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(54) Title: METHODS FOR DETECTING AAV

(57) Abstract: Provided herein are methods for determining the serotype of a virus particle and/or or determining the heterogeneity of a virus particle (*e.g.*, an AAV particle). In other embodiments, the invention provides methods to determine the heterogeneity of AAV particles. In some aspects, the invention provides viral particles (*e.g.*, rAAV particles) with improved stability and/or improved transduction efficiency by increasing the acetylation and/or deamidation of capsid proteins.

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METHODS FOR DETECTING AAV

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application Serial No. 62/375,314, filed August 15, 2016, 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: 159792014140SEQLIST.txt, date recorded: August 14, 2017, size: 51 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)

CLAIMS

What is claimed is:

1. 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.

2. 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.

3. 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.

4. The method of claim 3 wherein the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

5. The method of any one of claims 1-4, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

6. The method of any one of claims 1-5, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

7. The method of any one of claims 1-6, wherein the liquid chromatography is reverse phase liquid chromatography.

8. The method of claim 7, wherein the reverse phase chromatography is a C4 or C8 reverse chromatography.

9. The method of claim 8, wherein the chromatography uses a mobile phase A comprising formic acid in water.

10. The method of claim 9, wherein the mobile phase A comprises about 0.1% formic acid.

11. The method of any one of claims 8-10, wherein the chromatography comprises a mobile phase B comprising formic acid in acetonitrile.

12. The method of claim 11, wherein the mobile phase B comprises about 0.1% formic acid.

13. The method of claim 11 or 12, wherein the proportion of mobile phase B in the chromatography increases over time.

14. The method of claim 13, wherein the proportion of mobile phase B in the chromatography increases in a stepwise manner.

15. The method of claim 14, wherein mobile phase B increases from about 10% to about 20%, from about 20% to about 30%, and from about 30% to about 38%.

16. The method of claim 15, wherein 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.

17. The method of any one of claims 1-16, wherein the liquid chromatography is ultra-performance liquid chromatography (UPLC).

18. The method of any one of claims 1-17, wherein the mass spectrometry comprises a capillary voltage of about 3.5 kV.

19. The method of any one of claims 1-18, wherein the mass spectrometry comprises a sampling cone voltage of about 45 V.

20. The method of any one of claims 1-19 wherein the mass spectrometry comprises assisted calibration.

21. The method of claim 20, wherein sodium iodide is used as a calibrant.

22. The method of any one of claims 1-21, wherein the N-terminus of VP1 and/or VP3 is acetylated.

23. The method of any one of claims 1-22, wherein the AAV particle is a recombinant AAV (rAAV) particle.

24. The method of any one of claims 1-23, 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.

25. The method of claim 23 or 24, wherein the AAV capsid comprises a tyrosine mutation or a heparin binding mutation.

26. The method of any one of claims 1-25, wherein 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, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1) , an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

27. The method of any one of claims 1-26, wherein 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.

28. The method of any one of claims 1-27, wherein the AAV particle comprises an AAV vector encoding a heterologous transgene.

29. 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.

30. 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.

31. 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.

32. The method of claim 31 wherein the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

33. The method of any one of claims 29-32, wherein the reduction is by subjecting the AAV particle to dithiothreitol, beta-mercaptoethanol, or tris(2-carboxyethyl)phosphine (TCEP).

34. The method of any one of claims 29-33, where in the alkylation is by subjecting the AAV particle to iodoacetic acid, iodoacetamide, or 4-vinylpyridine.

35. The method of any one of claims 29-34, wherein the digestion is an enzymatic digestion or a chemical digestion.

36. The method of claim 35, wherein the enzymatic digestion is an endopeptidase digestion.

37. The method of claim 35 or 36, wherein the enzymatic digestion is a trypsin digestion, a LysC digestion, an Asp-N digestion or a Glu-C digestion.

38. The method of claim 35, wherein the chemical digestion is cyanogen bromide digestion or an acid digestion.

39. The method of any one of claims 29-37, wherein the AAV particle is denatured with acetic acid, guanidine hydrochloride and/or an organic solvent.

40. The method of any one of claims 29-39, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

41. The method of any one of claims 29-40, wherein the liquid chromatography is reverse phase liquid chromatography.

42. The method of claim 41, wherein the reverse phase chromatography is a C18 reverse chromatography.

43. The method of claim 42, wherein the chromatography uses a mobile phase A comprising formic acid in water.

44. The method of claim 43, wherein the mobile phase A comprises about 0.1% formic acid.

45. The method of any one of claims 42-44, wherein the chromatography comprises a mobile phase B comprising formic acid in acetonitrile.

46. The method of claim 45, wherein the mobile phase B comprises about 0.1% formic acid.

47. The method of claim 45 or 46, wherein the proportion of mobile phase B in the chromatography increases over time.

48. The method of claim 47, wherein mobile phase B increases from about 2% to about 60%.

49. The method of claim 48, wherein mobile phase B increases from about 2% to about 60% in about 121 minutes.

50. The method of any one of claims 29-49, wherein the liquid chromatography is high-performance liquid chromatography (HPLC).

51. The method of any one of claims 29-50, wherein the mass spectrometry comprises a source voltage of about 2.5 kV.

52. The method of any one of claims 29-51, wherein the mass spectrometry comprises a capillary temperature of about 275 °C.

53. The method of any one of claims 29-52 wherein the S-lens RF level of the mass spectrometry comprises is about 55%.

54. The method of any one of claims 29-53, wherein the N-terminus of VP1 and/or VP3 is acetylated.

55. The method of any one of claims 29-54, wherein the AAV particle is a recombinant AAV (rAAV) particle.

56. The method of any one of claims 29-55, 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.

57. The method of claim 55 or 56, wherein the AAV capsid comprises a tyrosine mutation, a heparin binding mutation, or an HBKO mutation.

58. The method of any one of claims 29-57, wherein 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, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid.

59. The method of any one of claims 29-58, wherein 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.

60. The method of any one of claims 29-59, wherein the AAV particle comprises an AAV vector encoding a heterologous transgene.

61. A method of determining the serotype of an AAV particle comprising the method of any one of claims 1, 2 or 4-28 combined with the method of any one of claims 29, 30 or 32-59.

62. A method of determining heterogeneity of an AAV particle comprising the method of any one of claims 3-28 combined with the method of any one of claims 31-60.

63. 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.

64. The rAAV particle of claim 63, wherein the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation.

65. The rAAV particle of claim 63 or 64, wherein the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP1; and 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.

66. The rAAV particle of any one of claims 63-65, wherein the rAAV particle comprises an amino acid substitution at amino acid residue 2 of VP3; and 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.

67. The rAAV particle of any one of claims 63-66, wherein the amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr.

68. The rAAV particle of claim 67, wherein the amino acid residue 2 is substituted with Ser, Asp or Glu.

69. The rAAV particle of any one of claims 63-68, 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, rAAV2/HBoV1 serotype capsid, AAV2 HBKO, AAVPHP.B or AAVPHB.eB serotype capsid.

70. The rAAV particle of any one of claims 63-69, comprising a tyrosine mutation or a heparin binding mutation in the particle's capsid.

71. The rAAV particle of any one of claims 63-70, wherein the rAAV particle comprises a rAAV vector.

72. The rAAV particle of claim 71, wherein the rAAV vector comprises one or more AAV ITRs.

73. The rAAV particle of claim 71 or 72, wherein 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.

74. The rAAV particle of claim 72 or 73, wherein the AAV capsid and the AAV ITRs are derived from the same serotype.

75. The rAAV particle of claim 72 or 73, wherein the AAV capsid and the AAV ITRs are derived from different serotypes.

76. The rAAV particle of any one of claims 71-75, wherein the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

77. The rAAV particle of any one of claims 71-76, wherein the rAAV vector is a self-complementary vector.

78. The rAAV particle of claim 77, wherein the rAAV vector comprises a 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.

79. The rAAV particle of claim 78, wherein 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.

80. The rAAV particle of any one of claims 63-79, wherein 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.

81. The rAAV particle of claim 80, wherein the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions.

82. The rAAV particle of claim 81, wherein the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions.

83. The rAAV particle of claim 82, wherein the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus.

84. The rAAV particle of any one of claims 63-79, wherein 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.

85. The rAAV particle of claim 84, wherein the AAV producer cell comprises nucleic acid encoding AAV helper functions.

86. The rAAV particle of claim 84, wherein the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions.

87. The rAAV particle of claim 86, wherein the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus.

88. The rAAV particle of any one of claims 80-87, wherein 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.

89. A pharmaceutical composition comprising the rAAV particle of any one of claims 63-88.

90. A kit comprising the rAAV particle of any one of claims 63-87 or the pharmaceutical composition of claim 89.

91. An article of manufacture comprising the rAAV particle of any one of claims 63-87 or the pharmaceutical composition of claim 89.

92. 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.

93. The AAV capsid protein of claim 92, wherein the substitution results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation.

94. The AAV capsid protein of claim 92 or 93, wherein the AAV capsid protein is VP1 or VP3.

95. The AAV capsid protein of any one of claims 92-94, wherein amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr.

96. The AAV capsid protein of claim 93, wherein amino acid residue 2 is substituted with Ser, Asp or Glu.

97. The AAV capsid protein of any one of claims 92-96, wherein the amino acid substitution results in less deamidation of the AAV capsid.

98. The AAV capsid protein of any one of claims 92-97, comprising 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.

99. The rAAV capsid protein of any one of claims 92-98, wherein the AAV capsid protein further comprises a tyrosine mutation or a heparin binding mutation.

100. A method of improving the 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 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.

101. 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 alters N-terminal acetylation of VP1 and/or VP3, as compared to amino acid residue 2 of the parent VP1 and/or VP3.

102. A method of improving transduction of rAAV particles in a cell comprising substituting amino acid residue 2 of VP1 and/or VP3 of a parent VP1 and/or VP3; wherein substituting the 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.

103. The method of any one of claims 100-102, wherein substituting the amino acid residue 2 results in a higher frequency of N-terminal acetylation or a lower frequency of N-terminal acetylation.

104. The method of any one of claims 100-103, wherein amino acid residue 2 of VP1 is substituted.

105. The method of any one of claims 100-104, wherein amino acid residue 2 of VP3 is substituted.

106. The method of any one of claims 100-105, wherein amino acid residue 2 is substituted with Cys, Ser, Thr, Val, Gly, Asn, Asp, Glu, Ile, Leu, Phe, Gln, Lys, Met, Pro or Tyr.

107. The method of claim 106, wherein amino acid residue 2 is substituted with Ser, Asp or Glu.

108. The method of any one of claims 100-107, 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, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid

109. The method of any one of claims 100-108, wherein the rAAV particle's capsid further comprises a tyrosine mutation or a heparin binding mutation.

110. The method of any one of claims 100-109, wherein the rAAV particle comprises a rAAV vector.

111. The method of claim 110, wherein the rAAV vector comprises one or more AAV ITRs.

112. The method of claim 110 or 111, wherein 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.

113. The method of any one of claims 110-112, wherein the AAV capsid and the AAV ITRs are derived from the same serotype.

114. The method of any one of claims 110-112, wherein the AAV capsid and the AAV ITRs are derived from different serotypes.

115. The method of any one of claims 100-114, wherein the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

116. The method of any one of claims 110-115, wherein the rAAV vector is a self-complementary vector.

117. The method of claim 116, wherein 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.

118. The method of claim 117, wherein 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.

119. The method of any one of claims 100-118, wherein 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.

120. The method of claim 119, wherein the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions.

121. The method of claim 120, wherein the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions.

122. The method of claim 121, wherein the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus.

123. The method of any one of claims 100-118, wherein 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.

124. The method of claim 123, wherein the AAV producer cell comprises nucleic acid encoding AAV helper functions.

125. The method of claim 124, wherein the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions.

126. The method of claim 125, wherein the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus.

127. The method of any one of claims 119-126, wherein 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.

128. 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.

129. 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.

130. 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.

131. The method of claim 130, wherein the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

132. The method of any one of claims 128-131, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

133. The method of any one of claims 128-132, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

134. 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.

135. 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.

136. 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.

137. The method of claim 136, wherein the heterogeneity comprises one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids.

138. The method of any one of claims 134-137, wherein the liquid chromatography is reverse phase liquid chromatography, size exclusion chromatography, hydrophilic interaction liquid chromatography, or cation exchange chromatography.

139. The method of any one of claims 134-138, wherein the viral particle comprises a viral vector encoding a heterologous transgene.

140. The method of any one of claims 128-139, wherein the viral particle belongs to a viral family selected from the group consisting of *Adenoviridae*, *Parvoviridae*, *Retroviridae*, *Baculoviridae*, and *Herpesviridae*.

141. The method of claim 140, wherein 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*.

142. 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.

