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(54) **Titre : PROCÉDES DE CARACTÉRISATION DE PARTICULES VIRALES À L'AIDE DE CHROMATOGRAPHIE LIQUIDE
BIDIMENSIONNELLE-SPECTROMÉTRIE DE MASSE**

(54) **Title: METHODS FOR VIRAL PARTICLE CHARACTERIZATION USING TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY-
MASS SPECTROMETRY**

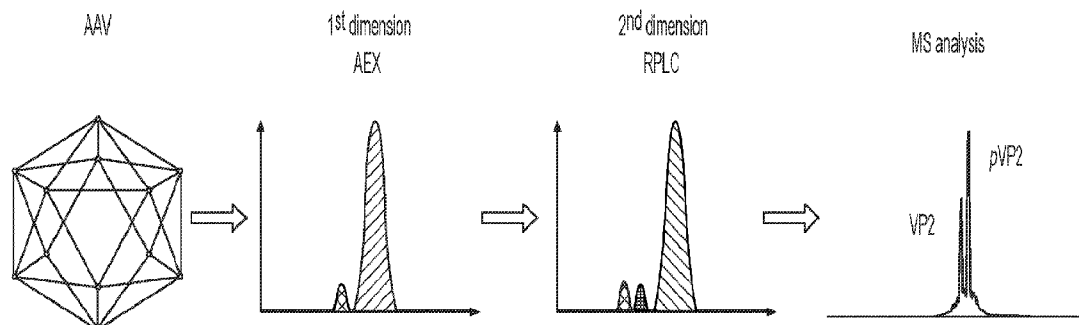


FIG. 2A

(57) **Abrégé/Abstract:**

Methods for identifying viral protein constituents and quantifying the relative abundance of such viral protein constituents in a sample of viral particles are disclosed. In embodiments, the methods include first-dimension chromatography to separate intact viral capsid components of the sample, online denaturation of the viral capsid components to produce intact viral proteins, second-dimension chromatography to separate the viral proteins, and mass spectrometry to determine the masses of the viral proteins and identify the viral protein constituents of the sample.



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Abstract:

Methods for identifying viral protein constituents and quantifying the relative abundance of such viral protein constituents in a sample of viral particles are disclosed. In embodiments, the methods include first-dimension chromatography to separate intact viral capsid components of the sample, online denaturation of the viral capsid components to produce intact viral proteins, second-dimension chromatography to separate the viral proteins, and mass spectrometry to determine the masses of the viral proteins and identify the viral protein constituents of the sample.

METHODS FOR VIRAL PARTICLE CHARACTERIZATION USING TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

FIELD OF THE INVENTION

[0001] The present invention relates to methods for characterization of quality attributes of viral particles (e.g., AAV capsids) using a two-dimensional liquid chromatography-mass spectrometry platform.

BACKGROUND

[0002] Adeno-associated virus (AAV), which is a non-enveloped, single-stranded DNA virus, has emerged as an attractive class of therapeutic agents to deliver genetic materials to host cells for gene therapy, due to its ability to transduce a wide range of species and tissue *in vivo*, low risk of immunotoxicity, and mild innate and adaptive immune responses. The complex nature of viral vectors such as AAV require specific analytical methods to enable product testing and characterization.

[0003] Existing analytical techniques often do not provide sufficient resolution for quantifying homogeneity for the production of clinical-grade viral vector preparations. Complete characterization of the constituent viral capsid proteins, such as the capsid proteins of AAV vectors, including their sequences and post-translational modifications (PTMs), is desirable to ensure product quality and consistency. Thus, methods are needed to determine the homogeneity of viral particles and identify various species of viral proteins within the viral particles.

BRIEF SUMMARY OF THE INVENTION

[0004] The present disclosure is directed to an online two-dimensional liquid chromatography-mass spectrometry (2DLC-MS) platform for viral particle (e.g., AAV) characterization, which can simultaneously perform characterization of the empty and full ratio and viral proteins by chromatographic separation of viral particles and viral proteins coupled with mass spectrometry. In exemplary embodiments, characterization of the empty and full ratio and viral proteins is performed by anion-exchange chromatography (AEX) and reverse-phase liquid chromatography (RPLC) coupled with mass spectrometry (MS), respectively.

[0005] In one aspect, the present disclosure provides a method for identifying viral protein constituents of a sample of viral particles, comprising: (a) subjecting the sample of viral particles to first-dimension chromatography to separate intact viral capsid components of the sample; (b) subjecting at least a portion of the intact viral capsid components to online denaturation to yield individual intact viral proteins; (c) subjecting the intact viral proteins to second-dimension

chromatography to separate the intact viral proteins; and (d) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of viral particles.

[0006] In some embodiments, the method further comprises selecting a portion of the separated intact viral capsid components, wherein subjecting at least a portion of the intact viral capsid components to online denaturation to yield individual viral proteins comprising subjected the selected portion of the separated intact viral capsid components to online denaturation.

[0007] In some embodiments, the sample of viral particles comprises adeno-associated virus (AAV) particles. In some cases, the AAV particles are of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, or AAV-PHP.S. In some cases, the AAV particles are of serotype AAV1. In some cases, the AAV particles are of serotype AAV5. In some cases, the AAV particles are of serotype AAV8.

[0008] In some embodiments, the intact viral capsid components comprise empty viral capsids and full viral capsids.

[0009] In some embodiments, the first-dimension chromatography comprises ion-exchange chromatography. In some cases, the ion-exchange chromatography is anion-exchange chromatography.

[0010] In some embodiments, the second-dimension chromatography comprises reverse-phase chromatography. In some embodiments, the second-dimension chromatography comprises hydrophilic interaction liquid chromatography.

[0011] In some embodiments, determining the masses of the separated intact viral proteins comprises subjecting the separated intact viral proteins to electrospray ionization mass spectrometry.

[0012] In some embodiments, the viral protein constituents comprise VP1, VP2 and/or VP3 of an AAV particle. In some cases, the viral protein constituents comprise post-translational variants of VP1, VP2 and/or VP3. In some cases, the post-translational variants of VP1, VP2 and/or VP3 comprise acetylated, phosphorylated and/or oxidized variants of VP1, VP2 and/or VP3. In some cases, the post-translational variants of VP1, VP2 and/or VP3 comprise fragments of VP1, VP2 and/or VP3 produced from cleavage of an aspartic acid-proline bond and/or cleavage of an aspartic acid-glycine bond.

[0013] In some embodiments, the method further comprises detecting the intact viral capsid components separated by the first dimension chromatography, and identifying a ratio of empty viral capsids to full and partially-full viral capsids.

[0014] In some embodiments, the method further comprises detecting the intact viral proteins separated by the second-dimension chromatography, and quantifying the relative abundance of the viral protein constituents of the sample of viral particles.

[0015] In some cases, the intact viral capsid components and/or the intact viral proteins are detected using an ultraviolet or fluorescence detector.

[0016] In one aspect, the present disclosure provides a method for identifying viral protein constituents of a sample of adeno-associated virus (AAV) particles, comprising: (a) subjecting the sample of AAV particles to anion-exchange chromatography to separate intact viral capsid components in the sample, wherein the intact viral capsid components comprise intact empty viral capsids and intact full viral capsids comprising a heterologous nucleic acid molecule; (b) selecting a portion of the intact viral capsid components for online desalting and denaturation; (c) subjecting the selected portion of the intact viral capsid components to online desalting and denaturation to yield individual intact viral proteins, wherein the intact individual viral proteins comprise VP1, VP2, VP3 and at least one variant of VP1, VP2 or VP3; (d) subjecting the intact viral proteins to reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography to separate the intact viral proteins; and (e) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of AAV particles.

[0017] In some embodiments, the intact viral proteins are subjected to reverse-phase liquid chromatography. In some embodiments, the intact viral proteins are subjected to hydrophilic interaction liquid chromatography.

[0018] In some embodiments, the method further comprises detecting the intact viral capsid components separated by the anion-exchange chromatography, and identifying a ratio of empty viral capsids to full and partially-full viral capsids.

[0019] In some embodiments, the method further comprises detecting the intact viral proteins separated by the reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography, and quantifying the relative abundance of the viral protein constituents of the sample of AAV particles.

[0020] In some embodiments, the AAV particles are of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, or AAV-PHP.S. In some cases, the AAV particles are of serotype AAV1. In some cases, the AAV particles are of serotype AAV5. In some cases, the AAV particles are of serotype AAV8.

[0021] In some embodiments, the at least one variant of VP1, VP2 or VP3 comprises a post-translational variant of VP1, VP2 or VP3. In some cases, the post-translational variant of VP1, VP2 or VP3 comprises an acetylated variant of VP1, VP2 or VP3. In some cases, the post-translational

variant of VP1, VP2 or VP3 comprises a phosphorylated variant of VP1, VP2 or VP3. In some cases, the post-translational variant of VP1, VP2 or VP3 comprises an oxidized variant of VP1, VP2 or VP3. In some cases, the post-translational variant of VP1, VP2 or VP3 comprises a fragment of VP1, VP2 or VP3 produced from cleavage of an aspartic acid-proline bond. In some cases, the post-translational variant of VP1, VP2 or VP3 comprises a fragment of VP1, VP2 or VP3 produced from cleavage of an aspartic acid-glycine bond.

[0022] In some embodiments, the intact viral capsid components and/or the intact viral proteins are detected using an ultraviolet or fluorescence detector.

[0023] In some embodiments, determining the masses of the separated intact viral proteins comprises subjecting the separated intact viral proteins to electrospray ionization mass spectrometry.

[0024] In some embodiments, the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using a first mobile phase comprising from 15 mM to 25 mM bis-tris-propane (BTP), from 250 mM to 1 M tetramethylammonium chloride (TMAC), and from 1 mM to 3 mM magnesium chloride at a pH of from 8 to 9. In some cases, the first mobile phase comprises 20 mM $\pm$ 2 mM BTP, 500 mM $\pm$ 50 mM TMAC, and 2 mM $\pm$ 0.2 mM MgCl₂ at a pH of 8.5 $\pm$ 0.1. In some embodiments, the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using the first mobile phase and a second mobile phase comprising from 15 mM to 25 mM bis-tris-propane (BTP), and from 1 mM to 3 mM magnesium chloride at a pH of from 8 to 9. In some cases, the second mobile phase comprises 20 mM $\pm$ 2 mM BTP, and 2 mM $\pm$ 0.2 mM MgCl₂ at a pH of 8.5 $\pm$ 0.1. In some embodiments, the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using the first mobile phase, the second mobile phase, and a third mobile phase comprising from 1.5 M to 2.5 M sodium chloride. In some cases, the third mobile phase comprises 2 M $\pm$ 0.1 M sodium chloride. In some embodiments, the separation of the intact viral capsid components is performed with a mobile phase gradient. In some cases, the mobile phase gradient comprises, in sequence: 10% first mobile phase and 90% second mobile phase for 1 minute; increasing the first mobile phase from 10% to 42%, and decreasing the second mobile phase from 90% to 58%, over a period of 20 minutes; 100% third mobile phase for 5 minutes; and 10% first mobile phase and 90% second mobile phase for 10 minutes.

[0025] In some embodiments, the method further comprises identifying an amount of intact empty viral capsids and an amount of full viral capsids in the sample, and determining a relative abundance of the intact empty viral capsids and intact full viral capsids in the sample.

[0026] In various embodiments, any of the features or components of embodiments discussed above or herein may be combined, and such combinations are encompassed within the scope of the present disclosure. Any specific value discussed above or herein may be combined with another related value discussed above or herein to recite a range with the values representing the upper and lower ends of the range, and such ranges are encompassed within the scope of the present disclosure.

[0027] Other embodiments will become apparent from a review of the ensuing detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] Figures 1A, 1B and 1C illustrate an AAV capsid comprising a heterologous nucleic acid molecule (e.g., a therapeutic gene or gene of interest (GOI)) (Fig. 1A); empty, partially-full and full capsids (Fig. 1B); and a AAV capsid composed of 60 copies of three viral proteins (VP1, VP2 and VP3) that give rise to a wide range of theoretical capsid stoichiometries (Fig. 1C).

[0029] Figures 2A and 2B illustrate an exemplary two-dimensional liquid chromatography-mass spectrometry system (2DLC-MS) in accordance with an embodiment of the present disclosure, in which viral capsids are separated in a first dimension and viral proteins are separated in a second dimension for mass spectral analysis.

