ion guide or S-lens. As is known in the art, an S-lens may be employed to focus the ion beam using radio frequency (RF), thereby increasing transmission of ions into the instrument. This may improve sensitivity (*e.g.,* for low-intensity ions) and/or improve the scan rate. In certain embodiments, the S-lens RF level of the mass spectrometry is about 55%. In certain embodiments, the S-lens RF level of the mass spectrometry is about 20% to about 100%.

[0117] In some embodiments, masses of viral capsid proteins may be determined, *e.g.,* based on LC/MS and/or LC/MS/MS data. In some embodiments, masses of VP1, VP2 and VP3 of an AAV particle, or of fragments of VP1, VP2 and VP3 of the AAV particle, may be determined, *e.g.,* based on LC/MS and/or LC/MS/MS data. Various methods to determine protein mass and/or identity from MS data are known in the art. For example, peptide mass fingerprinting may be used to determine protein sequence based on MS data, or proteins may be identified based on MS/MS data related to one or more constituent peptides. When using tandem MS, product ion scanning may be used to analyze m/z data related to one or more peptides of a protein of interest. Software known in the art may then be used, *e.g.,* to match identified peaks to reference or known peaks, to group peaks into isotopomer envelopes, and so forth. Peptide mass values may be compared to a database of known peptide sequences. For example, Mascot may be used to match observed peptides with theoretical database peptides, *e.g.,* resulting from application of a particular digest pattern to an *in silico* protein database. Other suitable software may include without limitation Proteome Discoverer, ProteinProspector, X!Tandem, Pepfinder, Bonics, or MassLynx™ (Waters). Other software suitable for various steps of MS data analysis may be found, *e.g.,* at www.ms-utils.org/wiki/pmwiki.php/Main/SoftwareList.

[0118] In some embodiments, a determined or calculated mass of the present disclosure (*e.g.,* the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a reference, *e.g.,* a theoretical mass of a VP1, VP2, and/or VP3 of one or more AAV serotypes. A reference of the present disclosure may include a theoretical mass of a VP1, VP2, and/or VP3 of one or more of any of the AAV serotypes described herein. For example, in some embodiments, the masses of VP1, VP2, and/or VP3 are compared to the theoretical masses of one or more of an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid (*see* US Patent No. 9,169,299), an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid (*see* US Patent No. 7,588,772), an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid. In some embodiments, a determined or calculated mass of the present disclosure (*e.g.,* the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a theoretical mass of a VP1, VP2, and/or VP3 of the corresponding AAV serotype.

[0119] In some embodiments, the methods of the present disclosure may include determining the heterogeneity of an AAV particle. In some embodiments, a deviation of one or more of the masses of VP1, VP2 and/or VP3 (*e.g.,* from a reference mass, such as a theoretical, predicted, or expected mass) is indicative of the AAV capsid heterogeneity. In some embodiments, heterogeneity may include one or more of the following, without limitation: mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0120] In some embodiments, the use of LC/MS and LC/MS/MS as described herein may be combined. In some embodiments, a method of determining the serotype of an AAV particle may include subjecting a denatured AAV particle to LC/MS (*e.g.,* as described herein) and determining the masses of VP1, VP2 and VP3 of the AAV particle; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS and determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (the specific combination of masses of fragments of

VP1, VP2 and VP3 are indicative of the AAV serotype). In some embodiments, a method of determining the heterogeneity of an AAV particle may include subjecting a denatured AAV particle to LC/MS (*e.g.,* as described herein), determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS, determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype (a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity).

[0121] In some embodiments, an AAV particle of the present disclosure may be acetylated. For example, in some embodiments, the N-terminus of VP1 and/or VP3 is acetylated. As described in greater detail below, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be mutated in order to determine its effect on N-terminal (Nt-) acetylation, as well as the functional consequences of affecting Nt-acetylation on AAV particle trafficking, transduction, and/or post-translational modification (*e.g.,* glycosylation, ubiquitination, and so forth). In some embodiments, the N-terminus of an AAV capsid protein (*e.g.,* VP1 or VP3) may refer to the first amino acid after the initiating methionine, which in some cases may be removed by, *e.g.,* a Met-aminopeptidase.

[0122] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a recombinant AAV or rAAV particle) comprises an amino acid substitution at amino acid residue 2 of VP1 and/or VP3. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of N-terminal acetylation as compared to a reference (*e.g.,* the parent AAV particle before the amino acid substitution, or an AAV particle with a different amino acid residue 2 of VP1 and/or VP3). In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. For example, in certain embodiments, the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In certain embodiments, the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal

acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, an amino acid substitution (*e.g.*, an amino acid substitution at amino acid residue 2 of VP1 or VP3) that "alters" N-terminal acetylation results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (*e.g.,* bearing tyrosine mutation or heparin binding mutations). Exemplary assays for N-terminal acetylation include without limitation mass spectrometry, isotope labeling (*e.g.,* with an isotope-labeled acetyl group or precursor thereof), Western blotting with an acetylation-specific antibody, and so forth. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0123] In some embodiments, an AAV particle of the present disclosure may be deamidated. For example, in some embodiments, N57 of VP1 and/or N382, N511, and/or N715 VP3 is deamidated. As described in greater detail below, an amino acid selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 of an AAV capsid protein (*e.g.*, VP1 or VP3) may be mutated in order to determine its effect on deamidation, as well as the functional consequences of affecting deamidation on AAV particle trafficking, transduction, and/or post-translational modification (*e.g.,* glycosylation, ubiquitination, and so forth).

[0124] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a recombinant AAV or rAAV particle) comprises an amino acid substitution at one or more amino acid residues selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and G716 of VP3. In some embodiments, the amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of deamidation as compared to a reference (*e.g.,* the parent AAV particle before the amino acid substitution, or an AAV particle with a different corresponding amino acid residue 2). In some embodiments, an amino acid substitution (*e.g.*, an

amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (*e.g.,* bearing tyrosine mutation or heparin binding mutations). Exemplary assays for deamidation include without limitation mass spectrometry, HPLC (see, *e.g.*, the ISOQUANT® isoaspartate detection kit from Promega), and so forth. In some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (*e.g.*, Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, A35 of VP1 is substituted with Asn and results in a higher frequency of deamidation as compared to deamidation of VP1 of a parent particle.

[0125] As used herein "N-acetylation" refers to a process whereby an acetyl group is covalently added to the amino group of the N-terminal amino acid of a protein. Typically, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the α-amino group of the first amino acid residue of the protein.

[0126] As used here in, "deamidation" refers to a chemical reaction in which an amide functional group in the side chain of asparagine or glutamine is removed or converted to another functional group. For example, asparagine may be converted to aspartic acid or isoaspartic acid. In other examples, glutamine is converted to glutamic acid or pyroglutamic acid (5-oxoproline).

[0127] In some embodiments, the AAV particle is N-acetlyated to a higher extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than

about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more N-acetyl groups compared to a parent AAV particle.

[0128] In some embodiments, the AAV particle N-acetlyated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less N-acetyl groups compared to a parent AAV particle.

[0129] In some embodiments, the AAV particle is deamidated to a higher extent compared to a parental AAV particle. In some embodiments, the AAV particle is more than about any of 5%,

10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more than a parent AAV particle.

[0130] In some embodiments, a capsid protein of AAV is deamidated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less than a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less than a parent AAV particle.

[0131] The invention provides any combination of N-acetlylation and deamidation. For example, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and

deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, or the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein.

IV. Vectors

[0132] In certain aspects, the invention relates to viral particles, suitable for use in any of the methods described herein, which may comprise AAV vectors (*e.g.,* rAAV vectors) or vectors derived from another virus. In some embodiments, the viral particle comprises a vector encoding a heterologous nucleic acid, *e.g.,* a heterologous transgene. In some embodiments, the AAV particle comprises an AAV vector genome encoding a heterologous nucleic acid, *e.g.,* a heterologous transgene.

[0133] The present invention contemplates the use of a recombinant viral genome for introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a rAAV viral particle. The recombinant viral genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

[0134] In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. A therapeutic polypeptide may, *e.g.,* supply a polypeptide and/or enzymatic activity that is

absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (*e.g.,* one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, *e.g.,* as a dominant-negative polypeptide.

[0135] The nucleic acids of the invention may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the invention. For polypeptides secreted by the cell that receives the vector; the polypeptide can be soluble (*i.e.*, not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

[0136] The nucleic acids if the invention (e.g. the AAV vector genome) may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TM, GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1), an anti-oxidant, an anti-angiogenic polypeptide, an anti-inflammatory polypeptide, and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may

encode a functional RNA, *e.g.*, shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (*e.g.*, Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, *e.g.*, HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, *e.g.*, when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Examples of anti-oxidants include without limitation SOD1; SOD2; Catalase; Sirtuins 1, 3, 4, or 5; NRF2; PGC1a; GCL (catalytic subunit); GCL (modifier subunit); adiponectin,; glutathione peroxidase 1; and neuroglobin. Examples of anti-angiogenic polypeptides include without limitation angiostatin, endostatin, PEDF, a soluble VEGF receptor, and a soluble PDGF receptor. Examples of anti-inflammatory polypeptides include without limitation IL-10, soluble IL17R, soluble TNF-R, TNF-R-Ig, an IL-1 inhibitor, and an IL18 inhibitor. Other examples of transgenes that may be used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, *e.g.*, Costantini L C, *et al.*, *Gene Therapy* (2000) 7, 93-109).

[0137] In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

[0138] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the

expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (*e.g.,* a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (*e.g., caspase-3, Beclin1, Ask1, PAR1, HIF1α, PUMA*, and/or any of the genes described in Fukuda, A.M. and Badaut, J. (2013) *Genes (Basel)* 4:435-456), Huntington's disease (mutant *HTT*), epilepsy (*e.g., SCN1A, NMDAR, ADK,* and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; *SOD1*), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (*e.g.,* a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, *e.g.,* more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

[0139] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder)

include Gaucher disease type 2 or type 3 (acid beta-glucosidase, *GBA*), GM1 gangliosidosis (beta-galactosidase-1, *GLB1*), Hunter disease (iduronate 2-sulfatase, *IDS*), Krabbe disease (galactosylceramidase, *GALC*), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, *MAN2B1*), β mannosidosis disease (beta-mannosidase, *MANBA*), metachromatic leukodystrophy disease (pseudoarylsulfatase A, *ARSA*), mucolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, *GNPTAB*), Niemann-Pick A disease (acid sphingomyelinase, *ASM*), Niemann-Pick C disease (Niemann-Pick C protein, *NPC1*), Pompe disease (acid alpha-1,4-glucosidase, *GAA*), Sandhoff disease (hexosaminidase beta subunit, *HEXB*), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, *MPS3A*), Sanfilippo B disease (N-alpha-acetylglucosaminidase, *NAGLU*), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, *MPS3C*), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, *GNS*), Schindler disease (alpha-N-acetylgalactosaminidase, *NAGA*), Sly disease (beta-glucuronidase, *GUSB*), Tay-Sachs disease (hexosaminidase alpha subunit, *HEXA*), and Wolman disease (lysosomal acid lipase, *LIPA*).

[0140] Additional lysosomal storage diseases, as well as the defective enzyme associated with each disease, are listed in Table 1 below. In some embodiments, a disease listed in the table below is treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention that complements or otherwise compensates for the corresponding enzymatic defect.

Table 1. Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Aspartylglusoaminuria	Aspartylglucosaminidase
Fabry	Alpha-galactosidase A
Infantile Batten Disease (CNL1)	Palmitoyl protein thioesterase
Classic Late Infantile Batten Disease (CNL2)	Tripeptidyl peptidase
Juvenile Batten Disease (CNL3)	Lysosomal transmembrane protein
Batten, other forms (CNL4-CNL8)	multiple gene products
Cystinosis	Cysteine transporter
Farber	Acid ceramidase
Fucosidosis	Acid alpha-L-fucosidase

Galactosidosialidosis	Protective protein/cathepsin A
Gaucher types 1, 2, and 3	Acid beta-glucosidase
GM1 gangliosidosis	Acid beta-galactosidase
Hunter	Iduronate-2-sulfatase
Hurler-Scheie	Alpha-L-iduronidase
Krabbe	Galactocerebrosidase
alpha-mannosidosis	Acid alpha-mannosidase
beta-mannosidosis	Acid beta-mannosidase
Maroteaux-Lamy	Arylsulfatase B
Metachromatic leukodystrophy	Arylsulfatase A
Morquio A	N-acetylgalactosamine-6-sulfate
Morquio B	Acid beta-galactosidase
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase
Niemann-Pick A, B	Acid sphingomyelinase
Niemann-Pick C	NPC-1
Pompe acid	alpha-glucosidase
Sandhoff	beta-hexosaminidase B
Sanfilippo A	Heparan N-sulfatase
Sanfilippo B	alpha-N-acetylglucosaminidase
Sanfilippo C	Acetyl-CoA:alpha-glucoasaminide N-acetyltransferase
Sanfilippo D	N-acetylglucosamine-6-sulfate
Schindler disease	alpha-N-acetylgalactosaminidase
Schindler-Kanzaki	alpha-N-acetylgalactosaminidase
Sialidosis	alpha-neuramidase
Sly	beta-glucuronidase
Tay-Sachs	beta-hexosaminidase A
Wolman	Acid lipase

[0141] In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase- 1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit β-globin promoter (CAG promoter; Niwa *et al.*, *Gene*, 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EFl-alpha) promoter (Kim *et al.*, *Gene*, 1990, 91(2):217-23 and Guo *et al.*, *Gene Ther.*, 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β-actin (CBA)

promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the invention provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, *e.g.,* in U.S. PG Pub. 20140335054.

[0142] Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart *et al.*, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EFla promoter [Invitrogen].

[0143] Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No *et al., Proc. Natl. Acad. Sci.* USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen *et al., Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen *et al., Science*, 268:1766-1769 (1995), see also Harvey *et al., Curr. Opin. Chem. Biol.*, 2:512-518 (1998)), the RU486-inducible system (Wang *et al., Nat. Biotech.*, 15:239-243 (1997) and Wang *et al., Gene Ther.*, 4:432-441 (1997)) and the rapamycin-inducible system (Magari *et al., J. Clin. Invest.*, 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

[0144] In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene

should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression

[0145] In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*) are well known in the art.

[0146] In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

[0147] In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, *e.g.,* accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

V. Viral particles and methods of producing viral particles

[0148] Certain aspects of the present disclosure relate to recombinant viral particles (*e.g.,* rAAV particles).

[0149] Based on the guidance provided herein, the techniques of the present disclosure may suitably be adapted by one of skill in the art for use with a variety of different viruses.

[0150] In some embodiments, the virus is of the family *Adenoviridae*, which includes non-enveloped viruses typically known as Adenoviruses. In some embodiments, the virus is of the genus *Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus,* or *Siadenovirus*.

[0151] In some embodiments, the virus is of the family *Parvoviridae*, which includes non-enveloped viruses such as AAV and Bocaparvovirus. In some embodiments, the virus is of the subfamily *Densovirinae*. In some embodiments, the virus is of the genus *Ambidensovirus,*

Brevidensovirus, Hepandensovirus, Iteradensovirus, or *Penstyldensovirus*. In some embodiments, the virus is of the subfamily *Parvovirinae*. In some embodiments, the virus is of the genus *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* or *Tetraparvovirus*.

[0152] In some embodiments, the virus is of the family *Retroviridae*, which includes enveloped viruses including lentivirus. In some embodiments, the virus is of the subfamily *Orthoretrovirinae*. In some embodiments, the virus is of the genus *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus,* or *Lentivirus*. In some embodiments, the virus is of the subfamily *Spumaretrovirinae*. In some embodiments, the virus is of the genus *Spumavirus*.

[0153] In some embodiments, the virus is of the family *Baculoviridae*, which includes enveloped viruses including alphabaculovirus. In some embodiments, the virus is of the genus *Alphabaculovirus, Betabaculovirus, Deltabaculovirus,* or *Gammabaculovirus*.

[0154] In some embodiments, the virus is of the family *Herpesviridae*, which includes enveloped viruses such as the simplex viruses HSV-1 and HSV-2. In some embodiments, the virus is of the subfamily *Alphaherpesvirinae*. In some embodiments, the virus is of the genus *Iltovirus, Mardivirus, Simplexvirus,* or *Varicellovirus*. In some embodiments, the virus is of the subfamily *Betaherpesvirinae*. In some embodiments, the virus is of the genus *Cytomegalovirus, Muromegalovirus, Proboscivirus,* or *Roseolovirus*. In some embodiments, the virus is of the subfamily *Gammaherpesvirinae*. In some embodiments, the virus is of the genus *Lymphocryptovirus*, *Macavirus*, *Percavirus*, or *Rhadinovirus*.

[0155] In some embodiments, the virus is an AAV virus. In an AAV particle, a nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

[0156] In some embodiments, the viral particle comprises an AAV ITR sequence. For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. *See* Davidson *et al.*, *PNAS*, 2000, 97(7)3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the nucleic acid in the AAV (*e.g.,* an rAAV vector) comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0157] In some embodiments, a rAAV particle comprises an encapsulation protein selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 (*e.g.,* a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), AAV7, AAV8, AAVrh8, AAVrh8R, AAV9 (*e.g.,* a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), AAV10, AAVrh10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8

capsid, an AAV LK03 capsid, an AAV DJ capsid (*e.g.*, an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), AAV2 N587A capsid, AAV2 E548A capsid, AAV2 N708A capsid, AAV V708K capsid, goat AAV capsid, AAV1/AAV2 chimeric capsid, bovine AAV capsid, mouse AAV capsid, rAAV2/HBoV1 capsid, an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

[0158] Certain aspects of the present disclosure relate to an AAV (*e.g.*, a rAAV) capsid protein comprising an amino acid substitution at amino acid residue 2. In some embodiments, the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. As described herein, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be examined for effects on N-terminal acetylation, trafficking, transduction, and/or other post-translational modification(s) (*e.g.*, glycosylation, ubiquitination, and so forth). Any assay described herein for examining acetylation, or a functional consequence thereof related to AAV particles, may be used to assess N-terminal acetylation. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0159] Other aspects of the present disclosure relate to an AAV (*e.g.*, a rAAV) capsid protein comprising an amino acid substitution that alters deamidation. In some embodiments, an amino acid substitution (*e.g.*, an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. As described herein, a potential deamidation site of an AAV capsid protein (*e.g.*, VP1 or VP3) may be examined for effects on deamidation, trafficking, transduction, and/or other post-translational modification(s) (*e.g.*, glycosylation, ubiquitination, and so forth). Any assay

described herein for examining deamidation, or a functional consequence thereof related to AAV particles, may be used to assess deamidation.

[0160] Several potential deamidation sites are described herein. In some embodiments, an amino acid substitution that alters deamidation is selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3. For example, in some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (*e.g.*, Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

[0161] In some embodiments, the AAV capsid protein is VP1, VP2, or VP3. The AAV particle may comprise any of the exemplary AAV capsid serotypes described herein, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1. The AAV capsid protein may further comprise any of the capsid protein mutations described herein, such as tyrosine and/or heparin binding mutations.

[0162] Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described

herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (*e.g.,* using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (*e.g.,* room temperature, or 4°C, for thermal stability) or treated at a particular pH (*e.g.,* pH stability), and the like.

[0163] Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of

VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the assembly of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (*e.g.,* after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or

structural analysis to observe particle formation (*e.g.,* using electron microscopy), production of AAV capsid proteins (*e.g.,* as assayed by Western blotting), and the like.

[0164] Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the transduction of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using

various assays known in the art, including without limitation, the transduction efficiency assays described herein. In some embodiments, the invention provide methods of reducing the transduction of a rAAV particle; for example, by substituting amino acid residue 2 of VP1 and/or VP3.

[0165] Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%,

about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (*e.g.,* using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (*e.g.,* room temperature, or 4°C, for thermal stability) or treated at a particular pH (*e.g.,* pH stability), and the like.

[0166] Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about

100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (*e.g.,* after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (*e.g.,* using electron microscopy), production of AAV capsid proteins (*e.g.,* as assayed by Western blotting), and the like.

[0167] Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at

least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein.

[0168] In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome (*e.g.,* a self-complementary or self-complimenting rAAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first

nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

[0169] In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (*e.g.*, the right ITR). In some embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC GGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGGGCG – 3' (SEQ ID NO:8). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0170] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or a rAAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the rAAV particle.

[0171] In some embodiments, the AAV capsid comprises a mutation, *e.g.,* the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the rAAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, *e.g.,* Zhong L. *et al.*, (2008) *Proc Natl Acad Sci U S A* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, *et al., J. Virol.* 2004, 78(12):6381).

[0172] In some embodiments, a capsid protein comprises one or more amino acid substitutions at one or more positions that interact with a heparin sulfate proteoglycan or at one or more positions corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2. Heparan sulfate proteoglycan (HSPG) is known in the art to act as the cellular receptor for AAV2 particles (Summerford, C. and Samulski, R.J. (1998) *J. Virol.* 72(2):1438-45). Binding between an AAV2 particle and HSPG at the cell membrane serves to attach the particle to the cell. Other cell surface proteins such as fibroblast growth factor receptor and αvβ5 integrin may also facilitate cellular infection. After binding, an AAV2 particle may enter the cell through mechanisms including receptor mediated endocytosis via clathrin-coated pits. An AAV2 particle may be released from an endocytic vesicle upon endosomal acidification. This allows the AAV2 particle to travel to the perinuclear region and then the cell nucleus. AAV3 particles are also known to bind heparin (Rabinowitz, J.E., *et al.* (2002) *J. Virol.* 76(2):791-801).

[0173] The binding between AAV2 capsid proteins and HSPG is known to occur via electrostatic interactions between basic AAV2 capsid protein residues and negatively charged glycosaminoglycan residues (Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006; Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081). Specific capsid residues implicated in these interactions include R484, R487, K527, K532, R585, and R588. Mutations in these residues have been shown to reduce AAV2 binding to Hela cells and heparin itself (Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006; Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081; WO 2004/027019 A2, US Patent No. 7,629,322). Further, without wishing to be bound to theory, it is thought that amino acid substitution(s) at one or more of the residues corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2 may modulate the transduction properties of AAV capsid types that do not bind to HSPG, or may modulate the transduction properties of AAV capsid types independent from their ability to bind HSPG. In some embodiments, the one or more amino acid substitutions comprises a substitution at position R484, R487, K527, K532, R585 and/or R588 of VP1, VP2 and/or VP3, numbering based on VP1 of AAV2.

[0174] In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 25%,

about at least 50%, about at least 75%, or about at least 100%. In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions results in no detectable binding of the rAAV particle to the heparin sulfate proteoglycan compared to binding of a wild-type rAAV particle. Means to measure binding of AAV particles to HSPG are known in the art; *e.g.*, binding to a heparin sulfate chromatography media or binding to a cell known to express HSPG on its surface. For example, see Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006 and Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081. In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (*e.g.,* a cell in the eye or CNS) by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%,

about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (*e.g.,* a cell in the eye or CNS) by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). Means to measure transduction efficiency of AAV particles to a cell (*e.g.,* a cell in culture or part of a tissue) are known in the art. For example, a population of cells (*e.g.,* in culture or part of a tissue) may be infected with a concentration of rAAV particles containing a vector that, when expressed in the cells, produces an assayable reporter (*e.g.,* GFP fluorescence, sFLT production, etc.).

AAV capsid proteins

[0175] In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution at amino acid residue 2; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr.

In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid protein. Non-limiting examples of AAV capsid proteins of the invention include VP1 and/or VP3 of any of the following AAV serotypes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

Production of AAV particles

[0176] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE *et al.*, (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. *et al.*, (2002) *Human Gene Therapy* 13(16):1935-1943; Kotin, R. (2011) *Hum Mol Genet.* 20(R1): R2–R6). rAAV production cultures for the production of rAAV viral particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (*e.g.*, an AAV genome encoding GNPTAB); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent

No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (*e.g.*, *Spodoptera frugiperda* (Sf9) cells). In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0177] One method for producing rAAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (*e.g.*, using the calcium phosphate method) into a cell line (*e.g.*, HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

[0178] In some embodiments, rAAV particles may be produced by a producer cell line method (see Martin *et al.*, (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X.L. *et al.* (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (*e.g.*, a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (*e.g.*, GNPTAB). Cell lines may be screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (*e.g.*, an adenovirus or HSV) to initiate rAAV production. Virus may subsequently be harvested, adenovirus may be inactivated (*e.g.*, by heat) and/or removed, and the rAAV particles may be purified. As such, in some embodiments, the rAAV particle was produced by a producer

cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, the producer cell line method may be advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

[0179] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (*e.g.*, 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (*e.g.*, a human cell) have been identified (*see*, *e.g.*, Krysan, P.J. *et al.* (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (*e.g.*, using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, *see e.g.*, Kim, T.K. and Eberwine, J.H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

[0180] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into

a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (*e.g.*, through use of a selectable marker) may be used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids may be integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (*see, e.g.*, O'Gorman, S. *et al.* (1991) *Science* 251:1351-1355), Cre/loxP (*see, e.g.*, Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (*see, e.g.*, Groth, A.C. *et al.* (2000) *Proc. Natl. Acad. Sci.* 97:5995-6000).

[0181] In some embodiments, the producer cell line is derived from a primate cell line (*e.g.*, a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

[0182] In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For further description, *see, e.g.*, ATCC frequently asked questions document (available on the world wide web at atcc.org/Global/FAQs/9/1/Adapting%20a%20monolayer%20cell%20line%20to%20suspension-40.aspx) and references cited therein.

[0183] Suitable AAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, AAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of AAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of AAV in production cultures.

[0158] AAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, AAV production cultures include attachment- dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. AAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0159] AAV vector particles of the invention may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0160] In a further embodiment, the AAV particles are purified. The term "purified" as used herein includes a preparation of AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from. Thus, for example, isolated AAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity,

or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

[0161] In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0162] In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0163] AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify AAV particles are found, for example, in Xiao *et al.*, (1998) *Journal of Virology* 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948; and WO 2010/148143.

Pharmaceutical compositions

[0164] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a rAAV particle) is in a pharmaceutical composition. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to improve the stability and/or

improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to modulate the stability and/or the transduction efficiency of rAAV particles (*e.g.*, increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (*e.g.*, increase deamidation or decrease deamidation).

[0165] In some embodiments, the rAAV particle is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

[0166] Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable

solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

Kits and articles of manufacture

[0167] The present invention also provides kits or articles of manufacture comprising any of the rAAV particles and/or pharmaceutical compositions of the present disclosure. The kits or articles of manufacture may comprise any of the rAAV particles or rAAV particle compositions of the invention. In some embodiments the kits are used to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments the kits are used to modulate the stability and/or the transduction efficiency of rAAV particles (*e.g.*, increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (*e.g.*, increase deamidation or decrease deamidation).

[0168] In some embodiments, the kits or articles of manufacture further include instructions for administration of a composition of rAAV particles. The kits or articles of manufacture described herein may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

[0169] In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (*e.g.,* as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits

or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

EXAMPLES

[0170] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Direct LC/MS and LC/MS/MS for complete characterization of recombinant AAV viral capsid protein

[0171] Recombinant adeno-associated viruses (rAAVs) have become popular gene therapy vectors due to their nonpathogenic nature, ability to infect both dividing and non-dividing cells and long term gene expression. Currently, AAV-based gene therapies are used in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration.

[0172] AAV is a small and nonenveloped parvovirus with a single stranded DNA genome encapsulated in an icosahedra shell. Each capsid includes sixty copies of three viral capsid proteins VP1 (87kDa), VP2 (73kDa) and VP3 (62 kDa) in an approximately 1:1:10 ratio. The three viral capsid proteins are expressed from the same open reading frame by using alternative splicing and an atypical start codon and thus have overlapping sequences. VP1 has ~137 additional N-terminal amino acid residues compared to VP3 while VP2 has ~65 additional N-terminal amino acid residues compared to VP3. At least 13 AAV serotypes and ~ 150 gene sequences have been isolated from human and non-human primate tissues; AAV serotypes differ in the amino acid sequence of viral capsid proteins and their corresponding cellular receptors and co-receptors for targeting.

[0173] The AAV capsid, in addition to protecting the genome inside, plays an important role in mediating receptor binding, escape of virus from endosome, and transport of viral DNA into nucleus in the viral infection cycle, thus directly impacting viral infectivity. It has been shown that the VP1 N-terminus contains a phospholipase PLA2 domain (a.a. 52-97) which is critical in endosomal escaping of virus [1-3]. N-termini of VP1 and VP2 also contain three basic amino acid clusters as nuclear localization signals. These sequences are highly conserved among different AAV serotypes. Mutations of these amino acids have been shown to reduce or abolish infectivity completely [4]. In addition, each AAV serotype has corresponding sequence-specific receptors and co-receptors. For example, heparin sulfate proteoglycan was identified as a major receptor of AAV2 and several other co-receptors, including αVβ5 integrin, fibroblast growth factor receptor 1, and hepatocyte growth receptor have been identified [5-8]. Mutation analysis of AAV2 capsid proteins has identified a group of basic amino acids (Arginine484, 487, 585, and Lysine532) as a heparin-binding motif which contributes to the heparin and HeLa cell binding [9]. NGR domain in AAV2 was identified as an integrin α5β1 binding domain which is essential for viral cell entry [10]. In summary, viral capsid protein sequences are important in cellular targeting and trafficking in the viral infection cycle. Since different production conditions may cause different expression levels of viral capsid proteins, post-translational modifications, and truncations, the viral capsid proteins need to be characterized and monitored to ensure the product consistency in gene therapy development programs.

[0174] Traditionally, SDS-PAGE has been used to characterize the AAV viral capsid proteins, providing rough molecular weight information such as 87 kDa, 73kDa and 62 kDa. No sequence information was obtained from Edman sequencing, possibly due to the blocked N-termini of viral capsid proteins, except VP2. Although X-ray structures of multiple AAVs have been solved, only the VP3 region sequence was observed in the crystal structures. Fifteen N-terminal amino acid residues of VP3 were still missing in the X-ray structure, possibly due to its intrinsic disorder [11-13]. It is possible that the lack of information of VP1 and VP2 N-terminal regions in the atomic structure might be due to low stoichiometry of VP1 and VP2 in the capsid. In addition, N-termini of VP1 and VP2 are buried inside the capsid and are not accessible to antibodies in the native state as reported in the some literature [3, 14, 15]. Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs [16-18]. However, N-termini of VP1, VP2 and VP3 have not been

confirmed using this approach, since this method failed to obtain 100% sequence coverage of VPs due to the limited recovery of peptide from gel.

[0175] Direct analysis using MALDI-TOF MS was reported for several virus capsid proteins including tobacco mosaic virus U2 after dissociation with organic acid [19]. Direct peptide mapping after amide hydrogen exchange and mass spectrometry have been used to study the pH-induced structural changes in the capsid of brome mosaic virus (BMV) [20]. Since AAVs are nonenveloped viruses containing only capsid proteins and genome, AAVs capsids could be directly analyzed by RP-LC/MS of proteins and LC/MS/MS of peptide mapping to achieve 100% sequence coverage after capsid dissociation without SDS-PAGE separation. The DNA fragments could elute in the void volume and thus have no interference on protein/peptide detection by LC/MS. In order to investigate these methods, direct LC/MS of different types of AAVs after denaturation was used to monitor the protein sequence and post-translational modifications of AAV capsid proteins. As described herein, N-termini of VP1, VP2 and VP3 of AAVs have been confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in the different serotype of AAVs. Direct LC/MS/MS peptide mapping of AAVs has also been developed to provide sequence coverage of VP1, VP2 and VP3 and confirm the N-termini acetylation of VP1 and VP3.

Methods

Materials and Reagents

[0176] Dithiothreitol (DTT), 4-vinylpyridine, ultra-pure formic acid, acetic acid, guanidine-HCl, Tris-HCl and Tris base were purchased from Sigma Chemicals (St. Louis, MO). Amicon ultra-4 filters were purchased from Millipore (Billerica, MA). The porcine sequencing grade trypsin was purchased from Promega (Milwaukee, WI). Endoproteinase Lys-C and Asp-N were purchased from Roche (Germany). Slide-A-Lyzer cassettes with 10,000 MWCO were purchased from Pierce (Rockford, IL).

Vector production and purification

[0177] AAV vectors were produced using the transient triple transfection method as previously described (Xiao, 1998 #123). Briefly, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap and Ad helper plasmid). The

vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the CMV enhancer chicken beta actin hybrid promoter (CBA) as described (Miyazaki, 1989 #124) (Niwa, 1991 #125). The AAV rep / cap helpers contained rep sequences from AAV2 and serotype specific capsid sequences with the nomenclature, rep2 / cap2, rep2 / cap5, rep2 /cap7 etc. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). Purification of AAV was performed as described by Qu *et al* . (2007, *J. Virol. Methods* 140:183-192).

LC/MS intact protein analysis

[0178] The AAV virions were concentrated with an Amicon ultra-4 filter (10kDa MWCO) and denatured with 10% acetic acid followed by direct analysis in an Acquity UPLC −Xevo® QTOF MS instrument (Waters, Milford, MA). The separations were performed with a UPLC BEH C4 or C8 column (1.7 μm, 2.1mm i.d.) at a 0.25 ml/min flow rate. Mobile phase A was 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The final gradient was as follows: from 10% B to 20% B for 6 minutes, from 20% B to 30% B in 10 min, then from 30% to 38% B for 40 minutes. For MS the capillary voltage and sampling cone voltage were set at 3.5 kV and 45 V respectively. The mass spectra were acquired in the sensitivity mode with *m/z* range of 500-4000. Assisted calibration with sodium iodide as calibrant was performed for mass calibration. MaxEnt1 in Masslynx software was used for protein deconvolution.