143. The rAAV particle of claim 142, wherein the one or more amino acid substitutions is at amino acid residue 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 and alters deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

144. The rAAV particle of claim 142, wherein the one or more amino acid substitutions comprise a substitution of Asp at N57 of VP1, N382 of VP3, N511 of VP3, or N715 of VP3 and result in a higher frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

145. The rAAV particle of claim 142, wherein 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.

146. The rAAV particle of claim 142, wherein the one or more amino acid substitutions comprise a substitution of Asn at A35 of VP1and result in a higher frequency of deamidation as compared to deamidation of VP1 of the parent AAV particle.

147. The rAAV particle of claim 142, wherein the one or more amino acid substitutions is at G58 of VP1, G383 of VP3, G512 of VP3, or G716 of VP3 and result in a lower frequency of deamidation as compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

148. The rAAV particle of claim 147, wherein the G58 of VP1 is substituted with Asp.

149. The rAAV particle of any one of claims 142-148, wherein the rAAV particle is an AAV1 particle or an AAV2 particle.

150. A pharmaceutical composition comprising the rAAV particle of any one of claims 142-149.

151. A kit comprising the rAAV particle of any one of claims 142-149 or the pharmaceutical composition of claim 150.

152. An article of manufacture comprising the rAAV particle of any one of claims 142-149 or the pharmaceutical composition of claim 150.

153. 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.

154. A method of improving the stability 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.

155. A method of improving the assembly of rAAV particles in a cell comprising substituting one or more amino acid residues of 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 deamidation of VP1 and/or VP3 of the parent VP1 and/or VP3.

156. 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.

157. The method of any one of claims 154-156, wherein 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.

158. The method of any one of claims 154-157, wherein the parental Ala residue at position 35 of VP1 is substituted with Asn.

159. The method of any one of claims 154-158, wherein the parental Gly residue at position 58 of VP1 is substituted with Asp.

160. The method of any one of claims 154-159, 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, rAAV2/HBoV1, AAV2HBKO, AAVPHP.B, or AAVPHP.eB serotype capsid

161. The method of any one of claims 154-160, wherein the rAAV particle's capsid further comprises a tyrosine mutation or a heparin binding mutation.

162. The method of any one of claims 154-160, wherein the rAAV particle is an AAV1 particle or an AAV2 particle.

163. The method of any one of claims 154-162, wherein the rAAV particle comprises a rAAV vector.

164. The method of claim 163, wherein the rAAV vector comprises one or more AAV ITRs.

165. The method of claim 163 or 164, wherein 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.

166. The method of any one of claims 164-165, wherein the AAV capsid and the AAV ITRs are derived from the same serotype.

167. The method of any one of claims 164-166, wherein the AAV capsid and the AAV ITRs are derived from different serotypes.

168. The method of any one of claims 164-167, wherein the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

169. The method of any one of claims 164-168, wherein the rAAV vector is a self-complementary vector.

170. The method of claim 169, wherein 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.

171. The method of claim 170, wherein 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.

172. The method of any one of claims 164-171, wherein 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.

173. The method of claim 172, wherein the AAV helper functions are provided by transfecting the host cell with nucleic acid encoding the AAV helper functions.

174. The method of claim 173, wherein the AAV helper functions are provided by infecting the host cell with an AAV helper virus that provides the AAV helper functions.

175. The method of claim 174, wherein the AAV helper virus is an adenovirus, a herpes simplex virus or a baculovirus.

176. The method of any one of claims 164-175, wherein 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.

177. The method of claim 176, wherein the AAV producer cell comprises nucleic acid encoding AAV helper functions.

178. The method of claim 177, wherein the AAV helper functions are provided by infecting the AAV producer cells with an AAV helper virus that provides the AAV helper functions.

179. The method of claim 178, wherein the AAV helper virus is an adenovirus, a herpes simplex virus, or a baculovirus.

180. The method of any one of claims 172-179, wherein the AAV cap functions provide an amino acid substitution of VP1 and/or VP3, wherein the amino acid substitution of VP1 and/or VP3 alters deamidation compared to deamidation of VP1 and/or VP3 of the parent AAV particle.

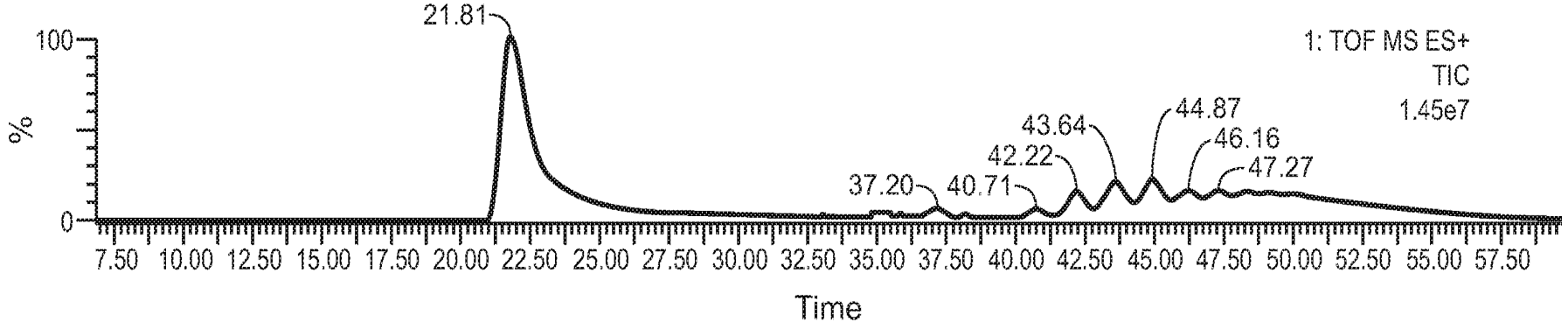


FIG. 1A

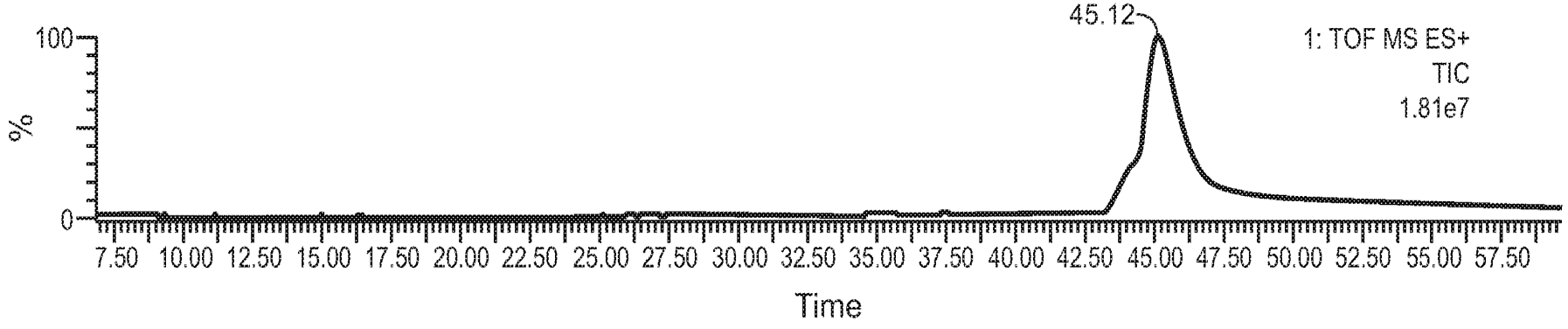


FIG. 1B

SUBSTITUTE SHEET (RULE 26)

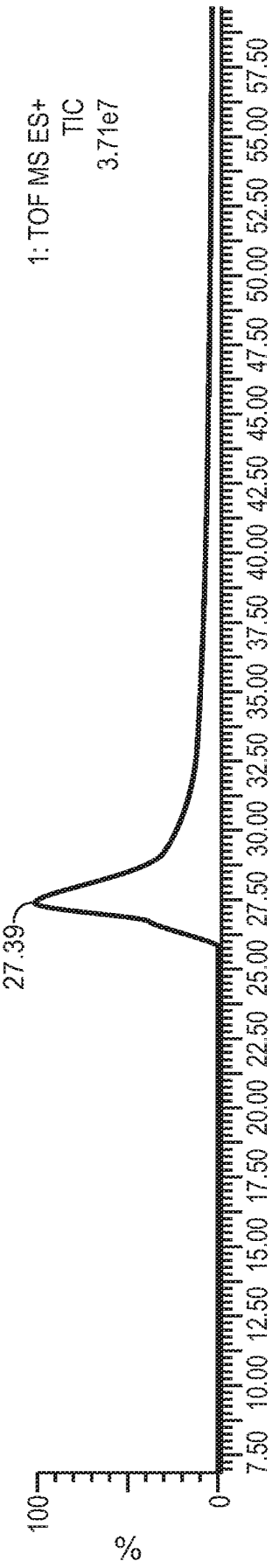


FIG. 1C

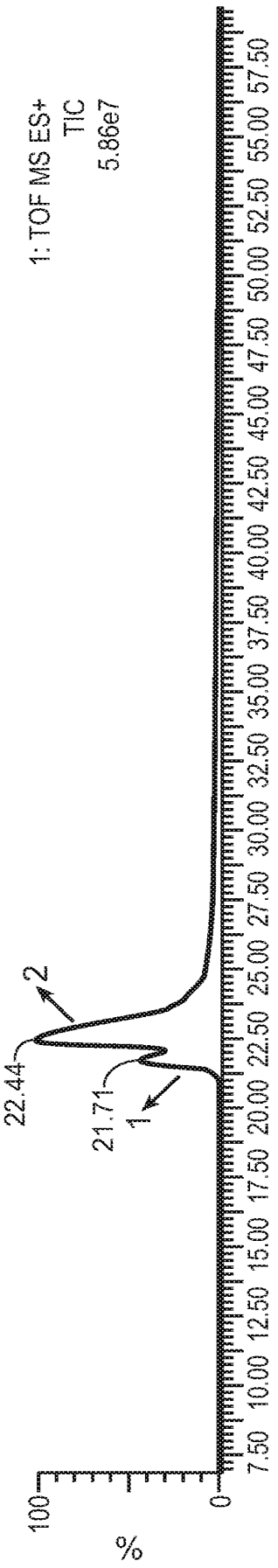


FIG. 1D

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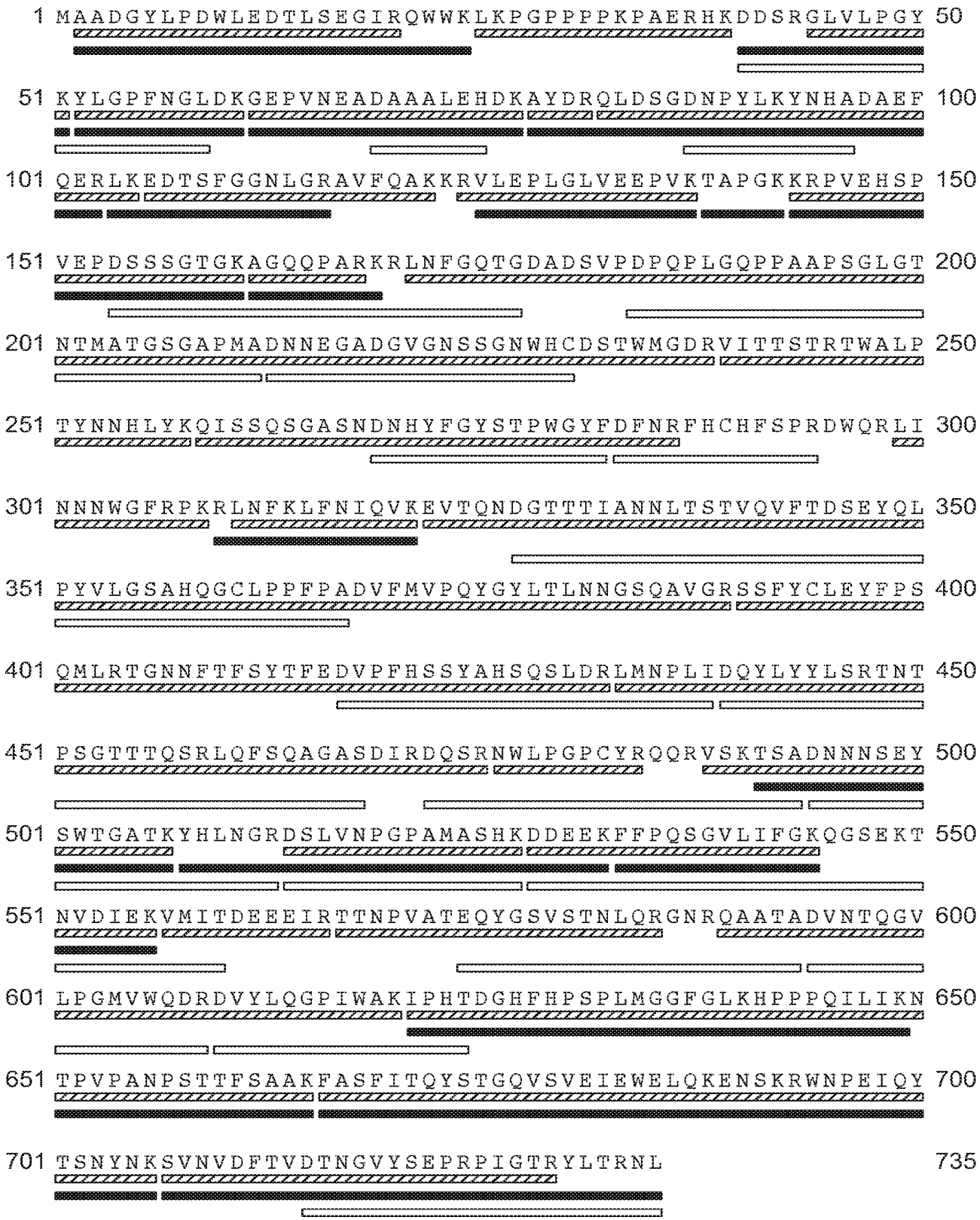