[0030] Figure 2C illustrates a valve setup for a 2DLC-MS system in accordance with an embodiment of the present disclosure. Part (a) illustrates a first position for the valve setup in which a second liquid chromatography flow is used to maintain the RPLC column temperature, and one fraction from the trapping loop enters the trap column for desalting and denaturation. Part (a) further illustrates a second position for the valve setup in which viral proteins from the denatured viral capsid (e.g., AAV) is migrated from the trap column to the analytical column (e.g., RPLC) for separation followed by mass spectral analysis. Parts (b) (c) (d) illustrate the separation of viral proteins with (part (c)) or without (part (b)) a trap column, and with different flow rates (0.2 mL/min in part (c); and 0.1 mL/min in part(d)). As shown, greater separation of the viral proteins is achieved with use of the trap column, and there is no significant change in the peaks with a change in flow rate. Part (e) shows that no salt adduct is observed in connection with the three AAV viral proteins (from deconvoluted spectra) using the exemplified valve setup.

[0031] Figure 2D shows a pair of chromatograms demonstrating that online denaturation (bottom chromatogram) provides for effective dissociation of the AAV viral proteins without the need for denaturation prior to sample injection.

[0032] Figure 2E shows raw and deconvoluted spectra of the peak representing high molecular weight species obtained from the 2DLC-MS system (for AAV8-GOI1), and confirming the identities of the high molecular weight species as the VP3 dimer and the VP2+VP3 heterodimer. Use of the trap column for online denaturation eliminated the high molecular weight species (compare Fig. 2C, parts (b) and (c), which show the presence of the high molecular weight species without the trap column, and its absence with the trap column, respectively).

[0033] Figures 3A and 3B are representative of chromatograms obtained from the first-dimension chromatography (e.g., AEX), and show separation of viral capsids (empty or containing a GOI) using either tetramethylammonium chloride or tetraethylammonium chloride (Fig. 3A) and from various AAV serotypes (Fig. 3B), respectively.

[0034] Figures 3C and 3D are representative of chromatograms and a mass spectrum obtained from the second-dimension chromatography (e.g., RPLC) and mass spectrometry, and show that viral proteins in AAV samples with or without a GOI can be effectively separated in both AAV8 and AAV1 samples, and that the GOI did not interfere with the separation (Fig. 3C), and that separation of viral proteins coupled with mass spectrometry can be used to identify the viral proteins (Fig. 3D).

[0035] Figure 4A illustrates a process in accordance with an embodiment of the present disclosure, showing (i) a chromatogram in which empty capsids (AAV8-Em) have been separated from capsids containing a gene of interest (AAV8-GOI), (ii) selection of a portion of the separated capsids for denaturation ("heart cutting"), (iii) a chromatogram in which viral proteins (VP1, VP2, VP3 and others) have been separated from one another, and (iv) a mass spectrum corresponding to the separated viral proteins.

[0036] Figure 4B illustrates multiple "heart cutting" of peaks following first-dimension chromatography (e.g., AEX), followed by second-dimension chromatography (e.g., RPLC) and mass spectrometry to identify and characterize the viral protein constituents of the peaks in an AAV8-GOI sample.

[0037] Figure 5 illustrates the effectiveness of the first-dimension chromatography in separating empty viral capsids from capsid containing a heterologous nucleic acid molecule (e.g., gene of interest or GOI). The chromatographic separation yields a ratio of viral capsids in which (i) the empty capsids, and (ii) the partially-full and full capsids are consistent with data produced from analytical ultracentrifugation (AUC) techniques.

[0038] Figure 6 illustrates the chromatographic separation of viral proteins via second-dimension chromatography and the relative quantity of each of VP1, VP2 and VP3 (of AAV).

[0039] Figures 7A and 7B illustrate the chromatographic separation of viral proteins (VP1, VP2 and VP3 of AAV) and post-translational variants of the viral proteins via second-dimension

chromatography. Fig. 7A shows labels for the abundant species, while Fig. 7B shows labels for the low-abundant species.

[0040] Figure 7C shows the identities of the species labeled in Figs. 7A and 7B for an AAV8 sample of viral particles, along with the observed mass and theoretical mass of each. “Ac” refers to acetylated, “P” refers to phosphorylated, “Clip (DP)” refers to a fragment produced by cleavage of an aspartic acid-proline bond, “Ox” refers to an oxidized, and “Clip (DG)” refers to a fragment produced by cleavage of an aspartic acid-glycine bond.

[0041] Figure 7D shows the identities of species for an AAV1 sample of viral particles in the same manner as Fig. 7C.

[0042] Figures 8A and 8B show mass spectra corresponding to the viral protein constituents of AAV8 or AAV1 capsids that are either empty or contain a gene of interest (GOI).

DETAILED DESCRIPTION

[0043] Before the present invention is described, it is to be understood that this invention is not limited to particular methods and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0044] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used herein, the term “about,” when used in reference to a particular recited numerical value, means that the value may vary from the recited value by no more than 1%. For example, as used herein, the expression “about 100” includes 99 and 101 and all values in between (e.g., 99.1, 99.2, 99.3, 99.4, etc.).

[0045] As used herein, the terms “include,” “includes,” and “including,” are meant to be non-limiting and are understood to mean “comprise,” “comprises,” and “comprising,” respectively.

[0046] Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All patents, applications and non-patent publications mentioned in this specification are incorporated herein by reference in their entireties.

Selected Abbreviations

[0047] 2DLC-MS – Two-Dimensional Liquid Chromatography-Mass Spectrometry

[0048] LC-MS – Liquid Chromatography-Mass Spectrometry

[0049] MS: Mass Spectrometry or Mass Spectrometer

- [0050]** ESI: Electrospray Ionization
- [0051]** rAAV: Recombinant AAV Particle or Capsid
- [0052]** AAV: Adeno-Associated Virus
- [0053]** LC: Liquid Chromatography
- [0054]** RPLC: Reverse Phase Liquid Chromatography
- [0055]** HILIC – Hydrophilic Interaction Liquid Chromatography
- [0056]** AEX – Anion Exchange Chromatography
- [0057]** IEX – Ion Exchange Chromatography
- [0058]** GOI – gene of interest
- [0059]** VP1 – Viral Protein 1 subunit of AAV
- [0060]** VP2 – Viral Protein 2 subunit of AAV
- [0061]** VP3 – Viral Protein 3 subunit of AAV
- [0062]** UV – Ultraviolet
- [0063]** FLR – Fluorescence

Definitions

[0064] “Intact viral capsid components” refer to viral capsids (*e.g.*, empty viral capsids, partially-full viral capsids, and/or full viral capsids) that are intact (*i.e.*, have not been denatured or otherwise broken down or disintegrated into their component parts (*e.g.*, different viral proteins) and retain the structural characteristics of a viral capsid (*e.g.*, the icosahedral conformation of an AAV capsid).

[0065] The terms “empty viral capsids” or “empty capsids” refer to capsids not containing a heterologous nucleic acid molecule (*e.g.*, a therapeutic gene), as illustrated in Fig. 1B.

[0066] The terms “partially-full viral capsids” or “partially full capsids” refer to capsids containing only a portion of a heterologous nucleic acid molecule (*e.g.*, a therapeutic gene), as illustrated in Fig. 1B.

[0067] The terms “full viral capsids” or “full capsids” refer to capsids containing a complete heterologous nucleic acid molecule (*e.g.*, a therapeutic gene or gene of interest), as illustrated in Fig. 1B.

[0068] The term “sample,” as used herein, refers to a mixture of viral particles (*e.g.*, AAV particles) that comprises at least one viral capsid component (*i.e.*, empty capsids, partially-full capsids, and/or full capsids), that is subjected to manipulation in accordance with the methods of the invention, including, for example, separating and analyzing.

[0069] The terms “analysis” or “analyzing,” are used interchangeably and refer to any of the various methods of separating, detecting, isolating, purifying and/or characterizing viral particles or

viral proteins of interest (e.g., AAV proteins). Examples include, but are not limited to, mass spectrometry, e.g., ESI-MS, liquid chromatography (e.g., AEX, RPLC or HILIC), and combinations thereof.

[0070] “Contacting,” as used herein, includes bringing together at least two substances in solution or solid phase, for example contacting a stationary phase of a chromatography material with a sample, such as a sample comprising viral particles or viral proteins.

[0071] “Intact mass analysis” as used herein includes experiments wherein a viral protein is characterized as an intact protein. Intact mass analysis can reduce sample preparation to a minimum.

[0072] As used herein, the term “liquid chromatography” refers to a process in which a chemical mixture carried by a liquid can be separated into components as a result of differential distribution of the chemical entities as they flow around or over a stationary liquid or solid phase. Non-limiting examples of liquid chromatography include reverse phase liquid chromatography, ion-exchange chromatography, size exclusion chromatography, affinity chromatography, and hydrophobic interaction chromatography.

[0073] As used herein, the term “mass spectrometer” refers to a device capable of detecting specific molecular species and accurately measuring their masses. The term can be meant to include any molecular detector into which a viral protein (e.g., AAV protein) may be eluted for detection and/or characterization. A mass spectrometer consists of three major parts: the ion source, the mass analyzer, and the detector. The role of the ion source is to create gas phase ions. Analyte atoms, molecules, or clusters can be transferred into gas phase and ionized either concurrently (as in electrospray ionization). The choice of ion source depends on the application. As used herein, the term “electrospray ionization” or “ESI” refers to the process of spray ionization in which either cations or anions in solution are transferred to the gas phase via formation and desolvation at atmospheric pressure of a stream of highly charged droplets that result from applying a potential difference between the tip of the electrospray emitter needle containing the solution and a counter electrode. There are three major steps in the production of gas-phase ions from electrolyte ions in solution. These are: (a) production of charged droplets at the ES infusion tip; (b) shrinkage of charged droplets by solvent evaporation and repeated droplet disintegrations leading to small highly charged droplets capable of producing gas-phase ions; and (c) the mechanism by which gas-phase ions are produced from very small and highly charged droplets. Stages (a)–(c) generally occur in the atmospheric pressure region of the apparatus.

[0074] As used herein, the term “electrospray ionization source” refers to an electrospray ionization system that can be compatible with a mass spectrometer used for mass analysis of viral

particles.

[0075] Native MS is a particular approach based on electrospray ionization in which the biological analytes are sprayed from a nondenaturing solvent. It is defined as the process whereby biomolecules, such as large biomolecules, and complexes thereof can be transferred from a three-dimensional, functional existence in a condensed liquid phase to the gas phase via the process of electrospray ionization mass spectrometry (ESI-MS).

[0076] The term “nanoelectrospray” or “nanospray” as used herein refers to electrospray ionization at a very low solvent flow rate, typically hundreds of nanoliters per minute of sample solution or lower, often without the use of an external solvent delivery.

[0077] As used herein, “mass analyzer” refers to a device that can separate species, that is, atoms, molecules, or clusters, according to their mass. Non-limiting examples of mass analyzers that could be employed for fast protein sequencing are time-of-flight (TOF), magnetic/electric sector, quadrupole mass filter (Q), quadrupole ion trap (QIT), orbitrap, Fourier transform ion cyclotron resonance (FTICR), and also the technique of accelerator mass spectrometry (AMS).

[0078] As used herein, “mass-to-charge ratio” or “ m/z ” is used to denote the dimensionless quantity formed by dividing the mass of an ion in unified atomic mass units by its charge number (regardless of sign).

[0079] As used herein, the term “quadrupole–Orbitrap hybrid mass spectrometer” refers to a hybrid system made by coupling a quadrupole mass spectrometer to an orbitrap mass analyzer. A tandem in-time experiment using the quadrupole–Orbitrap hybrid mass spectrometer begins with ejection of all ions except those within a selected, narrow m/z range from the quadrupole mass spectrometer. The selected ions can be inserted into orbitrap and fragmented most often by low-energy CID. Fragments within the m/z acceptance range of the trap should remain in the trap, and an MS-MS spectrum can be obtained.

[0080] “Adeno-associated virus” or “AAV” is a non-pathogenic parvovirus, with single-stranded DNA, a genome of approximately 4.7 kb, not enveloped and has icosahedric conformation. AAV was first discovered in 1965 as a contaminant of adenovirus preparations. AAV belongs to the *Dependovirus* genus and *Parvoviridae* family, requiring helper functions from either herpes virus or adenovirus for replication. In the absence of helper virus, AAV can set up latency by integrating into human chromosome 19 at the 19q13.4 location. The AAV genome consists of two open reading frames (ORF), one for each of two AAV genes, Rep and Cap. The AAV DNA ends have a 145-bp inverted terminal repeat (ITR), and the 125 terminal bases are palindromic, leading to a characteristic T-shaped hairpin structure.