Enzymatic digestions of AAV2 VPs

[0179] The concentrated AAV2 virions were denatured with 6 M Guanidine-HCl, 0.1 M Tris at pH 8.5. The proteins were reduced with 30 mM DTT at 55 °C for 1 hour in darkness and alkylated with 0.07% 4-vinylpyridine at room temperature for 2 hours. The reactions were quenched by the addition of 1M DTT. The samples were dialyzed with Slide-A-Lyzer cassettes (10,000 MWCO) against 25 mM Tris buffer at pH 8.5 for ~18 hours. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or Lys-C at 1:50 or Asp-N at 1:100 enzyme: protein ratio (wt/wt) for 18 hours at 37 °C, respectively.

LC/MS/MS peptide mapping

[0180] Nano LC/MS/MS was performed in using a NanoAcquity HPLC system (Waters, Milford, MA) in conjunction with an Orbitrap Velos mass spectrometer (Thermo-Fisher

Scientific, Waltham, MA) using home packed nanoLC column(75 μm x10mm) with Magic C18 with packing material(5 μm, Bruker, Billerica, MA) at a 300 nl/min flow rate. The mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The gradient was from 2% B to 60% B in 121min.

[0181] The source parameters for velos were as follows: source voltage: 2.5 kv, capillary temperature 275 °C; S-lens RF level: 55%. Data were acquired using the top-ten data dependent method with accurate ms at 60,000 resolution and 10 MS/MS in ion trap. Mascot was used for database searching against AAV2 viral capsid protein sequences. MS tolerance of 10 ppm and ms/ms tolerance of 0.8 Da were used for the database search.

UPLC/MS/MS peptide mapping

[0182] The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. A BEH300 C18 column (2.1 x150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe mode in the mass range of 200-2000.

Results

AAV denaturation method

[0183] AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs. Heat denaturation can lead to protein precipitation and as a result reverse phase columns are easily clogged and over pressurized. Denaturation with high salt requires an additional desalting step before LC/MS analysis. Denaturing with 10% acetic acid was used for the LC/MS intact protein analysis, as it allowed for clean mass spectrum. For peptide mapping, either 0.1% RapiGest or 6 M Guanidine HCl can be used as a denaturing reagent.

Intact protein analysis method development

[0184] Initial intact protein analysis of AAV2 was performed using an UPLC BEH C4 column at fast gradient. Under this condition, only one single peak in the total ion chromatogram was observed, with a mass corresponding to VP3 (**FIG. 1A**). Without wishing to be bound by theory, it is thought that the absence of VP1 and VP2 is possibly due to low stoichiometry of VP1 and VP2 or suppression of VP1 and VP2 signals by VP3 if all VPs co-elute. Increasing injection or

column length, using a shallower gradient, and using alternative columns have been attempted in order to detect VP1 and VP2. Higher loading (1.7µg) with a shallower gradient at 0.5% B/min resulted in a shoulder peak on the left (**FIG. 1B**). The increase in column length from 10 cm to 15 cm did not enhance the separation of the shoulder peak (**FIG. 1C**). However, the shoulder peak was further separated from the main peak using a BEH C8 column, with improved signal intensities observed (**FIG. 1D**).

[0185] As a result, the VP1 and VP2 masses were obtained in this shoulder peak at the signal intensities shown in **FIG. 2A**. The masses of VP1 and VP3 correspond to a.a. 2-735(acetylation) and a.a. 204-735(acetylation), respectively (**FIGS. 2A&2B**). No acetylation was observed in VP2 (a.a.139-735). In addition, a minor peak with a smaller mass than VP3 was observed, with a mass corresponding to amino acid sequence 212-735 with one acetylation (**FIG. 2B**). These data are consistent with DNA sequences since VP3 contains two ATG initiation codons in AAV2: ***ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC*** (SEQ ID NO:1), resulting in two possible N termini (underlined): MATGSGAPMAD (SEQ ID NO:2). The N-terminal methionine residues were not present in both VP1 and VP3 as measured by intact protein analysis. The acetylation of VP1 and VP3 is not a method-induced artifact (denaturation of AAV by 10% acetic acid) since acetylation of VP1 and VP3 is also observed in an AAV preparation using an alternative denature method without acetic acid. The intact protein data also confirmed that no glycosylation was present in the viral capsid proteins, even though several N-linked consensus sequences are present [16].

LC/MS/MS peptide mapping

[0186] To further confirm the N-termini and acetylation observed in the intact protein analysis, peptide mapping was performed using multiple enzymes and analyzed using multiple instruments. Various sample preparation methods, including denaturation methods and desalting steps, have been evaluated. The final digestion method, including denaturation with 6M guanidine HCl, reduction and alkylation with 4-vinylpyridine, and dialysis using slide-A-lyzer followed by enzymatic digestion, created clean peptide mapping with low artificial modifications during the digestion process. As low as 5 µg starting material was tested, yielding complete sequence coverage using nano LC/MS/MS and UPLC/MS/MS.

[0187] Mascot search of tryptic digests from nano LC/MS/MS alone yielded 78% sequence coverage with an ion score 13 cut off as shown in **FIG. 3**. The two large missing tryptic peptides, T27 and T38 (boxed) from nano LC/MS/MS were found in the LC/MS in Xevo TOF MS with BEH C18 column (**FIG. 3)**. In addition, most of the T27 and T38 peptide sequences were further confirmed by nano LC/MS/MS of Asp-N digests as shown in Italics in **FIG. 3**. The complete N-terminal and C-terminal peptides were covered by Lys-C digests as underlined in **FIG. 3**. Therefore, 100% sequence coverage of VP1 was achieved through multiple enzyme digestions and two LC/MS/MS methods.

[0188] LC/MS/MS confirmed the N- and C-termini of VP1, VP2 and VP3 and N-terminal acetylation of VP1 and VP3 observed in the intact protein analysis. **FIGS. 4A-4C** show the MS/MS spectra of the VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO:4) (**FIG. 4A**), VP2 N-terminal Asp-N derived peptide (APGKKRPVEHSPVEP) (SEQ ID NO:15) (**FIG. 4B**), and VP3 N-terminal Asp-N peptide A(Ac)TGSGAPM (SEQ ID NO:5) (**FIG. 4C**). MS/MS has confirmed the location of acetylation at the N-terminal alanine residues in both VP1 and VP3 peptides. The presence of unmodified y18 and y17 ions, and all detected b ions with 42Da mass shift in **FIG. 4A** indicates the 42 da-modification is located in N-terminal of VP1. Similarly, the presence of unmodified y3 to y8 ions in **FIG. 4C** confirmed the location of acetylation at the N-terminal alanine residue.

Comparison of AAV VP N-termini

[0189] In addition to AAV2, AAV1, AAV5, AAV7, AAV9 and AAV Rh10 have also been analyzed by intact protein analysis. The theoretical and predicted masses of VPs in AAVs are shown in Table 2.

Table 2: Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV1	VP1	1-736	2(ac)-736	81286	81291
	VP2	138-736	139-736	66093	66098
	VP3	203-736	204(ac)-736	59517	59520
AAV2	VP1	1-735	2(ac)-735	81856	81856
	VP2	138-735	139-735	66488	66488
	VP3	203-735	204(ac)-735	59974	59974
AAV5	VP1	1-724	2(ac)-724	80336	80336

	VP2	137-724	138-724	65283	65284
	VP3	193-724	194(ac)-724	59463	59463
AAV7	VP1	1-737	2(ac)-737	81564	81567
	VP2	138-737	139-737	66372	66374
	VP3	204-737	213(ac)-737	59101	59103
AAV9	VP1	1-736	2(ac)-736	81291	81288
	VP2	138-736	139-736	66210	66209
	VP3	203-736	204(ac)-736	59733	59733
AAVRh10	VP1	1-738	2(ac)-738	81455	81455
	VP2	138-738	139-738	66253	66252
	VP3	204-738	205(ac)-738	59634	59634

[0190] N-termini, as well as their posttranslational modifications, are highly conserved among the AAV serotypes analyzed, even though AAV5 is reported as the most diverse AAV serotype sequence, as shown in the sequence alignments in **FIG. 5**. In 11 out of 13 AAV serotypes, the N-termini of VP1 share an identical 13 amino acid residue sequence (MAADGYLPDWLED) (SEQ ID NO:6) while all 13 AAV serotypes have identical TAP… N-terminal sequences in VP2 (**FIG. 5)**. LC/MS of AAV2 indicated that T is missing in VP2 at protein level. The N-termini of VP3 are the most diverse among the three viral capsid proteins, with 8 out of 13 AAV serotypes sharing a MA… N-terminal sequence. Similar to AAV2, AAV1 and AAV Rh10 also have two ATG initiation codons with the first one as predominant N-terminal based on LC/MS intact protein analysis. Interestingly, though AAV7 has two potential initiation codons (**GTG**GCTGCAGGCGGTGGCGCACCA**ATG**GCAGACAATAAC…) (SEQ ID NO:7), the second initiation codon (ATG) was favorable based on the intact protein analysis: the VP3` with 213(ac)-737 was a predominant peak while VP3 with 203(ac)-737 was a minor peak.

Conclusions

Applications of LC/MS intact protein analysis and LC/MS/MS peptide mapping of AAV VPs in gene therapy research and development

[0191] These results demonstrate that direct LC/MS of different types of AAVs after denaturation was proved to be a simple and effective way to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. N-termini of VP1, VP2 and VP3 of AAVs were confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in different serotypes of AAVs. Direct

LC/MS/MS peptide mapping of AAVs was developed, provided 100% sequence coverage of VP1, VP2 and VP3, and confirmed the N-termini acetylation of VPs. The theoretical masses of predicted sequences of 13 AAV serotypes based on sequence alignment and intact protein analysis of several AAV serotypes are shown in Table 3.

Table 3: Predicted Sequences and Masses

	Predicted VP1 sequence	Mass(Da)	Predicted VP2 sequence	Mass(Da)	Predicted VP3 sequence	Mass(Da)
AAV1	2(ac)-736	81286	139-736	66093	204(ac)-736	59517
AAV2	2(ac)-735	81856	139-735	66488	204(ac)-735	59974
AAV3	2(ac)-736	81571	139-736	66319	204(ac)-736	59849
AAV4	2(ac)-734	80550	138-734	65626	198(ac)-734	59529
AAV5	2(ac)-724	80336	138-724	65283	194(ac)-724	59463
AAV6	2(ac)-736	81322	139-736	66096	204(ac)-736	59519
AAV7	2(ac)-737	81564	139-737	66372	213(ac)-737	59101
AAV8	2(ac)-738	81667	139-738	66519	205(ac)-738	59805
AAV9	2(ac)-736	81291	139-736	66210	204(ac)-736	59733
AAV10	2(ac)-738	81477	139-738	66271	205(ac)-738	59638
AAV11	2(ac)-733	80987	139-733	65794	198(ac)-733	59696
AAV12	2(ac)-742	82106	139-742	66905	207(ac)-742	59846
AAVRh10	2(ac)-738	81455	139-738	66253	205(ac)-738	59634

[0192] The accurate masses of VP1, VP2 and VP3 of each serotype are unique and therefore intact protein analysis can be used as an identity test to differentiate AAV capsid serotypes. Tables 4-6 show the mass differences of VPs among 13 common AAV serotypes. Shown in regular font are delta masses larger than 10, with delta masses less than 10 bolded.

Table 4: Mass Differences of VP1 Among 13 AAV Isotypes

	AAV1											
AAV2	570	AAV2										
AAV3	285	285	AAV3									
AAV4	736	1306	1021	AAV4								
AAV5	950	1520	1235	215	AAV5							
AAV6	36	534	249	772	987	AAV6						

AAV7	277	292	**8**	1013	1228	241	AAV7					
AAV8	381	189	96	1117	1332	345	104	AAV8				
AAV9	**5**	585	280	741	955	31	272	376	AAV9			
AAV10	191	379	94	927	1142	155	86	190	186	AAV10		
AAV11	299	869	584	436	651	335	577	681	304	490	AAV11	
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	AAV12
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651

Table 5: Mass Differences of VP2 among 13 AAV Isotypes

	AAV1											
AAV2	395	AAV2										
AAV3	226	169	AAV3									
AAV4	467	862	693	AAV4								
AAV5	810	1205	1036	343	AAV5							
AAV6	**2**	392	224	470	812	AAV6						
AAV7	278	116	52	746	1088	276	AAV7					
AAV8	425	31	199	893	1235	423	147	AAV8				
AAV9	117	278	109	584	927	115	161	308	AAV9			
AAV10	177	217	49	645	987	175	101	248	60	AAV10		
AAV11	299	694	525	168	511	301	578	725	416	476	AAV11	
AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	AAV12
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652

Table 6: Mass Differences of VP3 among 13 AAV Isotypes

	AAV1											
AAV2	457	AAV2										
AAV3	332	125	AAV3									
AAV4	12	445	320	AAV4								
AAV5	54	511	386	66	AAV5							
AAV6	**2**	455	330	**10**	56	AAV6						
AAV7	416	673	748	428	362	418	AAV7					
AAV8	288	169	44	276	342	286	704	AAV8				
AAV9	216	241	116	204	270	214	632	72	AAV9			
AAV10	121	336	211	109	175	119	537	167	95	AAV10		
AAV11	179	278	153	167	233	177	595	109	37	58	AAV11	
AAV12	329	128	**3**	317	383	327	745	41	113	208	150	AAV12
AAVRh10	117	340	215	105	171	115	533	171	99	**4**	62	212

[0193] No masses within 10 Da of all three VPs between two isotypes are observed. Even though both VP2 and VP3 have only a 2 Da difference between AAV1 and AAV6, the mass difference of VP1 between AAV1 and AAV6 is 36, significant enough to be distinguished by an accurate mass measurement. Therefore, intact protein measurement of VP1, VP2 and VP3 is highly specific as an identity test.

[0194] These results demonstrate that intact protein analysis and LC/MS/MS can be used to profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production. These two analyses can also be used to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications.

Example 2: The role of N terminal acetylation of AAV capsid proteins

[0195] Chemical modifications of cellular proteins are a common means of controlling their functions (Arnesen, T. (2006) *Virology* 353(2): 283–293). N-terminal acetylation (Nt-acetylation), which involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein (Brown, J.L. and Roberts, W.K. (1976) *J Biol Chem* 251: 1009–1014; Arnesen, T. *et al.* (2009) *Proc Natl Acad Sci U S A* 106: 8157–8162), is among the most abundant of protein modifications. Unlike most other protein modifications, Nt-acetylation is irreversible; it occurs mainly during the synthesis of the protein, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosomes (Gautschi, M. *et al.* (2003) *Mol Cell Biol* 23: 7403–7414; Pestana, A. and Pitot, H.C. (1975) *Biochemistry* 14: 1404–1412; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97). There are several distinct NATs in eukaryotes—NatA-NatF—each composed of one or more subunits and each acetylating a specific subgroup of N-termini depending on the amino acid sequence of the first few amino acids (Jornvall, H. (1975) *J Theor Biol* 55: 1–12; Persson, B. *et al.* (1985) *Eur J Biochem* 152: 523–527).

[0196] Experimental data indicate that proteins with acetylated N-termini are more stable *in vivo* than non-acetylated proteins; *i.e.*, Nt-acetylation protects proteins from degradation (Hershko, A. *et al.* (1984) *Proc Natl Acad Sci U S A* 81: 7021–7025). One explanation for this might be the discovery in 2004 that another N-terminal modification, ubiquitination, which involves direct attachment of the small protein ubiquitin to the N-terminal amino acid residue, promotes the subsequent degradation of the protein (Ben Saadon, R. *et al.* (2004) *J Biol Chem* 279: 41414–41421). Conversely, the Nt-acetylation signals can also be part of a quality control mechanism to degrade unfolded or misfolded proteins and to regulate *in vivo* protein stoichiometries (Hwang, C.S. *et al.* (2010) *Science* 327: 973–977).

[0197] A systematic analysis of the predicted N-terminal processing of cytosolic proteins, versus those destined to be sorted to the secretory pathway, revealed that the cytosolic proteins were profoundly biased in favor of processing, but there is an equal and opposite bias against such modification for secretory proteins (Forte, G.M.A. *et al.* (2011) *PLoS Biology*, 4 May 2011 Volume 9). Mutations in secretory signal sequences that lead to their acetylation result in mis-sorting to the cytosol in a manner that is dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that represent an extra layer of stringency in order to ensure that proteins destined to remain in the cytosol actually reside in the cytosol. The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are synthesized in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Proteins are modified by acetylation of their amino terminus at a very early stage in their synthesis. There is a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins are engineered to induce their acetylation their targeting to the ER was inhibited (Forte, G.M.A. *et al.* (2011) *PLoS Biology*, 4 May 2011 Volume 9).

[0198] The contractile proteins actin and tropomyosin have been shown to require NatB-mediated Nt-acetylation for proper function, specifically involving actin-tropomyosin binding and actomyosin regulation (Coulton, A.T. *et al.* (2010) *J Cell Sci* 123: 3235–3243; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97). Thus Nt acetylation of AAV capsid proteins may have importance in the transduction potential of rAAV vectors. If AAV vectors fail to gain entry into the nucleus, they consequently fail to transduce cells. The role of actin filaments and FKBP52 (FK506-binding protein p52) in the translocation of AAV capsids from the endosome to the nucleus is well defined (Zhao, W. *et al.* (2006) *Virology* 353(2): 283–293). Importantly, Nt-acetylation is essential for the functioning of actin filaments by modulating protein–protein interactions (Coulton, A.T. *et al.* (2010) *J Cell Sci* 123: 3235–3243; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97).

[0199] Though N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of Nt-acetylation on AAV capsid proteins is not well understood. The

predicted N-termini of VP1 and VP3 based on DNA sequencing are both methionine followed by alanine. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to Nt-acetylation of the resulting N-terminal alanine, valine, serine, threonine, and cysteine residues and that the acetylation of the N-terminus acts as a potential degradation signal [21]. Ubiquitination of viral capsid proteins was suggested as a potential signal for processing of the capsid at the time of virion disassembly [22]. The link between N-acetylation of VP1 and VP3 and viral capsid degradation and uncoating before the nuclear entry is further investigated.

[0200] To understand the functional implications of N-terminal acetylation with regard to AAV capsid proteins, site-directed mutagenesis of VP3 N-terminal initiation codons is used to generate AAV mutants.

Methods

[0201] AAV capsid proteins are generated with differing amino acids at the 2nd position to the initiating methionine (iMet X) to determine if Nt-acetylation is inhibited or reduced, and the functional consequences are then measured. The ability of the capsid proteins to be trafficked intra-cellularly and/or to acquire post translational modifications such as glycosylation is assessed, and whether this ability affects the infectivity of the assembled AAV particle is subsequently determined. In addition, the impact of acetylation on ubiquitination/degradation and targeting to the lysosome, ER, Golgi, or inner nuclear membrane is determined.

[0202] For example, to assay trafficking or targeting, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are fluorescently labeled and used to infect cells (*e.g.,* HeLa cells). These AAV particles are assayed for one or more of: time of viral particle uptake, colocalization of AAV particles with specific compartmental markers (*e.g.,* Golgi, ER, or lysosomal proteins or other markers), nuclear accumulation (*e.g.,* as assayed by colocalization with a nuclear marker or stain), and/or sensitivity of trafficking to specific inhibitors of early endosomal escape (such as bafilomycin A or ammonium chloride), as compared to fluorescently labeled wild-type AAV particles used to infect the same cell line (see, *e.g.,* Bartlett, J.S. *et al.* (2000) *J. Virol.* 74:2777-2785 for a description of such assays).

[0203] To assay infectivity, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (*e.g.,* HeLa cells), and their transduction efficiency is

compared to wild-type AAV particles (*e.g.*, having the same AAV serotype and infecting the same type of cells).

[0204] To assay glycosylation, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (*e.g.*, HeLa cells). AAV particles from infected cells are subjected to one or more assays including without limitation chemical detection of glycosylation (*e.g.*, applying a commercially available digoxigenin (DIG) glycan detection and/or fluorescent glycoprotein detection kit on denatured and electrophoretically separated capsid proteins) and mass spectrometry (*e.g.*, FT-ICR MS), as compared to wild-type AAV particles used to infect the same cell line (see, *e.g.*, Murray, S. *et al.* (2006) *J. Virol.* 80:6171-6176 for a description of such assays).

[0205] To assay ubiquitination, AAV particles with capsid proteins having a mutated 2nd position are used to infect cells (*e.g.*, HeLa cells). AAV particles are immunoprecipitated from infected cells with an anti-capsid antibody, then subjected to Western blotting with an anti-ubiquitin antibody and compared to wild-type AAV particles used to infect cells in the same manner. Mutant AAV particles may also be used in *in vitro* ubiquitination assays, as compared to wild-type AAV particles (see, *e.g.*, Yan, Z. *et al.* (2002) *J. Virol.* 76:2043-2053).

Example 3: The role of deamidation of AAV2 capsid proteins

[0206] Sequence analysis of the AAV2 capsid protein revealed potential deamidation sites, as underlined in the following amino acid sequence:

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYK***YLGPFNGLDKGEPVNEAD AAALEHDKAYDRQLDSGDNPYLKYNHADA***EFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTA PGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGA PMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEY QLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVP FHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSK TSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMIT DEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFH PSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTS NYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO:3).

[0207] In particular, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site), as bolded and italicized in the above sequence. The following experiments were aimed at exploring whether deamidation at N57 can lead to reduced potency and/or truncation of AAV2, as well as whether different AAV production methods may have different effects on deamidation. For example, the producer cell line method (see Martin *et al.*, (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X.L. *et al.* (1999) *Gene Ther.* 6:293-299) may induce a higher level of deamidation at N57, as compared to the triple transfection method. According to the crystal structure of AAV2, N57 is not shown; however, N382 and N511 are partially exposed, and N715 is fully exposed.

Methods

Enzymatic digestions of AAV1 and AAV2 VPs

[0208] 10 µg of each AAV1-EGFP or AAV2-EGFP material (generated from triple transfection as well as producer cell line process) were concentrated using Amicon filters (10 kDa MWCO), denatured with 6 M Guanidine-HCl, 50 mM Tris at pH 8.5. The proteins were reduced with 5 mM DTT at 60 °C for 30 minutes in darkness, alkylated with 15 mM iodoacetamide at room temperature for 30 minutes, and then buffer exchanged into 25 mM Tris pH 7.1 for digestion using Bio-Spin® 6 Tris micro-columns. After buffer exchange, the samples were split into two aliquots. Each aliquot was digested with trypsin at 1:25 or Asp-N at 1:50 enzyme: protein ratio (wt/wt) for 2 hours at 37 °C, respectively.

UPLC/MS/MS peptide mapping

[0209] The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. BEH300 C18 column (2.1 × 150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe resolution mode in the mass range of 50-2000.

Determination of deamidation levels in AAV VPs

[0210] The extracted ion chromatograms (XIC) of peptides containing NG sites (T9, T49, and T67 in AA1 and AAV2 VP) and their corresponding deamidated species were used for calculation of deamidation levels.

[0211] In order to compare AAV vectors produced by the triple transfection (TTx) and producer cell line (PCL) methods, AAV1 or AAV2 tagged with EGFP was produced using the TTx or PCL method. Truncated VP1 (tVP1) was found to be present in AAV2-EGFP produced by PCL, but not in the AAV2-EGFP produced by TTx. AAV1-EGFP was not found to have tVP1, regardless of the production method. The *in vitro* potency of AAV2 produced by the PCL method was also found to be reduced, as compared to AAV2 produced by TTx. Mutant N57K and N57Q AAV2 particles were also found to have reduced potency and disrupted Ca++ binding.

[0212] The following table provides the tryptic peptides that were analyzed to examine each potential deamidation site, as well as the corresponding residue.

Table 7: Tryptic peptides containing NG sites

Peptide (NG sequence underlined)	**Residue**
YLGPF**NG**LDK (SEQ ID NO: 9)	N57
EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMV PQYGYLTLN**NG**SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10)	N382
YNL**NG**R (AAV1) (SEQ ID NO: 11)	N511
YHL**NG**R (AAV2) (SEQ ID NO: 12)	N511
SANVDFTVDN**NG**LYTEPR (AAV1) (SEQ ID NO: 13)	N715
SVNVDFTVDT**NG**VYSEPR (AAV2) (SEQ ID NO: 14)	N715

[0213] As shown in Table 7, the T9 peptide YLGPF**NG**LDK (SEQ ID NO: 9) was used to monitor N57, the T38 peptide EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTL N**NG**SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10) was used to monitor N382, the T49 peptides YNL**NG**R (SEQ ID NO: 11) and YHL**NG**R (SEQ ID NO: 12) were used to monitor N511 in AAV1 or AAV2 (respectively), and the T67 peptides SANVDFTVDN**NG**LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT**NG**VYSEPR (SEQ ID NO: 14) were used to monitor N715 in AAV1 or AAV2 (respectively).

[0214] LC/MS/MS analysis was used to compare the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The results from the T9 peptide are shown in **FIGS. 6A & 6B**. The results from the T49 peptide are shown in **FIGS. 7A & 7B**. The results from the T67 peptide are shown in **FIGS. 8A & 8B**. These results are summarized in Table 8. The T38 peptide was not detected due to its size.

Table 8: Summary of LC/MS/MS results

		% Deamidation		
		N57	**N511**	**N715**
AAV1	**TTx**	7.9	30.9	18.1
	PCL	11.3	27.4	18.7
AAV2	**TTx**	6.7	39.6	27.4
	PCL	18.4	42.3	28.0

[0215] In particular, AAV2 produced by PCL showed nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx. These results suggest that deamidation decreases AAV potency, as the *in vitro* potency of AAV2 produced by PCL is reduced.

Conclusions

[0216] Taken together, Examples 1-3 demonstrate methods for analyzing intact proteins of viral particles (*e.g.*, AAV capsid proteins) using LC/MS. Molecular weights were measured accurately, and these techniques may be also used to assess N-termini and/or modifications of viral capsid proteins. Moreover, these methods are adaptable as capsid serotype identity assays useful in gene therapy, *e.g.*, as an analytical platform. These results further establish a correlation between capsid protein structure (*e.g.*, truncations, deamidation, etc.) and potency, suggesting that point mutations at key sites may be used to engineer more effective vectors.

Example 4: Elucidating the role of N terminal acetylation of AAV capsid proteins

[0217] As discussed above, the N-termini of AAV capsid proteins are highly conserved across serotypes (**FIG. 5**). The techniques described in Example 1 allow for interrogation of VP expression and posttranslational modifications. The role and biological significance of N-terminal acetylation of AAV capsid proteins was next examined.

Results

[0218] To elucidate the potential role of deacetylation of AAV capsid proteins, AAV5 deacetylation variants were tested. An AAV5 particle expressing eGFP under the CBA promoter (*AAV5-CBA-Egfp*) was compared to AAV5 variants with the amino acid adjacent to the initiating methionine (iMET) mutated for VP1 and VP3 (*deAC-AAV5-CBA-eGFPs*). Three amino acids predicted to have a low likelihood of acetylation by NatA, NatC, or NatD were chosen for generating variants: Gly, Leu, and Pro, as illustrated in Table 9 below.

Table 9: N-terminal acetylation frequency

N-term aa	**Transferase**	**NT-AC FREQUENCY**
MET-ALA *Normally found in VP1 & VP3*	NatA	High
MET-SER *Normally found in VP1 & VP3 for AAV5*	NatA	High
AAV variants		
MET-GLY	NatA	Low
MET-LEU	NatC	Low
MET-PRO	NatD/other	Low

[0219] The following AAV5 deacetylated (deAC) mutants were generated:

S2G*VP1* – Ser changed to Gly at position 2 in AAV5VP1
S2L*VP1* – Ser changed to Leu at position 2 in AAV5VP1
S2P*VP1* – Ser changed to Pro at position 2 in AAV5VP1
S2G*VP3* – Ser changed to Gly at position 2 in AAV5VP3
S2L*VP3* – Ser changed to Leu at position 2 in AAV5VP3
S2P*VP3* – Ser changed to Pro at position 2 in AAV5VP3
S2P*VP1/VP3* – Ser changed to Pro at position 2 in both AAV5 VP1 and VP3
S2G*VP1/VP3* – Ser changed to Gly at position 2 in both AAV5 VP1 and VP3
S2L*VP1/VP3* – Ser changed to Leu at position 2 in both AAV5 VP1 and VP3

[0220] These variants were generated using the TTX method as described above. All AAV5 variants showed good productivity, with yields greater than 10^{13} total VG. All AAV5 variants also showed the expected VP1:VP2:VP3 protein ratio by SYPRO protein gel analysis (**FIG. 9**).

Next, LC/MS was used to confirm that all AAV5 variants had decreased acetylation, as shown in Table 10.

Table 10: LC/MS analysis of AAV5 variant acetylation

	mutants	VP1 Theo.	VP1 Exp.	Δmass (VP1)	VP2 Theo.	VP2 Exp.	Δmass (VP2)	VP3 Theo.	VP3 Exp.	Δmass (VP3)	note
1	*deAC-AAV5 (S2GVP1)/ CBA-eGFP*	80234	nd		65283	65293	10	59463	59472	9	VP1 not detectable
2	*deAC-AAV5 (S2LVP1)/ CBA-eGFP*	80346	80501	181	65283	65292	9	59463	59471	8	VP1 incorrect
3	*deAC-AAV5 (S2GVP3)/ CBA-eGFP*	80234	nd		65253	65261	8	59391	59398	7	confirmed
4	*deAC-AAV5 (S2LVP3)/ CBA-eGFP*	80336	80363	27	65309	65309	0	59447	59620	173	VP3 incorrect
5	*deAC-AAV5 (S2PVP1VP3)/ CBA-eGFP*	80314	80324	10	65293	65300	7	59431	59438	7	confirmed
6	*deAC-AAV5 (S2GVP1VP3)/ CBA-eGFP*	80234	80243	9	65253	65261	8	59391	59398	7	confirmed
7	*deAC-AAV5 (S2PVP3)/ CBA-eGFP*	80336	80346	10	65293	65292	1	59431	59430	1	confirmed
8	*deAC-AAV5 (S2PVP1)/ CBA-eGFP*	80314	80313	1	65283	65291	8	59463	59470	7	confirmed
9	*deAC-AAV5 (S2L VP1VP3)/ CBA-eGFP*	80346	nd		65309	65318	9	59447	59629	182	VP3 incorrect

nd=not determined

[0221] These LC/MS analyses confirmed that AAV5 variants were deacetylated. The variants S2L*VP1*, S2L*VP3*, and S2L*VP1/VP3* all showed increased mass (increased from 173 to 182) in VP1 and VP3 proteins, suggesting that changing the second N-terminal amino acid to a leucine in VP1 or VP3 alters the protein, resulting in an increase in mass.

[0222] Next, AAV5 variants were assayed in an *in vitro* transduction assay using eGFP as a reporter gene (**FIG. 10**). The assay was designed to evaluate transduction by AAV5 deacetylated mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV5 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV5 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (**FIG. 10**). Vector genomes were quantified by TaqMan analysis.

[0223] **FIG. 11** shows that, based on vector genome analyses, AAV5 deacetylated mutant vectors infected all three test cell lines at similar, but reduced, levels as compared to the parental unmodified AAV5 particles. **FIG. 12** shows that AAV5 deacetylated mutant vectors all resulted in reduced eGFP expression in all three cell lines, as compared to transduction with parental unmodified AAV5.

Conclusions

[0224] As predicted, no acetylation was observed in N-terminal Ser to Pro/Leu/Gly mutant variants when examined by LC/MS. AAV5 deAC variants showed robust vector production, and AAV5 deAC variants infected cells at levels comparable to parental AAV5. However, functional protein levels in cells infected with deAC variants were greatly reduced when compared to the parental AAV5. These data suggest that tropism is minimally affected by a lack of N-terminal deacetylation in VP1/VP3, but downstream processing (*e.g.*, trafficking and/or degradation) is significantly affected. Since the variants tested demonstrated reduced *in vitro* activity, one of skill in the art may appreciate that variants characterized by reduced or eliminated acetylation could be employed, *inter alia*, when decreased levels of transduction are desirable.

Example 5: Assessment of deamidation of AAV capsid proteins

[0225] Examples 1 and 3 demonstrate techniques that allow the interrogation of post-translational modifications of AAV capsid proteins and explore the role of deamidation of the AAV2 capsid. The following Example tested whether deamidation reduces potency and/or induces truncation of capsid proteins, and whether different manufacturing processes can induce different levels of deamidation.

Methods

AAV particles were generated and deamidation status assayed as described in Example 3.

Results

[0226] As described in Example 3, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site) in VP1 of the AAV2 capsid. The N57/G58 motif is conserved across AAV serotypes (**FIG. 13**). Example 3 showed that AAV2 produced by PCL exhibited nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx (see **FIGS. 6A & 6B** and Table 8).

[0227] In examining VP1, VP2, and VP3 production by protein gels, a truncated VP1 protein (tVP1) was detected only in AAV2 capsid proteins produced by the PCL method (**FIG. 14**).

[0228] A series of AAV2 deamidation mutants was generated next. These mutants targeted the Gly residue in the canonical NG sequence. Mutations targeting the A35 residue (see **FIG. 13**), the N-terminal amino acid for tVP1 were also generated, as shown in Table 11. The pAF277 and pAF279 mutants bearing multiple mutations did not package.

Table 11: Deamidation mutants

Name	**mutation**	**avg drp/cell**
pAF274	G58K	4.54E+03
pAF275	G58D	5.00E+03
pAF276	G58Q	5.41E+03
pAF277	*G58,383,512,716K*	*1.2*
pAF278	A35N	6.89E+03
pAF279	*A35N, G58,383,512,765K*	*2.2*
293	-	0.9

PIM45	Control	6.28E+03

K = positive charge (basic)
D = negative charge (acidic)
Q = polar

[0229] Deamidation of variants were next analyzed by LC/MS as described in Example 3 above. The AAV2*A35N* and AAV2*G58D* variants had altered deamidation as compared to the parental AAV2 (**FIG. 15**). In particular, the AAV2*A35N* mutant had increased deamidation (17.8%) as compared to parental AAV2 (5.7%). The AAV2*G58D* variant had reduced deamidation (1.1%) as compared to parental AAV2. SYPRO protein gel analysis demonstrated that the AAV2 deamidation mutants exhibited the correct VP1:VP2:VP2 ratio (**FIG. 16**).