1 MAADGYLPDWLEDTLSEGIRQWWKLKPGPPPPKPAERHKDDSRGLVLPGY 50
51 KYLGPFNGLDKGEPVNEADAAALEHDKAYDRQLDSGDNPYLKYNHADAEF 100
101 QERLKEDTSFGGNLGRAVFQAKKRVLEPLGLVEEPVKTAPGKKRPVEHSP 150
151 VEPDSSSGTGKAGQQPARKRLNFGQTGDADSVPDPQPLGQPPAAPSGLGT 200
201 NTMATGSGAPMADNNEGADGVGNSSGNWHCDSTWMGDRVITTSTRTWALP 250
251 TYNNHLYKQISSQSGASNDNHYFGYSTPWGYFDFNRFHCHFSPRDWQRLI 300
301 NNNWGFRPKRLNFKLFNIQVKEVTQNDGTTTIANNLTSTVQVFTDSEYQL 350
351 PYVLGSAHQGCLPPFPADVFMVPQYGYLTLNNGSQAVGRSSFYCLEYFPS 400
401 QMLRTGNNFTFSYTFEDVPFHSSYAHSQSLDRLMNPLIDQYLYYLSRTNT 450
451 PSGTTTQSRLQFSQAGASDIRDQSRNWLPGPCYRQQRVSKTSADNNNSEY 500
501 SWTGATKYHLNGRDSLVNPGPAMASHKDDEEKFFPQSGVLIFGKQGSEKT 550
551 NVDIEKVMITDEEEIRTTNPVATEQYGSVSTNLQRGNRQAATADVNTQGV 600
601 LPGMVWQDRDVYLQGPIWAKIPHTDGHFHPSPLMGGFGLKHPPPQILIKN 650
651 TPVPANPSTTFSAAKFASFITQYSTGQVSVEIEWELQKENSKRWNPEIQY 700
701 TSNYNKSVNVDFTVDTNGVYSEPRPIGTRYLTRNL 735