[0081] 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.

[0082] A "recombinant viral particle" refers to a viral particle including one or more heterologous sequences (*e.g.*, a nucleic acid sequence not viral origin) that may be flanked by at least one viral nucleotide sequence.

[0083] A "recombinant AAV particle" refers to a adeno-associated viral particle including one or more heterologous sequences (*e.g.*, nucleic acid sequence not of AAV origin) that may be flanked by at least one, for example, two, AAV inverted terminal repeat sequences (ITRs). Such rAAV particles can be replicated and packaged 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).

[0084] A "viral particle" refers to a viral particle composed of at least one viral capsid protein and an encapsulated viral genome.

[0085] "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 particle is a heterologous nucleotide sequence with respect to the viral particle.

[0086] An "inverted terminal repeat" or "ITR" sequence is relatively short sequences found at the termini of viral genomes which are in opposite orientation. An "AAV inverted terminal repeat (ITR)" sequence, is an approximately 145-nucleotide sequence that is present at both termini of a single-stranded AAV genome.

[0087] The term "corresponding" is a relative term indicating similarity in position, purpose or structure. A mass spectral signal due to a particular peptide or protein is also referred to as a signal corresponding to the peptide or protein. In certain embodiments, a particular peptide sequence or set of amino acids, such as a protein, can be assigned to a corresponding peptide mass.

[0088] The term "isolated," as used herein, refers to a biological component (such as a nucleic acid, peptide, protein, lipid, viral particle or metabolite) that has been substantially separated,

produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs or is transgenically expressed.

[0089] The terms "peptide," "protein" and "polypeptide" refer, interchangeably, to a polymer of amino acids and/or amino acid analogs that are joined by peptide bonds or peptide bond mimetics. The twenty naturally-occurring amino acids and their single-letter and three-letter designations are as follows: Alanine A Ala; Cysteine C Cys; Aspartic Acid D Asp; Glutamic acid E Glu; Phenylalanine F Phe; Glycine G Gly; Histidine H His; Isoleucine I Ile; Lysine K Lys; Leucine L Leu; Methionine M Met; Asparagine N Asn; Proline P Pro; Glutamine Q Gln; Arginine R Arg; Serine S Ser; Threonine T Thr; Valine V Val; Tryptophan W Trp; and Tyrosine Y Tyr.

[0090] References to a mass of an amino acid means the monoisotopic mass or average mass of an amino acid at a given isotopic abundance, such as a natural abundance. In some examples, the mass of an amino acid can be skewed, for example, by labeling an amino acid with an isotope. Some degree of variability around the average mass of an amino acid is expected for individual single amino acids based on the exact isotopic composition of the amino acid. The masses, including monoisotopic and average masses for amino acids are easily obtainable by one of ordinary skill in the art.

[0091] Similarly, references to a mass of a peptide or protein means the monoisotopic mass or average mass of a peptide or protein at a given isotopic abundance, such as a natural abundance. In some examples, the mass of a peptide can be skewed, for example, by labeling one or more amino acids in the peptide or protein with an isotope. Some degree of variability around the average mass of a peptide is expected for individual single peptides based on the exact isotopic composition of the peptide. The mass of a particular peptide can be determined by one of ordinary skill in the art.

[0092] 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.

[0093] A "recombinant viral vector" refers to a recombinant polynucleotide vector including one or more heterologous sequences (i.e., nucleic acid sequence not of viral origin).

[0094] The term "hydrophilic interaction chromatography" or HILIC is intended to include a process employing a hydrophilic stationary phase and a hydrophobic organic mobile phase in which hydrophilic compounds are retained longer than hydrophobic compounds. In certain embodiments, the process utilizes a water-miscible solvent mobile phase.

The term "reverse-phase liquid chromatography" or RPLC is intended to include a process that separates analytes based on nonpolar interactions between analytes and a stationary phase (e.g., substrate). The nonpolar analyte associates with and is retained by the nonpolar stationary phase. Adsorption strengths increase with analyte nonpolarity, and the interaction between the nonpolar

analyte and the nonpolar stationary phase (relative to the mobile phase) increases the elution time. Use of more nonpolar solvents in the mobile phase will decrease the retention time of the analytes, while more polar solvents tend to increase retention times.

[0095] The term "anion-exchange chromatography" or AEX is intended to include a process that separates substances based on their charges using an ion-exchange resin containing positively charged groups, such as diethyl-aminoethyl groups. In solution, the resin is coated with positively charged counter-ions.

General Description

[0096] The present disclosure provides two-dimensional liquid chromatography and native mass spectrometry (MS) methods that provide sensitive and rapid identification and quantitative characterization of the viral protein constituents of a sample of viral particles (e.g., AAV particles). Complete characterization of the viral protein constituents of viral particle compositions, such as the viral protein constituents of viral capsid components of a sample of AAV particles, is necessary to ensure product quality and consistency to maintain safety and efficacy of the compositions.

[0097] Recombinant viral vector compositions (e.g., AAV vector compositions) can contain varying levels of viral proteins and post-translational modifications of such viral proteins arising from various production, purification and storage conditions. The present methods provide analytical techniques to identify and quantitate ratios of viral capsid components in a sample of viral particles, and to identify and quantitate viral protein constituents of the viral particles, including low-abundant viral protein constituents comprising acetylated, phosphorylated, oxidized, and fragmented variants of viral proteins.

Methods for Identifying and Quantifying Viral Protein Constituents

[0098] Aspects of the disclosure are directed to methods for identifying and quantifying viral protein constituents in a sample of viral particles (e.g., recombinant AAV particles) in a two dimensional liquid chromatography-mass spectrometry (2DLC-MS) system.

[0099] In some cases, the method comprises: (a) subjecting the sample of viral particles to first-dimension chromatography to separate intact viral capsid components of the sample; (b) subjecting at least a portion of the intact viral capsid components to online denaturation to yield individual intact viral proteins; (c) subjecting the intact viral proteins to second-dimension chromatography to separate the intact viral proteins; and (d) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of viral particles.

[0100] In some cases, the method comprises: (a) subjecting the sample of AAV particles to anion-exchange chromatography to separate intact viral capsid components in the sample, wherein

the intact viral capsid components comprise intact empty viral capsids and intact full viral capsids comprising a heterologous nucleic acid molecule; (b) selecting a portion of the intact viral capsid components for online desalting and denaturation; (c) subjecting the selected portion of the intact viral capsid components to online desalting and denaturation to yield individual intact viral proteins, wherein the intact individual viral proteins comprise VP1, VP2, VP3 and at least one variant of VP1, VP2 or VP3; (d) subjecting the intact viral proteins to reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography to separate the intact viral proteins; and (e) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of AAV particles.

[0101] In various embodiments of the methods, the viral protein constituents comprise viral proteins and post-translational variants of the viral proteins. For example, in compositions of AAV particles, the viral protein constituents comprise viral proteins VP1, VP2 and VP3, and post-translational variants of VP1, VP2 and/or VP3, including, in some cases, acetylated, phosphorylated and/or oxidized variants of VP1, VP2 and/or VP3, and/or fragments of VP1, VP2 and/or VP3 produced from cleavage of a peptide bond (e.g., cleavage of an aspartic acid-proline bond and/or cleavage of an aspartic acid-glycine bond).

[0102] In the methods disclosed herein, the 2DLC-MS system is exemplified by the schematic illustrated in Fig. 2A and 2B. In the example shown in Fig. 2B, the 2DLC-MS system 100 includes a first-dimension liquid chromatography column 102 (e.g., an AEX column) into which the sample of viral particles 101 (e.g., AAV particles) is introduced to separate the viral capsid components of the sample from one another, a detector 104 (e.g., a FLR detector) for detecting the eluate from the first-dimension column 102, peak-picking or heart-cutting software 106 to enable selection of a portion of the eluted and separated viral capsid components of the sample, a trapping loop 108 for online desalting and denaturation, and for temporarily storing the selected viral capsid components, which are to be transferred to the second-dimension chromatography column, a second-dimension chromatography column 110 (e.g., a RPLC column) into which the selected viral capsid components are transferred to yield intact viral proteins from the viral capsid (e.g., via a starting mobile phase) before a gradient is applied to separate the intact viral proteins from one another, a detector 112 (e.g., a FLR detector) for detecting the eluate from the second-dimension column 110, and a mass spectrometer 114 (e.g., an ESI-MS) to determine the mass of the separated viral proteins and thereby identify the viral protein constituents of the sample of viral particles 101 in a mass spectrum 116. One advantage of the 2DLC-MS system discussed herein is the capability of incorporating MS-incompatible salts for high-resolution separation in the first dimension, before using MS-compatible reagents in the second dimension for MS characterization. This advantage

can be achieved, for example, using the valve setup exemplified in Fig. 2C. As shown in Fig. 2D, online denaturation allows for efficient separation of the viral proteins.

[0103] In various embodiments of the methods discussed herein, separation of the viral capsid components (e.g., empty and full capsids) in the first-dimension chromatography can be used to determine the relative quantities of the capsid components within the sample of viral particles. In the context of AEX chromatography, for example, empty viral capsids (*i.e.*, those not containing a heterologous nucleic acid molecule) will elute before partially-full or full viral capsids because the negatively charged nucleic acids (e.g., DNA) encapsulated within the partially-full and full viral capsids result in lower isoelectric point (pI) values and higher affinity for the AEX resins, which are positively charged. An example of this separation is illustrated in Fig. 3B, which shows the AAV1, AAV5 and AAV8 empty capsids eluting prior to the capsids containing a gene of interest (GOI). Such separation can be achieved using, for example, tetramethylammonium chloride or tetraethylammonium chloride, as shown in Fig. 3A. As illustrated in Fig. 5, detection of the eluted viral capsid components (e.g., via a fluorescence detector) can then be used to determine the ratio of empty capsids to capsids containing a GOI. These data are consistent with data generated from AUC measurements, which is generally regarded as a state-of-art technique for determining relative quantities of empty, partial, and full viral capsid components (note that the partially-full and full viral capsid components are combined in the AEX column of the table shown in Fig. 5).

[0104] In various embodiments, the methods of the present disclosure can be used to determine the identity and stoichiometry of various viral protein constituents contained within the viral particles of a sample subjected to the 2DLC-MS system. In embodiments, the separated viral capsid components from the first-dimension chromatography are subjected to denaturation to yield intact viral proteins that formerly comprised the viral capsid (e.g., VP1, VP2 and VP3 of an AAV capsid). The intact viral proteins are then subjected to second-dimension chromatography to separate the viral proteins, which may include modified variants of the viral proteins (e.g., post-translational variants arising naturally, or from production, purification or storage conditions). Examples of this separation are illustrated in Fig. 3C, which shows that the presence of a GOI does not impact the separation of viral proteins, and in Fig. 3D (top), which shows the viral proteins of an empty AAV8 capsid that have been separated on a RPLC column. The chromatogram in Fig. 3D shows the peaks for the three natural viral proteins of an AAV capsid (VP1, VP2 and VP3) as well as for a variant of VP3 produced from cleavage of a peptide bond (unspecified).

[0105] The separated viral proteins are then subjected to mass spectrometry to ascertain the identities of the various viral proteins. An example mass spectrum is illustrated in Fig. 3D (bottom), which shows the identification of a VP2 viral protein and a phosphorylated VP2 viral protein (from

an AAV capsid). Further identification of the relative ratios of the viral proteins and identification of viral proteins and variants is illustrated in Figs. 6, 7A, 7B, 7C and 7D.

[0106] In embodiments of the methods discussed herein, it is also possible to select a subset of the viral capsid components separated in the first-dimension chromatography for denaturation and separation/analysis in the second-dimension chromatography and mass spectrometry portions of the 2DLC-MS system. As illustrated in Fig. 4A, “heart-cutting” can be performed to select a specified portion of eluate from the first-dimension chromatography for further processing in the second-dimension chromatography, and subsequent mass spectrometry. This technique enables improved resolution and analysis of specific components, such as low-abundant species of viral proteins that may be present in the sample under investigation. Multiple “heart cutting” can also be performed to analyze various peaks from the first-dimension chromatography, as shown in Fig. 4B.