[0230] Next, AAV2 deamidation variants were assayed in an *in vitro* transduction assay using eGFP as a reporter gene (**FIG. 17**). The assay was designed to evaluate transduction by AAV2 deamidation mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV2 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/µg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/µg protein) represents the efficiency at which the AAV2 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (**FIG. 17**). Vector genomes were quantified by TaqMan analysis.

[0231] Vector genome analysis indicated that AAV2 deamidation mutant variants infected all cell lines tested at levels comparable to that of parental AAV2 vectors (**FIG. 18**). Importantly, the AAV2*A35N* variant was found to be more potent than the parental AAV2 vector for transduction in all three cell lines (**FIG. 19**). The AAV2*G58D* variant was found to be more potent than the parental AAV2 vector in HuH7 cells (**FIG. 19**).

Conclusions

[0232] In summary, AAV2 deamidation mutant vectors infect cells at levels comparable to the parent AAV2 particles (*e.g.*, comparable vg/µg cellular protein). However, based on analysis of eGFP levels in transduced cells, the AAV2*A35N* variant had higher potency than the parental AAV2 in all cell lines tested, and the AAV2*G58D* variant had higher potency than the parental

AAV2 in HuH7 cells (a liver-derived cell line). These results suggest that the A35N mutation may be effective in increasing vector potency for transducing many cell types, and that the G58D mutation may also be effective in increasing potency in certain cell types, *e.g.*, liver cells.

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SEQUENCES

All polypeptide sequences are presented N-terminal to C-terminal unless otherwise noted.

All nucleic sequences are presented 5' to 3' unless otherwise noted.

Nucleotide sequence of potential AAV2 VP3 initiation codons (ATG codons underlined)

ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC (SEQ ID NO:1)

Polypeptide sequence corresponding to potential AAV2 VP3 initiation codons (methionines underlined)

MATGSGAPMAD (SEQ ID NO:2)

AAV2 VP1 polypeptide sequence

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLDKGEPVNEADAAALEHDK
AYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPD
SSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCD
STWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRP
KRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQ
AVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQF
SQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGV
LIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGP
IWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKR
WNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO:3)

VP1 N-terminal tryptic peptide (N-terminal alanine is acetylated)

AADGYLPDWLEDTLSEGIR (SEQ ID NO:4)

VP3 N-terminal Asp-N peptide (N-terminal alanine is acetylated)

ATGSGAPM (SEQ ID NO:5)

Common VP1 N-terminal sequence

MAADGYLPDWLED (SEQ ID NO:6)

Nucleotide sequence of potential AAV7 VP3 initiation codons (start codons underlined)

GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC (SEQ ID NO:7)

Nucleotide sequence of mutated ITR

CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGGGC
G (SEQ ID NO:8)

METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed August 14, 2017, which claims the priority benefit of U.S. Provisional Application No. 62/375,314, filed August 15, 2016, the disclosure of each of which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

[0002] The content of the following submission on ASCII text file is incorporated herein by reference in its entirety: a computer readable form (CRF) of the Sequence Listing (file name: 159792014100SEQLIST.TXT, date recorded: February 13, 2019, size: 52 KB).

FIELD OF THE INVENTION

[0003] The present invention relates to methods for serotyping and/or determining the heterogeneity of a viral particle (*e.g.*, an adeno-associated virus (AAV) particle) using mass determination, *e.g.,* by employing liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). In some aspects, the present invention relates to methods to improve the stability of AAV particles.

BACKGROUND OF THE INVENTION

[0004] Complete characterization of the viral capsid proteins of viral vectors (*e.g.*, AAV vectors), including their sequence and post-translation modifications, is desired in gene therapy research and development since viral capsid proteins (VPs) are critical for viral infectivity.

[0005] Viral vector products such as recombinant Adeno-Associated Virus (rAAV) products are typically identified using molecular tools targeting the nucleic acid transgene. These methods may include polymerase chain reaction (PCR) targeting transgene-specific sequences

and Restriction Fragment Length Polymorphism (RFLP) techniques. As rAAV technologies evolve, many facilities are beginning to investigate multiple AAV capsid serotypes encoding their therapeutic transgene in an effort to improve targeted tissue tropism.

[0006] Traditional molecular identification methods identify products containing unique transgenes but are unable to discern those that have differing AAV capsid serotypes. Currently, most AAV serotype identity tests are based on SDS-PAGE banding patterns, an antibody-based ELISA, or a Western blot assay. However, the banding patterns and antibodies are not specific enough to differentiate different AAV serotypes. Gel-LC/MS/MS has been reported as a capsid serotype identification method. However, this method involves multiple steps including SDS-PAGE, in-gel digestion, and LC/MS/MS and thus requires multiple days for the analysis while providing limited sequence coverage. Methods for identifying vectors such as rAAV vectors are of interest to gene therapy vectors (see, *e.g.,* U.S. PG Pub. No. US20110275529). Thus, it would be useful to have improved methods of characterizing viral particles.

[0007] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0008] Using rAAV as an example, described herein is the use of LC/MS as an analytical tool to specifically identify different viral capsid serotypes (*e.g.,* rAAV capsid serotypes). As part of viral characterization, LC/MS can be used to augment the molecular identification methods. This analytical combination can satisfy regulatory requirements by discerning both the identity of the product's therapeutic transgene and the identity of the capsid serotype. This method can be used *e.g.,* as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in recombinant AAV gene therapy development. It can also be used to confirm VP sequences in capsid engineering research. In addition, this technique can be used to study the impact of post translation modifications, such as N terminal acetylation of viral capsid proteins, on transfection potency and intracellular protein trafficking.

[0009] The methods described herein can also be used to design AAV particles for greater stability and/or improved transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is acetylated to a higher extent compared to a wild type AAV capsid. In some embodiments, the methods can be used to design AAV particles with reduced transduction efficiency; for example by altering the amino acid residue at position 2 of VP1 and/or VP3 of the AAV capsid such that the amino acid at position 2 is deacetylated to a higher or lower extent compared to a wild type AAV capsid.

[0010] In some aspects, the invention provides a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle; wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype. In some embodiments, the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

[0011] In some aspects, the invention provides a method of determining the heterogeneity of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of one or more capsid proteins of the viral particle, and d) comparing the masses of step c) with the theoretical masses of the one or more capsid proteins of the virus serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0012] In some embodiments of the above aspects, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the viral particle comprises a viral vector encoding a heterologous transgene.

[0013] In some aspects, the invention provide a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to

reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of the one or more capsid proteins of the viral particle; wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype. In some embodiments, the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

[0014] In some aspects, the invention provides a method of determining the heterogeneity of a serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to reduction and/or alkylation, c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle, d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of the one or more capsid proteins of the viral particle, and f) comparing the masses of step e) with the theoretical masses of fragments of the one or more capsid proteins of the viral serotype; wherein a deviation of one or more of the masses of the one or more capsid proteins are indicative of the viral capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

[0015] As shown herein the methods can be performed in the absence of a gel separation step (*e.g.,* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)).

[0016] In some embodiments of the above aspects and embodiments, the viral particle comprises a viral vector encoding a heterologous transgene. In some embodiments, the viral particle belongs to a viral family selected from the group consisting of *Adenoviridae*, *Parvoviridae*, *Retroviridae*, *Baculoviridae*, and *Herpesviridae*. In some embodiments, the viral particle belongs to a viral genus selected from the group consisting of *Atadenovirus,*

Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus, Ambidensovirus, Brevidensovirus, Hepandensovirus, Iteradensovirus, Penstyldensovirus, Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus, Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus, Lentivirus, Spumavirus, Alphabaculovirus, Betabaculovirus, Deltabaculovirus, Gammabaculovirus, Iltovirus, Mardivirus, Simplexvirus, Varicellovirus, Cytomegalovirus, Muromegalovirus, Proboscivirus, Roseolovirus, Lymphocryptovirus, *Macavirus*, *Percavirus*, and *Rhadinovirus*.

[0017] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

[0018] In some aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and d) comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0019] In some embodiments of the above aspects and embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent. In some embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C4 or C8

reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, the proportion of mobile phase B in the chromatography increases in a stepwise manner. In some embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In some embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In some embodiments, the liquid chromatography is ultra-performance liquid chromatography (UPLC).

[0020] In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

[0021] In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an

AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

[0022] In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

[0023] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype. In some embodiments, the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

[0024] In some aspects, the invention provides a method of determining the heterogeneity of a serotype of an AAV particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or

more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity. In some embodiments, the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). In some embodiments, the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine. In some embodiments, the digestion is an enzymatic digestion or a chemical digestion. In some embodiments, the enzymatic digestion is an endopeptidase digestion. In some embodiments, the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion. In some embodiments, the chemical digestion is cyanogen bromide digestion or an acid digestion. In some embodiments, the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

[0025] In some embodiments of the above aspects and embodiments, the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography. In some embodiments, the liquid chromatography is reverse phase liquid chromatography. In some embodiments, the reverse phase chromatography is a C18 reverse chromatography. In some embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In some embodiments, the mobile phase A comprises about 0.1% formic acid. In some embodiments, the chromatography comprises a mobile phase B comprising formic acid in acetonitrile. In some embodiments, the mobile phase B comprises about 0.1% formic acid. In some embodiments, the proportion of mobile phase B in the chromatography increases over time. In some embodiments, mobile phase B increases from about 2% to about 60%. In some embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC).

[0026] In some embodiments of the above aspects and embodiments, the mass spectrometry comprises a capillary voltage of about 3.5 kV. In some embodiments, the mass spectrometry comprises a sampling cone voltage of about 45 V. In some embodiments, the mass spectrometry comprises assisted calibration. In some embodiments, sodium iodide is used as a calibrant.

[0027] In some embodiments of the above aspects and embodiments, the N-terminus of VP1 and/or VP3 is acetylated. In some embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In some embodiments, the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1). In some embodiments, the AAV capsid comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the masses of VP1, VP2, and VP3 are compared to the theoretical masses of one or more of AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1).

[0028] In some embodiments of the above aspects and embodiments, the viral particle comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene.

[0029] In some embodiments, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some

embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; wherein the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In some embodiments, the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; wherein the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu.

[0030] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO,, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0031] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked

by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0032] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0033] In some aspects, the invention provides a pharmaceutical composition comprising the rAAV particle as described herein. In some aspects, the invention provides a kit comprising the rAAV particle or the pharmaceutical composition as described herein. In some aspects, the invention provides an article of manufacture comprising the rAAV particle or the pharmaceutical composition as described herein.

[0034] In some aspects, the invention provides as AAV capsid protein comprising an amino acid substitution at amino acid residue 2 of a parent AAV capsid protein; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of

N-terminal acetylation. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 of the AAV capsid protein is substituted with Ser, Asp or Glu. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0035] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

[0036] In some aspects, the invention provides a method of improving stability of a rAAV particle comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein the substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 or a parental VP1 and/or VP3; wherein substituting amino acid residue 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3. In some embodiments, the substituted amino acid results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 is substituted. In some embodiments, the amino acid substitution at amino acid residue 2 of VP3 is substituted. In some embodiments, amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, amino acid residue 2 is substituted with Ser, Asp or Glu. In some aspects, the invention provides a method of reducing the transduction of

rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

[0037] In some embodiments of the above aspects and embodiments, the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0038] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0039] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the

host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0040] In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3 of a parent particle, residue numbering based on VP1 of AAV2; wherein the one or more amino acid substitutions alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution is at amino acid residue A35, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprises a substitution with Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3; and results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions comprise a N57K or a N57Q substitution and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitution comprise a substitution with Asp at A35 of VP1and results in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle. In some embodiments, the one or more amino acid

substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the G58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

[0041] In some aspects, the invention provides pharmaceutical compositions comprising AAV particles comprising one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides kits comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides articles of manufacture comprising AAV particles or compositions comprising AAV particles wherein the AAV particles comprise one or more amino acid substitutions at amino acid residue A35, N57, G58, N382, G383, N511, G512, N715, or G716 of VP1 or VP3, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution of a parent AAV capsid protein; wherein the amino acid substitution alters deamidation of the capsid compared to the parent AAV capsid protein.

[0042] In some aspects, the invention provides a method of improving the stability of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to

deamidation of VP1 and/or VP3 of the parent AAV particle. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In some embodiments, the one or more amino acid substitutions is at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle**.** In some embodiments, the parental Ala residue at position 35 of VP1 is substituted with Asn. In some embodiments, the parental Gly residue at position 58 of VP1 is substituted with Asp. In some embodiments, the rAAV particle is an AAV1 particle or an AAV2 particle.

[0043] In some embodiments, the invention provides a method of improving the stability, aseembly and/or transduction efficiency of a rAAV particle comprising substituting one or more amino acid residues, wherein the one or more amino acid residues is A35, N57, G58, N382, G383, N511, G512, N715, or G716, residue numbering based on VP1 of AAV2; wherein the amino acid substitution alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle as described above, wherein the AAV particle comprises an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, an AAV DJ8 capsid, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation. In some embodiments, the rAAV particle comprises a rAAV vector. In some embodiments, the rAAV vector comprises one or more AAV ITRs. In some embodiments, the rAAV vector comprises an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR. In some embodiments, the AAV capsid and the AAV ITRs are derived from the same serotype. In some embodiments, the AAV capsid and the AAV ITRs are derived from

different serotypes. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0044] In some embodiments of the above aspects and embodiments, the rAAV vector is a self-complementary vector. In some embodiments, the rAAV vector comprises first nucleic acid sequence encoding the transgene and a second nucleic acid sequence encoding a complement of the transgene, wherein the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length. In some embodiments, the first nucleic acid sequence and the second nucleic acid sequence are linked by a mutated AAV ITR, wherein the mutated AAV ITR comprises a deletion of the D region and comprises a mutation of the terminal resolution sequence.

[0045] In some embodiments of the above aspects and embodiments, the rAAV particle is produced by transfecting a host cell with nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus. In some embodiments, the rAAV particle is produced by an AAV producer cell comprising nucleic acid encoding the rAAV vector and nucleic acid encoding AAV rep and cap functions, and providing nucleic acid encoding AAV helper functions. In some embodiments, the AAV producer cell comprises nucleic acid encoding AAV helper functions. In some embodiments, the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions. In some embodiments, the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus. In some embodiments, the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution modulated deamidation of the capsid compared to the parent AAV particle.

BRIEF DESCRIPTION OF THE DRAWINGS

[0046] **FIGS. 1A-D** provide total ion Chromatograms of LC/MS of AAV2 VPs. **FIG. 1A:** 10cm long BEH C4 column with 1.7%/min gradient, **FIG. 1B:** 10cm long BEH C4 column

with 0.5%/min gradient; **FIG. 1C:** 15cm long BEH C4 column with 0.5%/min gradient, **FIG. 1D:** 15cm long BEH C8 column with 0.5%/min gradient.

[0047] **FIGS. 2A&B** provide deconvoluted mass spectra from **FIG. 1D** peak 1 (**FIG. 2A)** and **FIG. 1D** peak 2 (**FIG. 2B)**.

[0048] **FIG. 3** provides the sequence coverage of AAV2 VP1 (SEQ ID NO:3): green, tryptic peptides, blue, Lys-C peptides, pink, Asp-N peptides.

[0049] **FIGS. 4A-4C** provide MS/MS spectra of AAV2 VP N-terminal peptides. **FIG. 4A:** VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO: 4), **FIG. 4B** VP2 N-terminal Asp-N peptide APGKKRPVEHSPVEP (SEQ ID NO: 15). **FIG. 4C**: VP-3 N-terminal Asp-N derived peptide A(Ac)TGSGAPM (SEQ ID NO: 5).

[0050] **FIG. 5** provides the sequence alignment of 13 AAV serotypes black letter/white background: non-similar; blue letter/blue background: conservative; black letter/green background: block of similar; red letter/yellow background: identical; green letter/white background: weakly similar. AAVRh10 (SEQ ID NO: 17); AAV10 (SEQ ID NO: 18); AAV8 (SEQ ID NO: 19); AAV7 (SEQ ID NO: 20); AAV1 (SEQ ID NO: 21); AAV6 (SEQ ID NO: 22); AAV2 (SEQ ID NO: 23); AAV3 (SEQ ID NO: 24); AAV11 (SEQ ID NO: 25); AAV12 (SEQ ID NO: 26); AAV4 (SEQ ID NO: 27); AAV5 (SEQ ID NO: 28); AAV9 (SEQ ID NO: 29); Consensus (SEQ ID NO: 30).

[0051] **FIGS. 6A & 6B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T9 peptide YLGPF**NG**LDK (SEQ ID NO: 9) was used to monitor potential deamidation site N57 in both AAV1 and AAV2.

[0052] **FIGS. 7A & 7B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T49 peptides YNL**NG**R (SEQ ID NO: 11) and YHL**NG**R (SEQ ID NO: 12) were used to monitor potential deamidation site N511 in AAV1 and AAV2, respectively.

[0053] **FIGS. 8A & 8B** show the results of LC/MS/MS analysis comparing the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The T67 peptides SANVDFTVDN**NG**LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT**NG**VYSEPR

(SEQ ID NO: 14) were used to monitor potential deamidation site N715 in AAV1 and AAV2, respectively.

[0054] **FIG. 9** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV5 deacetylated mutant variants.

[0055] **FIG. 10** illustrates an *in vitro* transduction assay for testing transduction efficiency of AAV5 deacetylated variants.

[0056] **FIG. 11** shows the efficiency of cell entry by the indicated AAV5 deacetylated variants or parental unmodified AAV5, as measured by vector genome copies/µg protein. Three cell lines were used: 293, HeLa, and HuH7.

[0057] **FIG. 12** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV5 deacetylated variants as compared to transduction with parental unmodified AAV5. Three cell lines were used: 293, HeLa, and HuH7.

[0058] **FIG. 13** provides the sequence alignment of 13 AAV serotypes, highlighting the conserved N57G58 deamidation site and the A35 residue in AAV2. AAVRh10 (SEQ ID NO: 31); AAV10 (SEQ ID NO: 31); AAV8 (SEQ ID NO: 32); AAV7 (SEQ ID NO: 33); AAV1 (SEQ ID NO: 31); AAV6 (SEQ ID NO: 31); AAV2 (SEQ ID NO: 34); AAV3 (SEQ ID NO: 35); AAV11 (SEQ ID NO: 31); AAV12 (SEQ ID NO: 36); AAV4 (SEQ ID NO: 37); AAV5 (SEQ ID NO: 38); AAV9 (SEQ ID NO: 39); Consensus (SEQ ID NO: 40).

[0059] **FIG. 14** shows a protein gel of VP1, VP2, and VP3 capsid proteins from AAV1 or AAV2 particles produced by the PCL or TTx method. *highlights the truncated VP1 (tVP1) protein.

[0060] **FIG. 15** shows the results of LC/MS analysis of deamidation of the indicated AAV2 mutants, as compared to control AAV2 capsids.

[0061] **FIG. 16** shows the results of SYPRO protein gel analysis of production and VP1:VP2:VP3 ratio of AAV2 deamidation mutant variants.

[0062] **FIG. 17** illustrates an *in vitro* transduction assay for testing transduction efficiency of AAV2 deamidation variants.

[0063] **FIG. 18** shows the efficiency of cell entry by the indicated AAV2 deamidation variants or parental unmodified AAV2, as measured by vector genome copies/µg protein. Three cell lines were used: 293, HeLa, and HuH7.

[0064] **FIG. 19** shows eGFP expression (as measured by ELISA) by cells transduced with the indicated AAV2 deamidation variants as compared to transduction with parental unmodified AAV2. Three cell lines were used: 293, HeLa, and HuH7.

DETAILED DESCRIPTION

[0065] In some aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle(s) comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype.

[0066] In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle comprising: a) denaturing the AAV particle, b) injecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing the masses of step c) with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

[0067] In other aspects, the invention provides a method to determine the serotype of an adeno-associated virus (AAV) particle comprising a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle; wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype.

[0068] In other aspects, the invention provides a method of determining the heterogeneity of an AAV particle of a serotype comprising: a) denaturing the AAV particle, b) subjecting the denatured AAV particle to reduction and/or alkylation, c) injecting the denatured AAV particle

to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle, d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and f) comparing the masses of step e) with the theoretical masses of fragments of VP1, VP2 and VP3 of the AAV serotype; wherein a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity.

[0069] In some aspects, the invention provides a recombinant AAV (rAAV) particle comprising an amino acid substitution at amino acid residue 2 of VP1 and/or VP3; wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle.

[0070] In some aspects, the invention provides a method of improving the assembly of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetlylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3. In some aspects, the invention provides a method of improving the transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3; wherein the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3.

I. General Techniques

[0071] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual (*Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998);

Introduction to Cell and Tissue Culture (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. Definitions

[0072] A "vector," as used herein, refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0073] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate ($P\text{-}NH_2$) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0074] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0075] A "recombinant viral vector" refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of viral origin). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, *e.g.,* two, inverted terminal repeat sequences (ITRs).

[0076] A "recombinant AAV vector (rAAV vector)" refers to a polynucleotide vector comprising one or more heterologous sequences (*i.e.*, nucleic acid sequence not of AAV origin) that are flanked by at least one, *e.g.,* two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (*i.e.* AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (*e.g.*, in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a "pro-vector" which can be "rescued" by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a "recombinant adeno-associated viral particle (rAAV particle)".

[0077] An "rAAV virus" or "rAAV viral particle" refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

[0078] A "parent AAV particle" and "parent AAV capsid protein" as used herein in the context of comparing N-acetylation and/or deamidation refers to an AAV particle or capsid protein into which amino acid modifications are introduced to modulate N-acetlylation and/or deamidation (*e.g.,* an AAV particle/capsid protein that is the same as or similar to the AAV particle/capsid of the subject invention but does not comprise the mutations that modulate/alter N-aceytlation and/or deamidation as described herein). In some embodiments, the parent AAV particle is a recombinant AAV particle comprising a recombinant AAV genome. In some embodiments, the parent AAV capsid particle or parent AAV capsid protein comprises amino acid substitutions that affect other aspects of the AAV particle. For example, the parent AAV particle may comprise amino acid substitutions that affect the binding of AAV to its receptor, such as affecting binding of AAV2 to heparin sulfate proteoglycan (e.g. an AAV2 HBKO particle). An AAV2 HBKO particle can be mutated to introduce amino acid substitutions that modulate N-acetylation and/or deamidation. Such a mutated AAV particle may then be compared to the parent AAV2 HBKO particle in aspects of the invention as described herein. A parent AAV capsid protein may include a parent VP1 capsid protein, a parent VP2 capsid protein, or a VP3 capsid protein.

[0079] As used herein, the term "modulate" or "alter" in reference to a parent molecule means to change a feature of the parent molecule. For example, an AAV particle with altered N-acetlylation may show increased or decreased N-acetylation compared to the parent AAV particle and an AAV particle with altered deamidation may show increased or decreased deamidation compared to the parent AAV particle.

[0080] "Heterologous" means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (*e.g.*, a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0081] The term "transgene" refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In aspects, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0082] The terms "genome particles (gp)," "genome equivalents," or "genome copies" as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark *et al.* (1999) *Hum. Gene Ther.*, 10:1031-1039; Veldwijk *et al.* (2002) *Mol. Ther.*, 6:272-278.

[0083] The terms "infection unit (iu)," "infectious particle," or "replication unit," as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin *et al.* (1988) *J. Virol.*, 62:1963-1973.

[0084] The term "transducing unit (tu)" as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for example, in Xiao *et al.* (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher *et al.* (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0085] An "inverted terminal repeat" or "ITR" sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0086] An "AAV inverted terminal repeat (ITR)" sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also

contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0087] A "terminal resolution sequence" or "trs" is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0088] "AAV helper functions" refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0089] A "helper virus" for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0090] "Percent (%) sequence identity" with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various

ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, those described in Current Protocols in Molecular Biology (Ausubel *et al.*, eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y, where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z, where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

[0091] An "isolated" molecule (*e.g.*, nucleic acid or protein) or cell means it has been identified and separated and/or recovered from a component of its natural environment.

[0092] "Mass spectrometry" refers to the analytical chemistry technique of identifying an amount and/or type of a compound (*e.g.,* a polypeptide) by measuring the mass-to-charge ratio and abundance of gas-phase ions. The term "mass spectrometry" may be used interchangeably herein.

[0093] "Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, *e.g.,* of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

[0094] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter *per se*. For example, description referring to "about X" includes description of "X."

[0095] As used herein, the singular form of the articles "a," "an," and "the" includes plural references unless indicated otherwise.

[0096] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and/or "consisting essentially of" aspects and embodiments.

III. Methods

[0097] Certain aspects of the present disclosure relate to methods of determining the serotype of a viral particle. Other aspects of the present disclosure relate to methods of determining the heterogeneity of a viral particle. As described below, the accurate masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. These methods are based in part on the discovery described herein that direct LC/MS of different types of AAVs after denaturation may be used to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. Further, acetylations of N-termini of VP1 and VP3 may also be identified and/or monitored in different AAV serotypes. Based on these AAV results and the guidance provided herein, it is contemplated that such methods may readily be applied to profile a variety of viruses, *e.g.*, the viral families, subfamilies, and genera of the present disclosure. The methods of the present disclosure may find use, *e.g.,* in profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP

production, to confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications, and/or to monitor or detect heterogeneity of a viral particle or preparation.

[0098] In some embodiments, the methods include denaturing a viral particle. In some embodiments, a viral particle such as an AAV particle may be denatured using detergent, heat, high salt, or buffering with a low or high pH. In certain embodiments, an AAV particle may be denatured using acetic acid or guanidine hydrochloride. The skilled artisan will recognize that a variety of methods useful for promoting and/or monitoring protein denaturation are available in the art and may suitably select a denaturation method compatible with liquid chromatography/mass spectrometry. For example, if heat denaturation is used, care may be applied to avoid protein precipitation and reverse phase column clogging. Similarly, high salt denaturation may be coupled with a desalting step prior to LC/MS or LC/MS/MS. In other embodiments, high pH denaturation, low pH denaturation, or denaturation using organic solvents is used.

[0099] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to liquid chromatography/mass spectrometry (LC/MS). As is known in the art, LC/MS utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions. Such mass spectral data may be used to determine, *e.g.,* molecular weight or structure, identification of particles by mass, quantity, purity, and so forth. These data may represent properties of the detected ions such as signal strength (*e.g.,* abundance) over time (*e.g.,* retention time), or relative abundance over mass-to-charge ratio.

[0100] In some embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC; the term "ultra high performance liquid chromatography" or UHPLC may be used interchangeably herein). UPLC is known in the art as an LC technique that relies upon a column with reduced particle size (*e.g.,* less than 2μm) and increased flow velocity to improve chromatographic resolution, efficiency, peak capacity, and sensitivity (see, *e.g.,* Plumb, R. *et al.* (2004) *Rapid Commun. Mass Spectrom.* 18:2331-2337). In some embodiments, UPLC refers to the use of a column with a particle size less than 2μm in liquid chromatography. In some embodiments, UPLC refers to the use of a high linear solvent

velocity (*e.g.,* as observed when operating at 6000 psi or higher) in liquid chromatography. Exemplary UPLC machines are commercially available (*e.g.,* the ACQUITY UPLC® from Waters; Milford, MA).

[0101] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) may refer to electrospray ionization mass spectrometry (ESI-MS). ESI-MS is known in the art as a technique that uses electrical energy to analyze ions derived from a solution using mass spectrometry (see, *e.g.,* Yamashita, M. and Fenn, J.B. (1984) *J. Phys. Chem.* 88:4451-4459). Ionic species (or neutral species that are ionized in solution or in gaseous phase) are transferred from a solution to a gaseous phase by dispersal in an aerosol of charged droplets, followed by solvent evaporation that reduces the size of the charged droplets and sample ion ejection from the charge droplets as the solution is passed through a small capillary with a voltage relative to ground (*e.g.,* the wall of the surrounding chamber). In some embodiments, the capillary voltage is from about 2 kV to about 10 kV, or about 2.5 kV to about 6.0 kV. In certain embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) uses a capillary voltage of about 3.5 kV. In some embodiments the capillary voltage ranges from about 1 kV to about 10 kV. In other embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) may refer to matrix-assisted laser desorption/ionization (MALDI).

[0102] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) uses a sampling cone and/or skimmer, through which ions may pass before entering the analyzer. In some embodiments, *e.g.,* when applying voltage to the capillary as described above, the sample cone is held at a lower voltage than the capillary voltage. In certain embodiments, liquid chromatography (*e.g.,* used in LC/MS as described herein) uses a sampling cone voltage of about 45 V. In some embodiments the sampling cone voltage ranges from about 0 V to about 200 V.

[0103] In some embodiments, mass spectrometry (*e.g.,* used in LC/MS as described herein) uses assisted calibration. Calibration, when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (*e.g.,* a standard) for the purpose of calibrating the instrument with respect to mass detection (*e.g.,* m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak and/or position of a known standard (*e.g.,* a calibrant) to a specific mass-to-charge (m/z) ratio. Once calibrated,

the user may then perform mass spectrometry on a sample having one or more unknown compounds, or compounds present at an unknown concentration, within a certain degree of accuracy or error, and/or a desired level of reproducibility, *e.g.,* as compared to a previous or known experimental condition. Various calibrants are known in the art, including without limitation sodium iodide, sodium cesium iodide, polyethylene glycol, and perfluorotributylamine. In certain embodiments, sodium iodide is used as a calibrant. In some embodiments the calibrants are Glu-1-fibrinopeptide B and leucine encephalin peptide to lock mass during LC/MS operation.

[0104] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure, or subjecting digested fragments of a denatured viral particle of the present disclosure, to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS). As is known in the art, LC/MS/MS (the term "liquid chromatography-tandem mass spectrometry" may be used interchangeably herein) utilizes liquid chromatography for physical separation of ions and mass spectrometry for generation of mass spectral data from the ions, where the mass spectrometry uses multiple stages of mass (*e.g.,* m/z) separation, typically separated by a fragmentation step. For example, ions of interest within a range of m/z may be separated out in a first round of MS, fragmented, and then further separated based on individual m/z in a second round of MS. Ion fragmentation may include without limitation a technique such as collision-induced dissociation (CID), higher energy collision dissociation (HCD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD).

[0105] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to reduction and/or alkylation. Means to reduce the viral particle include but are not limited to treatment with dithiothreitol, β-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP). Means to alkylate the viral particle include but are not limited to treating the AAV particle with iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

[0106] In some embodiments, the methods include subjecting a denatured viral particle of the present disclosure to digestion, *e.g.,* to generate fragments of VP1, VP2 and/or VP3 of an AAV particle. For example, a denatured AAV particle may be subjected to digestion to generate peptide fragments that may be analyzed, *e.g.,* using LC for separation and MS/MS for analysis

(see below for greater description). In some embodiments, the digestion is an enzymatic digestion. In some embodiments, the digestion uses chemical digestion such as CNBr treatment of instrument fragmentation (e.g., top down). In some embodiments, the digestion uses chemical digestion such as acid digestion.

[0107] In some embodiments, the enzymatic digestion is an endopeptidase digestion. An endopeptidase may include any peptidase that catalyzes the proteolysis of peptide bonds of non-terminal amino acids of a polypeptide. Known endopeptidases may include, without limitation, trypsin, chymotrypsin, AspN, Glu-C, LysC, pepsin, thermolysin, glutamyl endopeptidase, elastase, and neprilysin. In certain embodiments, the enzymatic digestion is a trypsin digestion or a LysC digestion.

[0108] In some embodiments, the liquid chromatography (*e.g.,* used in LC/MS or LC/MS/MS as described herein) is reverse phase liquid chromatography (the terms "reversed phase liquid chromatography" or RPLC may be used interchangeably herein with reverse phase liquid chromatography). As is known in the art, reverse phase liquid chromatography may refer to a chromatographic separation using a hydrophobic stationary phase (*e.g.,* a support or substrate such as a column) to adsorb hydrophobic molecules in a polar mobile phase. By decreasing the polarity of the mobile phase (*e.g.,* by adding an organic solvent), one may achieve gradient separation of molecules by hydrophobicity, since more hydrophobic molecules will stay on the column in higher concentrations of organic solvent due to stronger hydrophobic interactions with the column. In some embodiment, separation is by capillary electrophoresis (CE), size exclusion chromatography (SEC), ion exchange chromatography (IEC) such as cation exchange chromatography, hydrophobic interaction chromatography (HIC), hydrophilic interaction liquid chromatography (HILIC), but not limited to on-line LC/MS such as offline separation before MS; e.g., tips, columns; plates or cartridges.

[0109] Generally, a stationary phase suitable for reverse phase liquid chromatography (*e.g.,* a hydrophobic moiety) may be coupled to a support including without limitation a column or resin packed with particles or beads (*e.g.,* porous silica particles or polystyrene). A variety of hydrophobic stationary phases are known in the art, including without limitation hydrophobic alkyl chains, octyl or octadecyl silyl moieties, cyano moieties, and amino moieties. In some

embodiments, the stationary phase may include a hydrophobic alkyl chain of a particular length, such as C4, C8, or C18. In certain embodiments, the reverse phase chromatography is a C4 or C8 reverse chromatography (*e.g.,* reverse phase chromatography utilizing a C4 or C8 stationary phase). One of skill in the art may suitably select a stationary phase based on the molecule of interest (*e.g.,* a denatured AAV particle or fragment thereof).