FIG. 3

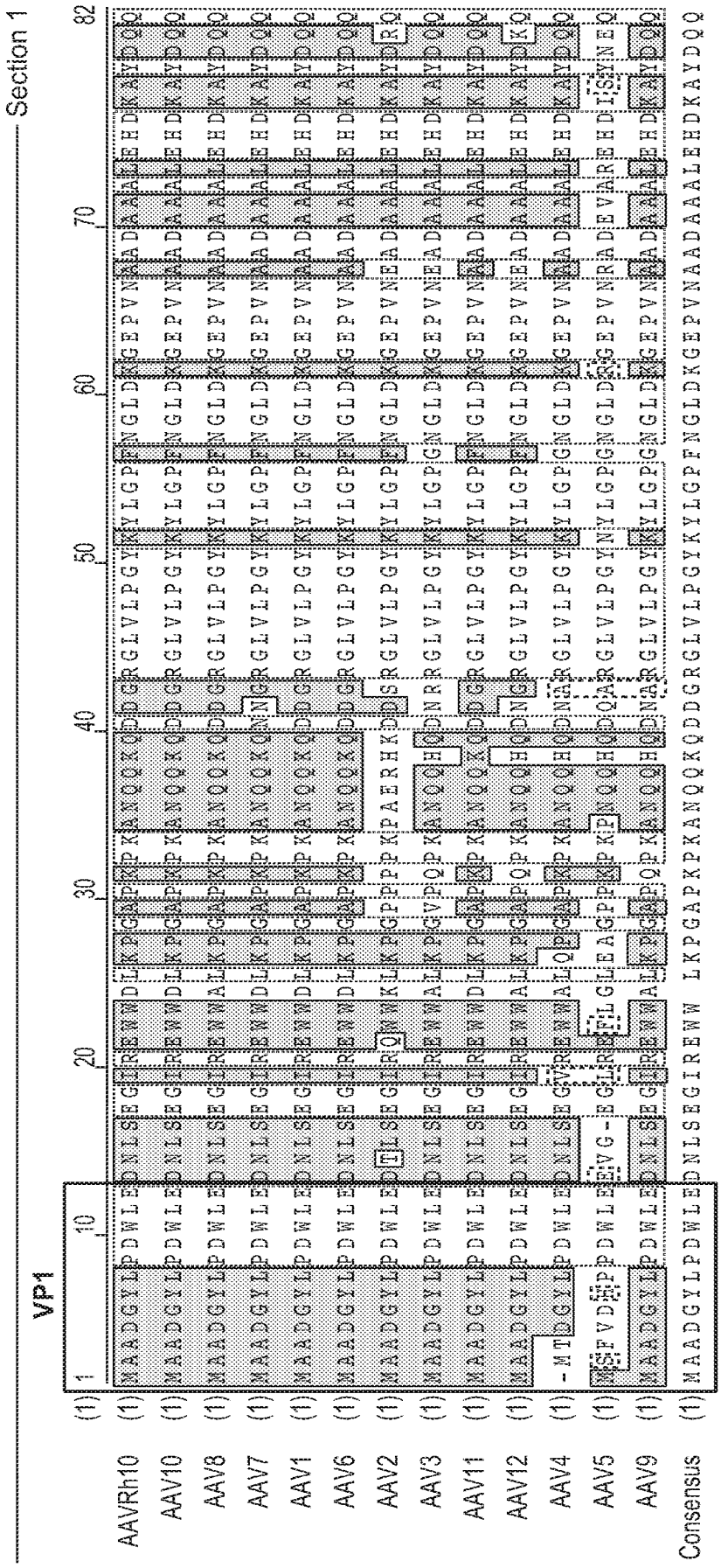


FIG. 5A

Sarepta Exhibit 1002, page 483

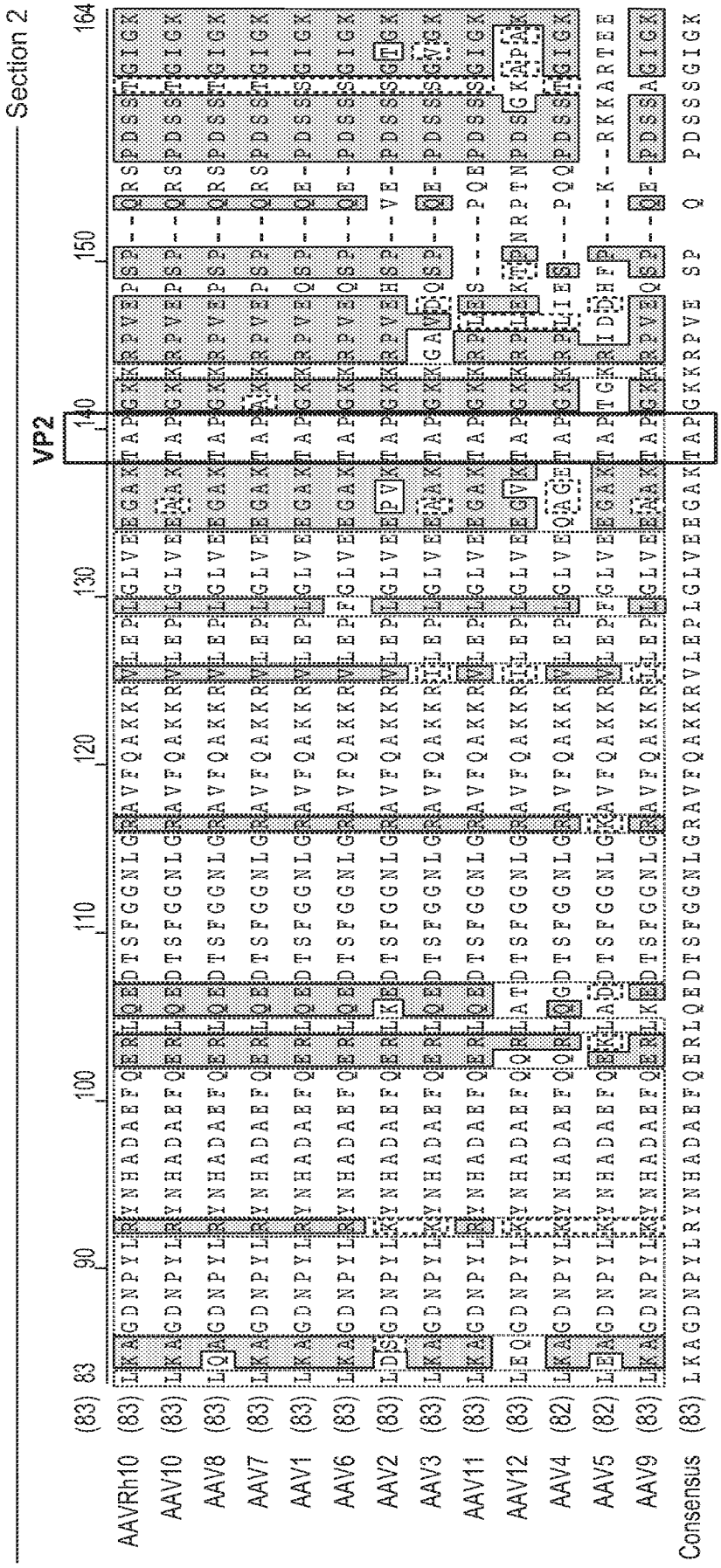


FIG. 5B

Sarepta Exhibit 1002, page 484

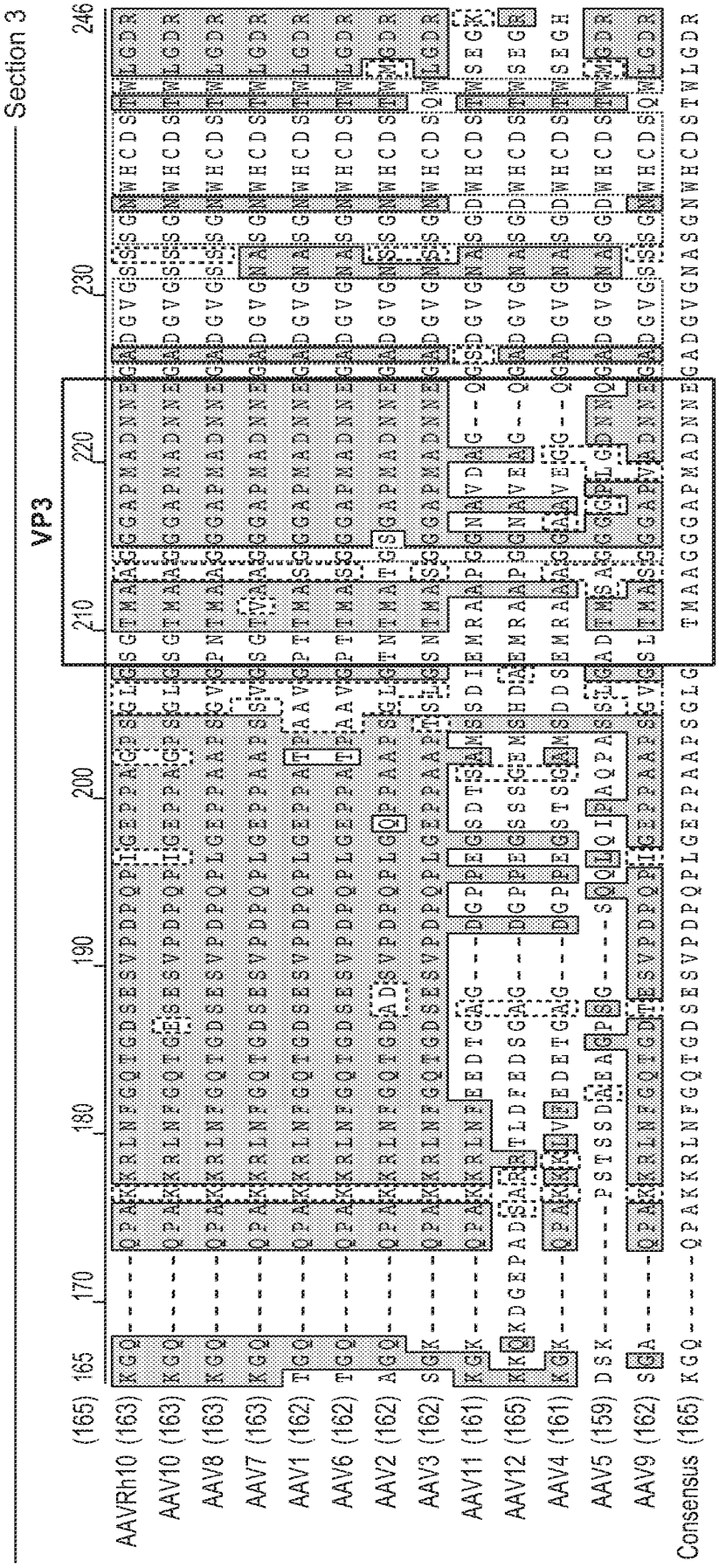


FIG. 5C

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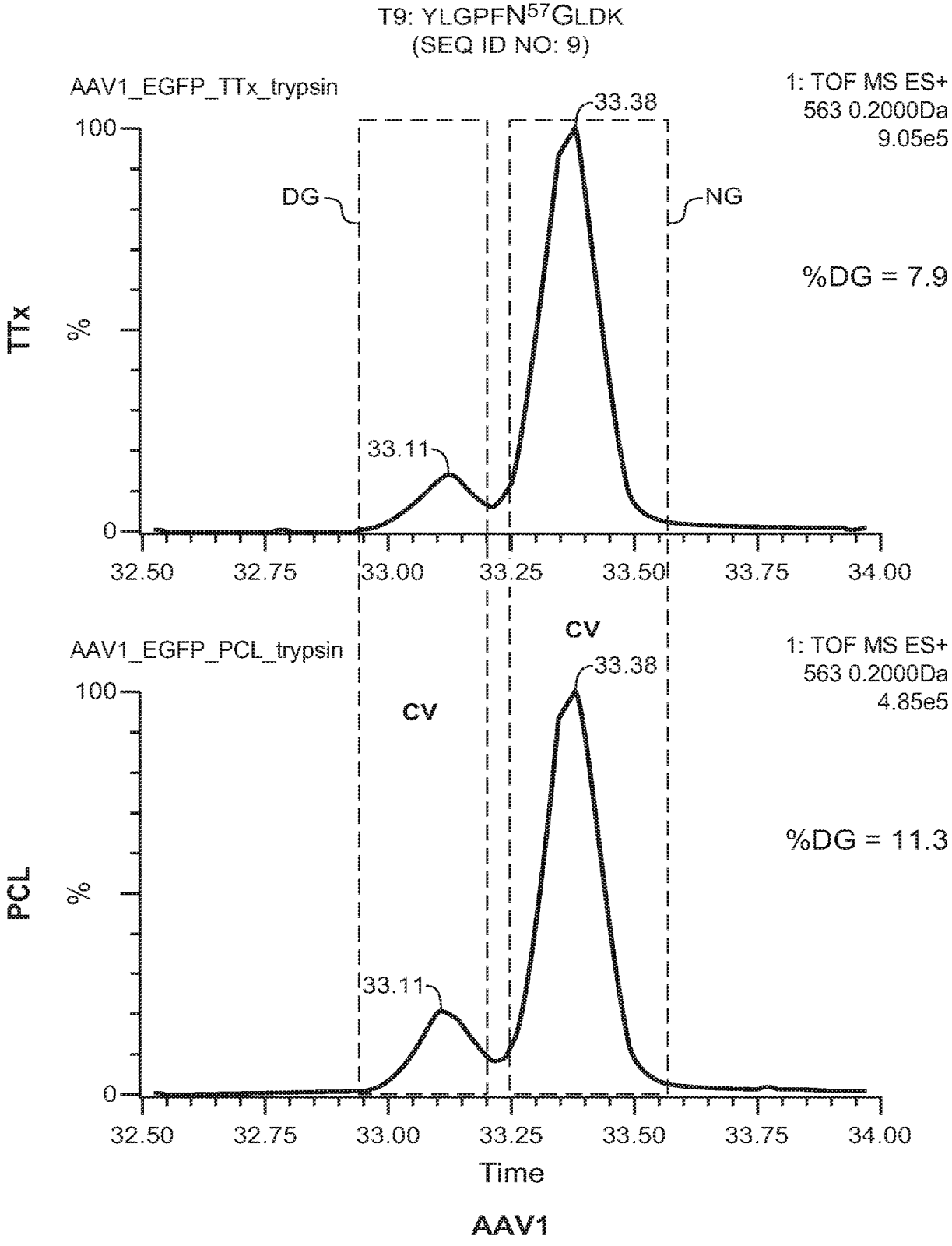

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
9.05e5
33.38
DG
NG
%DG = 7.9
TTx
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
563 0.2000Da
4.85e5
CV
CV
33.38
%DG = 11.3
PCL
%
100
0
33.11
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV1

FIG. 6A

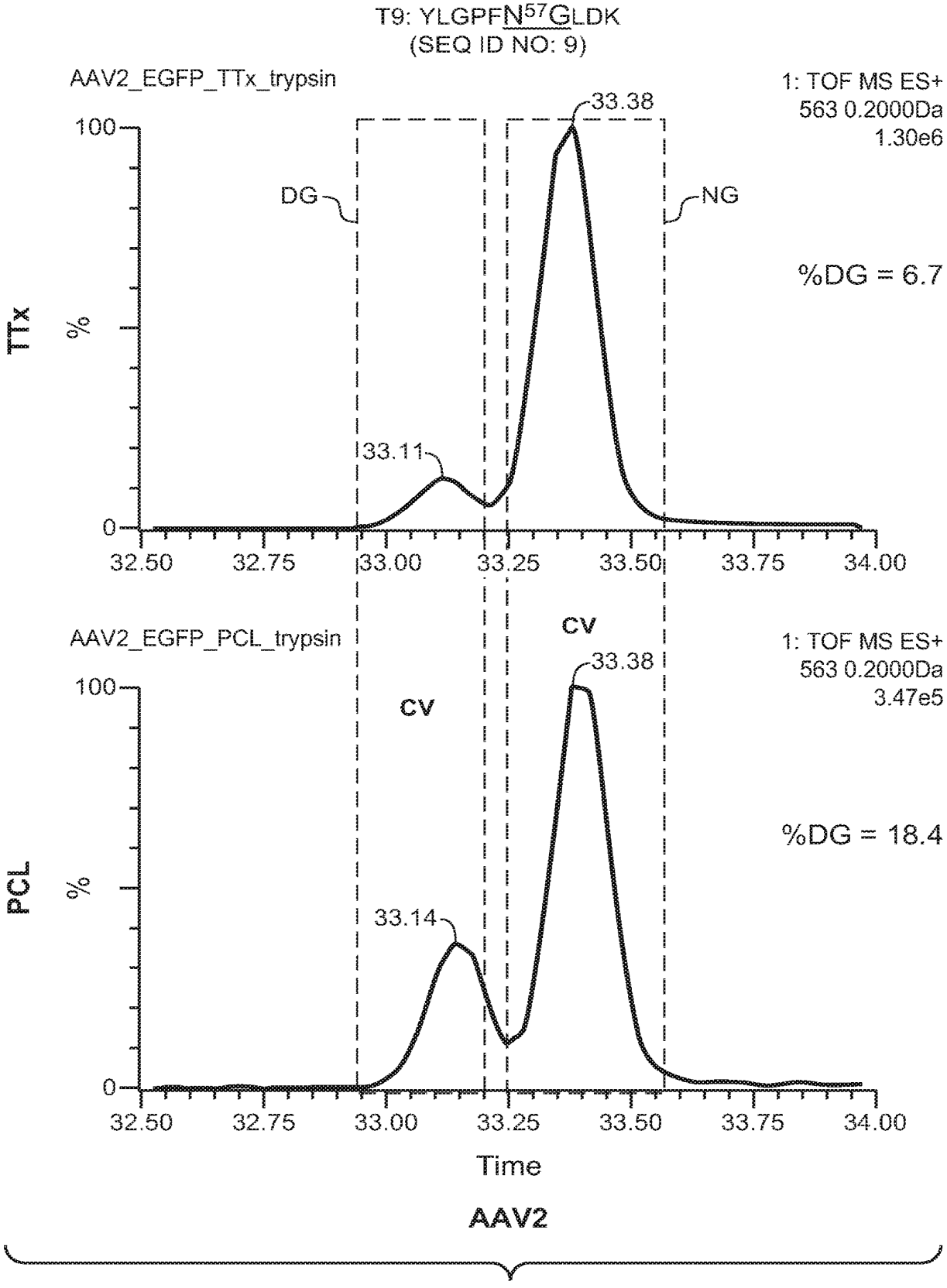

T9: YLGPFN57GLDK
(SEQ ID NO: 9)
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
563 0.2000Da
1.30e6
33.38
DG
NG
%DG = 6.7
TTx
%
33.11
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
AAV2_EGFP_PCL_trypsin
CV
1: TOF MS ES+
563 0.2000Da
3.47e5
33.38
CV
%DG = 18.4
PCL
%
33.14
100
0
32.50
32.75
33.00
33.25
33.50
33.75
34.00
Time
AAV2

FIG. 6B

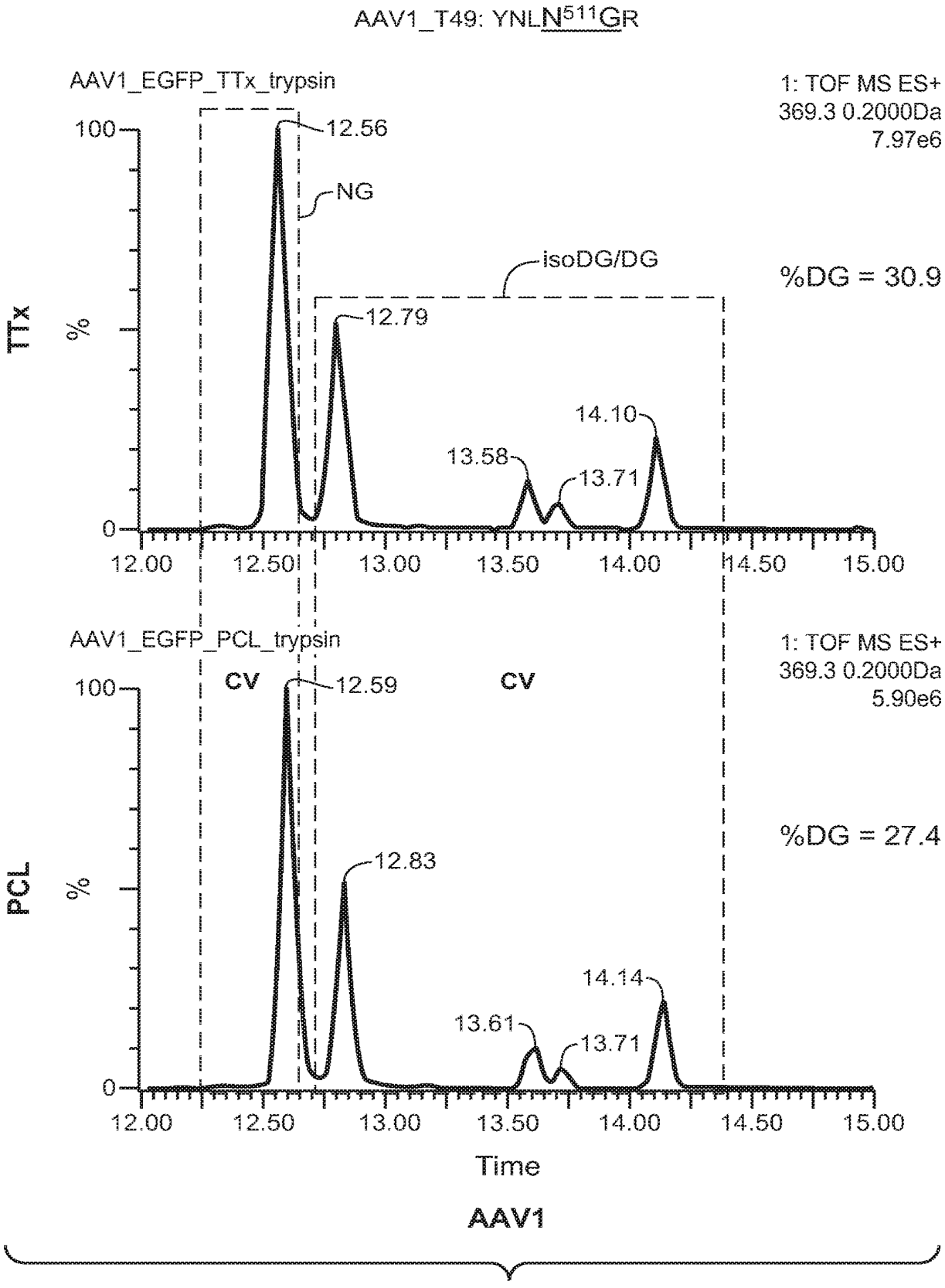

AAV1_T49: YNLN511GR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
369.3 0.2000Da
7.97e6
100
%
0
12.56
NG
isoDG/DG
%DG = 30.9
12.79
14.10
13.58
13.71
12.00
12.50
13.00
13.50
14.00
14.50
15.00
TTx
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
369.3 0.2000Da
5.90e6
CV
12.59
CV
100
%
0
%DG = 27.4
12.83
14.14
13.61
13.71
12.00
12.50
13.00
13.50
14.00
14.50
15.00
PCL
Time
AAV1