[0107] The methods discussed herein include subjection a sample of viral proteins to reverse phase liquid chromatography (RPLC) or hydrophilic interaction liquid chromatography (HILIC) to separate the protein components of the viral capsid of the viral particles, such as viral particles of interest where information about the capsid is desired. In embodiments, a RPLC or a HILIC column is contacted with the intact viral proteins following first-dimension chromatography and denaturation. In certain embodiments the method includes determining the masses of protein components of the viral capsid to identify the protein components separated by the second-dimension chromatography (e.g., RPLC or HILIC), for example, using mass spectrometry techniques, such as those described herein. In embodiments, the method includes calculating the relative abundance of the protein components of the viral capsid from the separation to determine the stoichiometry of protein components of a viral capsid of a viral particle, for example using ultraviolet (UV) detection or fluorescence (FLR) detection of the protein components of the viral capsid as they are eluted from the RPLC or HILIC column. For example, the area of a UV or FLR peak can be used to determine the relative abundance of the capsid proteins and used to calculate the stoichiometry of the capsid proteins in the viral capsid. In another example, the peak height and/or peak UV or FLR intensity is used to determine relative abundance. In some embodiments, the retention time of the different proteins on the second-dimension chromatography column (e.g., RPLC or HILIC) is determined as a function of the mobile phase used and, in subsequent analysis this retention time can be used to determine the proteins and relative abundance of the proteins from the viral particle without the need to determine the mass and/or identity of the proteins every time a determination of stoichiometry is made, e.g. a standard value or values can be developed. In some cases, the second dimension chromatography column can be used for both denaturation and separation of the viral protein components. In some cases, the methods discussed herein can be

used to determine the serotype of a viral particle. For example, the masses of VP1, VP2 and VP3 of each AAV serotype are unique and can be used to identify or differentiate AAV capsid serotypes. In addition, the separated capsid proteins can be subjected to downstream analysis, such as a determination of protein sequence and post-translational modifications of the capsid proteins, for example with accurate mass measurement at the intact protein level.

[0108] In some embodiments of the methods discussed herein, the methods can be used to determine the heterogeneity of protein components in a capsid of a viral particle. In embodiments, the method includes subjecting the viral particle to first-dimension chromatography to separate the viral capsid components, and subjecting at least a portion of the viral capsid components to second-dimension chromatography to separate the protein components of the viral particle capsid. In embodiments, the method includes determining the masses of protein components of the viral capsid. In some cases, the masses of the protein components of the viral capsid are compared with theoretical masses of the viral capsid. A deviation of one or more of the masses of protein components of the viral capsid indicates that one or more proteins of the capsid are heterogeneous. Conversely, no deviation would indicate that the proteins of the capsid are homogeneous. In embodiments, heterogeneity is due to one or more of mixed serotypes, variant capsids, capsid amino acid substitutions, truncated capsids, or modified capsids. In some embodiments, the determination of the stoichiometry of protein components of a viral capsid of a viral particle and the determination of the heterogeneity of protein components in a capsid of a viral particle are done on the same sample.

[0109] In certain embodiments, the viral particle is an adeno-associated virus (AAV) particle and the methods disclosed can be used to determine the identity (and optionally stoichiometry) of protein components in a capsid of an AAV particle and/or heterogeneity of protein components in a capsid of an AAV particle. In embodiments, the protein components of the protein capsid comprise VP1, VP2 and VP3 of an AAV particle, as well as one or more variants of VP1, VP2 or VP3. In embodiments, the AAV particle is a recombinant AAV (rAAV) particle. In embodiments, the AAV particle includes an AAV vector encoding a heterologous transgene. 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, or variants thereof, of the AAV particle) may be compared with a reference, for example, a theoretical mass of a VP1, VP2, and/or VP3, or variants thereof, of one or more AAV serotypes. A reference may include a theoretical mass of a VP1, VP2, and/or VP3, or variants thereof, of one or more of any of the AAV serotypes. For example, in some embodiments, the masses of VP1, VP2, and/or VP3, or variants thereof, are compared to 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 AAV 10 capsid, an AAV 11 capsid, an AAV 12 capsid, or a variant thereof. In some embodiments, a determined or calculated mass (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.

Viral Particles

[0110] In certain aspects, the viral particle is an AAV particle and the methods disclosed can be used to determine the relative abundance of viral capsid components in a sample of AAV particles, as well as the identity and stoichiometry of viral protein constituents of a viral capsid. The AAV particles may be recombinant AAV (rAAV) particles. The rAAV particle includes an AAV vector encoding a heterologous transgene or heterologous nucleic acid molecule.

[0111] In certain aspects, the AAV particles include 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 AAV11 capsid, an AAV 12 capsid, or a variant thereof. In certain aspects, the AAV particles are of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, or AAV-PHP.S. In some embodiments, the AAV particles are of serotype AAV1 or AAV8.

[0112] While AAV was the model viral particle for this disclosure, it is contemplated that the disclosed methods can be applied to characterize a variety of viruses, for example, the viral families, subfamilies, and genera. The methods of the present disclosure may find use, for example, in characterizing viral particles to monitor or detect relative abundance of viral capsid components, and identities and stoichiometries of viral protein components of the viral capsids, in a composition of viral particles during production, purification or storage of such compositions.

[0113] In exemplary embodiments, the viral particle belongs to a viral family selected from the group consisting of Adenoviridae, Parvoviridae, Retroviridae, Baculoviridae, and Herpesviridae.

[0114] In certain aspects, the viral particle belongs to a viral genus selected from the group consisting of Atadenovirus, Aviadenovirus, Ichtadenovirus, Mastadenovirus, Siadenovirus, Ambidensovirus, Brevidensovirus, Hepadensovirus, 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.

[0115] In certain aspects, the Retroviridae is Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), Spumavirus, Friend virus, Murine Stem Cell Virus (MSCV) Rous Sarcoma Virus (RSV), human T cell leukemia viruses, Human Immunodeficiency Viruse (HIV), feline immunodeficiency virus (FIV), equine immunodeficiency virus (EIV), visna-maedi virus; caprine arthritis-encephalitis virus; equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); or simian immunodeficiency virus (SIV).

[0116] In some aspects, the viral particle (e.g., AAV particle) contains a heterologous nucleic acid molecule (e.g., a therapeutic gene or gene of interest). In some aspects, the heterologous nucleic acid molecule 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 .beta.-globin promoter and the elongation factor 1-alpha promoter (EF1-alpha) promoter. In some aspects, the promoter comprises a human .beta.-glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken .beta.-actin (CBA) promoter. The promoter can be a constitutive, inducible or repressible promoter. In some aspects, the invention provides a recombinant vector comprising a nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. In some cases, 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 aspect, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

Two Dimensional Liquid Chromatography-Mass Spectrometry (2DLC-MS) System

[0117] The methods disclosed herein include subjecting a viral particle to two-dimensional liquid chromatography/mass spectrometry (2DLC-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, for example, 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. The exemplary 2DLC-MS system illustrated in Fig. 2B can be used to determine relative abundance of viral capsid components in a sample of viral particles, and to identify and quantify viral protein constituents of the viral capsids (or a portion thereof). However, modifications to the illustrated exemplary system can also be employed to determine relative abundance of intact viral capsid components, and to identify and quantify viral protein constituents of the viral capsids.

[0118] Non-limiting examples of the first-dimension and second-dimension liquid chromatography columns 102 and 110 (see Fig. 2B) include reverse phase liquid chromatography, ion-exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophilic-interaction chromatography, and hydrophobic chromatography. Liquid chromatography, including HPLC, can be used to separate components of a sample of viral particles into viral capsid components, and to separate viral protein components of the viral capsids for further analysis. In some embodiments, the first-dimension chromatography comprises anion-exchange chromatography, and the second dimension chromatography comprises reverse-phase liquid chromatography. In some embodiments, the first-dimension chromatography comprises anion-exchange chromatography, and the second dimension chromatography comprises hydrophilic interaction liquid chromatography.

[0119] In various embodiments, the first-dimension chromatography comprises anion-exchange chromatography employing a mobile phase A containing 20 mM bis-tris propane in water, and a mobile phase B containing 20 mM bis-tris propane and 1 M tetraalkylammonium salt (e.g., tetramethylammonium chloride or tetraethylammonium chloride). In some cases, the tetraalkylammonium salt is present at a concentration of from about 0.1 M to about 10 M. In various embodiments, the tetraalkylammonium salt is present at a concentration of about 0.5 M, about 0.6 M, about 0.7 M, about 0.8 M, about 0.9 M, about 1 M, about 1.1 M, about 1.2 M, about 1.3 M, about 1.4 M, about 1.5 M, about 1.6 M, about 1.7 M, about 1.8 M, about 1.9 M, about 2 M, about 2.5 M, about 3 M, about 3.5 M, about 4 M, about 4.5 M, about 5 M, about 6 M, about 7 M, about 8 M, about 9 M, or about 10 M. In some embodiments, mobile phase A, mobile phase B, or both mobile phase A and mobile phase B comprise about 1 M sodium chloride. In various embodiments, the sodium chloride is present at a concentration of from about 0.1 M to about 10 M. In various embodiments, the sodium chloride is present at a concentration of about 0.5 M, about 0.6 M, about 0.7 M, about 0.8 M, about 0.9 M, about 1 M, about 1.1 M, about 1.2 M, about 1.3 M, about 1.4 M,

about 1.5 M, about 1.6 M, about 1.7 M, about 1.8 M, about 1.9 M, about 2 M, about 2.5 M, about 3 M, about 3.5 M, about 4 M, about 4.5 M, about 5 M, about 6 M, about 7 M, about 8 M, about 9 M, or about 10 M. In some embodiments, mobile phase A or mobile phase B, or both, contain a halide salt of an alkali metal or an alkaline earth metal (e.g., chloride, bromide or iodide salts of sodium, potassium, lithium, calcium or magnesium) at any of the concentration noted above. In some cases, the pH of the mobile phases is from about 7 to about 12. In some cases, the pH of the mobile phases is from about 8 to about 11. In some cases, the pH of the mobile phases is about 9, about 9.1, about 9.2, about 9.3, about 9.4, about 9.5, about 9.6, about 9.7, about 9.8, about 9.9, or about 10. In some embodiments, the flow rate is about 0.1 mL/min or about 0.2 mL/min or about 0.3 mL/min.

[0120] In various embodiments, the second-dimension chromatography comprises reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography. In some cases, the second-dimension chromatography comprises reverse-phase liquid chromatography employing a mobile phase A containing 0.1% to 0.5% difluoroacetic acid (DFA) in water, and a mobile phase B containing 0.1% to 0.5% DFA in acetonitrile (ACN). In various embodiments, the DFA concentration is about 0.1%, about 0.15%, about 0.2%, about 0.25%, about 0.3%, about 0.35%, about 0.4%, about 0.45%, or about 0.5%. In some embodiments, the flow rate is about 0.1 mL/min or about 0.2 mL/min or about 0.3 mL/min.

[0121] In various embodiments, the eluate from the first-dimension and/or second-dimension chromatography is detected using UV or FLR detectors. In some cases, the FLR detectors utilized an excitation wavelength of from about 260 nm to about 300 nm (e.g., about 280 nm) and an emission wavelength of from about 310 to about 370 nm (e.g., about 330 nm or about 350 nm).

[0122] In various embodiments, denaturation of the viral capsid components (or portion thereof) is performed with about 10% acetic acid. In some embodiments, denaturation is accomplished in the second-dimension chromatography column by applying a starting mobile phase for a period of time (e.g., about 10 min.) before applying a gradient to separate the intact viral proteins produced from the denaturation process. In some embodiments, the starting mobile phase comprises 80% mobile phase A and 20% mobile phase B, wherein mobile phase A comprises 0.1% to 0.5% difluoroacetic acid (DFA) in water, and mobile phase B comprises 0.1% to 0.5% DFA in acetonitrile.