[0110] A variety of mobile phases suitable for reverse phase liquid chromatography are known in the art. As described above, a reverse phase liquid chromatography mobile phase may include a mixture of organic (*e.g.,* hydrophobic) and aqueous (*e.g.,* polar) solvents. Increasing the proportion of organic solvent increases its power to elute hydrophobic compounds from the stationary phase. Compound retention and/or selectivity may be altered, *e.g.,* by changing the type or exposure of the stationary phase, adding polar reagents such as end capping reagents, altering the temperature, and/or altering mobile phase characteristics such as the proportion of organic solvent, pH, buffers, and the type of organic solvent used. In some embodiments, the polar component of the mobile phase may include without limitation water or an aqueous buffer. In some embodiments, the polar component of the mobile phase may include without limitation acetonitrile, methanol, ethanol, isopropyl alcohol, tetrahydrofuran (THF), and formic acid.

[0111] In some embodiments, two or more mobile phases may be used (*e.g.,* mobile phase A, mobile phase B, etc.) in a gradient or proportion of interest. In certain embodiments, the chromatography uses a mobile phase A comprising formic acid in water. In certain embodiments, the mobile phase A comprises about 0.1% formic acid. In certain embodiments, the mobile phase A comprises about 0.1% to about 5% formic acid. In certain embodiments, the chromatography uses a mobile phase B comprising formic acid in acetonitrile. In certain embodiments, the mobile phase B comprises about 0.1% formic acid.

[0112] In some embodiments, the proportion of mobile phase B in the chromatography increases over time. For example, the proportion of mobile phase B in the chromatography may be increased in a stepwise manner. In certain embodiments, mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%. In other embodiments, mobile phase B increases from about 2% to about 60%. In other embodiments, mobile phase B increases from about 2% to about 100% from about 1 min to about 200 min In

some embodiments, the remainder of the mobile phase is a second mobile phase of the present disclosure, *e.g.,* mobile phase A. In certain embodiments, mobile phase B increases from about 10% to about 20% in about 6 minutes, from about 20% to about 30% in about 10 minutes, and from about 30% to about 38% in about 40 minutes. In other embodiments, mobile phase B increases from about 2% to about 60% in about 121 minutes. One of skill in the art may suitably adjust the mobile phase of interest and the gradient timing used based on the desired chromatographic separation and/or analyte of interest.

[0113] In some embodiments, the liquid chromatography is high-performance liquid chromatography (HPLC). HPLC is known in the art as a form of liquid chromatography in which a liquid solvent containing a sample is pressurized as it passes through a column containing solid phase. While traditional or low pressure LC may use gravity to pass a mobile phase through the solid phase, HPLC uses pumps to apply a pressure to the mobile phase and typically uses a solid phase with smaller particles to increase resolution. In some embodiments, the HPLC uses a pressure of between about 50 bar and about 350 bar. In some embodiments, reversed phase HPLC may be used to concentrate and/or desalt proteins (*e.g.,* AAV capsid proteins) for MS analysis.

[0114] In some embodiments, one or more parameters including without limitation source voltage, capillary temperature, ESI voltage (if using ESI-MS), CID energy, and the number of MS/MS events may be adjusted, *e.g.,* in LC/MS/MS as used herein, based on the findings described herein. In some embodiments, mass spectrometry (*e.g.,* used in LC/MS/MS as described herein) uses a source voltage (*e.g.*, capillary voltage) of about 2.5 kV. In some embodiments, mass spectrometry (*e.g.,* used in LC/MS/MS as described herein) uses a capillary temperature of about 275°C. In some embodiments, the capillary temperature ranges from about 20°C to about 400°C.

[0115] A variety of mass analyzers suitable for LC/MS and/or LC/MS/MS are known in the art, including without limitation time-of-flight (TOF) analyzers, quadrupole mass filters, quadrupole TOF (QTOF), and ion traps (*e.g.,* a Fourier transform-based mass spectrometer or an Orbitrap). In Orbitrap, a barrel-like outer electrode at ground potential and a spindle-like central electrode are used to trap ions in trajectories rotating elliptically around the central electrode with

oscillations along the central axis, confined by the balance of centrifugal and electrostatic forces. The use of such instruments employs a Fourier transform operation to convert a time domain signal (*e.g.,* frequency) from detection of image current into a high resolution mass measurement (*e.g.,* nano LC/MS/MS). Further descriptions and details may be found, *e.g.,* in Scheltema, R.A. *et al.* (2014) *Mol. Cell Proteomics* 13:3698-3708; Perry, R.H. *et al.* (2008) *Mass. Spectrom. Rev.* 27:661-699; and Scigelova, M. *et al.* (2011) *Mol. Cell Proteomics* 10:M111.009431.

[0116] As described above, in some embodiments, the MS includes nano LC/MS/MS, *e.g.,* using an Orbitrap mass analyzer. In some embodiments, the ion source may include a stacked-ring ion guide or S-lens. As is known in the art, an S-lens may be employed to focus the ion beam using radio frequency (RF), thereby increasing transmission of ions into the instrument. This may improve sensitivity (*e.g.,* for low-intensity ions) and/or improve the scan rate. In certain embodiments, the S-lens RF level of the mass spectrometry is about 55%. In certain embodiments, the S-lens RF level of the mass spectrometry is about 20% to about 100%.

[0117] In some embodiments, masses of viral capsid proteins may be determined, *e.g.,* based on LC/MS and/or LC/MS/MS data. In some embodiments, masses of VP1, VP2 and VP3 of an AAV particle, or of fragments of VP1, VP2 and VP3 of the AAV particle, may be determined, *e.g.,* based on LC/MS and/or LC/MS/MS data. Various methods to determine protein mass and/or identity from MS data are known in the art. For example, peptide mass fingerprinting may be used to determine protein sequence based on MS data, or proteins may be identified based on MS/MS data related to one or more constituent peptides. When using tandem MS, product ion scanning may be used to analyze m/z data related to one or more peptides of a protein of interest. Software known in the art may then be used, *e.g.,* to match identified peaks to reference or known peaks, to group peaks into isotopomer envelopes, and so forth. Peptide mass values may be compared to a database of known peptide sequences. For example, Mascot may be used to match observed peptides with theoretical database peptides, *e.g.,* resulting from application of a particular digest pattern to an *in silico* protein database. Other suitable software may include without limitation Proteome Discoverer, ProteinProspector, X!Tandem, Pepfinder, Bonics, or MassLynx™ (Waters). Other software suitable for various steps of MS data analysis may be found, *e.g.,* at www.ms-utils.org/wiki/pmwiki.php/Main/SoftwareList.

[0118] In some embodiments, a determined or calculated mass of the present disclosure (*e.g.,* the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a reference, *e.g.,* a theoretical mass of a VP1, VP2, and/or VP3 of one or more AAV serotypes. A reference of the present disclosure may include a theoretical mass of a VP1, VP2, and/or VP3 of one or more of any of the AAV serotypes described herein. For example, in some embodiments, the masses of VP1, VP2, and/or VP3 are compared to the theoretical masses of one or more of an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid (*see* US Patent No. 9,169,299), an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid (*see* US Patent No. 7,588,772), an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, or a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid. In some embodiments, a determined or calculated mass of the present disclosure (*e.g.,* the determined or calculated mass of VP1, VP2 and/or VP3 of the AAV particle) may be compared with a theoretical mass of a VP1, VP2, and/or VP3 of the corresponding AAV serotype.

[0119] In some embodiments, the methods of the present disclosure may include determining the heterogeneity of an AAV particle. In some embodiments, a deviation of one or more of the masses of VP1, VP2 and/or VP3 (*e.g.,* from a reference mass, such as a theoretical, predicted, or expected mass) is indicative of the AAV capsid heterogeneity. In some embodiments, heterogeneity may include one or more of the following, without limitation: mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

[0120] In some embodiments, the use of LC/MS and LC/MS/MS as described herein may be combined. In some embodiments, a method of determining the serotype of an AAV particle may include subjecting a denatured AAV particle to LC/MS (*e.g.,* as described herein) and determining the masses of VP1, VP2 and VP3 of the AAV particle; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS and determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (the specific combination of masses of fragments of

VP1, VP2 and VP3 are indicative of the AAV serotype). In some embodiments, a method of determining the heterogeneity of an AAV particle may include subjecting a denatured AAV particle to LC/MS (*e.g.,* as described herein), determining the masses of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype; as well as subjecting fragments of VP1, VP2 and/or VP3 to LC/MS/MS, determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle, and comparing these masses with the theoretical masses of VP1, VP2 and VP3 of the AAV serotype (a deviation of one or more of the masses of VP1, VP2 or VP3 are indicative of the AAV capsid heterogeneity).

[0121] In some embodiments, an AAV particle of the present disclosure may be acetylated. For example, in some embodiments, the N-terminus of VP1 and/or VP3 is acetylated. As described in greater detail below, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be mutated in order to determine its effect on N-terminal (Nt-) acetylation, as well as the functional consequences of affecting Nt-acetylation on AAV particle trafficking, transduction, and/or post-translational modification (*e.g.,* glycosylation, ubiquitination, and so forth). In some embodiments, the N-terminus of an AAV capsid protein (*e.g.,* VP1 or VP3) may refer to the first amino acid after the initiating methionine, which in some cases may be removed by, *e.g.,* a Met-aminopeptidase.

[0122] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a recombinant AAV or rAAV particle) comprises an amino acid substitution at amino acid residue 2 of VP1 and/or VP3. In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of N-terminal acetylation as compared to a reference (*e.g.,* the parent AAV particle before the amino acid substitution, or an AAV particle with a different amino acid residue 2 of VP1 and/or VP3). In some embodiments, the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. For example, in certain embodiments, the amino acid substitution at amino acid residue 2 of VP1 alters N-terminal acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP1 of the parent AAV particle. In certain embodiments, the amino acid substitution at amino acid residue 2 of VP3 alters N-terminal

acetylation as compared to N-terminal acetylation at amino acid residue 2 of VP3 of the parent AAV particle. In some embodiments, an amino acid substitution (*e.g.*, an amino acid substitution at amino acid residue 2 of VP1 or VP3) that "alters" N-terminal acetylation results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (*e.g.,* bearing tyrosine mutation or heparin binding mutations). Exemplary assays for N-terminal acetylation include without limitation mass spectrometry, isotope labeling (*e.g.,* with an isotope-labeled acetyl group or precursor thereof), Western blotting with an acetylation-specific antibody, and so forth. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0123] In some embodiments, an AAV particle of the present disclosure may be deamidated. For example, in some embodiments, N57 of VP1 and/or N382, N511, and/or N715 VP3 is deamidated. As described in greater detail below, an amino acid selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 of an AAV capsid protein (*e.g.*, VP1 or VP3) may be mutated in order to determine its effect on deamidation, as well as the functional consequences of affecting deamidation on AAV particle trafficking, transduction, and/or post-translational modification (*e.g.,* glycosylation, ubiquitination, and so forth).

[0124] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a recombinant AAV or rAAV particle) comprises an amino acid substitution at one or more amino acid residues selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and G716 of VP3. In some embodiments, the amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 leads to a VP1 and/or VP3 with a different frequency or proportion of deamidation as compared to a reference (*e.g.,* the parent AAV particle before the amino acid substitution, or an AAV particle with a different corresponding amino acid residue 2). In some embodiments, an amino acid substitution (*e.g.*, an

amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. The VP1 and/or VP3 may belong to any of the exemplary AAV serotypes described herein, including variants or hybrids thereof (*e.g.,* bearing tyrosine mutation or heparin binding mutations). Exemplary assays for deamidation include without limitation mass spectrometry, HPLC (see, *e.g.*, the ISOQUANT® isoaspartate detection kit from Promega), and so forth. In some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (*e.g.*, Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, A35 of VP1 is substituted with Asn and results in a higher frequency of deamidation as compared to deamidation of VP1 of a parent particle.

[0125] As used herein "N-acetylation" refers to a process whereby an acetyl group is covalently added to the amino group of the N-terminal amino acid of a protein. Typically, N-terminal acetyltransferases (NATs) transfer an acetyl group from acetyl-coenzyme A (Ac-CoA) to the α-amino group of the first amino acid residue of the protein.

[0126] As used here in, "deamidation" refers to a chemical reaction in which an amide functional group in the side chain of asparagine or glutamine is removed or converted to another functional group. For example, asparagine may be converted to aspartic acid or isoaspartic acid. In other examples, glutamine is converted to glutamic acid or pyroglutamic acid (5-oxoproline).

[0127] In some embodiments, the AAV particle is N-acetlyated to a higher extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than

about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold more N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more N-acetyl groups compared to a parent AAV particle.

[0128] In some embodiments, the AAV particle N-acetlyated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle comprises more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less N-acetyl groups compared to a parent AAV particle. In some embodiments, the AAV particle comprises between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less N-acetyl groups compared to a parent AAV particle.

[0129] In some embodiments, the AAV particle is deamidated to a higher extent compared to a parental AAV particle. In some embodiments, the AAV particle is more than about any of 5%,

10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% more deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% more than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold more than a parent AAV particle.

[0130] In some embodiments, a capsid protein of AAV is deamidated to a lower extent compared to a parental AAV capsid protein. In some embodiments, the AAV particle is more than about any of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% less deamidated compared to a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 5%-10%, 10%-15%, 15%-20%, 20%-25%, 25%-30%, 30%-35%, 35%-40%, 40%-55%, 45%-50%, 50%-55%, 55%-60%, 60%-65%, 65%-70%, 70%-75%, 75%-80%, 80%-85%, 85%-90%, 90%-95%, 95%-100%, 5-25%, 25-50%, 50-75%, 75%-100%, 5-50% or 50%-100% less than a parent AAV particle. In some embodiments, the AAV particle is deamidated more than about any of 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 500-fold, or 1000-fold less than a parent AAV particle. In some embodiments, the AAV particle is deamidated between about any of 2-fold to 3-fold, 3-fold to 4-fold, 4-fold to 5-fold, 5-fold to 10-fold, 10-fold to 25-fold, 25-fold to 50-fold, 50-fold to 100-fold, 100-fold to 500-fold, 500-fold to 1000-fold, 2-fold to 10-fold, 10-fold to 100-fold, or 100-fold to 1000-fold less than a parent AAV particle.

[0131] The invention provides any combination of N-acetlylation and deamidation. For example, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and

deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a higher extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to the same extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a higher extent than a parent AAV capsid protein, the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to the same extent than a parent AAV capsid protein, or the AAV capsid protein may be N-acetylated to a lower extent than a parent AAV capsid protein and deamidated to a lower extent than a parent AAV capsid protein.

IV. Vectors

[0132] In certain aspects, the invention relates to viral particles, suitable for use in any of the methods described herein, which may comprise AAV vectors (*e.g.,* rAAV vectors) or vectors derived from another virus. In some embodiments, the viral particle comprises a vector encoding a heterologous nucleic acid, *e.g.,* a heterologous transgene. In some embodiments, the AAV particle comprises an AAV vector genome encoding a heterologous nucleic acid, *e.g.,* a heterologous transgene.

[0133] The present invention contemplates the use of a recombinant viral genome for introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a rAAV viral particle. The recombinant viral genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

[0134] In some embodiments, the heterologous nucleic acid encodes a therapeutic polypeptide. A therapeutic polypeptide may, *e.g.,* supply a polypeptide and/or enzymatic activity that is

absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (*e.g.,* one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, *e.g.,* as a dominant-negative polypeptide.

[0135] The nucleic acids of the invention may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the invention. For polypeptides secreted by the cell that receives the vector; the polypeptide can be soluble (*i.e.*, not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

[0136] The nucleic acids if the invention (e.g. the AAV vector genome) may comprise as a transgene, a nucleic acid encoding a protein or functional RNA that modulates or treats a CNS-associated disorder. The following is a non-limiting list of genes associated with CNS-associated disorders: neuronal apoptosis inhibitory protein (NAIP), nerve growth factor (NGF), glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), ciliary neurotrophic factor (CNTF), tyrosine hydroxylase (TM, GTP-cyclohydrolase (GTPCH), aspartoacylase (ASPA), superoxide dismutase (SOD1), an anti-oxidant, an anti-angiogenic polypeptide, an anti-inflammatory polypeptide, and amino acid decorboxylase (AADC). For example, a useful transgene in the treatment of Parkinson's disease encodes TH, which is a rate limiting enzyme in the synthesis of dopamine. A transgene encoding GTPCII, which generates the TII cofactor tetrahydrobiopterin, may also be used in the treatment of Parkinson's disease. A transgene encoding GDNF or BDNF, or AADC, which facilitates conversion of L-Dopa to DA, may also be used for the treatment of Parkinson's disease. For the treatment of ALS, a useful transgene may encode: GDNF, BDNF or CNTF. Also for the treatment of ALS, a useful transgene may

encode a functional RNA, *e.g.*, shRNA, miRNA, that inhibits the expression of SOD1. For the treatment of ischemia a useful transgene may encode NAIP or NGF. A transgene encoding Beta-glucuronidase (GUS) may be useful for the treatment of certain lysosomal storage diseases (*e.g.*, Mucopolysacharidosis type VII (MPS VII)). A transgene encoding a prodrug activation gene, *e.g.*, HSV-Thymidine kinase which converts ganciclovir to a toxic nucleotide which disrupts DNA synthesis and leads to cell death, may be useful for treating certain cancers, *e.g.*, when administered in combination with the prodrug. A transgene encoding an endogenous opioid, such a β-endorphin may be useful for treating pain. Examples of anti-oxidants include without limitation SOD1; SOD2; Catalase; Sirtuins 1, 3, 4, or 5; NRF2; PGC1a; GCL (catalytic subunit); GCL (modifier subunit); adiponectin,; glutathione peroxidase 1; and neuroglobin. Examples of anti-angiogenic polypeptides include without limitation angiostatin, endostatin, PEDF, a soluble VEGF receptor, and a soluble PDGF receptor. Examples of anti-inflammatory polypeptides include without limitation IL-10, soluble IL17R, soluble TNF-R, TNF-R-Ig, an IL-1 inhibitor, and an IL18 inhibitor. Other examples of transgenes that may be used in the rAAV vectors of the invention will be apparent to the skilled artisan (See, *e.g.*, Costantini L C, *et al.*, *Gene Therapy* (2000) 7, 93-109).

[0137] In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid. In some embodiments, a therapeutic nucleic acid may include without limitation an siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNAzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the invention. For example, the nucleic acids of the invention may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

[0138] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a therapeutic polypeptide or therapeutic nucleic acid may be used to reduce or eliminate the

expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (*e.g.,* a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (*e.g., caspase-3, Beclin1, Ask1, PAR1, HIF1α, PUMA*, and/or any of the genes described in Fukuda, A.M. and Badaut, J. (2013) *Genes (Basel)* 4:435-456), Huntington's disease (mutant *HTT*), epilepsy (*e.g., SCN1A, NMDAR, ADK,* and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; *SOD1*), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (*e.g.,* a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the invention may include those that involve large areas of the cortex, *e.g.,* more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

[0139] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the invention that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder)

include Gaucher disease type 2 or type 3 (acid beta-glucosidase, *GBA*), GM1 gangliosidosis (beta-galactosidase-1, *GLB1*), Hunter disease (iduronate 2-sulfatase, *IDS*), Krabbe disease (galactosylceramidase, *GALC*), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, *MAN2B1*), β mannosidosis disease (beta-mannosidase, *MANBA*), metachromatic leukodystrophy disease (pseudoarylsulfatase A, *ARSA*), mucolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, *GNPTAB*), Niemann-Pick A disease (acid sphingomyelinase, *ASM*), Niemann-Pick C disease (Niemann-Pick C protein, *NPC1*), Pompe disease (acid alpha-1,4-glucosidase, *GAA*), Sandhoff disease (hexosaminidase beta subunit, *HEXB*), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, *MPS3A*), Sanfilippo B disease (N-alpha-acetylglucosaminidase, *NAGLU*), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, *MPS3C*), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, *GNS*), Schindler disease (alpha-N-acetylgalactosaminidase, *NAGA*), Sly disease (beta-glucuronidase, *GUSB*), Tay-Sachs disease (hexosaminidase alpha subunit, *HEXA*), and Wolman disease (lysosomal acid lipase, *LIPA*).

[0140] Additional lysosomal storage diseases, as well as the defective enzyme associated with each disease, are listed in Table 1 below. In some embodiments, a disease listed in the table below is treated by a therapeutic polypeptide or therapeutic nucleic acid of the invention that complements or otherwise compensates for the corresponding enzymatic defect.

Table 1. Lysosomal storage disorders and associated defective enzymes.

Lysosomal storage disease	Defective enzyme
Aspartylglusoaminuria	Aspartylglucosaminidase
Fabry	Alpha-galactosidase A
Infantile Batten Disease (CNL1)	Palmitoyl protein thioesterase
Classic Late Infantile Batten Disease (CNL2)	Tripeptidyl peptidase
Juvenile Batten Disease (CNL3)	Lysosomal transmembrane protein
Batten, other forms (CNL4-CNL8)	multiple gene products
Cystinosis	Cysteine transporter
Farber	Acid ceramidase
Fucosidosis	Acid alpha-L-fucosidase

Galactosidosialidosis	Protective protein/cathepsin A
Gaucher types 1, 2, and 3	Acid beta-glucosidase
GM1 gangliosidosis	Acid beta-galactosidase
Hunter	Iduronate-2-sulfatase
Hurler-Scheie	Alpha-L-iduronidase
Krabbe	Galactocerebrosidase
alpha-mannosidosis	Acid alpha-mannosidase
beta-mannosidosis	Acid beta-mannosidase
Maroteaux-Lamy	Arylsulfatase B
Metachromatic leukodystrophy	Arylsulfatase A
Morquio A	N-acetylgalactosamine-6-sulfate
Morquio B	Acid beta-galactosidase
Mucolipidosis II/III	N-acetylglucosamine-1-phosphotransferase
Niemann-Pick A, B	Acid sphingomyelinase
Niemann-Pick C	NPC-1
Pompe acid	alpha-glucosidase
Sandhoff	beta-hexosaminidase B
Sanfilippo A	Heparan N-sulfatase
Sanfilippo B	alpha-N-acetylglucosaminidase
Sanfilippo C	Acetyl-CoA:alpha-glucoasaminide N-acetyltransferase
Sanfilippo D	N-acetylglucosamine-6-sulfate
Schindler disease	alpha-N-acetylgalactosaminidase
Schindler-Kanzaki	alpha-N-acetylgalactosaminidase
Sialidosis	alpha-neuramidase
Sly	beta-glucuronidase
Tay-Sachs	beta-hexosaminidase A
Wolman	Acid lipase

[0141] In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase- 1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit β-globin promoter (CAG promoter; Niwa *et al.*, *Gene*, 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EFl-alpha) promoter (Kim *et al.*, *Gene*, 1990, 91(2):217-23 and Guo *et al.*, *Gene Ther.*, 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β-actin (CBA)

promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the invention provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, *e.g.,* in U.S. PG Pub. 20140335054.

[0142] Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, *e.g.*, Boshart *et al.*, Cell, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EFla promoter [Invitrogen].

[0143] Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, *e.g.*, acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothionine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No *et al., Proc. Natl. Acad. Sci.* USA, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen *et al., Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen *et al., Science*, 268:1766-1769 (1995), see also Harvey *et al., Curr. Opin. Chem. Biol.*, 2:512-518 (1998)), the RU486-inducible system (Wang *et al., Nat. Biotech.*, 15:239-243 (1997) and Wang *et al., Gene Ther.*, 4:432-441 (1997)) and the rapamycin-inducible system (Magari *et al., J. Clin. Invest.*, 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, *e.g.*, temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

[0144] In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene

should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression

[0145] In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some cases, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (*e.g.*, promoters, enhancers, *etc.*) are well known in the art.

[0146] In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

[0147] In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, *e.g.,* accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

V. Viral particles and methods of producing viral particles

[0148] Certain aspects of the present disclosure relate to recombinant viral particles (*e.g.,* rAAV particles).

[0149] Based on the guidance provided herein, the techniques of the present disclosure may suitably be adapted by one of skill in the art for use with a variety of different viruses.

[0150] In some embodiments, the virus is of the family *Adenoviridae*, which includes non-enveloped viruses typically known as Adenoviruses. In some embodiments, the virus is of the genus *Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus,* or *Siadenovirus*.

[0151] In some embodiments, the virus is of the family *Parvoviridae*, which includes non-enveloped viruses such as AAV and Bocaparvovirus. In some embodiments, the virus is of the subfamily *Densovirinae*. In some embodiments, the virus is of the genus *Ambidensovirus,*

Brevidensovirus, Hepandensovirus, Iteradensovirus, or *Penstyldensovirus*. In some embodiments, the virus is of the subfamily *Parvovirinae*. In some embodiments, the virus is of the genus *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* or *Tetraparvovirus*.

[0152] In some embodiments, the virus is of the family *Retroviridae*, which includes enveloped viruses including lentivirus. In some embodiments, the virus is of the subfamily *Orthoretrovirinae*. In some embodiments, the virus is of the genus *Alpharetrovirus, Betaretrovirus, Deltaretrovirus, Epsilonretrovirus, Gammaretrovirus,* or *Lentivirus*. In some embodiments, the virus is of the subfamily *Spumaretrovirinae*. In some embodiments, the virus is of the genus *Spumavirus*.

[0153] In some embodiments, the virus is of the family *Baculoviridae*, which includes enveloped viruses including alphabaculovirus. In some embodiments, the virus is of the genus *Alphabaculovirus, Betabaculovirus, Deltabaculovirus,* or *Gammabaculovirus*.

[0154] In some embodiments, the virus is of the family *Herpesviridae*, which includes enveloped viruses such as the simplex viruses HSV-1 and HSV-2. In some embodiments, the virus is of the subfamily *Alphaherpesvirinae*. In some embodiments, the virus is of the genus *Iltovirus, Mardivirus, Simplexvirus,* or *Varicellovirus*. In some embodiments, the virus is of the subfamily *Betaherpesvirinae*. In some embodiments, the virus is of the genus *Cytomegalovirus, Muromegalovirus, Proboscivirus,* or *Roseolovirus*. In some embodiments, the virus is of the subfamily *Gammaherpesvirinae*. In some embodiments, the virus is of the genus *Lymphocryptovirus*, *Macavirus*, *Percavirus*, or *Rhadinovirus*.

[0155] In some embodiments, the virus is an AAV virus. In an AAV particle, a nucleic acid is encapsidated in the AAV particle. The AAV particle also comprises capsid proteins. In some embodiments, the nucleic acid comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

[0156] In some embodiments, the viral particle comprises an AAV ITR sequence. For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By "functional AAV ITR sequences" it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. *See* Davidson *et al.*, *PNAS*, 2000, 97(7)3428-32; Passini *et al.*, *J. Virol.*, 2003, 77(12):7034-40; and Pechan *et al.*, *Gene Ther.*, 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some aspects of the invention, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection by the rAAV. AAV ITRs for use in the vectors of the invention need not have a wild-type nucleotide sequence (*e.g.*, as described in Kotin, *Hum. Gene Ther.*, 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao *et al.*, *PNAS*, 2002, 99(18): 11854-6; Gao *et al.*, *PNAS*, 2003, 100(10):6081-6; and Bossis *et al.*, *J. Virol.*, 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present invention. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the nucleic acid in the AAV (*e.g.,* an rAAV vector) comprises an ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0157] In some embodiments, a rAAV particle comprises an encapsulation protein selected from AAV1, AAV2, AAV3, AAV4, AAV5, AAV6 (*e.g.,* a wild-type AAV6 capsid, or a variant AAV6 capsid such as ShH10, as described in U.S. PG Pub. 2012/0164106), AAV7, AAV8, AAVrh8, AAVrh8R, AAV9 (*e.g.,* a wild-type AAV9 capsid, or a modified AAV9 capsid as described in U.S. PG Pub. 2013/0323226), AAV10, AAVrh10, AAV11, AAV12, a tyrosine capsid mutant, a heparin binding capsid mutant, an AAV2R471A capsid, an AAVAAV2/2-7m8

capsid, an AAV LK03 capsid, an AAV DJ capsid (*e.g.*, an AAV-DJ/8 capsid, an AAV-DJ/9 capsid, or any other of the capsids described in U.S. PG Pub. 2012/0066783), AAV2 N587A capsid, AAV2 E548A capsid, AAV2 N708A capsid, AAV V708K capsid, goat AAV capsid, AAV1/AAV2 chimeric capsid, bovine AAV capsid, mouse AAV capsid, rAAV2/HBoV1 capsid, an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or an AAV capsid described in U.S. Pat. No. 8,283,151 or International Publication No. WO/2003/042397. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

[0158] Certain aspects of the present disclosure relate to an AAV (*e.g.*, a rAAV) capsid protein comprising an amino acid substitution at amino acid residue 2. In some embodiments, the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. As described herein, the amino acid at the 2nd position to the initiating methionine (iMet X) of an AAV capsid protein may be examined for effects on N-terminal acetylation, trafficking, transduction, and/or other post-translational modification(s) (*e.g.*, glycosylation, ubiquitination, and so forth). Any assay described herein for examining acetylation, or a functional consequence thereof related to AAV particles, may be used to assess N-terminal acetylation. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0159] Other aspects of the present disclosure relate to an AAV (*e.g.*, a rAAV) capsid protein comprising an amino acid substitution that alters deamidation. In some embodiments, an amino acid substitution (*e.g.*, an amino acid substitution at A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3) that "alters" deamidation results in a higher frequency of deamidation or a lower frequency of deamidation, *e.g.*, as compared to a VP1 or VP3 without the substitution, such as the parental VP1 or VP3. As described herein, a potential deamidation site of an AAV capsid protein (*e.g.*, VP1 or VP3) may be examined for effects on deamidation, trafficking, transduction, and/or other post-translational modification(s) (*e.g.*, glycosylation, ubiquitination, and so forth). Any assay

described herein for examining deamidation, or a functional consequence thereof related to AAV particles, may be used to assess deamidation.

[0160] Several potential deamidation sites are described herein. In some embodiments, an amino acid substitution that alters deamidation is selected from A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3. For example, in some embodiments, N57 of VP1, N382 of VP3, N511 of VP3, and/or N715 of VP3 is substituted with Asp, and the amino acid substitution results in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In other embodiments, the amino acid substitution is N57K or N57Q, and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle. In yet other embodiments, G58 of VP1, G383 of VP3, G512 of VP3, and/or G716 of VP3 is substituted with an amino acid that is not Gly (*e.g.*, Ala, Arg, Asn, Asp, Cys, Glu, Gln, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, or Val), and the amino acid substitution results in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

[0161] In some embodiments, the AAV capsid protein is VP1, VP2, or VP3. The AAV particle may comprise any of the exemplary AAV capsid serotypes described herein, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1. The AAV capsid protein may further comprise any of the capsid protein mutations described herein, such as tyrosine and/or heparin binding mutations.

[0162] Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described

herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (*e.g.,* using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (*e.g.,* room temperature, or 4°C, for thermal stability) or treated at a particular pH (*e.g.,* pH stability), and the like.

[0163] Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of

VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the assembly of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (*e.g.,* after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or

structural analysis to observe particle formation (*e.g.,* using electron microscopy), production of AAV capsid proteins (*e.g.,* as assayed by Western blotting), and the like.

[0164] Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting amino acid residue 2 of VP1 and/or VP3, *e.g.,* as described herein. For example, in some embodiments, amino acid residue 2 of VP1 is substituted. In other embodiments, amino acid residue 2 of VP3 is substituted. In some embodiments, the substituted amino acid at position 2 is N-acetylated at a higher frequency than amino acid residue 2 of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the transduction of a rAAV particle with a substituted amino acid at position 2 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting amino acid residue 2 of VP1 and/or VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using

various assays known in the art, including without limitation, the transduction efficiency assays described herein. In some embodiments, the invention provide methods of reducing the transduction of a rAAV particle; for example, by substituting amino acid residue 2 of VP1 and/or VP3.

[0165] Other aspects of the present disclosure relate to methods of improving the stability of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the stability of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%,

about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to stability of a rAAV particle comprising a wild-type capsid. AAV particle stability may be measured using various assays known in the art, including without limitation differential scanning fluorescence (DSF), differential scanning calorimetry (DSC), other thermal denaturation assays, susceptibility to proteolysis, imaging or structural analysis to observe denaturation (*e.g.,* using electron microscopy), transduction efficiency or another functional assay on AAV particle compositions kept for a designated time interval at a particular temperature (*e.g.,* room temperature, or 4°C, for thermal stability) or treated at a particular pH (*e.g.,* pH stability), and the like.

[0166] Other aspects of the present disclosure relate to methods of improving the assembly of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the assembly of a rAAV particle by any one of about 10% to about 100%, about 20% to about

100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to assembly of a rAAV particle comprising a wild-type capsid. AAV particle assembly may be measured using various assays known in the art, including without limitation, measuring particle production amount and/or rate, quantifying capsid production (*e.g.,* after purification using any of the methods described herein), assaying production of complete vectors vs. empty capsids, measuring transduction efficiency, imaging or structural analysis to observe particle formation (*e.g.,* using electron microscopy), production of AAV capsid proteins (*e.g.,* as assayed by Western blotting), and the like.