FIG. 7A

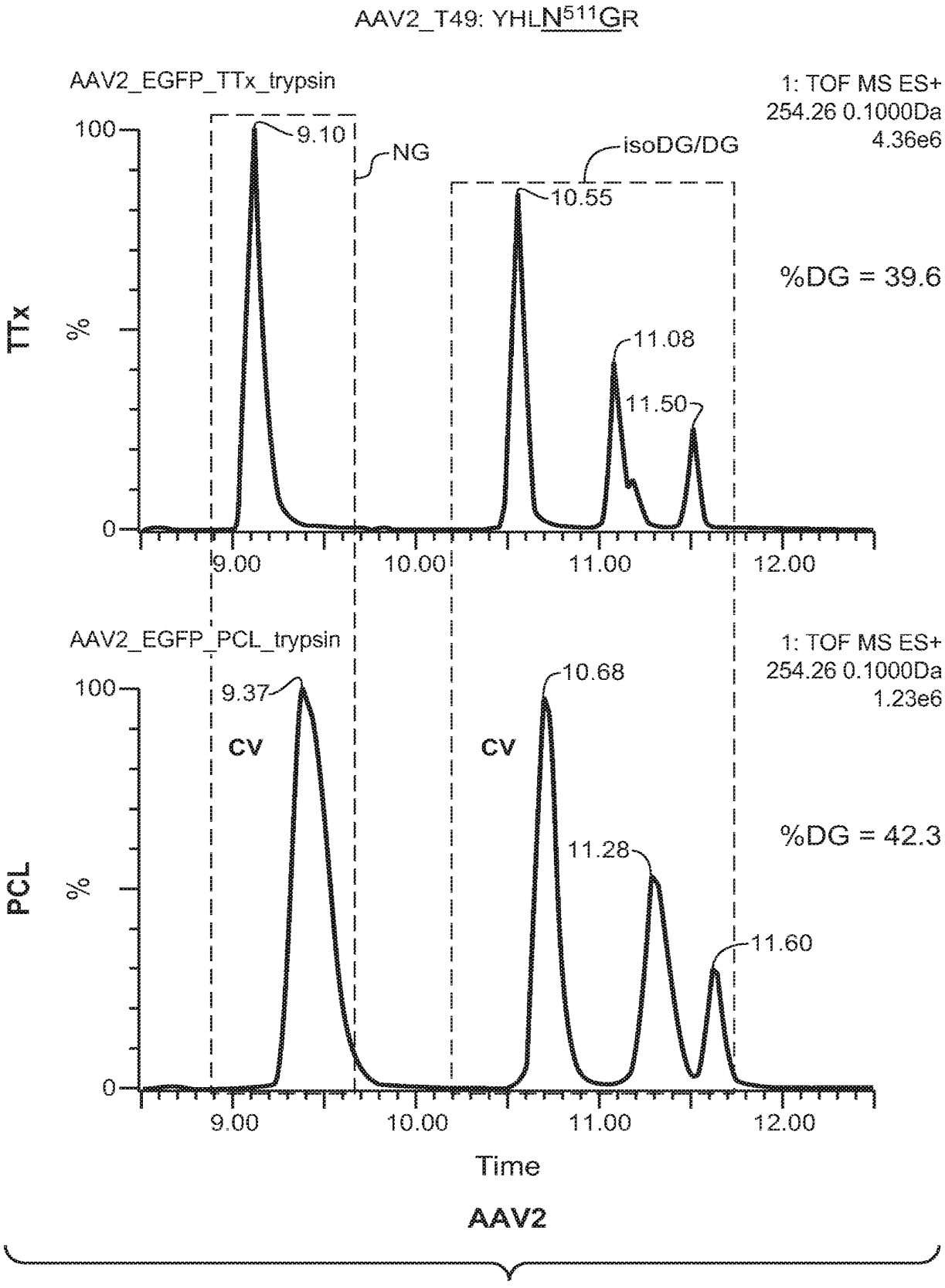

AAV2_T49: YHLN511GR
AAV2_EGFP_TTx_trypsin
1: TOF MS ES+
254.26 0.1000Da
4.36e6
100
%
0
9.10
NG
isoDG/DG
10.55
%DG = 39.6
11.08
11.50
9.00
10.00
11.00
12.00
TTx
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
254.26 0.1000Da
1.23e6
100
%
0
9.37
CV
CV
10.68
%DG = 42.3
11.28
11.60
9.00
10.00
11.00
12.00
PCL
Time
AAV2

FIG. 7B

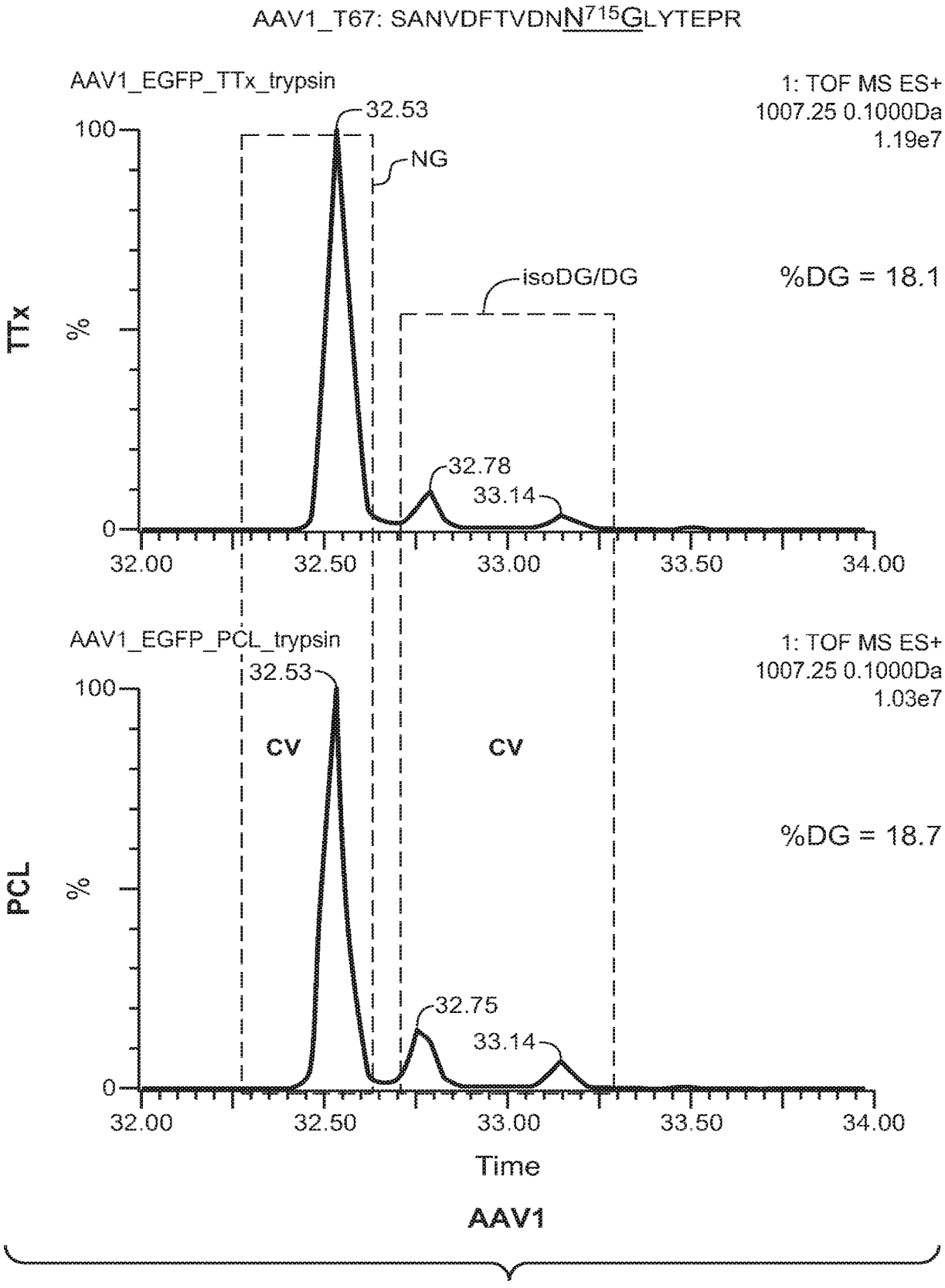

AAV1_T67: SANVDFTVDNN715GLYTEPR
AAV1_EGFP_TTx_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.19e7
32.53
NG
isoDG/DG
%DG = 18.1
32.78
33.14
TTx
%
100
0
32.00
32.50
33.00
33.50
34.00
AAV1_EGFP_PCL_trypsin
1: TOF MS ES+
1007.25 0.1000Da
1.03e7
32.53
CV
CV
%DG = 18.7
32.75
33.14
PCL
%
100
0
32.00
32.50
33.00
33.50
34.00
Time
AAV1