[0123] In some embodiments, the mobile phase of the first-dimension chromatography and/or the second-dimension chromatography is an aqueous mobile phase. In exemplary embodiments, the mobile phase used to elute the viral proteins from the second-dimension chromatography is a mobile phase that is compatible with a mass spectrometer. In some exemplary embodiments, the mobile phase used in the first or second-dimension liquid chromatography column can include

water, acetonitrile, difluoroacetic acid, or combinations thereof. The mobile phase may include buffers with or without ion pairing agents, e.g., acetonitrile and water. Ion pairing agents include acetate, diifluoroacetic acid and salts. Gradients of the buffers can be used, for example, if two buffers are used, the concentration or percentage of the first buffer can decrease while the concentration or percentage of the second buffer increases over the course of the chromatography run. For example, the percentage of the first buffer can decrease from about 100%, about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 50%, about 45%, or about 40% to about 0%, about 1%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, or about 40% over the course of the chromatography run. As another example, the percentage of the second buffer can increase from about 0%, about 1%, about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, or about 40% to about 100%, about 99%, about 95%, about 90%, about 85%, about 80%, about 75%, about 70%, about 65%, about 60%, about 50%, about 45%, or about 40% over the course of the same run. In certain aspects, the proportion of mobile phase A in the chromatography increases over time. Optionally, the concentration or percentage of the first and second buffer can return to their starting values at the end of the chromatography run. The percentages can change gradually as a linear gradient or in a non-linear (e.g., stepwise) fashion. For example, the gradient can be multiphasic, for example, biphasic, triphasic, etc.

[0124] In some exemplary embodiments, the mobile phase can have a flow rate through the liquid chromatography column of about 0.1 $\mu\text{L}/\text{min}$ to about 100 mL/min , or about 0.05 mL/min to about 5 mL/min . In some cases, the flow rate is about 0.05 mL/min , about 0.06 mL/min , about 0.07 mL/min , about 0.08 mL/min , about 0.09 mL/min , about 0.1 mL/min , about 0.11 mL/min , about 0.12 mL/min , about 0.13 mL/min , about 0.14 mL/min , about 0.15 mL/min , about 0.16 mL/min , about 0.17 mL/min , about 0.18 mL/min , about 0.19 mL/min , about 0.2 mL/min , about 0.21 mL/min , about 0.22 mL/min , about 0.23 mL/min , about 0.24 mL/min , about 0.25 mL/min , about 0.26 mL/min , about 0.27 mL/min , about 0.28 mL/min , about 0.29 mL/min , about 0.3 mL/min , about 0.4 mL/min , about 0.5 mL/min , about 0.6 mL/min , about 0.7 mL/min , about 0.8 mL/min , about 0.9 mL/min , about 1 mL/min , about 2 mL/min , about 3 mL/min , about 4 mL/min , about 5 mL/min , about 6 mL/min , about 7 mL/min , about 8 mL/min , about 9 mL/min , or about 10 mL/min . In some cases, the flow rate is 0.1 mL/min . In some cases, the flow rate is 0.2 mL/min .

[0125] In some aspects, mass spectrometry (e.g., used in 2DLC-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. Ionic species, including 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. Subsequently, solvent evaporation is conducted to reduce the size of the charged droplets. Then, sample ion is ejected from the charge droplets as the solution passing through a small capillary with a voltage relative to ground. For example, the wall of the surrounding ESI chamber is performed by mixing the sample with volatile acid and organic solvent and infusing it through a conductive needle charged with high voltage. The charged droplets that are sprayed (or ejected) from the needle end are directed into the mass spectrometer, and are dried up by heat and vacuum as they fly in. After the drops dry, the remaining charged molecules are directed by electromagnetic lenses into the mass detector and mass analyzed. In one aspect, the eluted sample is deposited directly from the capillary into an electrospray nozzle, for example, the capillary functions as the sample loader. In another aspect, the capillary itself functions as both the extraction device and the electrospray nozzle.

[0126] In some exemplary embodiments, the electrospray ionization emitter comprises multiple emitter nozzles, such as at least two, at least three, at least four, at least five, at least six, at least seven, at least eight emitter nozzles, such as two, three, four, five, six, seven or eight emitter nozzles. In some exemplary embodiments, the electrospray ionization emitter is a M3 emitter from Newomics (Berkeley, CA) which includes 8 emitter nozzles.

[0127] In some exemplary embodiments, other ionization modes are used for example, turbospray ionization mass spectrometry, nanospray ionization mass spectrometry, thermospray ionization mass spectrometry, sonic spray ionization mass spectrometry, SELDI-MS and MALDI-MS. In general, an advantage of these methods (like ESI-MS) is that they allow for the "just-in-time" purification of sample and direct introduction into the ionizing environment. It is important to note that the various ionization and detection modes introduce their own constraints on the nature of the desorption solution used, and it is important that the desorption solution be compatible with both. For example, the sample matrix in many applications must have low ionic strength, or reside within a particular pH range, etc. In ESI, salt in the sample can prevent detection by lowering the ionization or by clogging the nozzle. This problem can be addressed by presenting the analyte in low salt and/or by the use of a volatile salt. In the case of MALDI, the analyte should be in a solvent compatible with spotting on the target and with the ionization matrix employed.

[0128] In some exemplary embodiments, the electrospray ionization source provides an electrospray with a solvent flow rate of from about 1 $\mu\text{L}/\text{min}$ to about 20 $\mu\text{L}/\text{min}$. In various embodiments, the flow rate into the ESI emitter is about 1 $\mu\text{L}/\text{min}$, about 2 $\mu\text{L}/\text{min}$, about 3 $\mu\text{L}/\text{min}$, about 4 $\mu\text{L}/\text{min}$, about 5 $\mu\text{L}/\text{min}$, about 6 $\mu\text{L}/\text{min}$, about 7 $\mu\text{L}/\text{min}$, about 8 $\mu\text{L}/\text{min}$, about 9 $\mu\text{L}/\text{min}$,

about 10 $\mu\text{L}/\text{min}$, about 11 $\mu\text{L}/\text{min}$, about 12 $\mu\text{L}/\text{min}$, about 13 $\mu\text{L}/\text{min}$, about 14 $\mu\text{L}/\text{min}$, about 15 $\mu\text{L}/\text{min}$, about 16 $\mu\text{L}/\text{min}$, about 17 $\mu\text{L}/\text{min}$, about 18 $\mu\text{L}/\text{min}$, about 19 $\mu\text{L}/\text{min}$, or about 20 $\mu\text{L}/\text{min}$.

[0129] The mass spectrometer can be a native ESI mass spectrometry system. In some exemplary embodiments, the mass spectrometer can be a quadrupole–Orbitrap hybrid mass spectrometer. The quadrupole–Orbitrap hybrid mass spectrometer can be Q Exactive™ Focus Hybrid Quadrupole–Orbitrap™ Mass Spectrometer, Q Exactive™ Plus Hybrid Quadrupole–Orbitrap™ Mass Spectrometer, Q Exactive™ BioPharma Platform, Q Exactive™ UHMR Hybrid Quadrupole–Orbitrap™ Mass Spectrometer, Q Exactive™ HF Hybrid Quadrupole–Orbitrap™ Mass Spectrometer, Q Exactive™ HF-X Hybrid Quadrupole–Orbitrap™ Mass Spectrometer, and Q Exactive™ Hybrid Quadrupole–Orbitrap™ Mass Spectrometer. In some exemplary embodiments, the mass spectrometry system is a Thermo Exactive EMR mass spectrometer. The mass spectrometry system can also contain an ultraviolet light detector.

[0130] A variety of mass analyzers suitable for LC/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.

EXAMPLES

[0131] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1: Characterization of Viral Protein Constituents of AAV Capsids

[0132] AAV empty and full samples of different serotypes were prepared in-house. Empty and full AAV samples were mixed and analyzed directly using an Agilent 1290 Infinity II 2D-LC system. In the first dimension, the empty and full AAV capsids were separated by a ProPac SAX-10 column (Thermo Scientific). In the second dimension, the AAV capsid was first denatured and desalted, and

the viral proteins were separated by an ACQUITY UPLC Protein BEH C4 column (Waters Corporation). MS analysis of viral proteins were performed on a Q Exactive™ Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific), and the MS data was analyzed using Xcalibur (Thermo Scientific) and Intact Mass™ (Protein Metrics Inc.).

Chemicals and Reagents

[0133] Unless otherwise stated, all chemicals and reagents were acquired from MilliporeSigma (Burlington, MA, USA). Empty and full capsids of three AAV serotypes (AAV8, AAV5, and AAV1) were produced in-house at Regeneron Pharmaceuticals Inc. (Tarrytown, NY, USA), and the detailed sample information and concentrations are shown in Table 1, below. Acetonitrile (ACN) was acquired from Thermo Fisher Scientific (Waltham, MA, USA). Difluoroacetic acid (DFA) was purchased from Waters Corporations (Milford, MA, USA). Deionized water (Milli-Q water) was obtained from a Milli-Q integral water purification system (MilliporeSigma).

[0134] Table 1: Concentration of AAV Samples

Sample	Concentration
AAV8 – Empty	3.07×10^{13} capsids/mL
AAV8 – GOI1	2.70×10^{13} vg/mL
AAV8 – GOI2	2.70×10^{13} vg/mL
AAV5 – Empty	3.32×10^{13} capsids/mL
AAV5 – GOI1	4.61×10^{13} vg/mL
AAV1 – Empty	2.54×10^{13} capsids/mL
AAV1 – GOI1	1.82×10^{13} vg/mL

vg/mL – viral genome/milliliter

Anion-exchange chromatography (AEX) experiment

[0135] AAV samples were directly analyzed by AEX without any sample pretreatment. The AEX separation was performed using a Thermo ProPac SAX-10 column (10 μ m, 2 mm x 250 mm) (Thermo Fisher Scientific) on an ACQUITY UPLC I-Class system (Waters Corporation) equipped with fluorescence detector. Mobile phase A (MPA) contained 20 mM bis-tris propane in Milli-Q water, and mobile phase B (MPB) was contained 20 mM bis-tris propane, and 1 M of either tetramethylammonium chloride (TMAC) or tetraethylammonium chloride (TEAC) in Milli-Q water. Both MPA and MPB were adjusted to pH 9.5 using hydrochloric acid. The flow rate for AEX was 0.2 mL/min, and the gradient consisted of 10% to 30% MPB from 0 to 10 min, 30% to 90% MPB from 10 to 10.1 min, and 90% MPB until 12 min. MPB was reduced to 10% from 12 to 12.1 min, and then maintained at 10% until the end of the 20 min gradient. For all AEX analyses, 1 μ L of the sample was injected. Data was recorded using a fluorescence detector with excitation (Ex) and emission (Em) wavelengths of 280 nm and 350 nm, respectively.

Reverse-phase liquid chromatography (RPLC) experiment

[0136] AAV samples were denatured with 10% acetic acid for 10 minutes prior to RPLC analysis. The RPLC experiment was performed using an ACQUITY UPLC Protein BEH C4 column (1.7 μ m, 300 Å, 2.1 mm x 150 mm) (Waters Corporation) on an ACQUITY UPLC I-Class system (Waters Corporation) equipped with a fluorescence detector. MPA was prepared with 0.1% DFA in Milli-Q water and MPB was prepared with 0.1% DFA in acetonitrile. The gradient was run at 0.2 mL/min starting with 20% to 32% MPB from 0 to 1 min, followed by 32% to 36% MPB from 1 to 16 min, 36% to 80% MPB from 20 to 21.5 min, 80% to 20% MPB from 21.5 to 22 min, and then 20% MPB until the end of the 30 min gradient. For all RPLC analyses, 1 μ L of the sample was injected. Data was acquired using a fluorescence detector with 280 nm Ex wavelength and 350 nm Em wavelength.

Two-dimensional liquid chromatography (2DLC) conditions

[0137] The 2DLC experiment was performed on an Agilent 1290 Infinity II 2D-LC System (Agilent Technologies, Santa Clara, CA, USA). The AEX gradient was applied to the first dimension at a flow rate of 0.1 mL/min instead of 0.2 mL/min.. At this lower flow rate, the 40 μ L trapping loop allowed for 0.4 seconds of sample trapping. 6 μ L of the AAV8 sample containing the gene of interest 1 (GOI1) was injected. Heart-cutting was performed using the time-based mode for high-resolution sampling, where peaks were selected based on the UV spectrum at 280 nm wavelength. The sample was then transferred to the second-dimension RP column from the trapping loop. The RPLC gradient was also applied to the second dimension, with the addition of 10-minute holding period at 80% MPA to remove the MS-incompatible salt used for AEX separation and online denaturation of intact viral capsids. An ACQUITY UPLC Protein BEH C4 column (1.7 μ m, 300 Å,

2.1 mm x 50 mm) (Waters Corporation) was used as a trap column prior to the analytical column with 150 mm in length. A divert valve was utilized to direct flow to waste during denaturation and salt removal and to the analytical column for downstream analysis. To maintain the temperature of the analytical column, an additional LC pump was used to keep the starting RPLC mobile phase at 0.05 mL/min.