[0167] Other aspects of the present disclosure relate to methods of improving the transduction of a rAAV particle. In some embodiments, the methods include substituting an amino acid of VP1 and/or VP3 that alters deamidation, *e.g.,* as described herein. For example, in some embodiments, A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 is substituted. In some embodiments, the substituted amino acid is deamidated at a higher frequency than the amino acid residue of the parent VP1 and/or VP3, *e.g.,* as described herein. In some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at

least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 100%. In some embodiments, the stability of a rAAV particle with a substituted A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, or G716 of VP3 may be compared to a wild-type or parental AAV capsid, *e.g.,* of the same serotype. For example, in some embodiments, substituting A35 of VP1, N57 of VP1, G58 of VP1, N382 of VP3, G383 of VP3, N511 of VP3, G512 of VP3, N715 of VP3, and/or G716 of VP3 improves the transduction of a rAAV particle by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, *e.g.,* as compared to transduction of a rAAV particle comprising a wild-type capsid. AAV particle transduction may be measured using various assays known in the art, including without limitation, the transduction efficiency assays described herein.

[0168] In some aspects, the invention provides viral particles comprising a recombinant self-complementing genome (*e.g.,* a self-complementary or self-complimenting rAAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing AAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., *et al.*, (2003) *Gene Ther* 10:2105-2111, each of which are incorporated herein by reference in its entirety. A rAAV comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (*e.g.*, complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first

nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

[0169] In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (*e.g.*, the right ITR). In some embodiments, the ITR comprises the polynucleotide sequence 5'-CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC GGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGGGCG – 3' (SEQ ID NO:8). The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating an AAV viral genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in 5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0170] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (*e.g.*, a diseased tissue). A rAAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, a rAAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or a rAAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the rAAV particle.

[0171] In some embodiments, the AAV capsid comprises a mutation, *e.g.,* the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the rAAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, *e.g.,* Zhong L. *et al.*, (2008) *Proc Natl Acad Sci U S A* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, *et al., J. Virol.* 2004, 78(12):6381).

[0172] In some embodiments, a capsid protein comprises one or more amino acid substitutions at one or more positions that interact with a heparin sulfate proteoglycan or at one or more positions corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2. Heparan sulfate proteoglycan (HSPG) is known in the art to act as the cellular receptor for AAV2 particles (Summerford, C. and Samulski, R.J. (1998) *J. Virol.* 72(2):1438-45). Binding between an AAV2 particle and HSPG at the cell membrane serves to attach the particle to the cell. Other cell surface proteins such as fibroblast growth factor receptor and αvβ5 integrin may also facilitate cellular infection. After binding, an AAV2 particle may enter the cell through mechanisms including receptor mediated endocytosis via clathrin-coated pits. An AAV2 particle may be released from an endocytic vesicle upon endosomal acidification. This allows the AAV2 particle to travel to the perinuclear region and then the cell nucleus. AAV3 particles are also known to bind heparin (Rabinowitz, J.E., *et al.* (2002) *J. Virol.* 76(2):791-801).

[0173] The binding between AAV2 capsid proteins and HSPG is known to occur via electrostatic interactions between basic AAV2 capsid protein residues and negatively charged glycosaminoglycan residues (Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006; Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081). Specific capsid residues implicated in these interactions include R484, R487, K527, K532, R585, and R588. Mutations in these residues have been shown to reduce AAV2 binding to Hela cells and heparin itself (Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006; Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081; WO 2004/027019 A2, US Patent No. 7,629,322). Further, without wishing to be bound to theory, it is thought that amino acid substitution(s) at one or more of the residues corresponding to amino acids 484, 487, 527, 532, 585 or 588, numbering based on VP1 numbering of AAV2 may modulate the transduction properties of AAV capsid types that do not bind to HSPG, or may modulate the transduction properties of AAV capsid types independent from their ability to bind HSPG. In some embodiments, the one or more amino acid substitutions comprises a substitution at position R484, R487, K527, K532, R585 and/or R588 of VP1, VP2 and/or VP3, numbering based on VP1 of AAV2.

[0174] In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 25%,

about at least 50%, about at least 75%, or about at least 100%. In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%, about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions reduce binding of the rAAV particle to the heparin sulfate proteoglycan by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to binding of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions results in no detectable binding of the rAAV particle to the heparin sulfate proteoglycan compared to binding of a wild-type rAAV particle. Means to measure binding of AAV particles to HSPG are known in the art; *e.g.*, binding to a heparin sulfate chromatography media or binding to a cell known to express HSPG on its surface. For example, see Opie, SR *et al.*, (2003) *J. Virol.* 77:6995-7006 and Kern, A *et al.*, (2003) *J. Virol.* 77:11072-11081. In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (*e.g.,* a cell in the eye or CNS) by about at least 10%, about at least 15%, about at least 20%, about at least 25%, about at least 30%, about at least 35%, about at least 40%, about at least 45%, about at least 50%, about at least 55%,

about at least 60%, about at least 65%, about at least 70%, about at least 75%, about at least 80%, about at least 85%, about at least 90%, about at least 95%, or about at least 100% (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). In some embodiments, the one or more amino acid substitutions improve the transduction efficiency of the rAAV particle to a cell (*e.g.,* a cell in the eye or CNS) by any one of about 10% to about 100%, about 20% to about 100%, about 30% to about 100%, about 40% to about 100%, about 50% to about 100%, about 60% to about 100%, about 70% to about 100%, about 80% to about 100%, about 90% to about 100%, about 10% to about 90%, about 20% to about 90%, about 30% to about 90%, about 40% to about 90%, about 50% to about 90%, about 60% to about 90%, about 70% to about 90%, about 80% to about 90%, about 10% to about 80%, about 20% to about 80%, about 30% to about 80%, about 40% to about 80%, about 50% to about 80%, about 60% to about 80%, about 70% to about 80%, about 10% to about 70%, about 20% to about 70%, about 30% to about 70%, about 40% to about 70%, about 50% to about 70%, about 60% to about 70%, about 10% to about 60%, about 20% to about 60%, about 30% to about 60%, about 40% to about 60%, about 50% to about 60%, about 10% to about 50%, about 20% to about 50%, about 30% to about 50%, about 40% to about 50%, about 10% to about 40%, about 20% to about 40%, about 30% to about 40%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 20%, (as compared to transduction efficiency of a rAAV particle comprising a wild-type capsid). Means to measure transduction efficiency of AAV particles to a cell (*e.g.,* a cell in culture or part of a tissue) are known in the art. For example, a population of cells (*e.g.,* in culture or part of a tissue) may be infected with a concentration of rAAV particles containing a vector that, when expressed in the cells, produces an assayable reporter (*e.g.,* GFP fluorescence, sFLT production, etc.).

AAV capsid proteins

[0175] In some aspects, the invention provides an AAV capsid protein comprising an amino acid substitution at amino acid residue 2; wherein the amino acid substitution at amino acid residue 2 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of the parent AAV capsid protein. In some embodiments, the AAV capsid protein is VP1 or VP3. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr.

In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid protein. Non-limiting examples of AAV capsid proteins of the invention include VP1 and/or VP3 of any of the following AAV serotypes: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV2/2-7m8, AAV DJ, AAV DJ8, AAV2 N587A, AAV2 E548A, AAV2 N708A, AAV V708K, goat AAV, AAV1/AAV2 chimeric, bovine AAV, mouse AAV, or rAAV2/HBoV1 serotype capsid. In some embodiments, the AAV capsid further comprises a tyrosine mutation or a heparin binding mutation.

Production of AAV particles

[0176] Numerous methods are known in the art for production of rAAV vectors, including transfection, stable cell line production, and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE *et al.*, (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. *et al.*, (2002) *Human Gene Therapy* 13(16):1935-1943; Kotin, R. (2011) *Hum Mol Genet.* 20(R1): R2–R6). rAAV production cultures for the production of rAAV viral particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences (*e.g.*, an AAV genome encoding GNPTAB); and 5) suitable media and media components to support rAAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV vector genome as long as the rep gene products may function to replicated and package the rAAV genome. Suitable media known in the art may be used for the production of rAAV vectors. These media include, without limitation, media produced by Hyclone Laboratories and JRH including Modified Eagle Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), custom formulations such as those described in U.S. Patent No. 6,566,118, and Sf-900 II SFM media as described in U.S. Patent

No. 6,723,551, each of which is incorporated herein by reference in its entirety, particularly with respect to custom media formulations for use in production of recombinant AAV vectors. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (*e.g.*, *Spodoptera frugiperda* (Sf9) cells). In some embodiments, the AAV cap functions provide an amino acid substitution at amino acid residue 2 of VP1 and/or VP3, wherein the amino acid substitution at amino acid residue 2 of VP1 and/or VP3 alters N-terminal acetylation compared to N-terminal acetylation at amino acid residue 2 of VP1 and/or VP3 of the parent AAV particle. In some embodiments, amino acid residue 2 of the AAV capsid protein (*e.g.*, VP1 or VP3) is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr. In some embodiments, the amino acid substitution results in less deamidation of the AAV capsid.

[0177] One method for producing rAAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (*e.g.*, using the calcium phosphate method) into a cell line (*e.g.*, HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the rAAV particle was produced by triple transfection of a nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing rAAV particles.

[0178] In some embodiments, rAAV particles may be produced by a producer cell line method (see Martin *et al.*, (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X.L. *et al.* (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (*e.g.*, a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence (*e.g.*, GNPTAB). Cell lines may be screened to select a lead clone for rAAV production, which may then be expanded to a production bioreactor and infected with a helper virus (*e.g.*, an adenovirus or HSV) to initiate rAAV production. Virus may subsequently be harvested, adenovirus may be inactivated (*e.g.*, by heat) and/or removed, and the rAAV particles may be purified. As such, in some embodiments, the rAAV particle was produced by a producer

cell line comprising one or more of nucleic acid encoding the rAAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions. As described herein, the producer cell line method may be advantageous for the production of rAAV particles with an oversized genome, as compared to the triple transfection method.

[0179] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (*e.g.*, 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate autonomously in a cell (*e.g.*, a human cell) have been identified (*see*, *e.g.*, Krysan, P.J. *et al.* (1989) *Mol. Cell Biol.* 9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (*e.g.*, using a cationic polymer such as DEAE-dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, *see e.g.*, Kim, T.K. and Eberwine, J.H. (2010) *Anal. Bioanal. Chem.* 397:3173-3178).

[0180] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the rAAV genome are stably integrated into the genome of the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into

a cell on different plasmids. In some embodiments, the plasmid(s) may contain a selectable marker (*e.g.*, an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Methods for stable integration of nucleic acids into a variety of host cell lines are known in the art. For example, repeated selection (*e.g.*, through use of a selectable marker) may be used to select for cells that have integrated a nucleic acid containing a selectable marker (and AAV cap and rep genes and/or a rAAV genome). In other embodiments, nucleic acids may be integrated in a site-specific manner into a cell line to generate a producer cell line. Several site-specific recombination systems are known in the art, such as FLP/FRT (*see, e.g.*, O'Gorman, S. *et al.* (1991) *Science* 251:1351-1355), Cre/loxP (*see, e.g.*, Sauer, B. and Henderson, N. (1988) *Proc. Natl. Acad. Sci.* 85:5166-5170), and phi C31-att (*see, e.g.*, Groth, A.C. *et al.* (2000) *Proc. Natl. Acad. Sci.* 97:5995-6000).

[0181] In some embodiments, the producer cell line is derived from a primate cell line (*e.g.*, a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the oversized rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

[0182] In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage. For further description, *see, e.g.*, ATCC frequently asked questions document (available on the world wide web at atcc.org/Global/FAQs/9/1/Adapting%20a%20monolayer%20cell%20line%20to%20suspension-40.aspx) and references cited therein.

[0183] Suitable AAV production culture media of the present invention may be supplemented with serum or serum-derived recombinant proteins at a level of 0.5%-20% (v/v or w/v). Alternatively, as is known in the art, AAV vectors may be produced in serum-free conditions which may also be referred to as media with no animal-derived products. One of ordinary skill in the art may appreciate that commercial or custom media designed to support production of AAV vectors may also be supplemented with one or more cell culture components know in the art, including without limitation glucose, vitamins, amino acids, and or growth factors, in order to increase the titer of AAV in production cultures.

[0158] AAV production cultures can be grown under a variety of conditions (over a wide temperature range, for varying lengths of time, and the like) suitable to the particular host cell being utilized. As is known in the art, AAV production cultures include attachment- dependent cultures which can be cultured in suitable attachment-dependent vessels such as, for example, roller bottles, hollow fiber filters, microcarriers, and packed-bed or fluidized-bed bioreactors. AAV vector production cultures may also include suspension-adapted host cells such as HeLa, 293, and SF-9 cells which can be cultured in a variety of ways including, for example, spinner flasks, stirred tank bioreactors, and disposable systems such as the Wave bag system.

[0159] AAV vector particles of the invention may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Patent No. 6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0160] In a further embodiment, the AAV particles are purified. The term "purified" as used herein includes a preparation of AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from. Thus, for example, isolated AAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity,

or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

[0161] In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including, for example, a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 μm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 μm or greater pore size known in the art.

[0162] In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0163] AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below. Methods to purify AAV particles are found, for example, in Xiao *et al.*, (1998) *Journal of Virology* 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948; and WO 2010/148143.

Pharmaceutical compositions

[0164] In some embodiments, an AAV particle of the present disclosure (*e.g.,* a rAAV particle) is in a pharmaceutical composition. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to improve the stability and/or

improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments, the pharmaceutical composition comprises rAAV particles modified to modulate the stability and/or the transduction efficiency of rAAV particles (*e.g.*, increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (*e.g.*, increase deamidation or decrease deamidation).

[0165] In some embodiments, the rAAV particle is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents, wetting and emulsifying agents, salts for varying osmolarity, encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, *e.g.*, Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

[0166] Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable

solutions. The pharmaceutical composition may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution.

Kits and articles of manufacture

[0167] The present invention also provides kits or articles of manufacture comprising any of the rAAV particles and/or pharmaceutical compositions of the present disclosure. The kits or articles of manufacture may comprise any of the rAAV particles or rAAV particle compositions of the invention. In some embodiments the kits are used to improve the stability and/or improve the transduction efficiency of rAAV particles; for example, for use in substituting the amino acid residue at position 2 of VP1 and/or VP3 to improve acetylation of rAAV capsid proteins. In some embodiments the kits are used to modulate the stability and/or the transduction efficiency of rAAV particles (*e.g.*, increase stability and/or transduction efficiency or decrease stability and/or transduction efficiency); for example, for use in substituting the amino acid residues that modulate deamidation (*e.g.*, increase deamidation or decrease deamidation).

[0168] In some embodiments, the kits or articles of manufacture further include instructions for administration of a composition of rAAV particles. The kits or articles of manufacture described herein may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (*e.g.*, sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

[0169] In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (*e.g.,* as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J. 1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits

or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

EXAMPLES

[0170] The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

Example 1: Direct LC/MS and LC/MS/MS for complete characterization of recombinant AAV viral capsid protein

[0171] Recombinant adeno-associated viruses (rAAVs) have become popular gene therapy vectors due to their nonpathogenic nature, ability to infect both dividing and non-dividing cells and long term gene expression. Currently, AAV-based gene therapies are used in clinical trials for numerous disease targets, such as muscular dystrophy, hemophilia, Parkinson's disease, Leber's congenital amaurosis and macular degeneration.

[0172] AAV is a small and nonenveloped parvovirus with a single stranded DNA genome encapsulated in an icosahedra shell. Each capsid includes sixty copies of three viral capsid proteins VP1 (87kDa), VP2 (73kDa) and VP3 (62 kDa) in an approximately 1:1:10 ratio. The three viral capsid proteins are expressed from the same open reading frame by using alternative splicing and an atypical start codon and thus have overlapping sequences. VP1 has ~137 additional N-terminal amino acid residues compared to VP3 while VP2 has ~65 additional N-terminal amino acid residues compared to VP3. At least 13 AAV serotypes and ~ 150 gene sequences have been isolated from human and non-human primate tissues; AAV serotypes differ in the amino acid sequence of viral capsid proteins and their corresponding cellular receptors and co-receptors for targeting.

[0173] The AAV capsid, in addition to protecting the genome inside, plays an important role in mediating receptor binding, escape of virus from endosome, and transport of viral DNA into nucleus in the viral infection cycle, thus directly impacting viral infectivity. It has been shown that the VP1 N-terminus contains a phospholipase PLA2 domain (a.a. 52-97) which is critical in endosomal escaping of virus [1-3]. N-termini of VP1 and VP2 also contain three basic amino acid clusters as nuclear localization signals. These sequences are highly conserved among different AAV serotypes. Mutations of these amino acids have been shown to reduce or abolish infectivity completely [4]. In addition, each AAV serotype has corresponding sequence-specific receptors and co-receptors. For example, heparin sulfate proteoglycan was identified as a major receptor of AAV2 and several other co-receptors, including αVβ5 integrin, fibroblast growth factor receptor 1, and hepatocyte growth receptor have been identified [5-8]. Mutation analysis of AAV2 capsid proteins has identified a group of basic amino acids (Arginine484, 487, 585, and Lysine532) as a heparin-binding motif which contributes to the heparin and HeLa cell binding [9]. NGR domain in AAV2 was identified as an integrin α5β1 binding domain which is essential for viral cell entry [10]. In summary, viral capsid protein sequences are important in cellular targeting and trafficking in the viral infection cycle. Since different production conditions may cause different expression levels of viral capsid proteins, post-translational modifications, and truncations, the viral capsid proteins need to be characterized and monitored to ensure the product consistency in gene therapy development programs.

[0174] Traditionally, SDS-PAGE has been used to characterize the AAV viral capsid proteins, providing rough molecular weight information such as 87 kDa, 73kDa and 62 kDa. No sequence information was obtained from Edman sequencing, possibly due to the blocked N-termini of viral capsid proteins, except VP2. Although X-ray structures of multiple AAVs have been solved, only the VP3 region sequence was observed in the crystal structures. Fifteen N-terminal amino acid residues of VP3 were still missing in the X-ray structure, possibly due to its intrinsic disorder [11-13]. It is possible that the lack of information of VP1 and VP2 N-terminal regions in the atomic structure might be due to low stoichiometry of VP1 and VP2 in the capsid. In addition, N-termini of VP1 and VP2 are buried inside the capsid and are not accessible to antibodies in the native state as reported in the some literature [3, 14, 15]. Conventionally, a Gel-LC/MS method (SDS-PAGE, in-gel tryptic digestion and LC/MS/MS) was used in characterization of VPs [16-18]. However, N-termini of VP1, VP2 and VP3 have not been

confirmed using this approach, since this method failed to obtain 100% sequence coverage of VPs due to the limited recovery of peptide from gel.

[0175] Direct analysis using MALDI-TOF MS was reported for several virus capsid proteins including tobacco mosaic virus U2 after dissociation with organic acid [19]. Direct peptide mapping after amide hydrogen exchange and mass spectrometry have been used to study the pH-induced structural changes in the capsid of brome mosaic virus (BMV) [20]. Since AAVs are nonenveloped viruses containing only capsid proteins and genome, AAVs capsids could be directly analyzed by RP-LC/MS of proteins and LC/MS/MS of peptide mapping to achieve 100% sequence coverage after capsid dissociation without SDS-PAGE separation. The DNA fragments could elute in the void volume and thus have no interference on protein/peptide detection by LC/MS. In order to investigate these methods, direct LC/MS of different types of AAVs after denaturation was used to monitor the protein sequence and post-translational modifications of AAV capsid proteins. As described herein, N-termini of VP1, VP2 and VP3 of AAVs have been confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in the different serotype of AAVs. Direct LC/MS/MS peptide mapping of AAVs has also been developed to provide sequence coverage of VP1, VP2 and VP3 and confirm the N-termini acetylation of VP1 and VP3.

Methods

Materials and Reagents

[0176] Dithiothreitol (DTT), 4-vinylpyridine, ultra-pure formic acid, acetic acid, guanidine-HCl, Tris-HCl and Tris base were purchased from Sigma Chemicals (St. Louis, MO). Amicon ultra-4 filters were purchased from Millipore (Billerica, MA). The porcine sequencing grade trypsin was purchased from Promega (Milwaukee, WI). Endoproteinase Lys-C and Asp-N were purchased from Roche (Germany). Slide-A-Lyzer cassettes with 10,000 MWCO were purchased from Pierce (Rockford, IL).

Vector production and purification

[0177] AAV vectors were produced using the transient triple transfection method as previously described (Xiao, 1998 #123). Briefly, HEK293 cells were transfected using polyethyleneimine, PEI, and a 1:1:1 ratio of three plasmids (ITR vector, AAV rep/cap and Ad helper plasmid). The

vector plasmid contains the vector genome CBA-EGFP and ITR sequences from AAV2. EGFP expression is driven by the CMV enhancer chicken beta actin hybrid promoter (CBA) as described (Miyazaki, 1989 #124) (Niwa, 1991 #125). The AAV rep / cap helpers contained rep sequences from AAV2 and serotype specific capsid sequences with the nomenclature, rep2 / cap2, rep2 / cap5, rep2 /cap7 etc. The pAd helper used was pHelper (Stratagene/Agilent Technologies, Santa Clara, CA). Purification of AAV was performed as described by Qu *et al* . (2007, *J. Virol. Methods* 140:183-192).

LC/MS intact protein analysis

[0178] The AAV virions were concentrated with an Amicon ultra-4 filter (10kDa MWCO) and denatured with 10% acetic acid followed by direct analysis in an Acquity UPLC −Xevo® QTOF MS instrument (Waters, Milford, MA). The separations were performed with a UPLC BEH C4 or C8 column (1.7 μm, 2.1mm i.d.) at a 0.25 ml/min flow rate. Mobile phase A was 0.1% formic acid in water while mobile phase B was 0.1% formic acid in acetonitrile. The final gradient was as follows: from 10% B to 20% B for 6 minutes, from 20% B to 30% B in 10 min, then from 30% to 38% B for 40 minutes. For MS the capillary voltage and sampling cone voltage were set at 3.5 kV and 45 V respectively. The mass spectra were acquired in the sensitivity mode with *m/z* range of 500-4000. Assisted calibration with sodium iodide as calibrant was performed for mass calibration. MaxEnt1 in Masslynx software was used for protein deconvolution.

Enzymatic digestions of AAV2 VPs

[0179] The concentrated AAV2 virions were denatured with 6 M Guanidine-HCl, 0.1 M Tris at pH 8.5. The proteins were reduced with 30 mM DTT at 55 °C for 1 hour in darkness and alkylated with 0.07% 4-vinylpyridine at room temperature for 2 hours. The reactions were quenched by the addition of 1M DTT. The samples were dialyzed with Slide-A-Lyzer cassettes (10,000 MWCO) against 25 mM Tris buffer at pH 8.5 for ~18 hours. After dialysis, the samples were split into three aliquots. Each aliquot was digested with trypsin at 1:25 or Lys-C at 1:50 or Asp-N at 1:100 enzyme: protein ratio (wt/wt) for 18 hours at 37 °C, respectively.

LC/MS/MS peptide mapping

[0180] Nano LC/MS/MS was performed in using a NanoAcquity HPLC system (Waters, Milford, MA) in conjunction with an Orbitrap Velos mass spectrometer (Thermo-Fisher

Scientific, Waltham, MA) using home packed nanoLC column(75 µm x10mm) with Magic C18 with packing material(5 µm, Bruker, Billerica, MA) at a 300 nl/min flow rate. The mobile phases A and B were 0.1% formic acid in water and acetonitrile, respectively. The gradient was from 2% B to 60% B in 121min.

[0181] The source parameters for velos were as follows: source voltage: 2.5 kv, capillary temperature 275 °C; S-lens RF level: 55%. Data were acquired using the top-ten data dependent method with accurate ms at 60,000 resolution and 10 MS/MS in ion trap. Mascot was used for database searching against AAV2 viral capsid protein sequences. MS tolerance of 10 ppm and ms/ms tolerance of 0.8 Da were used for the database search.

UPLC/MS/MS peptide mapping

[0182] The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. A BEH300 C18 column (2.1 x150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe mode in the mass range of 200-2000.

Results

AAV denaturation method

[0183] AAVs can be denatured through a number of methods using detergent, heat, high salt, or buffer with low or high pHs. Heat denaturation can lead to protein precipitation and as a result reverse phase columns are easily clogged and over pressurized. Denaturation with high salt requires an additional desalting step before LC/MS analysis. Denaturing with 10% acetic acid was used for the LC/MS intact protein analysis, as it allowed for clean mass spectrum. For peptide mapping, either 0.1% RapiGest or 6 M Guanidine HCl can be used as a denaturing reagent.

Intact protein analysis method development

[0184] Initial intact protein analysis of AAV2 was performed using an UPLC BEH C4 column at fast gradient. Under this condition, only one single peak in the total ion chromatogram was observed, with a mass corresponding to VP3 (**FIG. 1A**). Without wishing to be bound by theory, it is thought that the absence of VP1 and VP2 is possibly due to low stoichiometry of VP1 and VP2 or suppression of VP1 and VP2 signals by VP3 if all VPs co-elute. Increasing injection or

column length, using a shallower gradient, and using alternative columns have been attempted in order to detect VP1 and VP2. Higher loading (1.7µg) with a shallower gradient at 0.5% B/min resulted in a shoulder peak on the left (**FIG. 1B**). The increase in column length from 10 cm to 15 cm did not enhance the separation of the shoulder peak (**FIG. 1C**). However, the shoulder peak was further separated from the main peak using a BEH C8 column, with improved signal intensities observed (**FIG. 1D**).

[0185] As a result, the VP1 and VP2 masses were obtained in this shoulder peak at the signal intensities shown in **FIG. 2A**. The masses of VP1 and VP3 correspond to a.a. 2-735(acetylation) and a.a. 204-735(acetylation), respectively (**FIGS. 2A&2B**). No acetylation was observed in VP2 (a.a.139-735). In addition, a minor peak with a smaller mass than VP3 was observed, with a mass corresponding to amino acid sequence 212-735 with one acetylation (**FIG. 2B**). These data are consistent with DNA sequences since VP3 contains two ATG initiation codons in AAV2: ***ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC*** (SEQ ID NO:1)**,** resulting in two possible N termini (underlined): MATGSGAPMAD (SEQ ID NO:2). The N-terminal methionine residues were not present in both VP1 and VP3 as measured by intact protein analysis. The acetylation of VP1 and VP3 is not a method-induced artifact (denaturation of AAV by 10% acetic acid) since acetylation of VP1 and VP3 is also observed in an AAV preparation using an alternative denature method without acetic acid. The intact protein data also confirmed that no glycosylation was present in the viral capsid proteins, even though several N-linked consensus sequences are present [16].

LC/MS/MS peptide mapping

[0186] To further confirm the N-termini and acetylation observed in the intact protein analysis, peptide mapping was performed using multiple enzymes and analyzed using multiple instruments. Various sample preparation methods, including denaturation methods and desalting steps, have been evaluated. The final digestion method, including denaturation with 6M guanidine HCl, reduction and alkylation with 4-vinylpyridine, and dialysis using slide-A-lyzer followed by enzymatic digestion, created clean peptide mapping with low artificial modifications during the digestion process. As low as 5 µg starting material was tested, yielding complete sequence coverage using nano LC/MS/MS and UPLC/MS/MS.

[0187] Mascot search of tryptic digests from nano LC/MS/MS alone yielded 78% sequence coverage with an ion score 13 cut off as shown in **FIG. 3**. The two large missing tryptic peptides, T27 and T38 (boxed) from nano LC/MS/MS were found in the LC/MS in Xevo TOF MS with BEH C18 column (**FIG. 3)**. In addition, most of the T27 and T38 peptide sequences were further confirmed by nano LC/MS/MS of Asp-N digests as shown in Italics in **FIG. 3**. The complete N-terminal and C-terminal peptides were covered by Lys-C digests as underlined in **FIG. 3**. Therefore, 100% sequence coverage of VP1 was achieved through multiple enzyme digestions and two LC/MS/MS methods.

[0188] LC/MS/MS confirmed the N- and C-termini of VP1, VP2 and VP3 and N-terminal acetylation of VP1 and VP3 observed in the intact protein analysis. **FIGS. 4A-4C** show the MS/MS spectra of the VP1 N-terminal tryptic peptide A(Ac)ADGYLPDWLEDTLSEGIR (SEQ ID NO:4) (**FIG. 4A**), VP2 N-terminal Asp-N derived peptide (APGKKRPVEHSPVEP) (SEQ ID NO:15) (**FIG. 4B**), and VP3 N-terminal Asp-N peptide A(Ac)TGSGAPM (SEQ ID NO:5) (**FIG. 4C**). MS/MS has confirmed the location of acetylation at the N-terminal alanine residues in both VP1 and VP3 peptides. The presence of unmodified y18 and y17 ions, and all detected b ions with 42Da mass shift in **FIG. 4A** indicates the 42 da-modification is located in N-terminal of VP1. Similarly, the presence of unmodified y3 to y8 ions in **FIG. 4C** confirmed the location of acetylation at the N-terminal alanine residue.

Comparison of AAV VP N-termini

[0189] In addition to AAV2, AAV1, AAV5, AAV7, AAV9 and AAV Rh10 have also been analyzed by intact protein analysis. The theoretical and predicted masses of VPs in AAVs are shown in Table 2.

Table 2: Theoretical Mass vs Experimental Mass for AAV VPs

Serotype	Isoform	Predicted amino acid sequence	Actual amino acid sequence	Theoretical Ms.(Da)	Experimental Ms.(Da)
AAV1	VP1	1-736	2(ac)-736	81286	81291
	VP2	138-736	139-736	66093	66098
	VP3	203-736	204(ac)-736	59517	59520
AAV2	VP1	1-735	2(ac)-735	81856	81856
	VP2	138-735	139-735	66488	66488
	VP3	203-735	204(ac)-735	59974	59974
AAV5	VP1	1-724	2(ac)-724	80336	80336

	VP2	137-724	138-724	65283	65284
	VP3	193-724	194(ac)-724	59463	59463
AAV7	VP1	1-737	2(ac)-737	81564	81567
	VP2	138-737	139-737	66372	66374
	VP3	204-737	213(ac)-737	59101	59103
AAV9	VP1	1-736	2(ac)-736	81291	81288
	VP2	138-736	139-736	66210	66209
	VP3	203-736	204(ac)-736	59733	59733
AAVRh10	VP1	1-738	2(ac)-738	81455	81455
	VP2	138-738	139-738	66253	66252
	VP3	204-738	205(ac)-738	59634	59634

[0190] N-termini, as well as their posttranslational modifications, are highly conserved among the AAV serotypes analyzed, even though AAV5 is reported as the most diverse AAV serotype sequence, as shown in the sequence alignments in **FIG. 5**. In 11 out of 13 AAV serotypes, the N-termini of VP1 share an identical 13 amino acid residue sequence (MAADGYLPDWLED) (SEQ ID NO:6) while all 13 AAV serotypes have identical TAP… N-terminal sequences in VP2 (**FIG. 5)**. LC/MS of AAV2 indicated that T is missing in VP2 at protein level. The N-termini of VP3 are the most diverse among the three viral capsid proteins, with 8 out of 13 AAV serotypes sharing a MA… N-terminal sequence. Similar to AAV2, AAV1 and AAV Rh10 also have two ATG initiation codons with the first one as predominant N-terminal based on LC/MS intact protein analysis. Interestingly, though AAV7 has two potential initiation codons (**GTG**GCTGCAGGCGGTGGCGCACCA**ATG**GCAGACAATAAC…) (SEQ ID NO:7), the second initiation codon (ATG) was favorable based on the intact protein analysis: the VP3` with 213(ac)-737 was a predominant peak while VP3 with 203(ac)-737 was a minor peak.

Conclusions

Applications of LC/MS intact protein analysis and LC/MS/MS peptide mapping of AAV VPs in gene therapy research and development

[0191] These results demonstrate that direct LC/MS of different types of AAVs after denaturation was proved to be a simple and effective way to monitor the protein sequence and post-translational modifications with accurate mass measurement in the intact protein level. N-termini of VP1, VP2 and VP3 of AAVs were confirmed by mass spectrometry. Acetylations of N-termini of VP1 and VP3 were also identified in different serotypes of AAVs. Direct

LC/MS/MS peptide mapping of AAVs was developed, provided 100% sequence coverage of VP1, VP2 and VP3, and confirmed the N-termini acetylation of VPs. The theoretical masses of predicted sequences of 13 AAV serotypes based on sequence alignment and intact protein analysis of several AAV serotypes are shown in Table 3.

Table 3: Predicted Sequences and Masses

	Predicted VP1 sequence	Mass(Da)	Predicted VP2 sequence	Mass(Da)	Predicted VP3 sequence	Mass(Da)
AAV1	2(ac)-736	81286	139-736	66093	204(ac)-736	59517
AAV2	2(ac)-735	81856	139-735	66488	204(ac)-735	59974
AAV3	2(ac)-736	81571	139-736	66319	204(ac)-736	59849
AAV4	2(ac)-734	80550	138-734	65626	198(ac)-734	59529
AAV5	2(ac)-724	80336	138-724	65283	194(ac)-724	59463
AAV6	2(ac)-736	81322	139-736	66096	204(ac)-736	59519
AAV7	2(ac)-737	81564	139-737	66372	213(ac)-737	59101
AAV8	2(ac)-738	81667	139-738	66519	205(ac)-738	59805
AAV9	2(ac)-736	81291	139-736	66210	204(ac)-736	59733
AAV10	2(ac)-738	81477	139-738	66271	205(ac)-738	59638
AAV11	2(ac)-733	80987	139-733	65794	198(ac)-733	59696
AAV12	2(ac)-742	82106	139-742	66905	207(ac)-742	59846
AAVRh10	2(ac)-738	81455	139-738	66253	205(ac)-738	59634

[0192] The accurate masses of VP1, VP2 and VP3 of each serotype are unique and therefore intact protein analysis can be used as an identity test to differentiate AAV capsid serotypes. Tables 4-6 show the mass differences of VPs among 13 common AAV serotypes. Shown in regular font are delta masses larger than 10, with delta masses less than 10 bolded.