FIG. 8A

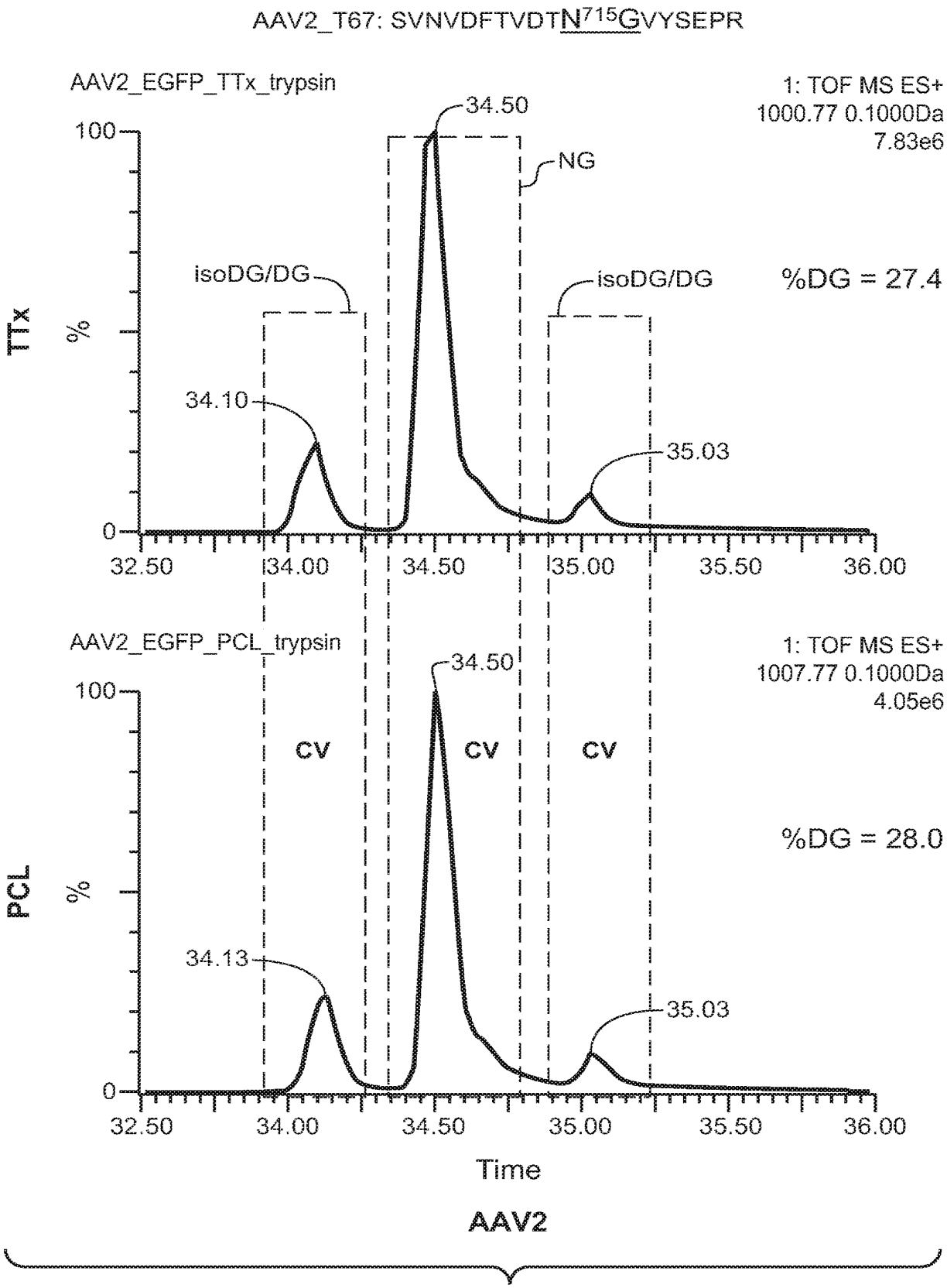
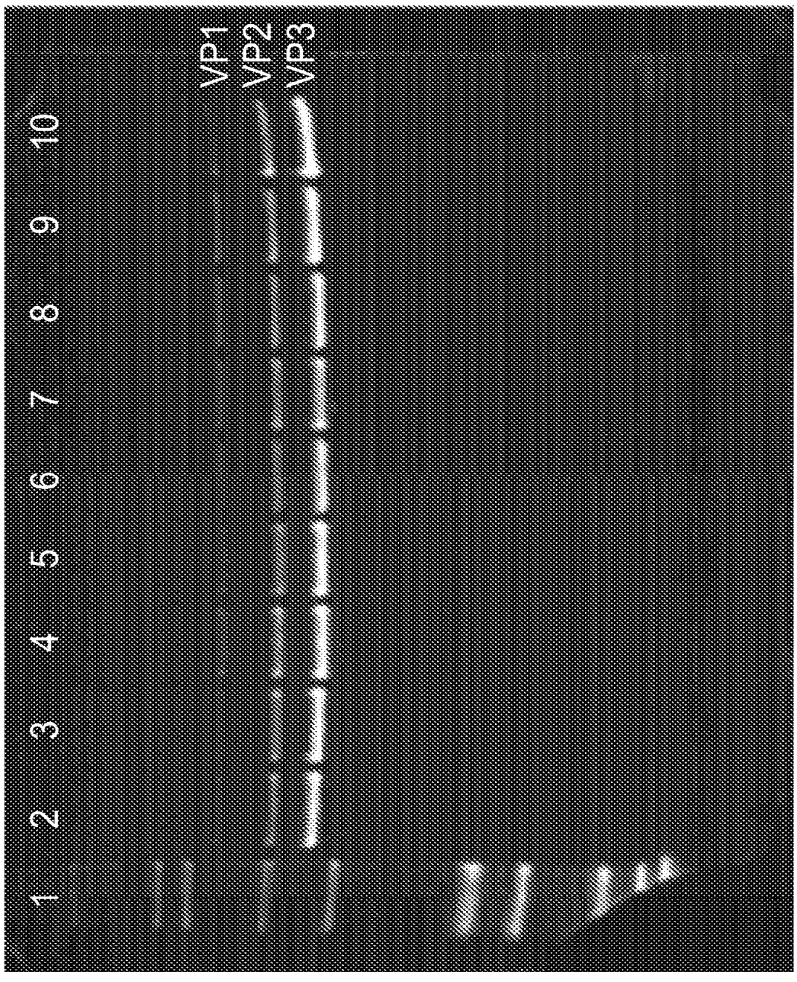

AAV2_T67: SVNVDFTVDTN715GVYSEPR
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7.83e6
34.50
NG
isoDG/DG
isoDG/DG
%DG = 27.4
TTx
%
34.10
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
AAV2_EGFP_PCL_trypsin
1: TOF MS ES+
1007.77 0.1000Da
4.05e6
34.50
CV
CV
CV
%DG = 28.0
PCL
%
34.13
35.03
100
0
32.50
34.00
34.50
35.00
35.50
36.00
Time
AAV2

FIG. 8B



Deacetylation AAV5 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAC-AAV5 (S2G)/CBA-eGFP X17052C
3	deAC-AAV5 (S2L)/CBA-eGFP X17052D
4	deAC-AAV5 (S2GVP3)/CBA-eGFP X17058D
5	deAC-AAV5 (S2LVP3)/CBA-eGFP X17065A
6	deAC-AAV5 (S2PVP1VP3)/CBA-eGFP X17065B
7	deAC-AAV5 (S2GVP1VP3)/CBA-eGFP X17065C
8	deAC-AAV5 (S2PVP3)/CBA-eGFP X17065D
9	deAC-AAV5 (S2P)/CBA-eGFP X17065E
10	deAC-AAV5 (S2LVP1VP3)/CBA-eGFP X17072B

(~1E10 VGs/Lane)

FIG. 9

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Sarepta Exhibit 1002, page 493

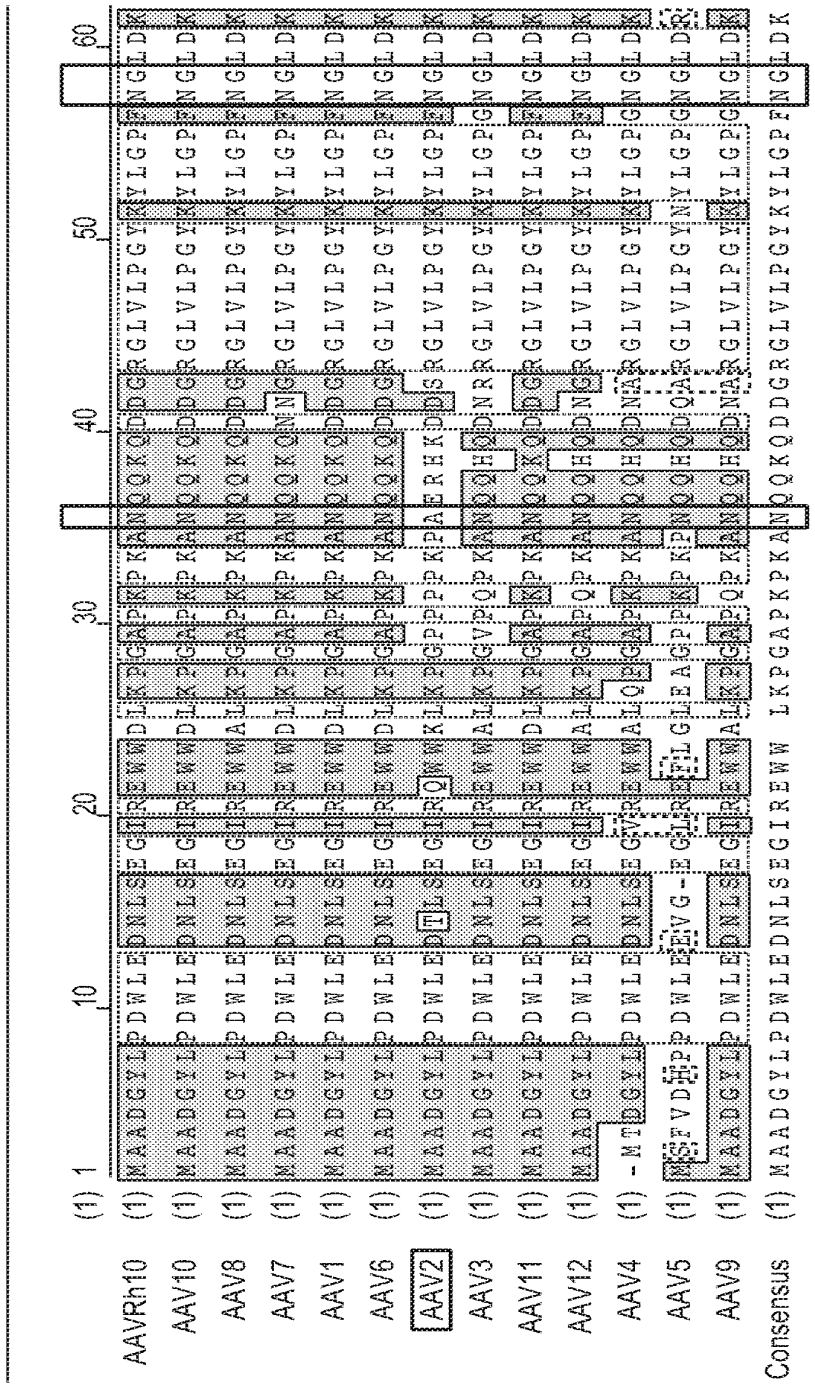
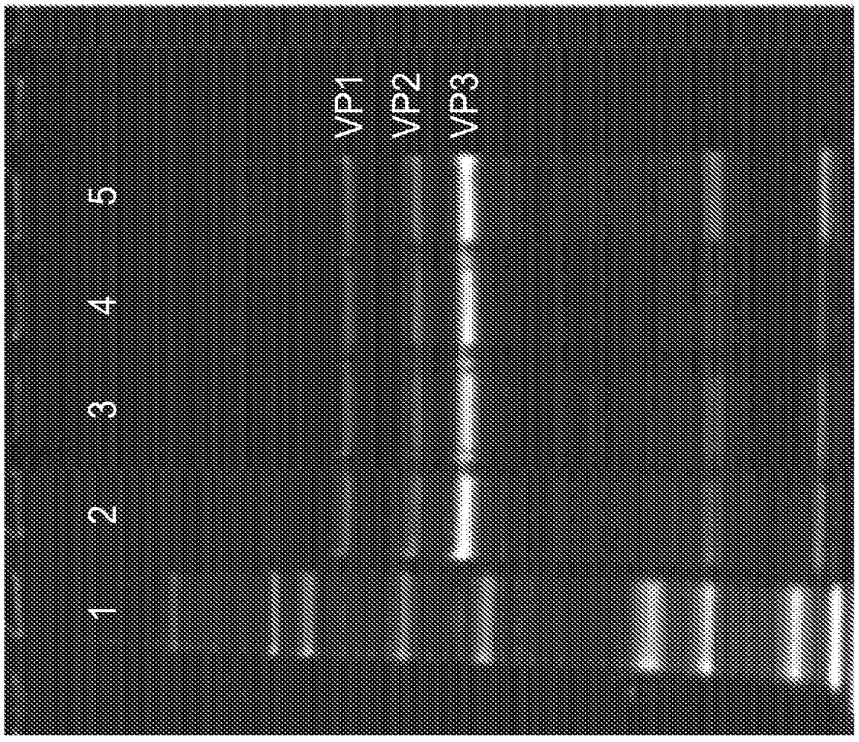


FIG. 13

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Deamidation AAV2 Mutant Vectors

Lane	Sample
1	Mark12 Marker
2	deAMID-AAV2 (N57K)/CBA-eGFP X17079B
3	deAMID-AAV2 (N57D)/CBA-eGFP X17079C
4	deAMID-AAV2 (N57Q)/CBA-eGFP X17079D
5	deAMID-AAV2 (A35N)/CBA-eGFP X17079E

(~5E10 VGs/Lane)