Mass spectrometry (MS) Data Acquisition

[0138] RPLC-MS data was acquired using a Thermo Scientific Q Exactive™ Plus Hybrid Quadrupole-Orbitrap mass spectrometer (Bremen, Germany). For data acquisition, the resolution was set at 17,500, AGC target at 3e6, and maximum injection time at 500 ms. The spray voltage was set at 3.8 kV and S-lens RF level was at 50. The sheath and auxiliary gas flow were 40 and 15, respectively, and the capillary temperature and auxiliary gas heater temperatures were both set at 250 °C. Spectra were acquired from 1,000 to 3,000 *m/z*.

[0139] All 2DLC-MS data were acquired on a Thermo Scientific Orbitrap Exploris 480 mass spectrometer (Bremen, Germany) equipped with a Thermo Scientific NanoSpray Flex ion source. A nano flow splitter from Analytical Scientific Instruments (Richmond, CA, USA) was set at 50, and electrospray ionization emitter tips (CoAnn Technologies, Richland, WA, USA) were used for electrospray. For data acquisition, the resolution, AGC target, maximum injection time, and number of microscans were set at 15,000, 3e6, auto, and 3, respectively. The spray voltage was set to 2,200 V, RF lens level was at 50%, and the ion transfer tube temperature was set to 275 °C. Mass spectra were acquired from 1,000 to 3,000 *m/z*.

Data Analysis

[0140] For data acquired on a Waters instrument, the analysis was performed on the Empower 3 version 1.65. Mass spectrometry data were analyzed using Xcalibur 4.3.73.11. Data acquired on the Agilent instrument were analyzed using OpenLAB CDS ChemStation Edition Rev. C.01.07 SR2. Intact mass analysis was performed using Intact Mass™ version 3.11-1 (Protein Metrics Inc., Cupertino, CA, USA).

Results and Discussion

[0141] A 2DLC-MS platform (schematic illustrated in Fig. 2B) was utilized for AAV characterization. The method implemented high-resolution AEX in the first dimension for empty and full virus capsid separation (Figs. 3A, 3B and 5). Following online denaturation and desalting of MS-incompatible salt, the viral proteins were subjected to intact protein separation in the second RPLC dimension and intact protein characterization by MS (Figs. 3C, 3D, 6, 7A, 7B, 7C, 7D, 8A and 8B).

[0142] In the first dimension, the separation of empty and full AAV capsids was performed using AEX. With the salt gradient using either tetramethylammonium chloride and tetraethylammonium chloride compared to traditionally used sodium chloride, the empty and full AAV capsids were baseline resolved for all the tested samples. Empty capsids, and capsids containing a gene of interest (GOI) were separated for each of AAV1, AAV5 and AAV8 serotypes (Figs. 3A and 3B). The separation of empty and full capsids allowed for quantitation of the relative percentage of empty and full capsids in the samples (e.g., Fig. 5), which were consistent with those determined by AUC. Following high-resolution separation of empty and full capsids in the first dimension, online trapping was performed to select the peak of interest. Intact AAV capsid was denatured into individual viral proteins prior to the RPLC analysis in the second dimension. A starting mobile phase composition of the RPLC was used to both denature the AAV capsids through acidification and remove MS-incompatible salt used in the AEX separation. In the second dimension, the viral proteins were separated by RPLC using difluoroacetic acid as the ion-pairing reagent. MS analysis of the viral proteins revealed low-abundant species including unmodified, phosphorylated, and oxidative proteoforms. Additionally, differences in the phosphorylation levels of VP2 were observed among AAV samples (see, e.g., Figs. 7A, 7B, 7C, 7D, 8A and 8B).

[0143] The AAV capsid comprises three types of viral protein (VP) subunits, VP1, VP2 and VP3, totaling 60 copies in a ratio of 1:1:10 (VP1:VP2:VP3). These capsid proteins are alternatively spliced from one mRNA, and thus share a common sequence.

[0144] In the reverse-phase liquid chromatography-mass spectrometry (RPLC-MS) analysis, the major proteoforms included acetylated VP1 and its phosphorylated form, VP2 and its phosphorylated form, acetylated VP3, and VP3 clip species. Minor proteoforms included those arising from cleavage of an aspartic acid-proline (DP) bond. This DP bond cleavage generates species including acetylated VP1 clip, VP2 clip and its phosphorylated form, and acetylated VP3 clip, and a DP clip fragment. The clip species arise from cleavage of an aspartic acid-proline (DP) bond, which may be introduced during denaturation and separation. Additionally, unmodified and oxidized VP3 species were observed, along with additional acetylated VP3 clip species in which an aspartic acid-glycine (DG) bond was broken (Fig. 8A).

[0145] Similarly, RPLC-MS analysis for an AAV1-Empty capsid sample revealed the major proteoforms included acetylated VP1 and its phosphorylated form, VP2 and its phosphorylated form, acetylated VP3, and VP clip arising from DP bond cleavage. DP bond cleavage also generated proteoforms such as VP2 clip and its phosphorylated form, and acetylated VP3 clip, and clip fragment. Low-abundant oxidized proteoforms were detected for all three VPs. DG bond

cleavage was also observed, providing an additional acetylated VP3 clip species and a DG clip fragment (Fig. 8B).

[0146] In addition to proteoform identification, intact mass analysis also revealed differences in post-translational modification (PTM) level. Previous studies have shown Ser149 to be the major phosphorylation site in the AAV8 sequence. As the three viral proteins are alternatively cleaved, Ser149 was not included in the VP3 sequence. While the phosphorylation level in VP1 remained similar among three AAV8 samples, the phosphorylation level in VP2 varied significantly (Fig. 8A). Both AAV8 samples containing GOI showed elevated VP2 phosphorylation levels compared to the AAV8 sample without GOI. For AAV1, phosphorylation differences were not observed in viral proteins for the empty and full capsids (Fig. 8B).

[0147] The 2DLC-MS method demonstrated herein enabled high throughput and multi-attribute AAV characterization in a single system. In the first dimension, AEX provided high resolution separation of empty and full capsids using TMAC or TEAC. Online denaturation and desalting were achieved to dissociate the AAV capsids into viral proteins. In the second dimension, RPLC coupling to MS was used to characterize the viral proteins. Using this method, AAV samples were directly analyzed without sample pretreatment to minimize sample handling and avoid sample loss. The platform combined two characterization techniques in one analysis and provided good separation and high sensitivity, enabling detection of both major and minor viral protein proteoforms and fragments.

[0148] The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for identifying viral protein constituents of a sample of viral particles, comprising:
 - (a) subjecting the sample of viral particles to first-dimension chromatography to separate intact viral capsid components of the sample;
 - (b) subjecting at least a portion of the intact viral capsid components to online denaturation to yield individual intact viral proteins;
 - (c) subjecting the intact viral proteins to second-dimension chromatography to separate the intact viral proteins; and
 - (d) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of viral particles.
2. The method of claim 1, further comprising selecting a portion of the separated intact viral capsid components, wherein subjecting at least a portion of the intact viral capsid components to online denaturation to yield individual viral proteins comprising subjected the selected portion of the separated intact viral capsid components to online denaturation.
3. The method of claim 1 or 2, wherein sample of viral particles comprises adeno-associated virus (AAV) particles.
4. The method of claim 3, wherein the AAV particles are of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, or AAV-PHP.S.
5. The method of claim 4, wherein the AAV particles are of serotype AAV1 or AAV8.
6. The method of any one of claims 1-5, wherein the intact viral capsid components comprise empty viral capsids and full viral capsids.
7. The method of any one of claims 1-6, wherein the first-dimension chromatography comprises ion-exchange chromatography.
8. The method of claim 7, wherein the ion-exchange chromatography is anion-exchange chromatography.

9. The method of any one of claims 1-8, wherein the second-dimension chromatography comprises reverse-phase chromatography.
10. The method of any one of claims 1-8, wherein the second-dimension chromatography comprises hydrophilic interaction liquid chromatography.
11. The method of any one of claims 1-10, wherein determining the masses of the separated intact viral proteins comprises subjecting the separated intact viral proteins to electrospray ionization mass spectrometry.
12. The method of any one of claims 3-11, wherein the viral protein constituents comprise VP1, VP2 and/or VP3 of an AAV particle.
13. The method of claim 12, wherein the viral protein constituents comprise post-translational variants of VP1, VP2 and/or VP3.
14. The method of claim 13, wherein the post-translational variants of VP1, VP2 and/or VP3 comprise acetylated, phosphorylated and/or oxidized variants of VP1, VP2 and/or VP3.
15. The method of claim 13 or 14, wherein the post-translational variants of VP1, VP2 and/or VP3 comprise fragments of VP1, VP2 and/or VP3 produced from cleavage of an aspartic acid-proline bond and/or cleavage of an aspartic acid-glycine bond.
16. The method of any one of claims 1-15, further comprising detecting the intact viral capsid components separated by the first dimension chromatography, and identifying a ratio of empty viral capsids to full and partially-full viral capsids.
17. The method of any one of claims 1-16, further comprising detecting the intact viral proteins separated by the second-dimension chromatography, and quantifying the relative abundance of the viral protein constituents of the sample of viral particles.
18. The method of claim 16 or 17, wherein the intact viral capsid components and/or the intact viral proteins are detected using an ultraviolet or fluorescence detector.
19. A method for identifying viral protein constituents of a sample of adeno-associated virus (AAV) particles, comprising:
 - (a) subjecting the sample of AAV particles to anion-exchange chromatography to separate intact viral capsid components in the sample, wherein the intact viral capsid components

comprise intact empty viral capsids and intact full viral capsids comprising a heterologous nucleic acid molecule;

- (b) selecting a portion of the intact viral capsid components for online desalting and denaturation;
- (c) subjecting the selected portion of the intact viral capsid components to online desalting and denaturation to yield individual intact viral proteins, wherein the intact individual viral proteins comprise VP1, VP2, VP3 and at least one variant of VP1, VP2 or VP3;
- (d) subjecting the intact viral proteins to reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography to separate the intact viral proteins; and
- (e) determining the masses of the separated intact viral proteins to identify the viral protein constituents of the sample of AAV particles.

20. The method of claim 19, further comprising detecting the intact viral capsid components separated by the anion-exchange chromatography, and identifying a ratio of empty viral capsids to full and partially-full viral capsids.

21. The method of claim 19 or 20, further comprising detecting the intact viral proteins separated by the reverse-phase liquid chromatography or hydrophilic interaction liquid chromatography, and quantifying the relative abundance of the viral protein constituents of the sample of AAV particles.

22. The method of any one of claims 19-21, wherein the AAV particles are of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, or AAV-PHP.S.

23. The method of claim 22, wherein the AAV particles are of serotype AAV1 or AAV8.

24. The method of any one of claims 19-23, wherein the at least one variant of VP1, VP2 or VP3 comprises a post-translational variant of VP1, VP2 or VP3.

25. The method of claim 24, wherein the post-translational variant of VP1, VP2 or VP3 comprises an acetylated variant of VP1, VP2 or VP3.

26. The method of claim 24, wherein the post-translational variant of VP1, VP2 or VP3 comprises a phosphorylated variant of VP1, VP2 or VP3.

27. The method of claim 24, wherein the post-translational variant of VP1, VP2 or VP3 comprises an oxidized variant of VP1, VP2 or VP3.

28. The method of claim 24, wherein the post-translational variant of VP1, VP2 or VP3 comprises a fragment of VP1, VP2 or VP3 produced from cleavage of an aspartic acid-proline bond.

29. The method of claim 24, wherein the post-translational variant of VP1, VP2 or VP3 comprises a fragment of VP1, VP2 or VP3 produced from cleavage of an aspartic acid-glycine bond.

30. The method of any one of claims 20-29, wherein the intact viral capsid components and/or the intact viral proteins are detected using an ultraviolet or fluorescence detector.

31. The method of any one of claims 19-30, wherein determining the masses of the separated intact viral proteins comprises subjecting the separated intact viral proteins to electrospray ionization mass spectrometry.