Table 4: Mass Differences of VP1 Among 13 AAV Isotypes

	~~AAV1~~											
~~AAV2~~	~~570~~	~~AAV2~~										
~~AAV3~~	~~285~~	~~285~~	~~AAV3~~									
~~AAV4~~	~~736~~	~~1306~~	~~1021~~	~~AAV4~~								
~~AAV5~~	~~950~~	~~1520~~	~~1235~~	~~215~~	~~AAV5~~							
~~AAV6~~	~~36~~	~~534~~	~~249~~	~~772~~	~~987~~	~~AAV6~~						

~~AAV7~~	~~277~~	~~292~~	~~**8**~~	~~1013~~	~~1228~~	~~241~~	~~AAV7~~					
~~AAV8~~	~~381~~	~~189~~	~~96~~	~~1117~~	~~1332~~	~~345~~	~~104~~	~~AAV8~~				
~~AAV9~~	~~**5**~~	~~585~~	~~280~~	~~741~~	~~955~~	~~31~~	~~272~~	~~376~~	~~AAV9~~			
~~AAV10~~	~~191~~	~~379~~	~~94~~	~~927~~	~~1142~~	~~155~~	~~86~~	~~190~~	~~186~~	~~AAV10~~		
~~AAV11~~	~~299~~	~~869~~	~~584~~	~~436~~	~~651~~	~~335~~	~~577~~	~~681~~	~~304~~	~~490~~	~~AAV11~~	
~~AAV12~~	~~820~~	~~250~~	~~535~~	~~1555~~	~~1770~~	~~784~~	~~542~~	~~439~~	~~815~~	~~629~~	~~1119~~	~~AAV12~~
~~AAVRh10~~	~~169~~	~~401~~	~~116~~	~~905~~	~~1119~~	~~133~~	~~109~~	~~212~~	~~164~~	~~22~~	~~468~~	~~651~~

	AAV1											
AAV2	570	AAV2										
AAV3	285	285	AAV3									
AAV4	736	1306	1021	AAV4								
AAV5	950	1520	1235	215	AAV5							
AAV6	36	534	249	772	987	AAV6						
AAV7	277	292	**8**	1013	1228	241	AAV7					
AAV8	381	189	96	1117	1332	345	104	AAV8				
AAV9	**5**	585	280	741	955	31	272	376	AAV9			
AAV10	191	379	94	927	1142	155	86	190	186	AAV10		
AAV11	299	869	584	436	651	335	577	681	304	490	AAV11	
AAV12	820	250	535	1555	1770	784	542	439	815	629	1119	AAV12
AAVRh10	169	401	116	905	1119	133	109	212	164	22	468	651

Table 5: Mass Differences of VP2 among 13 AAV Isotypes

	~~AAV1~~											
~~AAV2~~	~~395~~	~~AAV2~~										
~~AAV3~~	~~226~~	~~169~~	~~AAV3~~									
~~AAV4~~	~~467~~	~~862~~	~~693~~	~~AAV4~~								
~~AAV5~~	~~810~~	~~1205~~	~~1036~~	~~343~~	~~AAV5~~							
~~AAV6~~	~~**2**~~	~~392~~	~~224~~	~~470~~	~~812~~	~~AAV6~~						
~~AAV7~~	~~278~~	~~116~~	~~52~~	~~746~~	~~1088~~	~~276~~	~~AAV7~~					
~~AAV8~~	~~425~~	~~31~~	~~199~~	~~893~~	~~1235~~	~~423~~	~~147~~	~~AAV8~~				
~~AAV9~~	~~117~~	~~278~~	~~109~~	~~584~~	~~927~~	~~115~~	~~161~~	~~308~~	~~AAV9~~			
~~AAV10~~	~~177~~	~~217~~	~~49~~	~~645~~	~~987~~	~~175~~	~~101~~	~~248~~	~~60~~	~~AAV10~~		
~~AAV11~~	~~299~~	~~694~~	~~525~~	~~168~~	~~511~~	~~301~~	~~578~~	~~725~~	~~416~~	~~476~~	~~AAV11~~	
~~AAV12~~	~~812~~	~~417~~	~~586~~	~~1279~~	~~1622~~	~~810~~	~~533~~	~~386~~	~~695~~	~~635~~	~~1111~~	~~AAV12~~
~~AAVRh10~~	~~160~~	~~235~~	~~66~~	~~627~~	~~970~~	~~157~~	~~119~~	~~266~~	~~43~~	~~18~~	~~459~~	~~652~~

	AAV1											
AAV2	395	AAV2										
AAV3	226	169	AAV3									
AAV4	467	862	693	AAV4								
AAV5	810	1205	1036	343	AAV5							
AAV6	**2**	392	224	470	812	AAV6						
AAV7	278	116	52	746	1088	276	AAV7					
AAV8	425	31	199	893	1235	423	147	AAV8				
AAV9	117	278	109	584	927	115	161	308	AAV9			
AAV10	177	217	49	645	987	175	101	248	60	AAV10		
AAV11	299	694	525	168	511	301	578	725	416	476	AAV11	

AAV12	812	417	586	1279	1622	810	533	386	695	635	1111	AAV12
AAVRh10	160	235	66	627	970	157	119	266	43	18	459	652

Table 6: Mass Differences of VP3 among 13 AAV Isotypes

	~~AAV1~~											
~~AAV2~~	~~457~~	~~AAV2~~										
~~AAV3~~	~~332~~	~~125~~	~~AAV3~~									
~~AAV4~~	~~12~~	~~445~~	~~320~~	~~AAV4~~								
~~AAV5~~	~~54~~	~~511~~	~~386~~	~~66~~	~~AAV5~~							
~~AAV6~~	~~**2**~~	~~455~~	~~330~~	~~**10**~~	~~56~~	~~AAV6~~						
~~AAV7~~	~~416~~	~~673~~	~~748~~	~~428~~	~~362~~	~~418~~	~~AAV7~~					
~~AAV8~~	~~288~~	~~169~~	~~44~~	~~276~~	~~342~~	~~286~~	~~704~~	~~AAV8~~				
~~AAV9~~	~~216~~	~~241~~	~~116~~	~~204~~	~~270~~	~~214~~	~~632~~	~~72~~	~~AAV9~~			
~~AAV10~~	~~121~~	~~336~~	~~211~~	~~109~~	~~175~~	~~119~~	~~537~~	~~167~~	~~95~~	~~AAV10~~		
~~AAV11~~	~~179~~	~~278~~	~~153~~	~~167~~	~~233~~	~~177~~	~~595~~	~~109~~	~~37~~	~~58~~	~~AAV11~~	
~~AAV12~~	~~329~~	~~128~~	~~**3**~~	~~317~~	~~383~~	~~327~~	~~745~~	~~41~~	~~113~~	~~208~~	~~150~~	~~AAV12~~
~~AAVRh10~~	~~117~~	~~340~~	~~215~~	~~105~~	~~171~~	~~115~~	~~533~~	~~171~~	~~99~~	~~**4**~~	~~62~~	~~212~~

	AAV1											
AAV2	457	AAV2										
AAV3	332	125	AAV3									
AAV4	12	445	320	AAV4								
AAV5	54	511	386	66	AAV5							
AAV6	**2**	455	330	**10**	56	AAV6						
AAV7	416	673	748	428	362	418	AAV7					
AAV8	288	169	44	276	342	286	704	AAV8				
AAV9	216	241	116	204	270	214	632	72	AAV9			
AAV10	121	336	211	109	175	119	537	167	95	AAV10		
AAV11	179	278	153	167	233	177	595	109	37	58	AAV11	
AAV12	329	128	**3**	317	383	327	745	41	113	208	150	AAV12
AAVRh10	117	340	215	105	171	115	533	171	99	**4**	62	212

[0193] No masses within 10 Da of all three VPs between two isotypes are observed. Even though both VP2 and VP3 have only a 2 Da difference between AAV1 and AAV6, the mass difference of VP1 between AAV1 and AAV6 is 36, significant enough to be distinguished by an accurate mass measurement. Therefore, intact protein measurement of VP1, VP2 and VP3 is highly specific as an identity test.

[0194] These results demonstrate that intact protein analysis and LC/MS/MS can be used to profile VPs to monitor VP expressions, posttranslational modifications, and truncations and to ensure product consistency during VLP production. These two analyses can also be used to

confirm site-direct mutagenesis or structural characterization for capsid protein engineering applications.

Example 2: The role of N terminal acetylation of AAV capsid proteins

[0195] Chemical modifications of cellular proteins are a common means of controlling their functions (Arnesen, T. (2006) *Virology* 353(2): 283–293). N-terminal acetylation (Nt-acetylation), which involves the transfer of an acetyl group from acetyl coenzyme A to the α-amino group of the first amino acid residue of a protein (Brown, J.L. and Roberts, W.K. (1976) *J Biol Chem* 251: 1009–1014; Arnesen, T. *et al.* (2009) *Proc Natl Acad Sci U S A* 106: 8157–8162), is among the most abundant of protein modifications. Unlike most other protein modifications, Nt-acetylation is irreversible; it occurs mainly during the synthesis of the protein, catalyzed by N-terminal acetyltransferases (NATs) associated with ribosomes (Gautschi, M. *et al.* (2003) *Mol Cell Biol* 23: 7403–7414; Pestana, A. and Pitot, H.C. (1975) *Biochemistry* 14: 1404–1412; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97). There are several distinct NATs in eukaryotes—NatA-NatF—each composed of one or more subunits and each acetylating a specific subgroup of N-termini depending on the amino acid sequence of the first few amino acids (Jornvall, H. (1975) *J Theor Biol* 55: 1–12; Persson, B. *et al.* (1985) *Eur J Biochem* 152: 523–527).

[0196] Experimental data indicate that proteins with acetylated N-termini are more stable *in vivo* than non-acetylated proteins; *i.e.*, Nt-acetylation protects proteins from degradation (Hershko, A. *et al.* (1984) *Proc Natl Acad Sci U S A* 81: 7021–7025). One explanation for this might be the discovery in 2004 that another N-terminal modification, ubiquitination, which involves direct attachment of the small protein ubiquitin to the N-terminal amino acid residue, promotes the subsequent degradation of the protein (Ben Saadon, R. *et al.* (2004) *J Biol Chem* 279: 41414–41421). Conversely, the Nt-acetylation signals can also be part of a quality control mechanism to degrade unfolded or misfolded proteins and to regulate *in vivo* protein stoichiometries (Hwang, C.S. *et al.* (2010) *Science* 327: 973–977).

[0197] A systematic analysis of the predicted N-terminal processing of cytosolic proteins, versus those destined to be sorted to the secretory pathway, revealed that the cytosolic proteins were profoundly biased in favor of processing, but there is an equal and opposite bias against

such modification for secretory proteins (Forte, G.M.A. *et al.* (2011) *PLoS Biology*, 4 May 2011 Volume 9). Mutations in secretory signal sequences that lead to their acetylation result in mis-sorting to the cytosol in a manner that is dependent upon the N-terminal processing machinery. Hence N-terminal acetylation represents an early determining step in the cellular sorting of nascent polypeptides that represent an extra layer of stringency in order to ensure that proteins destined to remain in the cytosol actually reside in the cytosol. The eukaryotic cell comprises several distinct compartments, called organelles, required to perform specific functions. The proteins in these compartments are synthesized in the cytoplasm and so require complex sorting mechanisms to ensure their delivery to the appropriate organelle. Proteins are modified by acetylation of their amino terminus at a very early stage in their synthesis. There is a profound difference between the likelihood of such a modification on cytoplasmic proteins and on those destined for one of the major organelles, the endoplasmic reticulum (ER): whereas cytoplasmic proteins are typically acetylated, those bound for the ER are largely unmodified. Moreover, when specific ER proteins are engineered to induce their acetylation their targeting to the ER was inhibited (Forte, G.M.A. *et al.* (2011) *PLoS Biology*, 4 May 2011 Volume 9).

[0198] The contractile proteins actin and tropomyosin have been shown to require NatB-mediated Nt-acetylation for proper function, specifically involving actin-tropomyosin binding and actomyosin regulation (Coulton, A.T. *et al.* (2010) *J Cell Sci* 123: 3235–3243; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97). Thus Nt acetylation of AAV capsid proteins may have importance in the transduction potential of rAAV vectors. If AAV vectors fail to gain entry into the nucleus, they consequently fail to transduce cells. The role of actin filaments and FKBP52 (FK506-binding protein p52) in the translocation of AAV capsids from the endosome to the nucleus is well defined (Zhao, W. *et al.* (2006) *Virology* 353(2): 283–293). Importantly, Nt-acetylation is essential for the functioning of actin filaments by modulating protein–protein interactions (Coulton, A.T. *et al.* (2010) *J Cell Sci* 123: 3235–3243; Polevoda, B. *et al.* (2003) *J Biol Chem* 278: 30686–97).

[0199] Though N-terminal acetylation of proteins is a widely known phenomenon, the biological significance of Nt-acetylation on AAV capsid proteins is not well understood. The predicted N-termini of VP1 and VP3 based on DNA sequencing are both methionine followed by alanine. It has been reported that removal of N-terminal methionine by Met-aminopeptidases frequently leads to Nt-acetylation of the resulting N-terminal alanine, valine, serine, threonine,

and cysteine residues and that the acetylation of the N-terminus acts as a potential degradation signal [21]. Ubiquitination of viral capsid proteins was suggested as a potential signal for processing of the capsid at the time of virion disassembly [22]. The link between N-acetylation of VP1 and VP3 and viral capsid degradation and uncoating before the nuclear entry is further investigated.

[0200] To understand the functional implications of N-terminal acetylation with regard to AAV capsid proteins, site-directed mutagenesis of VP3 N-terminal initiation codons is used to generate AAV mutants.

Methods

[0201] AAV capsid proteins are generated with differing amino acids at the 2nd position to the initiating methionine (iMet X) to determine if Nt-acetylation is inhibited or reduced, and the functional consequences are then measured. The ability of the capsid proteins to be trafficked intra-cellularly and/or to acquire post translational modifications such as glycosylation is assessed, and whether this ability affects the infectivity of the assembled AAV particle is subsequently determined. In addition, the impact of acetylation on ubiquitination/degradation and targeting to the lysosome, ER, Golgi, or inner nuclear membrane is determined.

[0202] For example, to assay trafficking or targeting, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are fluorescently labeled and used to infect cells (*e.g.,* HeLa cells). These AAV particles are assayed for one or more of: time of viral particle uptake, colocalization of AAV particles with specific compartmental markers (*e.g.,* Golgi, ER, or lysosomal proteins or other markers), nuclear accumulation (*e.g.,* as assayed by colocalization with a nuclear marker or stain), and/or sensitivity of trafficking to specific inhibitors of early endosomal escape (such as bafilomycin A or ammonium chloride), as compared to fluorescently labeled wild-type AAV particles used to infect the same cell line (see, *e.g.,* Bartlett, J.S. *et al.* (2000) *J. Virol.* 74:2777-2785 for a description of such assays).

[0203] To assay infectivity, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (*e.g.,* HeLa cells), and their transduction efficiency is compared to wild-type AAV particles (*e.g.,* having the same AAV serotype and infecting the same type of cells).

[0204] To assay glycosylation, AAV particles with capsid proteins having a mutated 2nd position (e.g., iMet X) are used to infect cells (*e.g.,* HeLa cells). AAV particles from infected cells are subjected to one or more assays including without limitation chemical detection of glycosylation (*e.g.,* applying a commercially available digoxigenin (DIG) glycan detection and/or fluorescent glycoprotein detection kit on denatured and electrophoretically separated capsid proteins) and mass spectrometry (*e.g.,* FT-ICR MS), as compared to wild-type AAV particles used to infect the same cell line (see, *e.g.,* Murray, S. *et al.* (2006) *J. Virol.* 80:6171-6176 for a description of such assays).

[0205] To assay ubiquitination, AAV particles with capsid proteins having a mutated 2nd position are used to infect cells (*e.g.,* HeLa cells). AAV particles are immunoprecipitated from infected cells with an anti-capsid antibody, then subjected to Western blotting with an anti-ubiquitin antibody and compared to wild-type AAV particles used to infect cells in the same manner. Mutant AAV particles may also be used in *in vitro* ubiquitination assays, as compared to wild-type AAV particles (see, *e.g.,* Yan, Z. *et al.* (2002) *J. Virol.* 76:2043-2053).

Example 3: The role of deamidation of AAV2 capsid proteins

[0206] Sequence analysis of the AAV2 capsid protein revealed potential deamidation sites, as underlined in the following amino acid sequence:

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYK***YLGPFNGLDKGEPVNEAD AAALEHDKAYDRQLDSGDNPYLKYNHADA***EFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTA PGKKRPVEHSPVEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGA PMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTP WGYFDFNRFHCHFSPRDWQRLINNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEY QLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVP FHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSK TSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKTNVDIEKVMIT DEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGPIWAKIPHTDGHFH PSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQYTS NYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO:3).

[0207] In particular, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site), as bolded and italicized in the above sequence. The following experiments were aimed at exploring whether deamidation at N57 can lead to reduced potency

and/or truncation of AAV2, as well as whether different AAV production methods may have different effects on deamidation. For example, the producer cell line method (see Martin *et al.*, (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X.L. *et al.* (1999) *Gene Ther.* 6:293-299) may induce a higher level of deamidation at N57, as compared to the triple transfection method. According to the crystal structure of AAV2, N57 is not shown; however, N382 and N511 are partially exposed, and N715 is fully exposed.

Methods

Enzymatic digestions of AAV1 and AAV2 VPs

[0208] 10 µg of each AAV1-EGFP or AAV2-EGFP material (generated from triple transfection as well as producer cell line process) were concentrated using Amicon filters (10 kDa MWCO), denatured with 6 M Guanidine-HCl, 50 mM Tris at pH 8.5. The proteins were reduced with 5 mM DTT at 60 °C for 30 minutes in darkness, alkylated with 15 mM iodoacetamide at room temperature for 30 minutes, and then buffer exchanged into 25 mM Tris pH 7.1 for digestion using Bio-Spin® 6 Tris micro-columns. After buffer exchange, the samples were split into two aliquots. Each aliquot was digested with trypsin at 1:25 or Asp-N at 1:50 enzyme: protein ratio (wt/wt) for 2 hours at 37 °C, respectively.

UPLC/MS/MS peptide mapping

[0209] The protein digests were also analyzed by UPLC/MS/MS in Acquity UPLC-Xevo qTOF MS. BEH300 C18 column (2.1 × 150 mm) was used for separation in the mobile phases with 0.1% formic acid in water/acetontitrile gradient at a flow rate 0.25 ml/min. The mass spectra were acquired in the positive MSe resolution mode in the mass range of 50-2000.

Determination of deamidation levels in AAV VPs

[0210] The extracted ion chromatograms (XIC) of peptides containing NG sites (T9, T49, and T67 in AA1 and AAV2 VP) and their corresponding deamidated species were used for calculation of deamidation levels.

[0211] In order to compare AAV vectors produced by the triple transfection (TTx) and producer cell line (PCL) methods, AAV1 or AAV2 tagged with EGFP was produced using the

TTx or PCL method. Truncated VP1 (tVP1) was found to be present in AAV2-EGFP produced by PCL, but not in the AAV2-EGFP produced by TTx. AAV1-EGFP was not found to have tVP1, regardless of the production method. The *in vitro* potency of AAV2 produced by the PCL method was also found to be reduced, as compared to AAV2 produced by TTx. Mutant N57K and N57Q AAV2 particles were also found to have reduced potency and disrupted Ca++ binding.

[0212] The following table provides the tryptic peptides that were analyzed to examine each potential deamidation site, as well as the corresponding residue.

Table 7: Tryptic peptides containing NG sites

Peptide (NG sequence underlined)	**Residue**
YLGPF**NG**LDK (SEQ ID NO: 9)	N57
EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMV PQYGYLTLN**NG**SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10)	N382
YNL**NG**R (AAV1) (SEQ ID NO: 11)	N511
YHL**NG**R (AAV2) (SEQ ID NO: 12)	N511
SANVDFTVDN**NG**LYTEPR (AAV1) (SEQ ID NO: 13)	N715
SVNVDFTVDT**NG**VYSEPR (AAV2) (SEQ ID NO: 14)	N715

[0213] As shown in Table 7, the T9 peptide YLGPF**NG**LDK (SEQ ID NO: 9) was used to monitor N57, the T38 peptide EVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTL N**NG**SQAVGRSSFYCLEYFPSQMLR (SEQ ID NO: 10) was used to monitor N382, the T49 peptides YNL**NG**R (SEQ ID NO: 11) and YHL**NG**R (SEQ ID NO: 12) were used to monitor N511 in AAV1 or AAV2 (respectively), and the T67 peptides SANVDFTVDN**NG**LYTEPR (SEQ ID NO: 13) and SVNVDFTVDT**NG**VYSEPR (SEQ ID NO: 14) were used to monitor N715 in AAV1 or AAV2 (respectively).

[0214] LC/MS/MS analysis was used to compare the percentage of deamidation in AAV1 and AAV2 particles produced by the TTx and PCL methods. The results from the T9 peptide are shown in **FIGS. 6A & 6B**. The results from the T49 peptide are shown in **FIGS. 7A & 7B**. The

results from the T67 peptide are shown in **FIGS. 8A & 8B**. These results are summarized in Table 8. The T38 peptide was not detected due to its size.

Table 8: Summary of LC/MS/MS results

		% Deamidation		
		N57	**N511**	**N715**
AAV1	**TTx**	7.9	30.9	18.1
	PCL	11.3	27.4	18.7
AAV2	**TTx**	6.7	39.6	27.4
	PCL	18.4	42.3	28.0

[0215] In particular, AAV2 produced by PCL showed nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx. These results suggest that deamidation decreases AAV potency, as the *in vitro* potency of AAV2 produced by PCL is reduced.

Conclusions

[0216] Taken together, Examples 1-3 demonstrate methods for analyzing intact proteins of viral particles (*e.g.*, AAV capsid proteins) using LC/MS. Molecular weights were measured accurately, and these techniques may be also used to assess N-termini and/or modifications of viral capsid proteins. Moreover, these methods are adaptable as capsid serotype identity assays useful in gene therapy, *e.g.*, as an analytical platform. These results further establish a correlation between capsid protein structure (*e.g.*, truncations, deamidation, etc.) and potency, suggesting that point mutations at key sites may be used to engineer more effective vectors.

Example 4: Elucidating the role of N terminal acetylation of AAV capsid proteins

[0217] As discussed above, the N-termini of AAV capsid proteins are highly conserved across serotypes (**FIG. 5**). The techniques described in Example 1 allow for interrogation of VP expression and posttranslational modifications. The role and biological significance of N-terminal acetylation of AAV capsid proteins was next examined.

Results

[0218] To elucidate the potential role of deacetylation of AAV capsid proteins, AAV5 deacetylation variants were tested. An AAV5 particle expressing eGFP under the CBA promoter

(*AAV5-CBA-Egfp*) was compared to AAV5 variants with the amino acid adjacent to the initiating methionine (iMET) mutated for VP1 and VP3 (*deAC-AAV5-CBA-eGFPs*). Three amino acids predicted to have a low likelihood of acetylation by NatA, NatC, or NatD were chosen for generating variants: Gly, Leu, and Pro, as illustrated in Table 9 below.

Table 9: N-terminal acetylation frequency

N-term aa	Transferase	NT-AC FREQUENCY
MET-ALA *Normally found in VP1 & VP3*	NatA	High
MET-SER *Normally found in VP1 & VP3 for AAV5*	NatA	High
AAV variants		
MET-GLY	NatA	Low
MET-LEU	NatC	Low
MET-PRO	NatD/other	Low

[0219] The following AAV5 deacetylated (deAC) mutants were generated:

S2G*VP1* – Ser changed to Gly at position 2 in AAV5VP1
S2L*VP1* – Ser changed to Leu at position 2 in AAV5VP1
S2P*VP1* – Ser changed to Pro at position 2 in AAV5VP1
S2G*VP3* – Ser changed to Gly at position 2 in AAV5VP3
S2L*VP3* – Ser changed to Leu at position 2 in AAV5VP3
S2P*VP3* – Ser changed to Pro at position 2 in AAV5VP3
S2P*VP1/VP3* – Ser changed to Pro at position 2 in both AAV5 VP1 and VP3
S2G*VP1/VP3* – Ser changed to Gly at position 2 in both AAV5 VP1 and VP3
S2L*VP1/VP3* – Ser changed to Leu at position 2 in both AAV5 VP1 and VP3

[0220] These variants were generated using the TTX method as described above. All AAV5 variants showed good productivity, with yields greater than 10^{13} total VG. All AAV5 variants also showed the expected VP1:VP2:VP3 protein ratio by SYPRO protein gel analysis (**FIG. 9**). Next, LC/MS was used to confirm that all AAV5 variants had decreased acetylation, as shown in Table 10.

Table 10: LC/MS analysis of AAV5 variant acetylation

	mutants	VP1 Theo.	VP1 Exp.	Δmass (VP1)	VP2 Theo.	VP2 Exp.	Δmass (VP2)	VP3 Theo.	VP3 Exp.	Δmass (VP3)	note
1	*deAC-AAV5 (S2GVP1)/ CBA-eGFP*	80234	nd		65283	65293	10	59463	59472	9	VP1 not detectable
2	*deAC-AAV5 (S2LVP1)/ CBA-eGFP*	80346	80501	181	65283	65292	9	59463	59471	8	VP1 incorrect
3	*deAC-AAV5 (S2GVP3)/ CBA-eGFP*	80234	nd		65253	65261	8	59391	59398	7	confirmed
4	*deAC-AAV5 (S2LVP3)/ CBA-eGFP*	80336	80363	27	65309	65309	0	59447	59620	173	VP3 incorrect
5	*deAC-AAV5 (S2PVP1VP3)/ CBA-eGFP*	80314	80324	10	65293	65300	7	59431	59438	7	confirmed
6	*deAC-AAV5 (S2GVP1VP3)/ CBA-eGFP*	80234	80243	9	65253	65261	8	59391	59398	7	confirmed
7	*deAC-AAV5 (S2PVP3)/ CBA-eGFP*	80336	80346	10	65293	65292	1	59431	59430	1	confirmed
8	*deAC-AAV5 (S2PVP1)/ CBA-eGFP*	80314	80313	1	65283	65291	8	59463	59470	7	confirmed
9	*deAC-AAV5 (S2L VP1VP3)/ CBA-eGFP*	80346	nd		65309	65318	9	59447	59629	182	VP3 incorrect

nd=not determined

[0221] These LC/MS analyses confirmed that AAV5 variants were deacetylated. The variants S2L*VP1*, S2L*VP3*, and S2L*VP1/VP3* all showed increased mass (increased from 173 to 182) in VP1 and VP3 proteins, suggesting that changing the second N-terminal amino acid to a leucine in VP1 or VP3 alters the protein, resulting in an increase in mass.

[0222] Next, AAV5 variants were assayed in an *in vitro* transduction assay using eGFP as a reporter gene (**FIG. 10**). The assay was designed to evaluate transduction by AAV5 deacetylated mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV5 particle. Three cell lines were used: 293, HuH7, and HeLa cells.

Following infection, cells were assayed to determine vector genome copy number (vg/µg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/µg protein) represents the efficiency at which the AAV5 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (**FIG. 10**). Vector genomes were quantified by TaqMan analysis.

[0223] **FIG. 11** shows that, based on vector genome analyses, AAV5 deacetylated mutant vectors infected all three test cell lines at similar, but reduced, levels as compared to the parental unmodified AAV5 particles. **FIG. 12** shows that AAV5 deacetylated mutant vectors all resulted in reduced eGFP expression in all three cell lines, as compared to transduction with parental unmodified AAV5.

Conclusions

[0224] As predicted, no acetylation was observed in N-terminal Ser to Pro/Leu/Gly mutant variants when examined by LC/MS. AAV5 deAC variants showed robust vector production, and AAV5 deAC variants infected cells at levels comparable to parental AAV5. However, functional protein levels in cells infected with deAC variants were greatly reduced when compared to the parental AAV5. These data suggest that tropism is minimally affected by a lack of N-terminal deacetylation in VP1/VP3, but downstream processing (*e.g.*, trafficking and/or degradation) is significantly affected. Since the variants tested demonstrated reduced *in vitro* activity, one of skill in the art may appreciate that variants characterized by reduced or eliminated acetylation could be employed, *inter alia*, when decreased levels of transduction are desirable.

Example 5: Assessment of deamidation of AAV capsid proteins

[0225] Examples 1 and 3 demonstrate techniques that allow the interrogation of post-translational modifications of AAV capsid proteins and explore the role of deamidation of the AAV2 capsid. The following Example tested whether deamidation reduces potency and/or induces truncation of capsid proteins, and whether different manufacturing processes can induce different levels of deamidation.

Methods

AAV particles were generated and deamidation status assayed as described in Example 3.

Results

[0226] As described in Example 3, a potential deamidation site is found at N57/G58 in the phospholipase A2 domain (Ca++ binding site) in VP1 of the AAV2 capsid. The N57/G58 motif is conserved across AAV serotypes (**FIG. 13**). Example 3 showed that AAV2 produced by PCL exhibited nearly a 3-fold increase in deamidation as compared to AAV2 produced by TTx (see **FIGS. 6A & 6B** and Table 8).

[0227] In examining VP1, VP2, and VP3 production by protein gels, a truncated VP1 protein (tVP1) was detected only in AAV2 capsid proteins produced by the PCL method (**FIG. 14**).

[0228] A series of AAV2 deamidation mutants was generated next. These mutants targeted the Gly residue in the canonical NG sequence. Mutations targeting the A35 residue (see **FIG. 13**), the N-terminal amino acid for tVP1 were also generated, as shown in Table 11. The pAF277 and pAF279 mutants bearing multiple mutations did not package.

Table 11: Deamidation mutants

Name	mutation	avg drp/cell
pAF274	G58K	4.54E+03
pAF275	G58D	5.00E+03
pAF276	G58Q	5.41E+03
pAF277	*G58,383,512,716K*	*1.2*
pAF278	A35N	6.89E+03
pAF279	*A35N, G58,383,512,765K*	*2.2*
293	-	0.9
PIM45	Control	6.28E+03

K = positive charge (basic)
D = negative charge (acidic)
Q = polar

[0229] Deamidation of variants were next analyzed by LC/MS as described in Example 3 above. The AAV2*A35N* and AAV2*G58D* variants had altered deamidation as compared to the

parental AAV2 (**FIG. 15**). In particular, the AAV2*A35N* mutant had increased deamidation (17.8%) as compared to parental AAV2 (5.7%). The AAV2*G58D* variant had reduced deamidation (1.1%) as compared to parental AAV2. SYPRO protein gel analysis demonstrated that the AAV2 deamidation mutants exhibited the correct VP1:VP2:VP2 ratio (**FIG. 16**).

[0230] Next, AAV2 deamidation variants were assayed in an *in vitro* transduction assay using eGFP as a reporter gene (**FIG. 17**). The assay was designed to evaluate transduction by AAV2 deamidation mutant variants at 10^6 multiplicity of infection (MOI), comparing each variant to the parental, unmodified AAV2 particle. Three cell lines were used: 293, HuH7, and HeLa cells. Following infection, cells were assayed to determine vector genome copy number (vg/μg cellular protein) and eGFP expression (by ELISA). Vector genome copy number (vg/μg protein) represents the efficiency at which the AAV2 variant enters the cell, and eGFP represents the efficiency of capsid intracellular trafficking, since transgene expression requires the capsid/vector DNA to efficiently traffic to the nucleus (**FIG. 17**). Vector genomes were quantified by TaqMan analysis.

[0231] Vector genome analysis indicated that AAV2 deamidation mutant variants infected all cell lines tested at levels comparable to that of parental AAV2 vectors (**FIG. 18**). Importantly, the AAV2*A35N* variant was found to be more potent than the parental AAV2 vector for transduction in all three cell lines (**FIG. 19**). The AAV2*G58D* variant was found to be more potent than the parental AAV2 vector in HuH7 cells (**FIG. 19**).

Conclusions

[0232] In summary, AAV2 deamidation mutant vectors infect cells at levels comparable to the parent AAV2 particles (*e.g.*, comparable vg/μg cellular protein). However, based on analysis of eGFP levels in transduced cells, the AAV2*A35N* variant had higher potency than the parental AAV2 in all cell lines tested, and the AAV2*G58D* variant had higher potency than the parental AAV2 in HuH7 cells (a liver-derived cell line). These results suggest that the A35N mutation may be effective in increasing vector potency for transducing many cell types, and that the G58D mutation may also be effective in increasing potency in certain cell types, *e.g.*, liver cells.

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SEQUENCES

All polypeptide sequences are presented N-terminal to C-terminal unless otherwise noted.

All nucleic sequences are presented 5' to 3' unless otherwise noted.

Nucleotide sequence of potential AAV2 VP3 initiation codons (ATG codons underlined)

ATGGCTACAGGCAGTGGCGCACCAATGGCAGAC (SEQ ID NO:1)

Polypeptide sequence corresponding to potential AAV2 VP3 initiation codons (methionines underlined)

MATGSGAPMAD (SEQ ID NO:2)

AAV2 VP1 polypeptide sequence

MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGYKYLGPFNGLDKGEPVNEADAAALEHDK
AYDRQLDSGDNPYLKYNHADAEFQERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSPVEPD
SSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGTNTMATGSGAPMADNNEGADGVGNSSGNWHCD
STWMGDRVITTSTRTWALPTYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLINNNWGFRP
KRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQLPYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQ
AVGRSSFYCLEYFPSQMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNTPSGTTTQSRLQF
SQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEYSWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGV
LIFGKQGSEKTNVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGVLPGMVWQDRDVYLQGP
IWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKNTPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKR
WNPEIQYTSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL (SEQ ID NO:3)

VP1 N-terminal tryptic peptide (N-terminal alanine is acetylated)

AADGYLPDWLEDTLSEGIR (SEQ ID NO:4)

VP3 N-terminal Asp-N peptide (N-terminal alanine is acetylated)

ATGSGAPM (SEQ ID NO:5)

Common VP1 N-terminal sequence

MAADGYLPDWLED (SEQ ID NO:6)

Nucleotide sequence of potential AAV7 VP3 initiation codons (start codons underlined)

GTGGCTGCAGGCGGTGGCGCACCAATGGCAGACAATAAC (SEQ ID NO:7)

Nucleotide sequence of mutated ITR

CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGGGC
G (SEQ ID NO:8)

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Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	15979-20141.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-7,588,772-B2		09-15-2009	Kay et al.	
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	4.	US-8,137,948-B2		03-20-2012	Qu et al.	
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	6.	US-9,169,299-B2		10-27-2015	Lisowski et al.	