FIG. 16

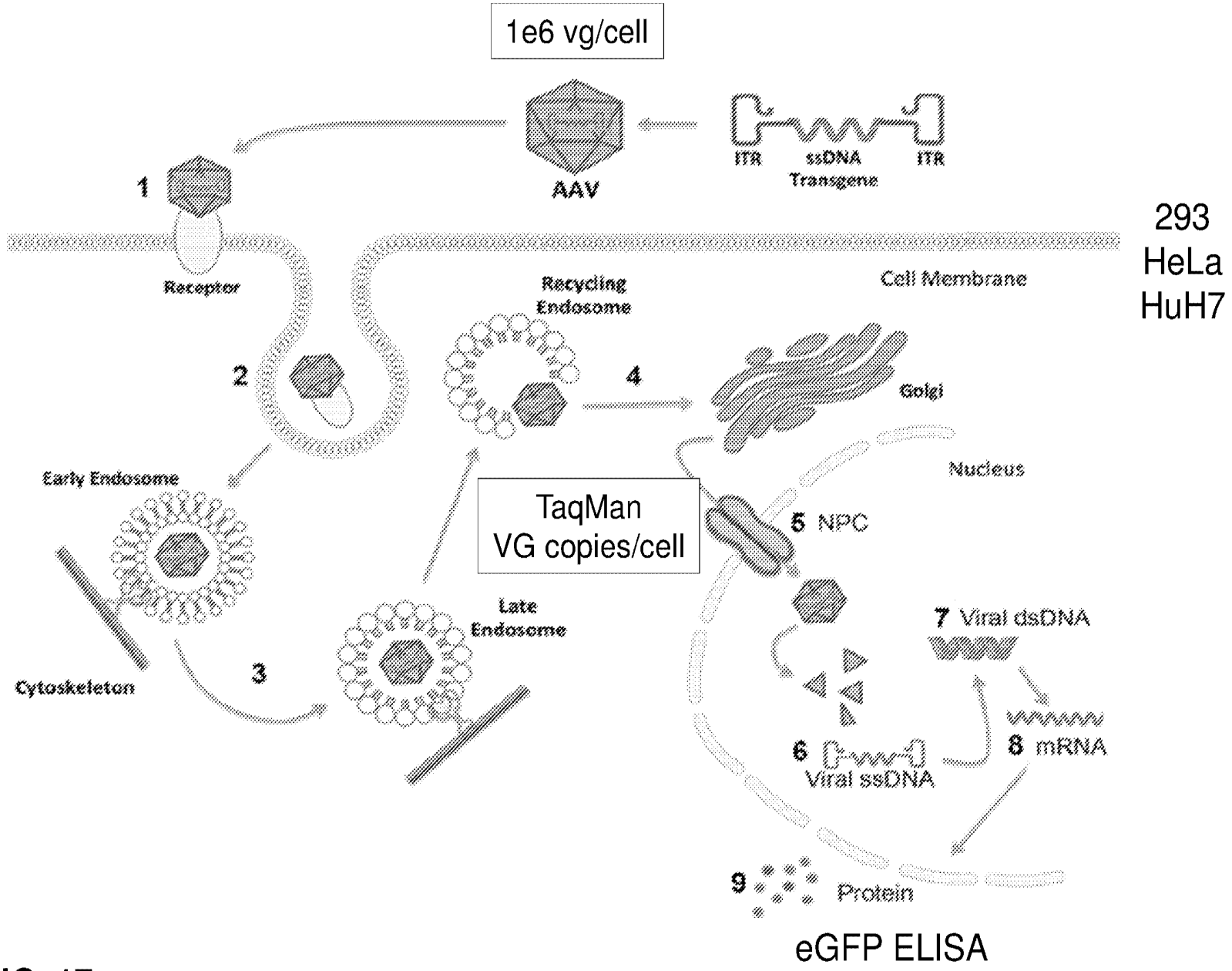

1e6 vg/cell
AAV
ITR
ssDNA
Transgene
ITR
1
Receptor
293
HeLa
HuH7
Cell Membrane
Recycling
Endosome
2
4
Golgi
Early Endosome
Nucleus
TaqMan
VG copies/cell
5 NPC
Late
Endosome
7 Viral dsDNA
Cytoskeleton
3
6
Viral ssDNA
8 mRNA
9
Protein
eGFP ELISA

FIG. 17

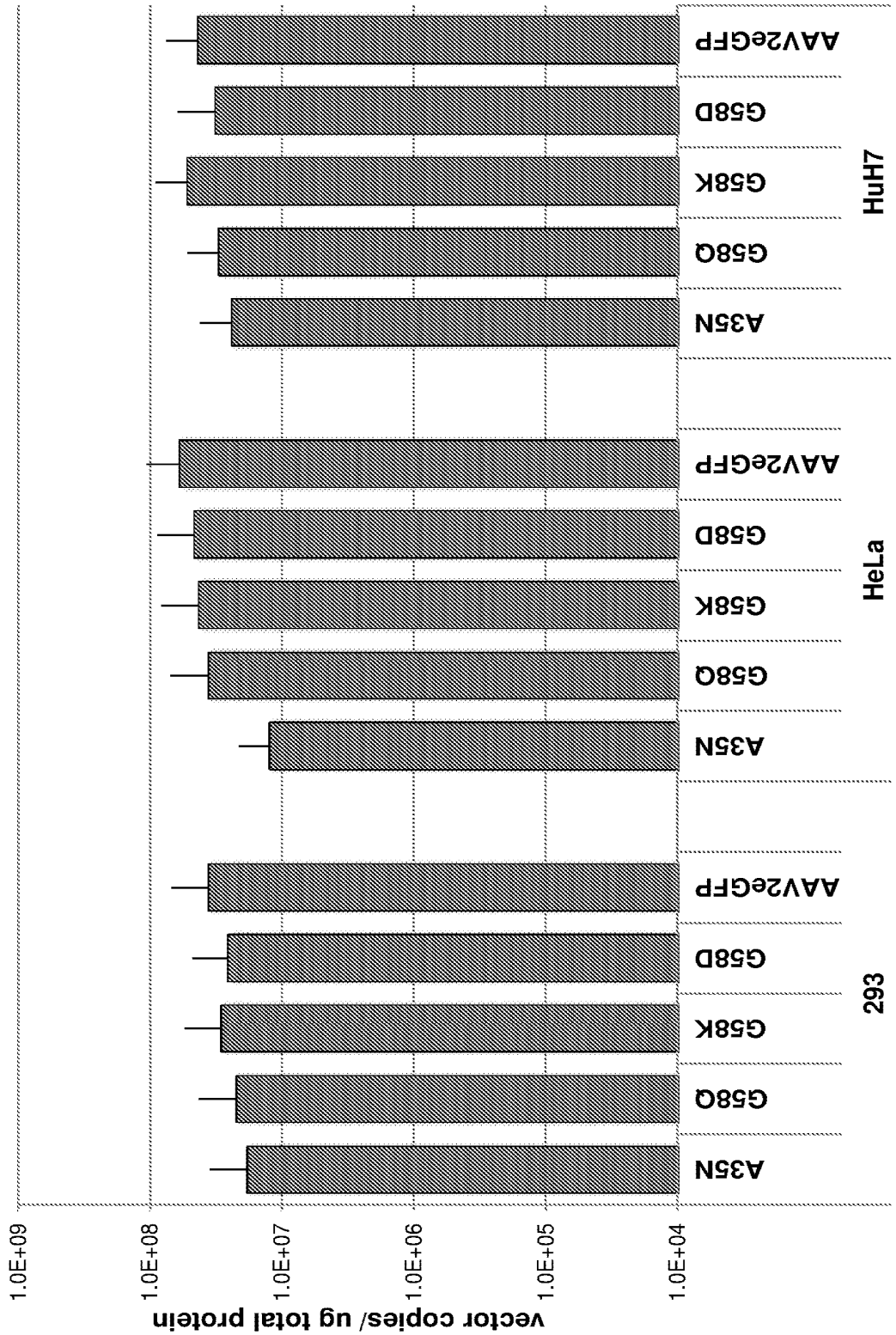
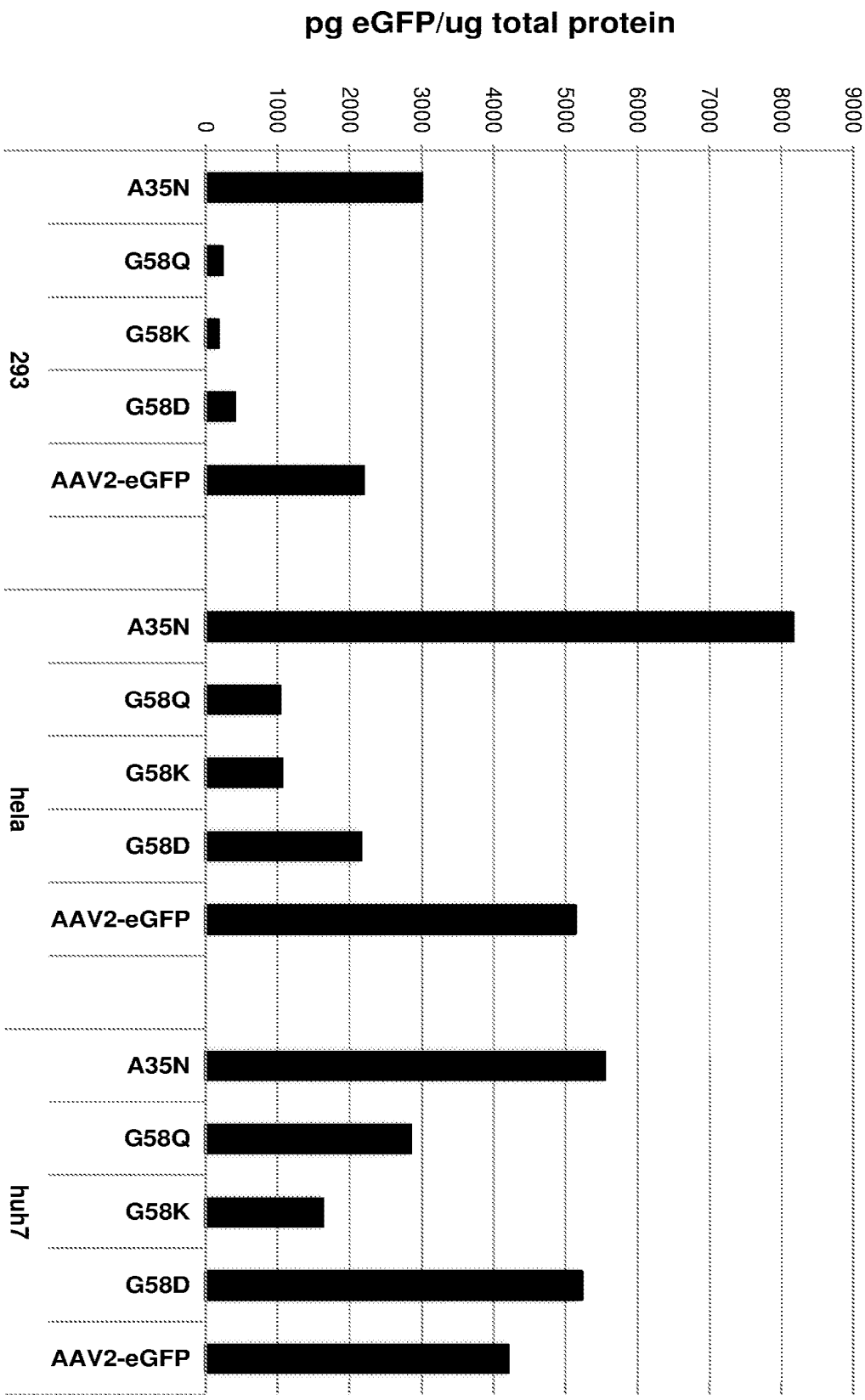

vector copies/ ug total protein
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
293
A35N
G58Q
G58K
G58D
AAV2eGFP
HeLa
A35N
G58Q
G58K
G58D
AAV2eGFP
HuH7
A35N
G58Q
G58K
G58D
AAV2eGFP

FIG. 18

FIG. 19



PCT/US2017/046814

WO 2018/035059

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Sequence Listing was accepted.
See attached Validation Report.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Wheat Jr, Scott (ASRC)
Timestamp: [year=2019; month=2; day=19; hr=12; min=50; sec=54; ms=369; ]
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Validated By CRFValidator v 1.0.5

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Output Set:

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Finished: 2019-02-14 16:48:38.434
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Total Errors: 1
No. of SeqIDs Defined: 40
Actual SeqID Count: 40

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W	213	Artificial or Unknown found in <213> in SEQ ID (21)

Input Set:

Output Set:

Started: 2019-02-14 16:48:37.446
Finished: 2019-02-14 16:48:38.434
Elapsed: 0 hr(s) 0 min(s) 0 sec(s) 988 ms
Total Warnings: 44
Total Errors: 1
No. of SeqIDs Defined: 40
Actual SeqID Count: 40

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W 447	n or Xaa used, for: SEQID(30) on line number 1017
W 447	n or Xaa used, for: SEQID(30) on line number 1019
W 447	n or Xaa used, for: SEQID(30) on line number 1023
W 447	n or Xaa used, for: SEQID(30) on line number 1025
W 447	n or Xaa used, for: SEQID(40) on line number 1220

SEQUENCE LISTING

<110> Genzyme Corporation
JIN, Xiaoying
O'RIORDAN, Catherine
LIU, Ling
ZHANG, Kate

<120> METHODS FOR DETECTING AAV

<130> 15979-20141.00

<140> US 16/325,653
<141> 2019-02-14

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Cys

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<210> 3

<211> 735
<212> PRT
<213> Adeno-associated virus 2

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            20   25   30
Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
        35   40   45
Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50   55   60
Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65   70   75   80
Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
                85   90   95
Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
            100   105   110
Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115   120   125
Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
    130   135   140
Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
145   150   155   160
Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
                165   170   175
Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
            180   185   190
Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
        195   200   205
Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
    210   215   220
Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg Val Ile
225   230   235   240
Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
                245   250   255
Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala Ser Asn Asp Asn His Tyr
            260   265   270
Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg Phe His
        275   280   285
Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn Asn Trp
    290   295   300
Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile Gln Val
305   310   315   320
Lys Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu
                325   330   335
Thr Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr
            340   345   350
Val Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp
        355   360   365
Val Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser
    370   375   380
Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser
385   390   395   400
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                405   410   415