32. The method of any one of claims 19-31, wherein the intact viral proteins are subjected to reverse-phase liquid chromatography.

33. The method of any one of claims 19-31, wherein the intact viral proteins are subjected to hydrophilic interaction liquid chromatography.

34. The method of any one of claims 8 or 19-31, wherein the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using a first mobile phase comprising from 15 mM to 25 mM bis-tris-propane (BTP), from 250 mM to 1 M tetramethylammonium chloride (TMAC), and from 1 mM to 3 mM magnesium chloride at a pH of from 8 to 9.

35. The method of claim 34, wherein the first mobile phase comprises 20 mM $\pm$ 2 mM BTP, 500 mM $\pm$ 50 mM TMAC, and 2 mM $\pm$ 0.2 mM MgCl_2 at a pH of 8.5 $\pm$ 0.1.

36. The method of claim 34 or 35, wherein the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using the first mobile phase

and a second mobile phase comprising from 15 mM to 25 mM bis-tris-propane (BTP), and from 1 mM to 3 mM magnesium chloride at a pH of from 8 to 9.

37. The method of claim 36, wherein the second mobile phase comprises 20 mM $\pm$ 2 mM BTP, and 2 mM $\pm$ 0.2 mM MgCl₂ at a pH of 8.5 $\pm$ 0.1.

38. The method of claim 36 or 37, wherein the intact viral capsid components of the sample subjected to anion-exchange chromatography are separated using the first mobile phase, the second mobile phase, and a third mobile phase comprising from 1.5 M to 2.5 M sodium chloride.

39. The method of claim 38, wherein the third mobile phase comprises 2 M $\pm$ 0.1 M sodium chloride.

40. The method of claim 38 or 39, wherein the separation of the intact viral capsid components is performed with a mobile phase gradient.

41. The method of claim 40, wherein the mobile phase gradient comprises, in sequence: 10% first mobile phase and 90% second mobile phase for 1 minute; increasing the first mobile phase from 10% to 42%, and decreasing the second mobile phase from 90% to 58%, over a period of 20 minutes; 100% third mobile phase for 5 minutes; and 10% first mobile phase and 90% second mobile phase for 10 minutes.

42. The method of any one of claims 34-41, further comprising identifying an amount of intact empty viral capsids and an amount of full viral capsids in the sample, and determining a relative abundance of the intact empty viral capsids and intact full viral capsids in the sample.

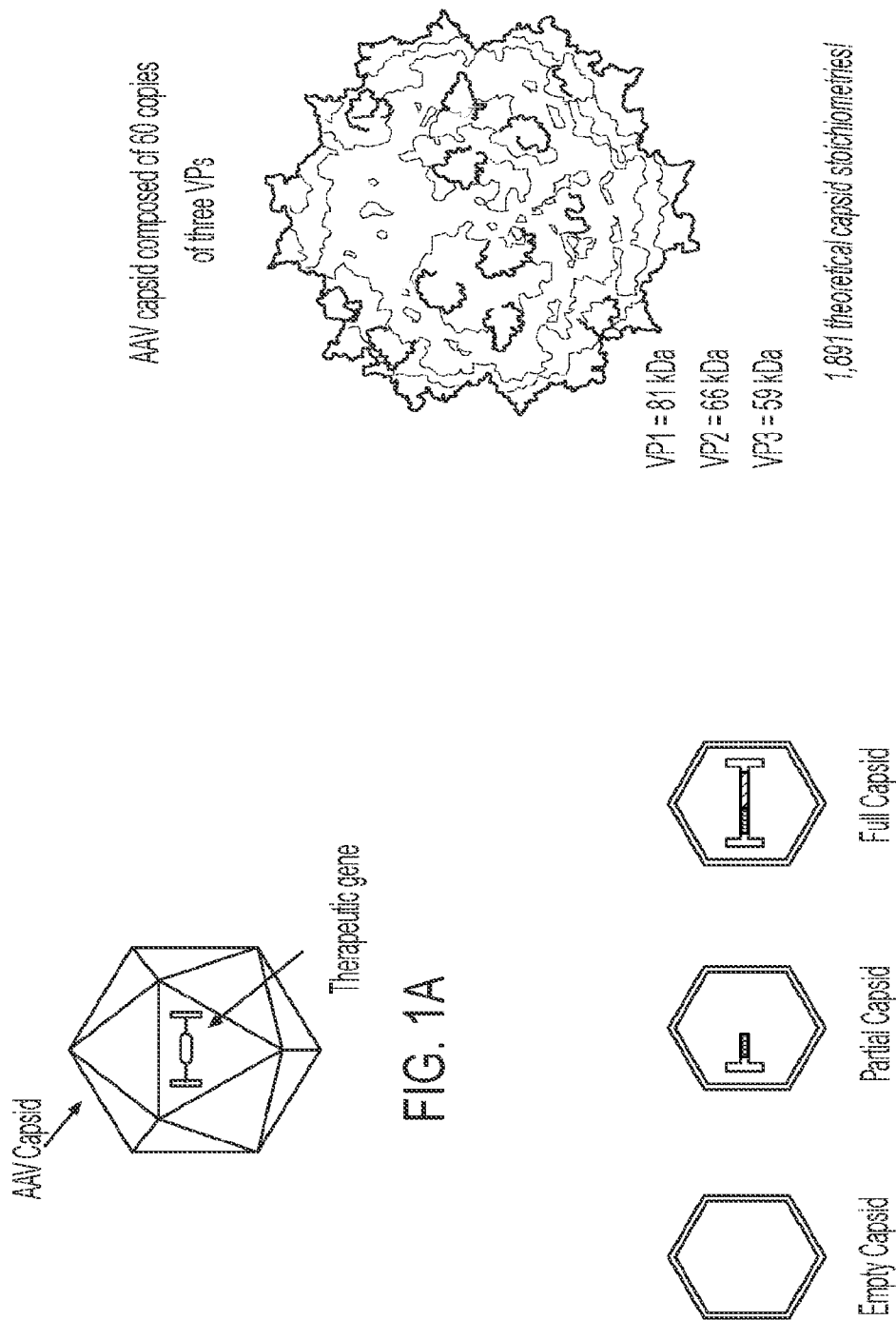


FIG. 1C

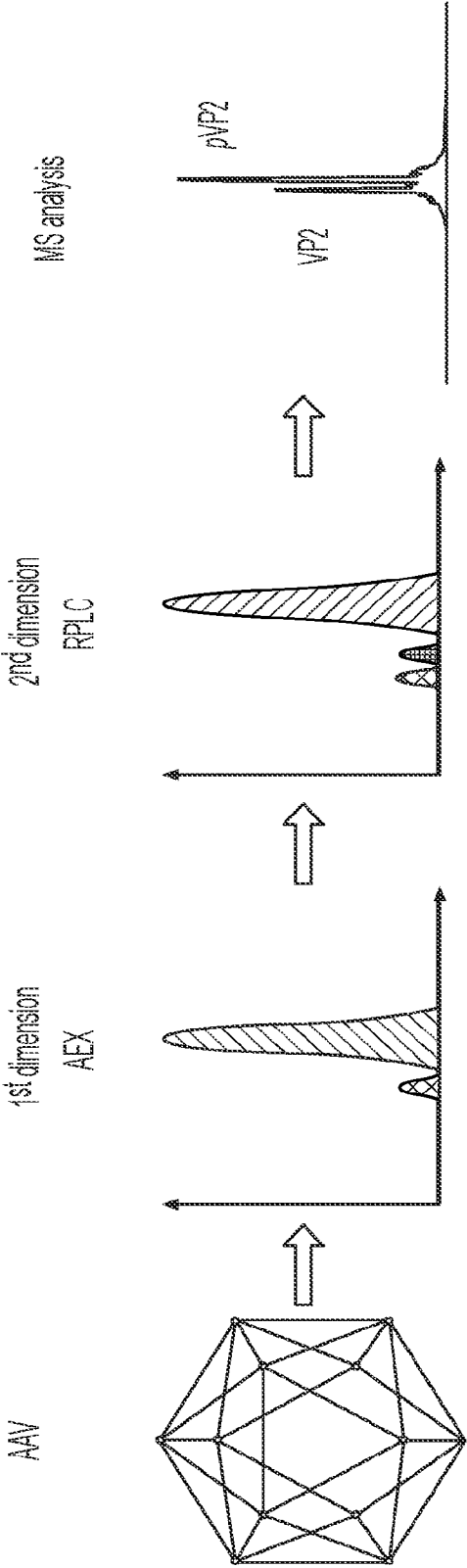


FIG. 2A

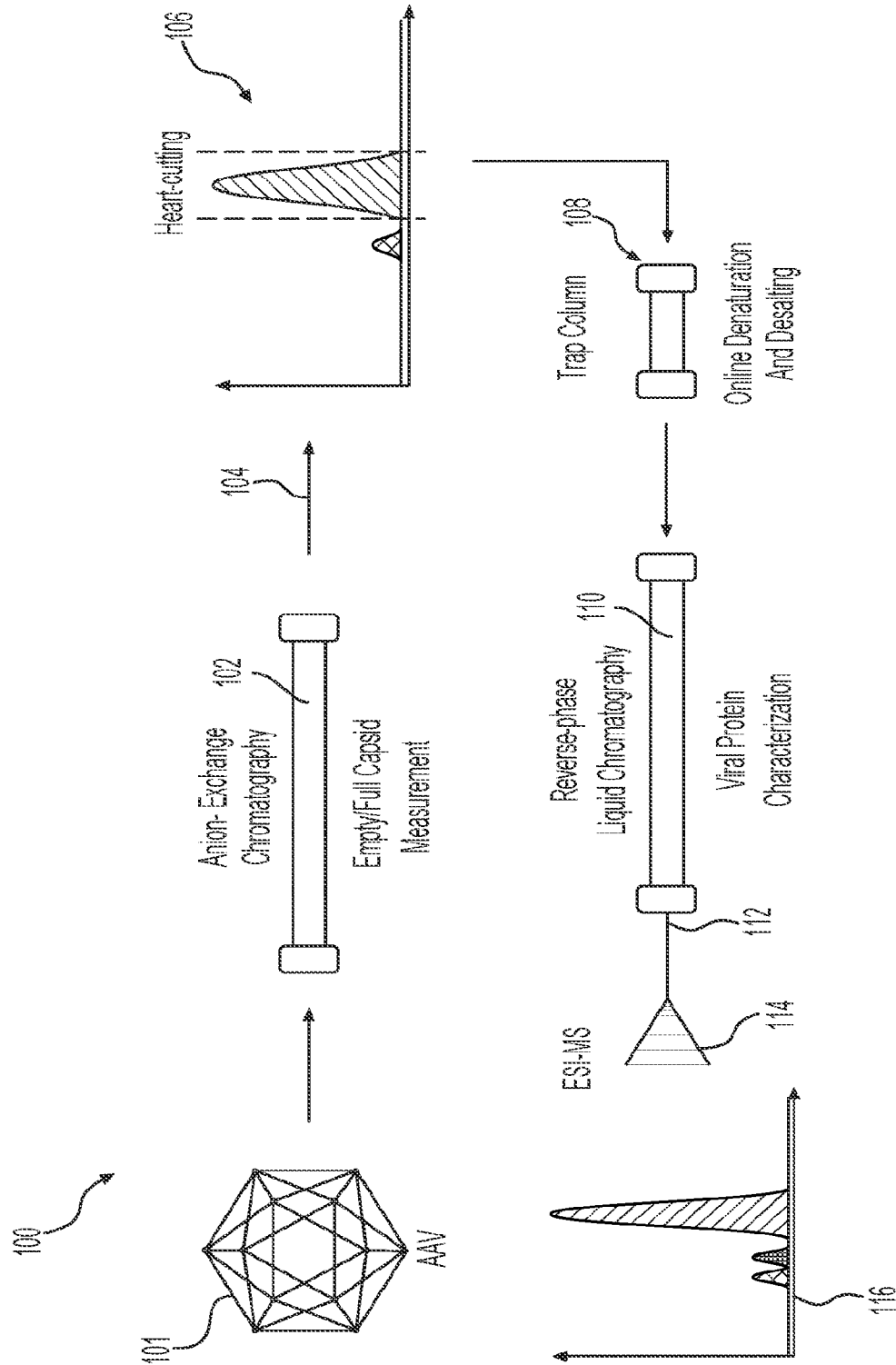
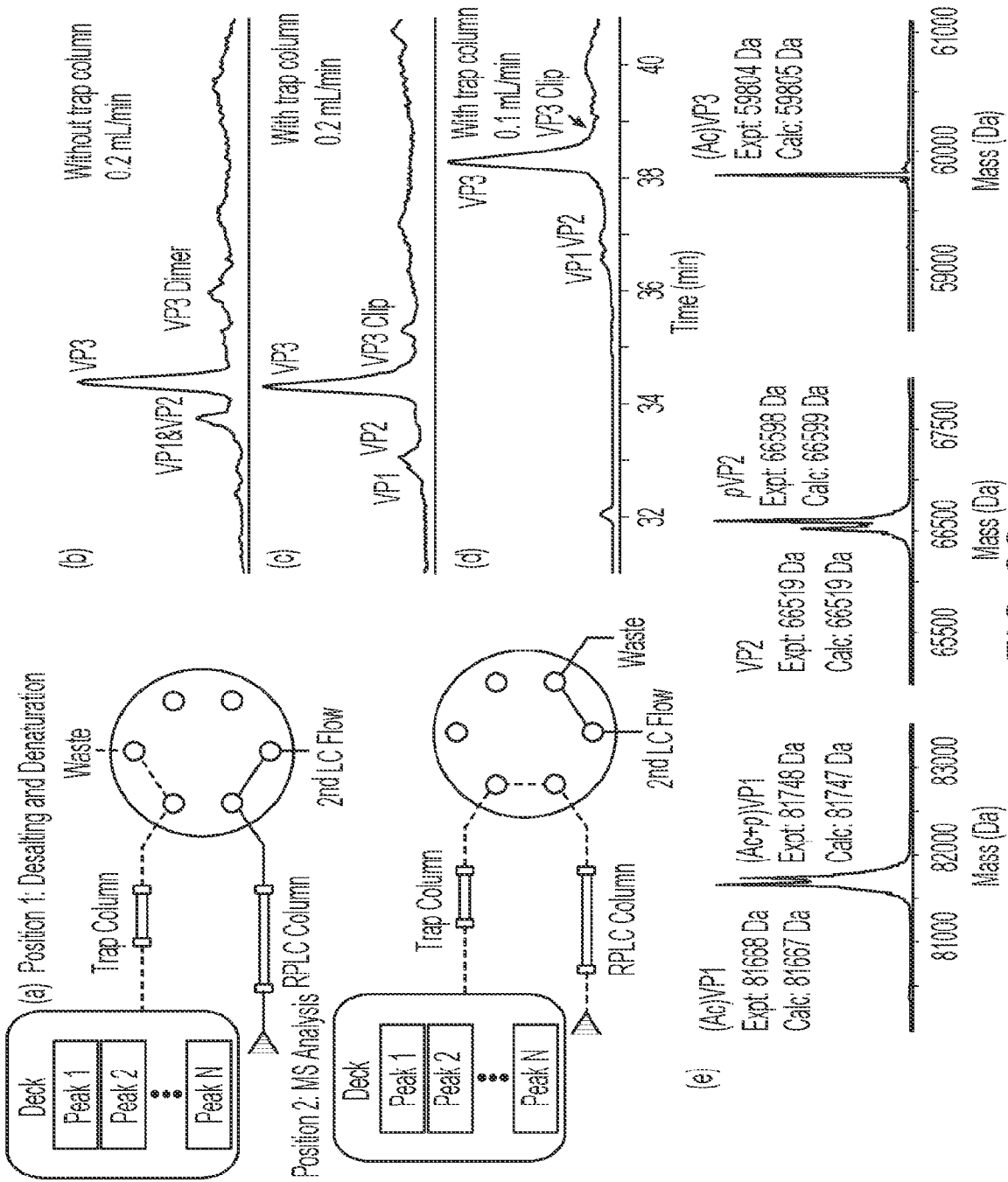