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Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	7.	US-2011/0275529-A1		11-10-2011	Heilbronn, R.	
	8.	US-2012/0164106-A1		03-15-2012	Kay et al.	
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Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	11.	WO-2003/042397-A2			05-22-2003	The Trustees of the University of Pennsylvania		
	12.	WO-2004/027019-A2			04-01-2004	University of Florida Research Foundation		
	13.	WO-2010/148143-A1			12-23-2010	Genzyme Corporation		
	14.	WO-2013/158879-A1			10-24-2013	The Children's Hospital of Philadelphia		

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
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EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2019-07-23
Name/Print	Brian A. Donahue	Registration Number	58,206

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Brian Arthur Donahue/Aurelia Caparas
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
CLAIMS IN EXCESS OF 20	1615	2	100	200
INDEPENDENT CLAIMS IN EXCESS OF 3	1614	11	460	5060
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**5460**

Electronic Acknowledgement Receipt

EFS ID:	36662812
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
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Attorney Docket Number:	15979-20141.00
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Payment Type	DA
Payment was successfully received in RAM	$5460
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	Multipart Description/PDF files in .zip description				
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	Preliminary Amendment		1	1	
	Specification		2	2	
	Claims		3	9	
	Applicant Arguments/Remarks Made in an Amendment		10	11	
Warnings:					
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Warnings:					
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3	Specification	Sub_Spec_-_Marked-up_copy.pdf	537390 3754627ad5fd281c3821b9bdae3c60bbcb021bdd	no	99
Warnings:					
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4	Information Disclosure Statement (IDS) Form (SB08)	IDS_SB08.pdf	167520 84d6ed54d3036093825283dfea8fb8d3912ae449	no	6
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5	Foreign Reference	WO_2003_042397_A2.pdf	20678974 7e10419a60942cf89835b58169759c41af6d0ada	no	461
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6	Foreign Reference	WO_2004_027019_A2_Part1.pdf	15293089 202d4b12bdbcf014f5d6f7ea5955ac71827b0dc7	no	90
Warnings:					
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7	Foreign Reference	WO_2004_027019_A2_Part2.pdf	11224396 01192759a77a39676900b884d9fd8291cd0a5741	no	90
Warnings:					
Information:					
8	Foreign Reference	WO_2010_148143_A1.pdf	3329252 e9f0c0a3b720edfa9e8b480b3327ded45b342821	no	63
Warnings:					
Information:					
9	Foreign Reference	WO-2013_158879_A1.pdf	7498336 0daac765baaff92aba66aa927437c29a54f8530c	no	60
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Information:					
10	Non Patent Literature	AHMADIANKIA_2013_813-821.pdf	1028248 4837f5f2d0454bb20e6e021b5ae6ed489d2ad84d	no	9
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Information:					
11	Non Patent Literature	ARNESEN_2009_8157-8162.pdf	659638 88271294bd6ebfb1e0f89b2e127646f06bf45116	no	6
Warnings:					
Information:					

12	Non Patent Literature	ASOKAN_2006_8961-8969.pdf	700350 25ef54bbd84c6999f7dcc5b88a81606d7b3711e7	no	9
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13	Non Patent Literature	BARK_2001_1774-1775.pdf	352289 55416f278fb5d9f01c21829dc44b799831dd507e	no	2
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Information:					
14	Non Patent Literature	BARTLETT_2000_2777-2785.pdf	970522 f7f977596b4c16e151db73a4393b9f0c210b0681	no	9
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15	Non Patent Literature	BEN-SAADON_2004_41414-41421.pdf	1310093 7f71352ca33ad647e0ce2c6c5455c3414b0647ee	no	9
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16	Non Patent Literature	BLEKER_2005_2528-2540.pdf	1198503 10d259790fa0fdec3e697f6240025543f1b01624	no	13
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17	Non Patent Literature	BOSSIS_2003_6799-6810.pdf	1132374 a45a0296b7eb5b00721bdb4bf8b9d99a19eb6760	no	12
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18	Non Patent Literature	BROWN_1976_1009-1014.pdf	1508128 5bd650fffed137369a7fe69051060730578c6ddf	no	7
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Information:					

19	Non Patent Literature	COULTON_2010_3235-3243.pdf	2078671 52cab6c6efdf88b5c01a3cdbca8036f7f8173591	no	23
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20	Non Patent Literature	DiMATTA_2012_6947-6958.pdf	1297231 96dcd795eea231a64f3712ba5fdde25f74043eb6	no	12
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21	Non Patent Literature	FORTE_2011_e1001073_13pgs.pdf	2053301 e80f510478c6316ddd0fb6e7128b88bbbbacd65d	no	13
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22	Non Patent Literature	GAO_2002_11854-11856.pdf	955952 609be4c0f38e49f0e9e22b5f5b61b5fe3da8dcf6	no	6
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23	Non Patent Literature	GAO_2003_6081-6086.pdf	644372 d46f01524ce9ec2a173dfc871d61805350d3b876	no	6
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Information:					
24	Non Patent Literature	GAO_2004_6381-6388.pdf	526109 d7b6b022a7d77dca2c0018e5956a7762cffee687	no	8
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25	Non Patent Literature	GAUTSCHI_2003_7403-7414.pdf	2012595 246440562807f532153939505d434596155239a6	no	12
Warnings:					
Information:					

26	Non Patent Literature	GIROD_2002_973-978.pdf	551106 4a98315bbf4103a708323a2ba4d1920d6dc1387c	no	6
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Information:					
27	Non Patent Literature	HERSHKO_1984_7021-7025.pdf	588661 35f508301ba66b45f9dbfa5b2712908d34d93916	no	5
Warnings:					
Information:					
28	Non Patent Literature	HWANG_2010_973-977.pdf	974160 d584af6626ab69ac756d3d629bdd4cdebfc2b7bd	no	13
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Information:					
29	Non Patent Literature	Jornvall_1975_1-12.pdf	8125406 6d9587fd4be8e5654ec9b12cf4e7a656419a2d74	no	12
Warnings:					
Information:					
30	Non Patent Literature	KASHIWAKURA_2005_609-614.pdf	518999 e46622aa1aa09d98824baba10e2b87b23d2b2839	no	6
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Information:					
31	Non Patent Literature	KERN_2003_11072-11081.pdf	704701 f110b77bc1a480b13e594c25a52ec6485f17bcef	no	10
Warnings:					
Information:					
32	Non Patent Literature	KRONENBERG_2005_5296-5303.pdf	582882 5ba89810fd1bbbcd7568d1ac78aa7b8e3d20739e	no	8
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Information:					

33	Non Patent Literature	MAJCHRZYKIEWICZ-KOEHORST_2015_75-83.pdf	674602 7fe3edf64f6338f82b3b018c0b374ca25198d4b0	no	9
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Information:					
34	Non Patent Literature	MURRAY_2006_6171-6176.pdf	616323 8500cc03f857125ef0f3f5483b5a10e0ce1272a7	no	6
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Information:					
35	Non Patent Literature	NAM_2007_12260-12271.pdf	865187 cfdadf9e30fe17b8ae7835a50d3363ca4e59c869	no	12
Warnings:					
Information:					
36	Non Patent Literature	OPIE_2003_6995-7006.pdf	1002111 e0bf5f93002ec59d117f1b3eb9d6cb86508f6c14	no	12
Warnings:					
Information:					
37	Non Patent Literature	PERRY_2008_661-699.pdf	4760554 a3c8753aae80d6570e96c5763ed4a11b6fab0b56	no	39
Warnings:					
Information:					
38	Non Patent Literature	PERSSON_1985_523-527.pdf	839226 4e75c9e12a4e793c673d9b05cbfeddc07a93b995	no	5
Warnings:					
Information:					
39	Non Patent Literature	PESTANA_1975_1404-1412.pdf	6899677 4fdd8d43e42985030cc2711966415a7d3e5f0f9c	no	9
Warnings:					
Information:					

40	Non Patent Literature	PLUMB_2004_2331-2337.pdf	258925 0373918c60e4ef865d05b9a8048bd5b741905ecb	no	7
Warnings:					
Information:					
41	Non Patent Literature	POLEVODA_2003_30686-30697.pdf	1765439 765e393bfe70d3f56ab7ce64ca66436cddbe5e67	no	13
Warnings:					
Information:					
42	Non Patent Literature	POPA-WAGNER_2012_9163-9174.pdf	1106662 e2277e9318094050782404e94fb5b0f62c7b067f	no	12
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43	Non Patent Literature	QING_1999_71-77.pdf	827852 c866f9bfb58e1c8ce3c87646b05865fff783f941	no	7
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Information:					
44	Non Patent Literature	QU_2007_183-192.pdf	1093809 affd815285b4d56005558f64f98585a07a76fbc0	no	10
Warnings:					
Information:					
45	Non Patent Literature	RABINOWITZ_2002_791-801.pdf	1029045 ff91629321b5b6a0847de9258257bc0d3926b3ba	no	11
Warnings:					
Information:					
46	Non Patent Literature	SALGANIK_2012_11877-11885.pdf	932431 4cfe5cab001cbb0400f93b036ebf6abc4dfbf674	no	9
Warnings:					
Information:					

47	Non Patent Literature	SANLIOGLU_2000_9184-9196.pdf	1133019 eeec108dfb0603f847a3cc8d0ab4fdd6b36adf2e	no	13
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Information:					
48	Non Patent Literature	SCHELTEMA_2014_3698-3708.pdf	1791373 a8c30790a8c3ac58b7df14e8f1533c62f79b1de0	no	11
Warnings:					
Information:					
49	Non Patent Literature	SCIGELOVA_2011_M111009431_19pgs.pdf	1954446 11660d25f7d01a1bced16878d6f4730955a2d2d3	no	19
Warnings:					
Information:					
50	Non Patent Literature	SONNTAG_2006_11040-11054.pdf	1575059 ff96f3a9f6aa369cecd4cd6a35adbbcf8b0b5fab	no	15
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Information:					
51	Non Patent Literature	STAHNKE_2011_77-83.pdf	899214 04e3106d6bb6db8c4fee3496552b9b2faa333e5d	no	7
Warnings:					
Information:					
52	Non Patent Literature	SUMMERFORD_1998_1438-1445.pdf	809731 2b068ad415ca109434e30b670e818f1cec15a7bd	no	8
Warnings:					
Information:					
53	Non Patent Literature	SUMMERFORD_1999_78-82.pdf	573695 612d13d40133eeeae33d597b493c7603bf6d55d4	no	5
Warnings:					
Information:					

54	Non Patent Literature	VANVLIET_2009_167-177.pdf	2632722 7a1624604a39bcdd0a0f98b4a16c493a520cd3fa	no	11
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Information:					
55	Non Patent Literature	WANG_2001_1234-1243.pdf	901356 e380559b4ad339a993e451b1cd34f8e0c508d796	no	10
Warnings:					
Information:					
56	Non Patent Literature	THOMAS_.pdf	469994 e13cbc430cc131cbe2c374eed6b06e831881f1e2	no	5
Warnings:					
Information:					
57	Fee Worksheet (SB06)	fee-info.pdf	33886 b33e045b7f359149c302acc70f6685e3add7dc90	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	125277237	

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronic Acknowledgement Receipt

EFS ID:	38653578
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	21-FEB-2020
Filing Date:	
Time Stamp:	16:52:43
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition for review by the Office of Petitions	Petition_Under_181_withdraw_holding_of_abd.pdf	105685 4f1b9e82bc4426d1d82eec8f593fd5e6ee242240	no	4

Warnings:

Information:					
2		Third_prelim.pdf	111687 e9e7f87062dfec0d26b78e26a178fb6003a83cc2	yes	10
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Preliminary Amendment		1	1	
	Claims		2	8	
	Applicant Arguments/Remarks Made in an Amendment		9	10	
Warnings:					
Information:					
3	Miscellaneous Incoming Letter	20190723_filing.pdf	12134908 99bdaa5c88a7c9145e8a113591505b451c87b4ce	no	229
Warnings:					
Information:					
		Total Files Size (in bytes):	12352280		

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

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MAILED

OCT 13 2020

INTERNATIONAL PATENT LEGAL ADM.

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto CA CALIFORNIA 94304

In re Application of :
GENZYME CORPORATION :
U.S. Application No.: 16/325,653 :
PCT Application No.: PCT/US2017/046814 : DECISION ON PETITION
Int. Filing Date: 14 August 2017 : UNDER 37 CFR 1.181
Priority Date: 15 August 2016 :
Attorney Docket No.: 15979-20141.00 :
For: METHODS FOR DETECTING AAV :

This decision is in response to applicant's petition under 37 CFR 1.181 to withdraw the holding of abandonment filed 21 February 2020 in the United States Patent and Trademark Office (USPTO). No petition fee is required.

BACKGROUND

On 14 August 2017, applicant filed international application PCT/US2017/046814 which claimed priority to an earlier application filed 15 August 2016. Pursuant to 37 CFR 1.495, the thirty-month period for paying the basic national fee in the United States was set to expire at midnight on 15 February 2019.

On 14 February 2019 applicant electronically filed for entry into the national stage in the United States, among other items, $1980 in fees including payment of the full U.S. basic national fee; an Application Data Sheet (ADS); a PTO-1390 transmittal letter and a preliminary amendment.

On 23 April 2019, applicant was mailed a notification to filed corrected papers indicating that a substitute specification was required as pages 81 and 82 of the specification contained shading. Applicant was also advised that payment of additional claims fees in the amount of $26,120 was due. Applicant was afforded two months to file a response and advised that this period could be extended pursuant to 37 CFR 1.136(a).

On 23 July 2019, applicant filed a response including a substitute specification; a preliminary amendment and a fee payment of $5460 which included a one month extension of time. Applicant's response was timely filed.

On 19 February 2020, applicant was mailed a "Notification of Abandonment" (Form PCT/DO/EO/909) indicating that the application was abandoned noting that applicant had failed to respond to provide a proper reply to the notification mailed 23 April 2019 within the time period set therein. Specifically, applicant was informed that the status of each claim must be provided after the specific claim numbering by the proper status identifiers. The claim identifier for claims 32-60 was "original."

On 21 February 2020, applicant filed the petition discussed herein arguing that claims 32-60 "were properly indicated as abandoned in the Remarks section of the reply...and respectfully request that the Notice of Abandonment be withdrawn."

DISCUSSION

Applicant contends that the application should not have been held abandoned as the Remarks section of the preliminary amendment indicated that claims 32-60 were cancelled. However, a review of the amended claims filed on 23 July 2019 finds that the amended claim listing does not comply with 37 CFR 1.121. 37 CFR 1.121 (c) states that, "In the claim listing, the status of every claim must be indicated after its claim number by using one of the following identifiers in a parenthetical expression: (Original), (Currently amended), (Canceled), (Withdrawn), (Previously presented), (New), and (Not entered)." (See also the Manual of Patent Examining Procedure (MPEP) section 1893.01(a)(4)). Therefore, the non-compliant amendment could be entered and the claims remain the original claims as filed.

In the present case however the holding of abandonment was improper. The concluding paragraph of the Remarks states that, "Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account no. 03-1952." The only fee not authorized was payment of the issue fee. Applicant therefore timely provided authorization to charge all fees due in connection with the response filed on 23 July 2019 and rather than holding the application abandoned the Office should have charged the required fees as authorized.

As such, it is proper to grant applicant's petition at this time.

It is noted that applicant has filed an additional preliminary amendment concurrently with the petition. In order to avoid abandonment the fees charged must calculated based on the original set of claims. The amended claims filed 21 February 2020 were filed after the expiration of all extendable time. Further, MPEP section 1893.01 (c) provides that, "A subsequently filed amendment canceling claims and/or eliminating multiple dependent claims will not entitle applicant to a refund of fees previously paid. See MPEP §§ 607 and 608."

CONCLUSION

For the reasons detailed above, applicant's petition under 37 CFR 1.181 is **GRANTED**.

The form PCT/DO/EO/909 mailed 19 February 2020 is hereby **VACATED**.

This application is being returned to the Office of PCT Operations, National Stage Processing for continued pre-examination processing including consideration of applicant's 23 July 2019 response and the charging of all required fees based on the original set of claims.

/ Derek A. Putonen /

Derek A. Putonen
Attorney Advisor
International Patent Legal Administration
571-272-3294



United States Patent and Trademark Office

Office of the Chief Financial Officer

Document Code:WFEE

User :72469

Sale Accounting Date:10/14/2020

Sale Item Reference Number	Effective Date
16325653	07/23/2019

Document Number	Fee Code	Fee Code Description	Amount Paid	Payment Method
I20200DD49562382	1614	INDEPENDENT CLAIMS IN EXCESS OF 3	$6,900.00	Deposit Account
I20200DD49562382	1615	CLAIMS IN EXCESS OF 20	$18,400.00	Deposit Account
I20200DD49562382	1616	MULTIPLE DEPENDENT CLAIMS	$820.00	Deposit Account

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/325,653

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	320
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	540
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	800
TOTAL CLAIMS (37 CFR 1.16(i))	22 minus 20 =	* 2			OR	x 100 =	200
INDEPENDENT CLAIMS (37 CFR 1.16(h))	14 minus 3 =	* 11				x 480 =	5280
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						420
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	7560

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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U.S. APPLICATION NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
16/325,653	Xiaoying JIN	15979-20141.00

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

INTERNATIONAL APPLICATION NO.	
PCT/US2017/046814	
I.A. FILING DATE	PRIORITY DATE
08/14/2017	08/15/2016

CONFIRMATION NO. 1002
371 ACCEPTANCE LETTER

OC000000121052292

Date Mailed: 11/04/2020

NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

02/14/2019
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 02/14/2019
- Copy of the International Search Report filed on 02/14/2019
- Preliminary Amendments filed on 02/21/2020
- Information Disclosure Statements filed on 07/23/2019
- Sequence Listing txt file (EFS or physical medium) filed on 02/14/2019
- Inventor's Oath or Declaration filed on 02/14/2019
- Sequence Listing (PDF or paper) filed on 02/14/2019
- U.S. Basic National Fees filed on 02/14/2019
- Substitute Specification filed on 07/23/2019
- Authorize Access to Search Results filed on 02/14/2019
- Priority Documents filed on 02/14/2019
- Power of Attorney filed on 02/14/2019
- Specification filed on 02/14/2019
- Claims filed on 02/14/2019
- Abstracts filed on 02/14/2019
- Drawings filed on 02/14/2019
- Authorization to Permit Access filed on 02/14/2019
- Application Data Sheet (37 CFR 1.76) filed on 02/14/2019

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

MARILYN J YOUNGER

Telephone: (571) 272-8183

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/325,653		Xiaoying JIN	15979-20141.00

CONFIRMATION NO. 1002

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

WITHDRAWAL NOTICE

OC00000121049777

Date Mailed: 11/04/2020

Letter Regarding a New Notice and/or the Status of the Application

If a new notice or Filing Receipt is enclosed, applicant may disregard the previous notice mailed on 02/19/2020. The time period for reply runs from the mail date of the new notice. Within the time period for reply, applicant is required to file a reply in compliance with the requirements set forth in the new notice to avoid abandonment of the application.

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web. https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If the reply is not filed electronically via EFS-Web, the reply must be accompanied by a copy of the new notice.

If the Office previously granted a petition to withdraw the holding of abandonment or a petition to revive under 37 CFR 1.137, the status of the application has been returned to pending status.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/mjyounger/

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
16/325,653	02/14/2019		33360	15979-20141.00	22	14

CONFIRMATION NO. 1002

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

FILING RECEIPT

OC000000121052291

Date Mailed: 11/04/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

Inventor(s)

Xiaoying JIN, Bridgewater, NJ;
Catherine O'RIORDAN, Bridgewater, NJ;
Lin LIU, Bridgewater, NJ;
Kate ZHANG, Bridgewater, NJ;

Applicant(s)

Genzyme Corporation, Cambridge, MA;

Power of Attorney: The patent practitioners associated with Customer Number 89300

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2017/046814 08/14/2017
which claims benefit of 62/375,314 08/15/2016

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 04/20/2019
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/325,653**
Projected Publication Date: 02/11/2021
Non-Publication Request: No
Early Publication Request: No
Title

METHODS FOR DETECTING AAV

Preliminary Class

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

LICENSE FOR FOREIGN FILING UNDER

Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

GRANTED

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

NOT GRANTED

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

SelectUSA

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
16325653	

Applicant(s) Xiaoying JIN

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
1	1		1			
2		1	---	---		
3	1		1			
4		1	---	---		
5		4	---	---		
6		(1)	---	---		
7		(1)	---	---		
8		(1)	---	---		
9		(1)	---	---		
10		(1)	---	---		
11		(1)	---	---		
12		(1)	---	---		
13		(1)	---	---		
14		(1)	---	---		
15		(1)	---	---		
16		(1)	---	---		
17		(1)	---	---		
18		(1)	---	---		
19		(1)	---	---		
20		(1)	---	---		
21		(1)	---	---		
22		(1)	---	---		
23		(1)	---	---		
24		(1)	---	---		
25		(1)	---	---		
26		(1)	---	---		
27		(1)	---	---		
28		(1)	---	---		
29	1		1			
30		1	---	---		
31	1		1			
32		1	---	---		
33		4	---	---		
34		(1)	---	---		
35		(1)	---	---		
36		(1)	---	---		
37		(1)	---	---		
38		(1)	---	---		
39		(1)	---	---		
40		(1)	---	---		
41		(1)	---	---		
42		(1)	---	---		
43		(1)	---	---		
44		(1)	---	---		
45		(1)	---	---		
46		(1)	---	---		
47		(1)	---	---		
48		(1)	---	---		
49		(1)	---	---		
50		(1)	---	---		

	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
51		(1)	---	---		
52		(1)	---	---		
53		(1)	---	---		
54		(1)	---	---		
55		(1)	---	---		
56		(1)	---	---		
57		(1)	---	---		
58		(1)	---	---		
59		(1)	---	---		
60		(1)	---	---		
61		(1)		1		
62		(1)		1		
63	1		1			
64		1	---	---		
65		2	---	---		
66		(1)	---	---		
67		(1)	---	---		
68		(1)	---	---		
69		(1)	---	---		
70		(1)	---	---		
71		(1)	---	---		
72		(1)	---	---		
73		(1)	---	---		
74		(1)	---	---		
75		(1)	---	---		
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80		(1)	---	---		
81		(1)	---	---		
82		(1)	---	---		
83		(1)	---	---		
84		(1)	---	---		
85		(1)	---	---		
86		(1)	---	---		
87		(1)	---	---		
88		(1)	---	---		
89		(1)		1		
90		(1)		1		
91		(1)		1		
92	1		1			
93		1	---	---		
94		2	---	---		
95		(1)	---	---		
96		1	---	---		
97		(1)	---	---		
98		(1)	---	---		
99		(1)	---	---		
100	1		1			

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
16325653	

Applicant(s) Xiaoying JIN

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT	
	Indep	Depend	Indep	Depend	Indep	Depend
101	1		---	---		
102	1		---	---		
103		3	---	---		
104		(1)	---	---		
105		(1)	---	---		
106		(1)	---	---		
107		(1)	---	---		
108		(1)	---	---		
109		(1)	---	---		
110		(1)	---	---		
111		(1)	---	---		
112		(1)	---	---		
113		(1)	---	---		
114		(1)	---	---		
115		(1)	---	---		
116		(1)	---	---		
117		(1)	---	---		
118		(1)	---	---		
119		(1)	---	---		
120		(1)	---	---		
121		(1)	---	---		
122		(1)	---	---		
123		(1)	---	---		
124		(1)	---	---		
125		(1)	---	---		
126		(1)	---	---		
127		(1)	---	---		
128	1		1			
129		1	---	---		
130	1		1			
131		1	---	---		
132		4	---	---		
133		(1)	---	---		
134	1		1			
135		1	---	---		
136	1		1			
137		1	---	---		
138		4	---	---		
139		(1)	---	---		
140		(1)	---	---		
141		(1)	---	---		
142	1		1			
143		1	---	---		
144		1	---	---		
145		1	---	---		
146		1	---	---		
147		1	---	---		
148		1	---	---		
149		7	---	---		
150		(1)		1		
Total Indep	18		14		0	
Total Depend	186	↵	8	↵	0	↵
Total Claims	204		22		0	

	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
151		(1)		1		
152		(1)		1		
153	1		1			
154	1		1			
155	1		---	---		
156	1		---	---		
157		3	---	---		
158		(1)	---	---		
159		(1)	---	---		
160		(1)	---	---		
161		(1)	---	---		
162		(1)	---	---		
163		(1)	---	---		
164		(1)	---	---		
165		(1)	---	---		
166		(1)	---	---		
167		(1)	---	---		
168		(1)	---	---		
169		(1)	---	---		
170		(1)	---	---		
171		(1)	---	---		
172		(1)	---	---		
173		(1)	---	---		
174		(1)	---	---		
175		(1)	---	---		
176		(1)	---	---		
177		(1)	---	---		
178		(1)	---	---		
179		(1)	---	---		
180		(1)	---	---		
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Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: Not Yet Assigned

Examiner: Not Yet Assigned

REQUEST FOR REFUND

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

On October 14, 2020, Applicant's Deposit Account was charged for Claim fees in the amount of $26,120. On July 23, 2019, we authorized the correct claim fees and extension fees to be charged to our deposit account in the amount of $5,460.

The particulars of this request are given below:

- On April 23, 2019 a Notification to File Corrected Papers was issued.
- On July 23, 2019 a Response to Notification to File Corrected Papers was filed. Claims were amended to remove multiple claim dependencies and to reduce the total number of claims. The Office was authorized to pay claims fees of $5,260 for two claims in excess of 20 claims and for 11 independent claims in excess of three independent claims. Claims 32-60 were inadvertently marked as "(original)" when it was quite clear that they were canceled. The "Remarks" section of the amendment indicated that claims 32-60 were canceled. On July 24, 2020, the Office accepted payment of $5,260 for claims fees.

- No Notice of Non-Compliant Amendment was mailed by the Office.

  MPEP 714 II.F(A) provides the following:

  > (A) A preliminary amendment filed after the filing date of the application, the technical support staff (TSS) will send the notice which sets a time period of two months for reply. No extensions of time are permitted. Failure to submit a timely reply will result in the application being examined without entry of the preliminary amendment. Applicant's reply is required to include the corrected section of the amendment.
  >
  > In the absence of notification by the Office, Applicant was unaware that the amendment was non-compliant.

- On February 19, 2020 a Notification of Abandonment was issued.
- On February 21, 2020 a Petition to withdraw holding of Abandonment was filed noting that the abandonment was unintentional. A supplemental preliminary amended was filed to correct the status of canceled claims 32-60.
- On October 13, 2020 the Petition was Granted; however, the notice of grant of the petition indicated that the claim amendments were files after the statutory period and as such, claims fees of $26,120 would be charged to Applicant's representative Deposit account.

Applicant respectfully requests that a total refund of **$26,120** be credited to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00** for the fees indicated below.

Accounting Date	Fee Code	37 CFR	Fee	Description
10-14-2020	1615	1.492(e)	$18,400.00	Each claim in excess of 20
10-14-2020	1614	1.492(d)	$6,900.00	Each independent claim in excess of 3
10-14-2020	1616	1.492(f)	$820.00	Multiple dependent claim

As Applicant made a good faith effort to reduce claims fees and the Office did not provide a Notice of Noncompliant Response, Applicant believes they are entitled to a refund of the claims fees of **$26,120**. Applicant further notes that the **$26,120** charged to the Deposit

Account did not consider the **$5,260** for claims fees previously charged to the Deposit Account. At a minimum, Applicant should be credited **$5,620**, but as discussed above, Applicant believes that they are due a credit of **$26,120**.

Dated: November 11, 2020

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-5632

Electronic Acknowledgement Receipt

EFS ID:	41092136
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	11-NOV-2020
Filing Date:	14-FEB-2019
Time Stamp:	13:14:52
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Refund Request	Req_for_Refund.pdf	105149 a81976815983f2618aeef2ade4451fb86969b0a3	no	3

Warnings:

Information:	
Total Files Size (in bytes):	105149

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00

CONFIRMATION NO. 1002

89300
Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

PUBLICATION NOTICE

OC000000123339518

Title:METHODS FOR DETECTING AAV

Publication No.US-2021-0041451-A1
Publication Date:02/11/2021

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 03/18/2021

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
03/18/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Office Action Summary	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 02/21/2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,3,29,31,61-63,89-92,100,128,130,134,136,142 and 150-154 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1,3,29,31,61-63,89-92,100,128,130,134,136,142 and 150-154 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date 07/23/2019.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

1. Preliminary Amendment filed on 02/21/2020 is acknowledged. Claims 2, 4-28, 30, 32-60, 64-88, 93-99, 101-127, 129, 131-133, 135, 137-141, 143-149 and 155-180 are cancelled. Claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142 and 150-154 are pending in the application.

Notice of Pre-AIA or AIA Status

2. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Election/Restrictions

3. REQUIREMENT FOR UNITY OF INVENTION

As provided in 37 CFR 1.475(a), a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in a national stage application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim. See 37 CFR 1.475(e).

When Claims Are Directed to Multiple Categories of Inventions:

As provided in 37 CFR 1.475 (b), a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and a process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

Otherwise, unity of invention might not be present. See 37 CFR 1.475 (c).

4. Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 1, 29, 61, 128 and 134, drawn to a method to determine the serotype of an adeno-associated virus (AAV) particle.

Group II, claim(s) 3, 31, 62, 130 and 136, drawn to a method of determining the heterogeneity of an AAV particle.

Group III, claim(s) 63, 89-92 and 100, drawn to a recombinant AAV (rAAV) particle.

Group IV, claim(s) 142 and 150-154, drawn to a recombinant AAV (rAAV) particle.

5. The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I-IV lack unity of invention because even though the inventions of these groups require the technical feature of an adeno-associated virus (AAV) particle, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet). Vliet discloses an adeno-associated virus (AAV) particle (abstract).

6. A telephone call was made to Brian Donahue on 03/08/2021 to request an oral election to the above restriction requirement, but did not result in an election being made.

7. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached on M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Field

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT
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Application Number	16/325,653
Filing Date	August 14, 2017 (Int'l)
First Named Inventor	Xiaoying JIN
Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	15979-20141.00

U.S. PATENTS

Examiner Initial*	Cite No	Patent Number	Kind Code[1]	Issue Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	1.	US-6,989,264-B2		01-24-2006	Atkinson et al.	
	2.	US-7,588,772-B2		09-15-2009	Kay et al.	
	3.	US-7,629,322-B2		12-08-2009	Kleinschmidt et al.	
	4.	US-8,137,948-B2		03-20-2012	Qu et al.	
	5.	US-8,283,151-B2		10-09-2012	Schmidt et al.	
	6.	US-9,169,299-B2		10-27-2015	Lisowski et al.	

U.S. PATENT APPLICATION PUBLICATIONS

Examiner Initial*	Cite No	Publication Number	Kind Code[1]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	7.	US-2011/0275529-A1		11-10-2011	Heilbronn, R.	
	8.	US-2012/0164106-A1		03-15-2012	Kay et al.	
	9.	US-2013/0323226-A1		06-28-2012	Schaffer et al.	
	10.	US-2012/0066783-A1		12-05-2013	Wilson et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initial*	Cite No	Country Code[2]	Foreign Document Number[3]	Kind Code[4]	Publication Date	Name of Patentee or Applicant of cited Document	Pages, Columns, Lines where Relevant Passages or Relevant Figures Appear	T[5]
	11.	WO	2003/042397-A2		05-22-2003	The Trustees of the University of Pennsylvania		
	12.	WO	2004/027019-A2		04-01-2004	University of Florida Research Foundation		
	13.	WO	2010/148143-A1		12-23-2010	Genzyme Corporation		
	14.	WO	2013/158879-A1		10-24-2013	The Children's Hospital of Philadelphia		

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[5]
	15.	AHMADIANKIA, A. et al. (July 1, 2013). "Generation of Helper Plasmids Encoding Mutant Adeno-Associated Virus Type 2 Capsid Proteins with Increased Resistance against Proteasomal Degradation", *Iran J Basic Med Sci* 16:813-821.	

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Examiner Name	Not Yet Assigned
Attorney Docket Number	15979-20141.00

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Art Unit	Not Yet Assigned
Examiner Name	Not Yet Assigned
Attorney Docket Number	15979-20141.00

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EXAMINER SIGNATURE			
Examiner Signature	/XIAOYUN R XU, Ph.D./	Date Considered	03/08/2021

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document.
[4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

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CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e) (2).

☐ See attached certification statement.