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Asp Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu Asp Arg
            420                 425                 430
Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser Arg Thr
        435                 440                 445
Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser Arg Leu Gln Phe Ser Gln
    450                 455                 460
Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser Arg Asn Trp Leu Pro Gly
465                 470                 475                 480
Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys Thr Ser Ala Asp Asn Asn
                485                 490                 495
Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr Lys Tyr His Leu Asn Gly
            500                 505                 510
Arg Asp Ser Leu Val Asn Pro Gly Pro Ala Met Ala Ser His Lys Asp
        515                 520                 525
Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly Val Leu Ile Phe Gly Lys
    530                 535                 540
Gln Gly Ser Glu Lys Thr Asn Val Asp Ile Glu Lys Val Met Ile Thr
545                 550                 555                 560
Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro Val Ala Thr Glu Gln Tyr
                565                 570                 575
Gly Ser Val Ser Thr Asn Leu Gln Arg Gly Asn Arg Gln Ala Ala Thr
            580                 585                 590
Ala Asp Val Asn Thr Gln Gly Val Leu Pro Gly Met Val Trp Gln Asp
        595                 600                 605
Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr
    610                 615                 620
Asp Gly His Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Leu Lys
625                 630                 635                 640
His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asn
                645                 650                 655
Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe Ala Ser Phe Ile Thr Gln
            660                 665                 670
Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys
        675                 680                 685
Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr
    690                 695                 700
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Gly Ile Arg

<210> 5
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<210> 6
<211> 13
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<220>
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<210> 7
<211> 39
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<220>
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            20   25   30
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<220>

<223> Synthetic Construct

<400> 8
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Gly Cys Thr Cys Gly Cys Thr Cys Gly Cys Thr Cys Ala Cys Thr Gly
            20   25   30
Ala Gly Gly Cys Cys Gly Gly Gly Cys Gly Ala Cys Cys Ala Ala Ala
        35   40   45
Gly Gly Thr Cys Gly Cys Cys Cys Ala Cys Gly Cys Cys Cys Gly Gly
    50   55   60
Gly Cys Thr Thr Thr Gly Cys Cys Cys Gly Gly Gly Cys Gly
65   70   75

<210> 9
<211> 10
<212> PRT
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<220>
<223> Synthetic Construct

<400> 9
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Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr Val
            20   25   30
Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp Val
        35   40   45
Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser Gln
    50   55   60
Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser Gln
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Met Leu Arg

<210> 11
<211> 6
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<210> 12
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<210> 14
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 1   5   10   15
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<210> 15
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<210> 16
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            20   25   30
Lys Pro Ala Glu Arg His Lys Asp Asp Ser Arg Gly Leu Val Leu Pro
        35   40   45
Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50   55   60
Val Asn Glu Ala Asp Ala Ala Ala Leu Glu His Asp Lys Ala Tyr Asp
65   70   75   80
Arg Gln Leu Asp Ser Gly Asp Asn Pro Tyr Leu Lys Tyr Asn His Ala
                85   90   95
Asp Ala Glu Phe Gln Glu Arg Leu Lys Glu Asp Thr Ser Phe Gly Gly
            100   105   110
Asn Leu Gly Arg Ala Val Phe Gln Ala Lys Lys Arg Val Leu Glu Pro
        115   120   125
Leu Gly Leu Val Glu Glu Pro Val Lys Thr Ala Pro Gly Lys Lys Arg
    130   135   140
Pro Val Glu His Ser Pro Val Glu Pro Asp Ser Ser Ser Gly Thr Gly
145   150   155   160
Lys Ala Gly Gln Gln Pro Ala Arg Lys Arg Leu Asn Phe Gly Gln Thr
                165   170   175
Gly Asp Ala Asp Ser Val Pro Asp Pro Gln Pro Leu Gly Gln Pro Pro
            180   185   190
Ala Ala Pro Ser Gly Leu Gly Thr Asn Thr Met Ala Thr Gly Ser Gly
        195   200   205
Ala Pro Met Ala Asp Asn Asn Glu Gly Ala Asp Gly Val Gly Asn Ser
    210   215   220
Ser Gly Asn Trp His Cys Asp Ser Thr Trp Met Gly Asp Arg Val Ile
225   230   235   240
Thr Thr Ser Thr Arg Thr Trp Ala Leu Pro Thr Tyr Asn Asn His Leu
                245   250   255
Tyr Lys Gln Ile Ser Ser Gln Ser Gly Ala Ser Asn Asp Asn His Tyr
            260   265   270
Phe Gly Tyr Ser Thr Pro Trp Gly Tyr Phe Asp Phe Asn Arg Phe His
        275   280   285
Cys His Phe Ser Pro Arg Asp Trp Gln Arg Leu Ile Asn Asn Asn Trp
    290   295   300
Gly Phe Arg Pro Lys Arg Leu Asn Phe Lys Leu Phe Asn Ile Gln Val
305   310   315   320
Lys Glu Val Thr Gln Asn Asp Gly Thr Thr Thr Ile Ala Asn Asn Leu
                325   330   335
Thr Ser Thr Val Gln Val Phe Thr Asp Ser Glu Tyr Gln Leu Pro Tyr
            340   345   350
Val Leu Gly Ser Ala His Gln Gly Cys Leu Pro Pro Phe Pro Ala Asp

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        355                 360                 365
Val Phe Met Val Pro Gln Tyr Gly Tyr Leu Thr Leu Asn Asn Gly Ser
    370                 375                 380
Gln Ala Val Gly Arg Ser Ser Phe Tyr Cys Leu Glu Tyr Phe Pro Ser
385                 390                 395                 400
Gln Met Leu Arg Thr Gly Asn Asn Phe Thr Phe Ser Tyr Thr Phe Glu
                405                 410                 415
Asp Val Pro Phe His Ser Ser Tyr Ala His Ser Gln Ser Leu Asp Arg
            420                 425                 430
Leu Met Asn Pro Leu Ile Asp Gln Tyr Leu Tyr Tyr Leu Ser Arg Thr
        435                 440                 445
Asn Thr Pro Ser Gly Thr Thr Thr Gln Ser Arg Leu Gln Phe Ser Gln
    450                 455                 460
Ala Gly Ala Ser Asp Ile Arg Asp Gln Ser Arg Asn Trp Leu Pro Gly
465                 470                 475                 480
Pro Cys Tyr Arg Gln Gln Arg Val Ser Lys Thr Ser Ala Asp Asn Asn
                485                 490                 495
Asn Ser Glu Tyr Ser Trp Thr Gly Ala Thr Lys Tyr His Leu Asn Gly
            500                 505                 510
Arg Asp Ser Leu Val Asn Pro Gly Pro Ala Met Ala Ser His Lys Asp
        515                 520                 525
Asp Glu Glu Lys Phe Phe Pro Gln Ser Gly Val Leu Ile Phe Gly Lys
    530                 535                 540
Gln Gly Ser Glu Lys Thr Asn Val Asp Ile Glu Lys Val Met Ile Thr
545                 550                 555                 560
Asp Glu Glu Glu Ile Arg Thr Thr Asn Pro Val Ala Thr Glu Gln Tyr
                565                 570                 575
Gly Ser Val Ser Thr Asn Leu Gln Arg Gly Asn Arg Gln Ala Ala Thr
            580                 585                 590
Ala Asp Val Asn Thr Gln Gly Val Leu Pro Gly Met Val Trp Gln Asp
        595                 600                 605
Arg Asp Val Tyr Leu Gln Gly Pro Ile Trp Ala Lys Ile Pro His Thr
    610                 615                 620
Asp Gly His Phe His Pro Ser Pro Leu Met Gly Gly Phe Gly Leu Lys
625                 630                 635                 640
His Pro Pro Pro Gln Ile Leu Ile Lys Asn Thr Pro Val Pro Ala Asn
                645                 650                 655
Pro Ser Thr Thr Phe Ser Ala Ala Lys Phe Ala Ser Phe Ile Thr Gln
            660                 665                 670
Tyr Ser Thr Gly Gln Val Ser Val Glu Ile Glu Trp Glu Leu Gln Lys
        675                 680                 685
Glu Asn Ser Lys Arg Trp Asn Pro Glu Ile Gln Tyr Thr Ser Asn Tyr
    690                 695                 700
Asn Lys Ser Val Asn Val Asp Phe Thr Val Asp Thr Asn Gly Val Tyr
705                 710                 715                 720
Ser Glu Pro Arg Pro Ile Gly Thr Arg Tyr Leu Thr Arg Asn Leu
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<220>
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<400> 17

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            20                  25                  30
Lys Ala Asn Gln Gln Lys Gln Asp Asp Gly Arg Gly Leu Val Leu Pro
        35                  40                  45
Gly Tyr Lys Tyr Leu Gly Pro Phe Asn Gly Leu Asp Lys Gly Glu Pro
    50                  55                  60
Val Asn Ala Ala Asp Ala A
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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	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	204 minus 20 =	* 184			OR	x 100 =	18400
INDEPENDENT CLAIMS (37 CFR 1.16(h))	18 minus 3 =	* 15				x 460 =	6900
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).						400
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							820
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	28100

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	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
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.

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 FORMALITIES LETTER



Date Mailed: 04/23/2019

NOTIFICATION TO FILE CORRECTED PAPERS UNDER 35 U.S.C. 371 IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

The following items have been submitted by the applicant or the International Bureau to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Priority Document
- 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 02/14/2019
- 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
- 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 required to correct the following defect(s) in the application papers:

The application is not in compliance with 37 CFR 1.52 and PCT Rule 11 because pages 81 and 82 contains shading in the tables.. Application papers (including any electronically submitted papers) must be presented in a form having sufficient clarity and contrast between the background of the paper and the writing thereon to permit the Office to electronically reproduce the papers by use of digital imaging and optical character recognition. See 37 CFR 1.52(a)(1)(v) and PCT Rules 11.2(a) and 11.9(d).

Applicant must furnish a substitute specification in compliance with 37 CFR 1.52 and 1.125(a), including the following:

- A substitute specification in clean form without markings. See MPEP 608.01(q).
- A marked-up copy of the substitute specification showing all changes relative to the immediate prior version of the specification of record. The text of any added subject matter must be shown

by underlining the added text. The text of any deleted matter must be shown by strike-through except that double brackets placed before and after the deleted characters may be used to show deletion of five or fewer consecutive characters. The text of any deleted subject matter must be shown by being placed within double brackets if strike-through cannot be easily perceived. See 37 CFR 1.125(c) and MPEP 608.01(q).

- A statement that the substitute specification contains no new matter. See 37 CFR 1.125(b).

The applicant needs to satisfy supplemental fees problems indicated below.

The following items **MUST** be furnished within the period set forth below:

- Additional claim fees of $**26120** as a undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**26120** for an Undiscounted Entity:

Total additional claim fee(s) for this application is $**26120**

- $**6900** for **15** independent claims over 3.
- $**18400** for **184** total claims over 20.
- $**820** for multiple dependent claim surcharge.

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

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)

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 you are not using EFS-Web to submit your reply, you must include a copy of this notice.

MARILYN J YOUNGER

Telephone: (571) 272-8183

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

OC000000107564333

Date Mailed: 04/23/2019

NOTICE OF ACCEPTANCE OF AUTHORIZATION TO PERMIT ACCESS TO APPLICATION VIA PRIORITY DOCUMENT EXCHANGE

This is in response to the applicant's authorization to permit access to the application-as-filed by participating offices under 37 CFR 1.14(h)(1) submitted on 02/14/2019.

The authorization to permit access to the application under 37 CFR 1.14(h)(1) is accepted.

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/

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

OC000000107564335

Date Mailed: 04/23/2019

NOTICE OF ACCEPTANCE OF AUTHORIZATION TO PERMIT ACCESS TO SEARCH RESULTS

This is in response to the applicant's authorization to permit access to the search results from the instant application under 37 CFR 1.14(h)(2) submitted on 02/14/2019.

The authorization to permit access to the search results under 37 CFR 1.14(h)(2) is accepted.

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/

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					
2		1				
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5		4				
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	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
51		(1)				
52		(1)				
53		(1)				
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100	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
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150		(1)				
Total Indep	18		0		0	
Total Depend	186	↵	0	↵	0	↵
Total Claims	204		0		0	

	*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend
151		(1)				
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157		3				
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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
Warnings:					
Information:					
3	Non Patent Literature	XIE_2002_10405-10410.pdf	955612 ea095eddb186d2ed85712e3dfeb9e9f4ef3c3b4a	no	6
Warnings:					
Information:					
4	Non Patent Literature	YAMASHITA_1984_4451-4459.pdf	6598702 8ac076a78f0280b2d0c4bb87a6d2b391570eb1ec	no	9
Warnings:					
Information:					
5	Non Patent Literature	YAN_2002_2043-2053.pdf	806952 bbbce76e612d20e1cbfff7b4ff083d4d30d9c639	no	11
Warnings:					
Information:					
6	Non Patent Literature	ZHAO_2006_283-293.pdf	1347796 fe67d699520d2b4eac648e052eebad4cbe5660af	no	19
Warnings:					
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)

INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Not for submission under 37 CFR 1.99)		
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EXAMINER SIGNATURE			
Examiner Signature		Date Considered	

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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).

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☐ 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.**