☐ The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Brian A. Donahue/	Date (YYYY-MM-DD)	2019-07-23
Name/Print	Brian A. Donahue	Registration Number	58,206

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /R.X./

Electronic Patent Application Fee Transmittal

Application Number:	16325653
Filing Date:	14-Feb-2019
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Filer:	Brian Arthur Donahue/Mei Leung
Attorney Docket Number:	15979-20141.00

Filed as Large Entity

Filing Fees for U.S. National Stage under 35 USC 371

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	220	220
Miscellaneous:				
			Total in USD ($)	**220**

Electronic Acknowledgement Receipt

EFS ID:	43018326
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Mei Leung
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	17-JUN-2021
Filing Date:	14-FEB-2019
Time Stamp:	19:30:29
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$220
RAM confirmation Number	E20216GJ30563707
Deposit Account	
Authorized User	

The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Response to Election / Restriction Filed	15979-2014100_Resp_Rest_Requirement.pdf	27145 a098751359a5ae891f24956f16958cb229ffa3be	no	3
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30879 15c5b90b34927cd97b77b73fb47063ccc516bc9c	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	58024		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653 | Confirmation No.: 1002

Filed: August 14, 2017 (Int'l) | Art Unit: 1797

For: METHODS FOR DETECTING AAV | Examiner: X. Xu

RESPONSE TO RESTRICTION REQUIREMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

This is in response to the restriction requirement set forth in the Office Action dated March 18, 2021, for which a response was due on May 18, 2021. We hereby petition and submit the fee for a one-month extension of time, thereby extending the deadline for response to June 18, 2021. Accordingly, this response is timely filed.

Remarks begin on page 2 of this paper.

REMARKS

Claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142, and 150-154 are currently pending in the present application and are subject to restriction.

Regarding the Restriction Requirement

The Examiner has required the Applicant to elect a single invention from the following groups:

- **Group I, claims 1, 29, 61, 128 and 134**, drawn to a method to determine the serotype of an adeno-associated virus (AAV) particle;
- **Group II, claims 3, 31, 62, 130 and 136**, drawn to a method of determining the heterogeneity of an AAV particle;
- **Group III, claims 63, 89-92 and 100**, drawn to a recombinant AAV (rAAV) particle; and
- **Group IV, claim(s) 142 and 150-154**, drawn to a recombinant AAV (rAAV) particle.

Applicants hereby elect **Group I**, claims 1, 29, 61, 128 and 134, without traverse.

Applicant's election is made without prejudice. Applicant expressly reserves the right under 35 U.S.C. § 121 to file a divisional application directed on the non-elected subject matter during the pendency of this application, or an application claiming priority from this application.

Applicants believe that they have addressed each of the elections as set forth in the action. If questions arise that may be handled via teleconference, the Examiner is invited to contact the undersigned in order to expedite prosecution of the present case.

Applicants hereby request examination on the merits of the elected subject matter.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: June 17, 2021

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-5632

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 07/09/2021

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER	
XU, XIAOYUN	

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
07/09/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Office Action Summary	Application No. 16/325,653	Applicant(s) JIN et al.	
	Examiner XIAOYUN R XU, Ph. D.	Art Unit 1797	AIA (FITF) Status Yes

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☑ Responsive to communication(s) filed on 06/17/2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1,3,29,31,61-63,89-92,100,128,130,134,136,142 and 150-154 is/are pending in the application.

5a) Of the above claim(s) 3,31,62-63,89-92,100,130,136,142 and 150-154 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1,29,61,128 and 134 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 02/14/2019 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

1. Applicant's election without traverse of group I comprising Claims 1, 29, 61, 128 and 134 in the reply filed on 06/17/2021 is acknowledged. Claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142 and 150-154 are pending in the application. Claims 3, 31, 62-63, 89-92, 100, 130, 136, 142 and 150-154 are withdrawn from consideration. Claims 1, 29, 61, 128 and 134 are considered on merits.

Notice of Pre-AIA or AIA Status

2. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Claim Rejections - 35 USC § 102

3. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

4. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale, or otherwise available to the public before the effective filing date of the claimed invention.

5. **Claim(s) 29, 61 and 134** is/are rejected under 35 U.S.C. 102(a)(1) as being anticipated Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet).

Regarding claim 29, Vliet teaches a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 171, par 1);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Regarding claim 61, Vliet teaches a method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured AAV particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle (page 169, par 5),

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle (page 169, par 6);

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Regarding claim 134, Vliet teaches a method to determine the serotype of a viral particle (abstract) comprising

a) denaturing the viral particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured viral particle to reduction and/or alkylation (page 169, par 5),

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle (page 169, par 5),

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS) (page 170, par 2), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle (pae 169, par 6);

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype (Table 2, page 171, par 1).

Claim Rejections - 35 USC § 103

6. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

7. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

8. The factual inquiries for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

9. **Claim 1 and 128** is/are rejected under 35 U.S.C. 103 as being unpatentable over Vliet et al. (Journal of Virological Methods, 2009, IDS) (Vliet) in view of Bark et al. (J. Am. Chem. Soc. 2001, IDS) (Bark).

Regarding claim 1, Vliet teaches a method to determine the serotype of an adeno-associated virus (AAV) particle (abstract) comprising

a) denaturing the AAV particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured and in-gel digested AAV particle to liquid chromatography/mass spectrometry (LC/MS) (page 169, par 6), and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle (page 171, par 1);

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype (Table 2, page 171, par 1).

Vliet does not specifically teach directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS). However, Bark teaches directly subjecting the denatured virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis." (page 1774, par 1); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion." (page 1774, par 2). "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins." (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), in order to save the time required for adequate proteolysis.

Regarding claim 128, Vliet teaches a method to determine the serotype of a viral particle (abstract) comprising

a) denaturing the viral particle (boiled for 3 min) (page 169, par 4),

b) subjecting the denatured and in-gel digested viral particle to liquid chromatography/mass spectrometry (LC/MS) (page 169, par 6), and

c) determining the masses of one or more capsid proteins of the viral particle (page 171, par 1);

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype (Table 2, page 171, par 1).

Vliet does not specifically teach directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS). However, Bark teaches directly subjecting the denatured virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) (Fig. 1, page 1774, par 4). Bark teaches that "The primary techniques for proteomic analysis consist of 2-D gel electrophoresis followed by proteolytic digestion, mass spectrometry, and computer-facilitated data analysis." (page 1774, par 1); "Two limitations of this method are the time required for adequate proteolysis and the resistance of some proteins to digestion." (page 1774, par 2). "We have taken advantage of thermal denaturation to generate a facile proteolysis method for identifying proteins." (page 1774, par 3). At time before the filing, it would have been obvious to one of ordinary skill in the art to directly subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), in order to save the time required for adequate proteolysis.

Conclusion

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to XIAOYUN R XU, Ph. D. whose telephone number is (571)270-5560. The examiner can normally be reached on M-F 8am-5pm.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Lyle Alexander can be reached on 571-272-1254. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see https://ppair-my.uspto.gov/pair/PrivatePair. Should you have questions on access to the Private

PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/XIAOYUN R XU, Ph.D./
Primary Examiner, Art Unit 1797

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CPC - Searched*		
Symbol	Date	Examiner
C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 (text limited)	06/22/2021	RX

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner
436	173 (text limited)	06/22/2021	RX

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
EAST (CPC C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848 ; USPC 436/173 with key words and inventor names) and Google	06/22/2021	RX

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

CLAIMS

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	06/22/2021								
	1	✓								
	2	-								
	3	N								
	4	-								
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Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021								
	43	-								
	44	-								
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	94	-								

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	Art Unit
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021								
	95	-								
	96	-								
	97	-								
	98	-								
	99	-								
	100	N								
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	145	-								
	146	-								

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	16/325,653	JIN et al.
	Examiner	**Art Unit**
	XIAOYUN R XU, Ph. D.	1797

CLAIM		DATE								
Final	Original	06/22/2021								
	147	-								
	148	-								
	149	-								
	150	N								
	151	N								
	152	N								
	153	N								
	154	N								
	155	-								
	156	-								
	157	-								
	158	-								
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	173	-								
	174	-								
	175	-								
	176	-								
	177	-								
	178	-								
	179	-								
	180	-								

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	9	(adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/06/22 12:01
S1	20	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 12:05
S2	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:19
S3	1	(US-20100129405-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:21
S4	1	(US-6989264-$ | US-7588772-$ | US-7629322-$ | US-8137948-$ | US-8283151-$ | US-9169299-$ | US-20110275529-$ | US-20120066783-$ | US-20120164106-$ | US-20130323226-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S5	1	16/325653.app.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:25
S6	13,645	adeno-associated virus AND ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123	US-PGPUB; USPAT; USOCR; FPRS;	ADJ	ON	2021/03/08 13:26

		OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	EPO; JPO; DERWENT; IBM_TDB			
S7	5,489	adeno-associated virus AND serotype and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27
S8	836	adeno-associated virus AND serotype and denature$1 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:27
S9	125	adeno-associated virus AND serotype and denature$1 and heterogeneity and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28
S10	91	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:28
S11	25	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29
S12	9	adeno-associated virus AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:29

		OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)				
S13	9	(adeno-associated virus or AAV)AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016" and ((C07K14/005 OR C12N15/86 OR C12N2750/14122 OR C12N2750/14123 OR C12N2750/14143 OR G01N2030/027 OR G01N2333/075 OR G01N30/72 OR G01N30/7233 OR G01N33/6848).CPC.)	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:30
S15	3	436/173.ccls. and (adeno-associated virus or AAV) AND serotype and denature$1 and heterogeneity and mass$2 and spectromet$2 and @ay< "2016"	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:31
S16	10	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:33
S17	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and deamidation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:34
S18	0	(WO-2003042397-$ | WO-2004027019-$ | WO-2010148143-$ | WO-2013158879-$).DID. and acetylation	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT; IBM_TDB	ADJ	ON	2021/03/08 13:34
S19	5	((("JIN") near3 ("Xiaoying"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S20	42	((("O'RIORDAN") near3 ("Catherine"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:35
S21	1,105	((("LIU") near3 ("Lin"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:36
S22	5	((("ZHANG") near3 ("Kate"))).INV.	US-PGPUB; USPAT; USOCR	ADJ	ON	2021/03/08 13:37

EAST Search History (Interference)

<This search history is empty>

6/22/2021 12:02:34 PM
C:\Users\xxu\Documents\EAST\Workspaces\16325653.wsp

UNITED STATES PATENT AND TRADEMARK OFFICE

USPTO Automated Interview Request (AIR)

Oct 5 2021

This paper requesting to schedule and/or conduct an interview is appropriate because:

This submission is requested to be accepted as an authorization for this interview to communicate via the internet. Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned concerning scheduling of the interview via video conference, instant messaging, or electronic mail, and to conduct the interview in accordance with office practice including video conferencing.

Name(s):
Brian Donahue

S-signature:
/BrianDonahue/

Registration Number:
58206

U.S. Application Number:
16325653

Confirmation Number:
1002

E-mail Address:
bdonahue@mofo.com

Phone Number:
+1 6507049157

Proposed Time of Interview:
10-14-2021 1:00 PM ET

Alternative Proposed Time(s) of Interview:
10-15-2021 1:00 PM ET

Alternative Proposed Time(s) of Interview:
10-18-2021 1:00 PM ET

Prefered Interview Type:
Telephonic

I am the applicant or applicant's representative for this application.

Topic for Discussion:
Bark reference

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
16/325,653	02/14/2019	Xiaoying JIN	15979-20141.00	1002

89300 7590 10/20/2021

Sanofi/Genzyme c/o Morrison & Foerster LLP
755 Page Mill Road
Palo Alto, CA 94304

EXAMINER
XU, XIAOYUN

ART UNIT	PAPER NUMBER
1797	

NOTIFICATION DATE	DELIVERY MODE
10/20/2021	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

EOfficePA@mofo.com
PatentDocket@mofo.com
pair_mofo@firsttofile.com

Applicant-Initiated Interview Summary	**Application No.** 16/325,653	**Applicant(s)** JIN et al.		
	Examiner XIAOYUN R XU, Ph. D.	**Art Unit** 1797	**AIA (First Inventor to File) Status** Yes	**Page** 1 of 1

All Participants (applicant, applicants representative, PTO personnel)	**Title**	**Type**
XIAOYUN R XU, Ph. D.	Primary Examiner	Telephonic
Brian Donahue	Attorney	

Date of Interview: 14 October 2021

Issues Discussed:

35 U.S.C. 103

Applicant and examiner discussed the claimed subject matter in relation to the prior art. No agreement is reached.

/XIAOYUN R XU, Ph.D./ Primary Examiner, Art Unit 1797	

Applicant is reminded that a complete written statement as to the substance of the interview must be made of record in the application file. It is the applicants responsibility to provide the written statement, unless the interview was initiated by the Examiner and the Examiner has indicated that a written summary will be provided. See MPEP 713.04
Please further see:
MPEP 713.04
Title 37 Code of Federal Regulations (CFR) § 1.133 Interviews, paragraph (b)
37 CFR § 1.2 Business to be transacted in writing

Applicant recordation instructions: The formal written reply to the last Office action must include the substance of the interview. (See MPEP section 713.04). If a reply to the last Office action has already been filed, applicant is given a non-extendable period of the longer of one month or thirty days from this interview date, or the mailing date of this interview summary form, whichever is later, to file a statement of the substance of the interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: X. Xu

REQUEST FOR REFUND

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

On July 24, 2019, Applicant's Deposit Account was charged for Claim fees in the amount of $5,260. On October 14, 2020 the USPTO deducted from our deposit account claim fees in the amount of $26,120 without taking into account the claim fees already paid.

The particulars of this request are given below:

- On July 23, 2019 a Response to Notification to File Corrected Papers was filed. Claims were amended to remove multiple claim dependencies and to reduce the total number of claims. The Office was authorized to pay claims fees of $5,260 for two claims in excess of 20 claims and for 11 independent claims in excess of three independent claims. On July 24, 2020, the Office accepted payment of $5,260 for claims fees.
- No Notice of Non-Compliant Amendment was mailed by the Office.
- On February 19, 2020 a Notification of Abandonment was issued.

- On February 21, 2020 a Petition to withdraw holding of Abandonment was filed noting that the abandonment was unintentional. A supplemental preliminary amended was filed to correct the status of canceled claims 32-60.
- On October 13, 2020 the Petition was Granted; however, the notice of grant of the petition indicated that the claim amendments were filed after the statutory period and as such, claims fees of $26,120.00 would be charged to Applicant's representative Deposit account.
- On October 14, 2020 claim fees in the amount of $26,120.00 was charged to our Deposit Account. This value did not account for the $5,260.00 claims fees that had already been charged to our account.

Applicants request a refund in the total amount of $5,260.00 for the overpayment of the excess claim fees on October 14, 2020. A table illustrating the fees discussed above is found below.

	Amount Permitted to be Charged by Applicants on July 24, 2019	**Amount charged by the USPTO on October 14, 2020, not subtracting the July 24, 2019 payment**	**Refund Request**
Independent Claims Fee Code 1614 37 CFR 1.492(d)	$5,060.00 (11 independent claims)	$6,900.00 (15 independent claims)	$5,060.00 (Overpayment)
Dependent Claims Fee Code 1615 37 CFR 1.492(e)	$200.00 (2 dependent claims)	$18,400.00 (184 dependent claims)	$200.00 (Overpayment)
Multiple Dependent Claims Fee Code 1616 37 CFR 1.49(f)		$820.00	
Total	$5,260.00	$26,120.00	**$5,260.00**

Please credit **$5,260.00** to **Deposit Account No. 03-1952** referencing docket number **15979-20141.00**.

Dated: October 26, 2021

Respectfully submitted,

Electronic signature: /Brian A. Donahue/
Brian A. Donahue
Registration No.: 58,206
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, CA 94304-1018
+1 (650) 813-5632

Electronic Acknowledgement Receipt

EFS ID:	44125863
Application Number:	16325653
International Application Number:	
Confirmation Number:	1002
Title of Invention:	METHODS FOR DETECTING AAV
First Named Inventor/Applicant Name:	Xiaoying JIN
Customer Number:	89300
Filer:	Brian Arthur Donahue/Aurelia Caparas
Filer Authorized By:	Brian Arthur Donahue
Attorney Docket Number:	15979-20141.00
Receipt Date:	26-OCT-2021
Filing Date:	14-FEB-2019
Time Stamp:	16:02:51
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Refund Request	20211026_Req_for_refund.pdf	106643 13f43709a62620e99e234fe3e4f2d8232bec2a8a	no	3

Warnings:

Information:	
Total Files Size (in bytes):	106643

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 16/325,653	Filing Date 02/14/2019	☐ To be Mailed

ENTITY: ☑ LARGE ☐ SMALL ☐ MICRO

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A		N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A		N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A		N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*		x $100 =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*		x $460 =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).				
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))					
* If the difference in column 1 is less than zero, enter "0" in column 2.				TOTAL	

APPLICATION AS AMENDED - PART II

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT	11/09/2021	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 43	Minus	** 204	= 0		x $100 =	0
	Independent (37 CFR 1.16(h))	* 5	Minus	*** 18	= 0		x $480 =	0
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	0

		(Column 1)		(Column 2)	(Column 3)			
AMENDMENT		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=		x $0 =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=		x $0 =	
	☐ Application Size Fee (37 CFR 1.16(s))							
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							
							TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.	LIE
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".	/BRENDA MURPHY/
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".	
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.	

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Docket No.: 15979-20141.00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Xiaoying JIN et al.

Application No.: 16/325,653

Filed: August 14, 2017 (Int'l)

For: METHODS FOR DETECTING AAV

Confirmation No.: 1002

Art Unit: 1797

Examiner: Xiaolin Xu

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Commissioner:

INTRODUCTORY COMMENTS

This is in response to the non-final Office Action dated July 9, 2021, for which a response was due on October 9, 2021. We hereby petition and submit the fee for a 1-month extension of time, thereby extending the deadline for response to November 9, 2021. Accordingly, this response is timely filed. Reconsideration and allowance of the pending claims, as amended, in light of the remarks presented herein are respectfully requested.

Amendments to the Claims are reflected in the listing of claims which begins on page **2** of this paper.

Remarks/Arguments begin on page **10** of this paper.

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions, and listings, of claims in the application:

In the claims:

Claim 1 (currently amended): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 2-28 (canceled)

Claim 29 (currently amended): A method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 30-60 (canceled)

Claim 61 (currently amended): A method of determining the serotype of an AAV particle comprising the method of claim 1 combined with a method to determine the serotype of an adeno-associated virus (AAV) particle comprising

a) denaturing the AAV particle,

b) subjecting the denatured AAV particle to reduction and/or alkylation,

c) subjecting the denatured AAV particle to digestion to generate fragments of VP1, VP2 and/or VP3 of the AAV particle,

d) subjecting the fragments of VP1, VP2 and/or VP3 to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of VP1, VP2 and VP3 of the AAV particle;

wherein the specific combination of masses of fragments of VP1, VP2 and VP3 are indicative of the AAV serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 62-127 (canceled)

Claim 128 (currently amended): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and

c) determining the masses of one or more capsid proteins of the viral particle;

wherein the specific combination of masses of the one or more capsid proteins are indicative of the virus serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 129-133 (canceled)

Claim 134 (currently amended): A method to determine the serotype of a viral particle comprising

a) denaturing the viral particle,

b) subjecting the denatured viral particle to reduction and/or alkylation,

c) subjecting the denatured viral particle to digestion to generate fragments of one or more capsid proteins of the viral particle,

d) subjecting the fragments of the one or more capsid proteins to liquid chromatography/mass spectrometry-mass spectrometry (LC/MS/MS), and

e) determining the masses of fragments of the one or more capsid proteins of the viral particle;

wherein the specific combination of masses of fragments of the one or more capsid proteins are indicative of the viral serotype,

and wherein the method is performed in the absence of a gel separation step.

Claims 135-180 (canceled)

Claim 181 (new): The method of claim 1, wherein the calculated masses of VP1, VP2 and VP3 are compared to the theoretical masses of VP1, VP2 and VP3 of one or more AAV serotypes.

Claim 182 (new): The method of claim 1, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride, and/or an organic solvent.

Claim 183 (new): The method of claim 1, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 184 (new): The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.

Claim 185 (new): The method of claim 184, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 186 (new): The method of claim 1, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 187 (new): The method of claim 1, wherein the mass spectrometry comprises assisted calibration.

Claim 188 (new): The method of claim 187, wherein sodium iodide is used as a calibrant.

Claim 189 (new): The method of claim 1, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 190 (new): The method of claim 1, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claim 191 (new): The method of claim 29, wherein the calculated masses of the fragments of VP1, VP2 and/or VP3 are compared to the theoretical masses of fragments of VP1, VP2 and/or VP3 of one or more AAV serotypes.

Claim 192 (new): The method of claim 29, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

Claim 193 (new): The method of claim 29, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 194 (new): The method of claim 1, wherein the liquid chromatography is reverse phase liquid chromatography.

Claim 195 (new): The method of claim 194, wherein the reverse phase chromatography is a C18 reverse chromatography.

Claim 196 (new): The method of claim 29, wherein the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP).

Claim 197 (new): The method of claim 29, wherein the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

Claim 198 (new): The method of claim 1, wherein the digestion is an endopeptidase digestion.

Claim 199 (new): The method of claim 198, wherein the endopeptidase digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion.

Claim 200 (new): The method of claim 29, wherein the AAV particle is a recombinant AAV (rAAV) particle.

Claim 201 (new): The method of claim 29, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid,

an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

Claim 202 (new): The method of claim 128, wherein the calculated masses of the one or more capsid proteins are compared to the theoretical masses of the one or more capsid proteins of one or more virus serotypes.

Claim 203 (new): The method of claim 128, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 204 (new): The method of claim 128, wherein the liquid chromatography is reverse phase chromatography.

Claim 205 (new): The method of claim 204, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

Claim 206 (new): The method of claim 128, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

Claim 207 (new): The method of claim 128, wherein the mass spectrometry comprises assisted calibration.

Claim 208 (new): The method of claim 207, wherein sodium iodide is used as a calibrant.

Claim 209 (new): The method of claim 128, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

Claim 210 (new): The method of claim 134, wherein the calculated masses of the fragments of the one or more capsid proteins are compared to the theoretical masses of fragments of the one or more capsid proteins of one or more viral serotypes.

Claim 211 (new): The method of claim 134, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

Claim 212 (new): The method of claim 134, wherein the liquid chromatography is reverse phase liquid chromatography.

Claim 213 (new): The method of claim 212, wherein the reverse phase chromatography is a C18 reverse chromatography.

Claim 214 (new): The method of claim 134, wherein the reduction is by subjecting the viral particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP).

Claim 215 (new): The method of claim 134, wherein the alkylation is by subjecting the viral particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

Claim 216 (new): The method of claim 134, wherein the digestion is an endopeptidase digestion.

Claim 217 (new): The method of claim 216, wherein the endopeptidase digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion.

Claim 218 (new): The method of claim 134, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

REMARKS

Claims 1, 3, 29, 31, 61-63, 89-92, 100, 128, 130, 134, 136, 142, and 150-154 were pending in the present application. Claims 3, 31, 62-63, 89-92, 100, 130, 136, 142, and 150-154 were withdrawn from consideration. By virtue of this response, claims 1, 29, 61, 128, and 134 have been amended, claims 3, 31, 62-63, 89-92, 100, 130, 136, 142, and 150-154 have been canceled, and new claims 181-218 have been added. Accordingly, claims 1, 29, 61, 128, 134, and 181-218 are currently under consideration.

Support for the amended claims may be found throughout the specification. Support for the amendment to claims 1, 29, 61, 128, and 134 may be found, for example, in paragraph [0015]. Support for new claim 181 may be found, for example in original claim 2. Support for new claim 182 may be found, for example in original claim 5. Support for new claim 183 may be found, for example in original claim 6. Support for new claim 184 may be found, for example in original claim 7. Support for new claim 185 may be found, for example in original claim 8. Support for new claim 186 may be found, for example in original claim 17. Support for new claim 187 may be found, for example in original claim 20. Support for new claim 188 may be found, for example in original claim 21. Support for new claim 189 may be found, for example in original claim 23. Support for new claim 190 may be found, for example in original claim 24. Support for new claim 191 may be found, for example in original claim 30. Support for new claim 192 may be found, for example in original claim 39. Support for new claim 193 may be found, for example in original claim 40. Support for new claim 194 may be found, for example in original claim 41. Support for new claim 195 may be found, for example in original claim 42. Support for new claim 196 may be found, for example in original claim 33. Support for new claim 197 may be found, for example in original claim 34. Support for new claim 198 may be found, for example in original claims 35 and 36. Support for new claim 199 may be found, for example in original claim 37. Support for new claim 200 may be found, for example in original claim 55. Support for new claim 201 may be found, for example in original claim 56. Support for new claim 202 may be found, for example in original claim 129. Support for new claim 203 may be found, for example in original claim 132. Support for new claim 204 may be found, for

example in original claim 132. Support for new claim 205 may be found, for example in original claim 8. Support for new claim 206 may be found, for example in original claim 17. Support for new claim 207 may be found, for example in original claim 20. Support for new claim 208 may be found, for example in original claim21. Support for new claim 209 may be found, for example in original claim 133. Support for new claim 210 may be found, for example in original claim 135. Support for new claim 211 may be found, for example in original claim 138. Support for new claim 212 may be found, for example in original claim 138. Support for new claim 213 may be found, for example in original claim 42. Support for new claim 214 may be found, for example in original claim 33. Support for new claim 215 may be found, for example in original claim 34. Support for new claim 216 may be found, for example in original claims 35 and 36. Support for new claim 217 may be found, for example in original claim 37. Support for new claim 218 may be found, for example in original claim 139. No new matter has been added.

With respect to all claim amendments, Applicant has not dedicated or abandoned any unclaimed subject matter and moreover has not acquiesced to any rejections and/or objections made by the patent office. Applicant reserves the right to pursue prosecution of any presently excluded claim embodiments in a future continuation and/or divisional application.

Applicant-Initiated Interview Summary

The Applicant and the Applicant's representative, Brian Donahue, thank Examiner Xu for his time and for his assistance during the Applicant-initiated Interview on October 14, 2021. The claimed subject matter in relation to the prior art was discussed. No agreement was reached. In accordance with MPEP 713.04, this response contains a summary of the substance of the interview.

Rejections under 35 U.S.C. § 102

Claims 29, 61 and 134 are rejected under 35 U.S.C. § 102(a)(1) as allegedly being anticipated Vliet *et al.* (*Journal of Virological Methods*, 2009) ("Vliet").

Applicant respectfully notes that claim 61 incorporates the limitations of claim 1, which the Examiner holds to be novel in view of Vliet. Therefore, pending claim 61 is novel in view of Vliet.

Nonetheless, without acquiescing to the Examiner's rejection, and solely for purposes of expediting prosecution, claims 29, 61, and 134 have been amended to recite "wherein the method is performed in the absence of a gel separation step."

Applicant respectfully submits that Vliet neither teaches nor suggests performing the disclosed method of serotype identification of adeno-associated viruses (AAVs) in the absence of a gel separation step. As such, claims 29, 61, and 134 are novel in view of Vliet.

In view of the foregoing, Applicant respectfully requests withdrawal of this § 102 rejection of the claims.

Rejections under 35 U.S.C. § 103

Claims 1 and 128 are rejected under 35 U.S.C. § 103 as allegedly being unpatentable over Vliet *et al.* (*Journal of Virological Methods*, 2009) ("Vliet") in view of Bark *et al.* (*J. Am. Chem. Soc.* 2001) ("Bark").

Applicant respectfully traverses.

Without acquiescing to the Examiner's rejection, and for purposes of expediting prosecution, claims 1 and 128 have been amended to recite "wherein the method is performed in the absence of a gel separation step."

Applicant respectfully submits that it would not have been obvious to a person of ordinary skill in the art to combine the teachings of Vliet and Bark with a reasonable expectation of success to arrive at the presently claimed invention. Further, there is no teaching, suggestion, or motivation in Vliet and Bark that would have led one of ordinary skill to modify or combine the references to arrive at the presently claimed invention.

First, Vliet discloses that samples of AAV particles "were boiled for 3 min, centrifuged briefly and loaded on a 10% polyacrylamide gel", after which SDS-PAGE was performed and "AAV bands were cut from the gel". The reducing, alkylating, and digestion steps were then performed on the cut slices of the gel containing the AAV capsid proteins, prior to elution of the resulting peptide fragments from the gel followed by LC/MS/MS analysis of the eluted fragments. *See* 2.5.1. SDS-PAGE, 2.5.2. In-gel digestion, and 2.5.3. Mass spectrometry. Vliet is completely silent regarding a method to determine the serotype of a viral particle comprising a) denaturing the viral particle, b) subjecting the denatured viral particle to liquid chromatography/mass spectrometry (LC/MS), and c) determining the masses of one or more capsid proteins of the viral particle, *wherein the method is performed in the absence of a gel separation step*, as require by the amended claims.

Further, Vliet teaches away from performing the disclosed method in the absence of a gel separation step. Vliet explains that "The SDS-PAGE gel functions as a separation step for the three overlapping AAV capsid proteins that comprise the virion as well as impurities that may be present in the sample; therefore, samples of varying levels of purity can be analyzed." *See* 3. Results. Therefore, one of ordinary skill in the art reading Vliet would not have a reasonable expectation of success in performing the disclosed method in the absence of a gel separation step, as required by the claims, since according to Vliet, this would not enable separation of the overlapping AAV capsid proteins and analysis of samples of varying levels of purity.

Bark does not cure the defects of Vliet. Bark is directed to the development of a method for high-temperature proteolytic digestion using a thermotolerant protease (thermolysin) to generate protein fragments for analysis via mass spectrometry. One of the proteins analyzed using the disclosed method is a capsid protein from the HK97 virus. The Examiner alleges that "Bark teaches directly subjecting the denatured virus particle (HK97) to liquid chromatography/mass spectrometry (LC/MS) (Fig. 1, page 1774, par 4)." *See* Office Action dated July 9, 2021, page 5. Applicant respectfully disagrees.

The method disclosed in Bark is directed to *thermolysin*-mediated proteolysis of proteins, and does not teach performing the method without a protease. Bark attempts to overcome the challenges associated with proteolysis in proteomic techniques by taking advantage of "thermal denaturation to generate a facile proteolysis method for identifying proteins." Importantly, Bark states that "The key to this method is the use of a thermophilic enzyme, thermolysin**,** that exhibits optimal activity at elevated temperatures." *See* page 1774, paragraph 3. Thermolysin is well-known in the art to be a thermostable protease. Nowhere in Bark does it describe directly subjecting denatured viral capsid proteins to LC/MS without first subjecting them to proteolytic digestion with a protease.

The Examiner cites Figure 1 of Bark as teaching directly subjecting the denatured HK97 virus particle to LC/MS. *See* Office Action dated July 9, 2021, page 5. Applicant respectfully notes that Figure 1 shows mass fragments of the HK97 viral capsid, and does not show the mass of an intact, non-fragmented viral capsid protein. The largest panel of Figure 1, with an x-axis ranging from 500 to 4000 m/z, shows a large peak at 1263 m/z. Bark explains that "The 1263 (1263.66) mass peptide fragment appears most rapidly during the digestion process. This corresponds to residues 162-181 or 173-182 in the capsid sequence." *See* page 1774, paragraph 5, emphasis added. Therefore, the 1263 m/z peak in Figure 1 corresponds to a peptide fragment of the capsid protein resulting from thermolysin digestion, and not the entire, undigested capsid protein. Figure 1 further contains an inset panel showing additional mass fragments, which includes a tilde over the peak at 1263 m/z to show that the peak has been cut off in the inset. The largest mass fragment shown in at 2467 m/z, which is well below the sequence-predicted mass of the intact HK97 gp5 capsid protein (42,243 Da; UniProt P49861). The Figure 1 caption also discusses the rapid thermolysin digestion conditions used to generate the detected fragments. While the Figure 1 caption points out that "Trypsin cleavage showed no significant fragmentation within this time", Figure 1 does not show a mass chromatograph in which the mass of the unfragmented HK97 gp5 capsid protein can be detected. Therefore, Bark does not disclose that the samples containing the trypsin-treated (unfragmented) capsid protein were subjected to LC/MS, or that the mass of the unfragmented capsid protein was thus determined. In view of the foregoing, Applicant respectfully submits that Bark does not teach or

suggest directly subjecting a viral particle to LC/MS and determining its mass without using a protease.

Further, Applicant respectfully submits that, even if Bark did teach directly subjecting a viral particle to LC/MS without using a protease, with which Applicant respectfully disagrees, one of skill in the art would not look to this teaching to overcome the deficiencies of Vliet to arrive at the claimed invention. HK97 is a phage with only one major capsid protein which interlinks to form the viral capsid. *See* page 1774, paragraphs 5 and 6 of Bark. HK97 stands in stark contrast to AAVs, which have three capsid proteins (VP1, VP2, and VP3) with serotype-dependent sequences and masses. As explained above, Vliet discusses the need for an SDS-PAGE gel for analyzing AAV, which "functions as a separation step for the three overlapping AAV capsid proteins." *See* 3. Results of Vliet. Therefore, one of ordinary skill in the art reading Bark would not have a reasonable expectation of success in combining the teachings of Vliet and Bark to determine the serotype of an AAV particle, since Bark discloses only a simple phage particle and do provide a means to analyze VP1, VP2, and VP3 in the absence of a gel separation step.

The Examiner alleges that, because Bark discusses the long times required for adequate proteolysis in proteomic techniques, it would have thus been obvious to perform the method without using a protease in order to save the time required for adequate proteolysis. *See* Office Action dated July 9, 2021, page 5. However, as discussed above, the authors of Bark do not teach or suggest bypassing proteolytic digestion and directly subjecting denatured viral particles to LC/MS. Instead, they propose to overcome the aforementioned challenges through the use of a thermotolerant thermolysin capable of rapid proteolytic digestion at high temperatures. Thus, Bark does not teach or suggest directly subjecting denatured viral particles to LC/MS without using a protease.

For at least the reasons above, Applicant respectfully submits that the claims, as amended, are not obvious in view of the teachings of Vliet and Bark. Therefore, Applicant requests that the rejection of claims 1 and 128 be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, Applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. **03-1952** referencing docket no. **15979-20141.00**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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Respectfully submitted,

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