FIG. 2B



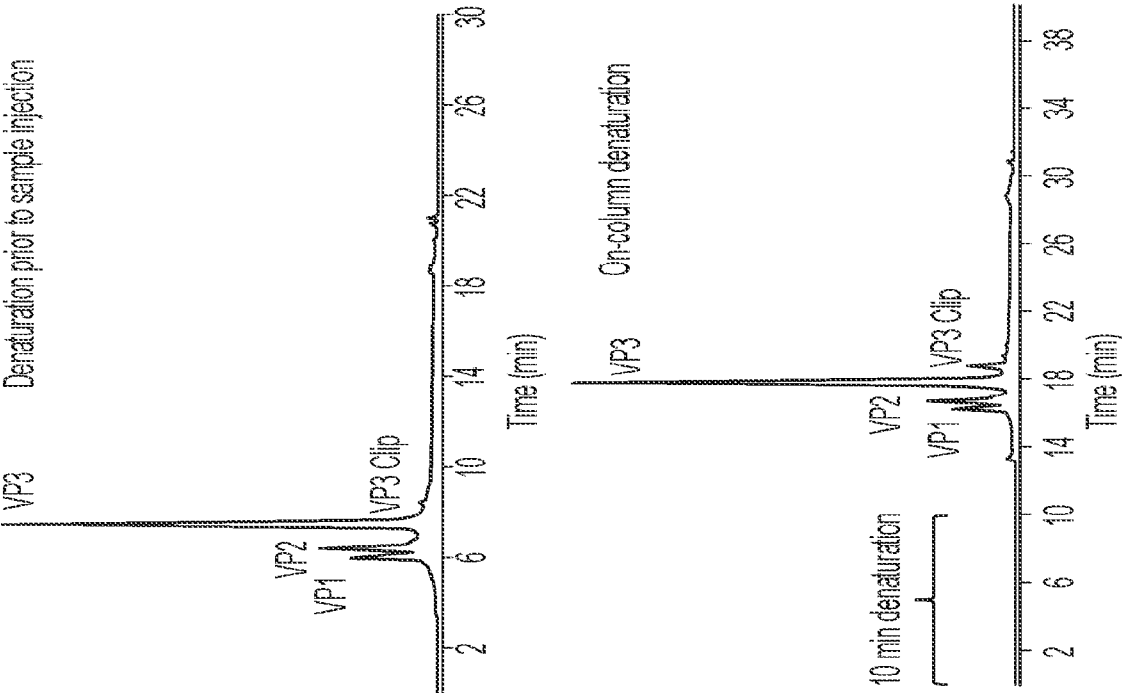


FIG. 2D

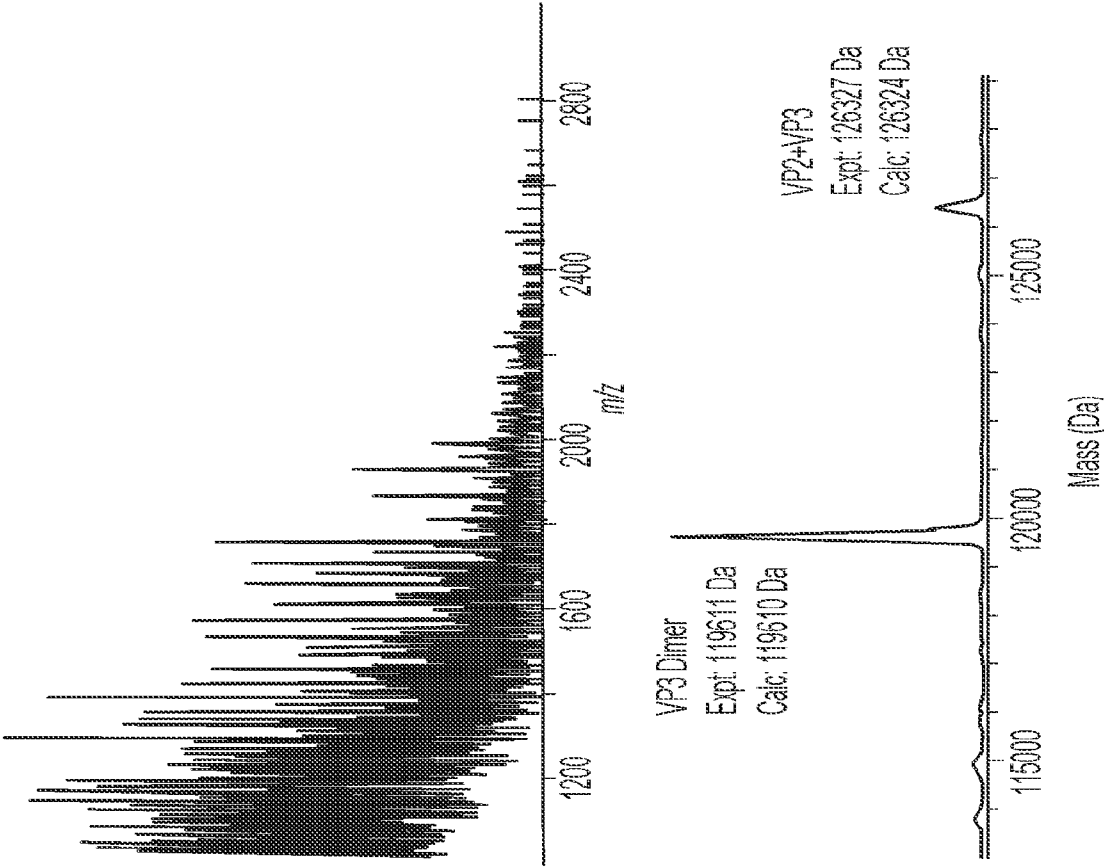


FIG. 2E

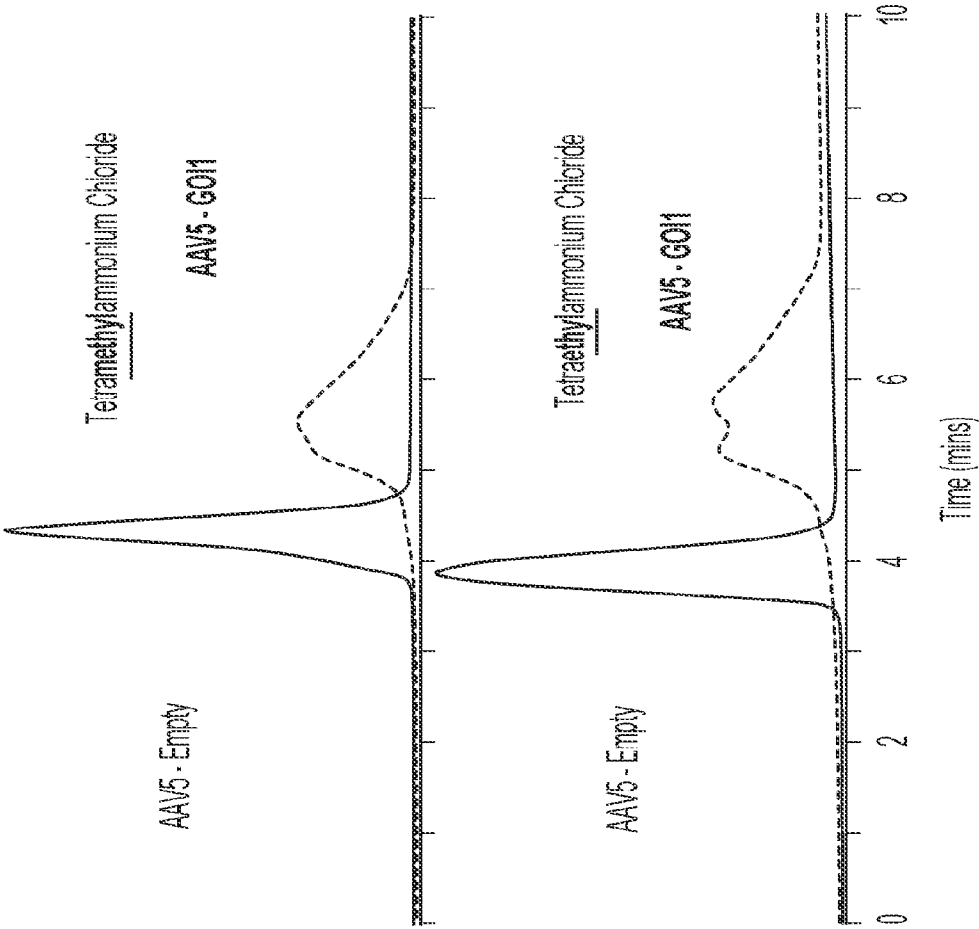


FIG. 3A

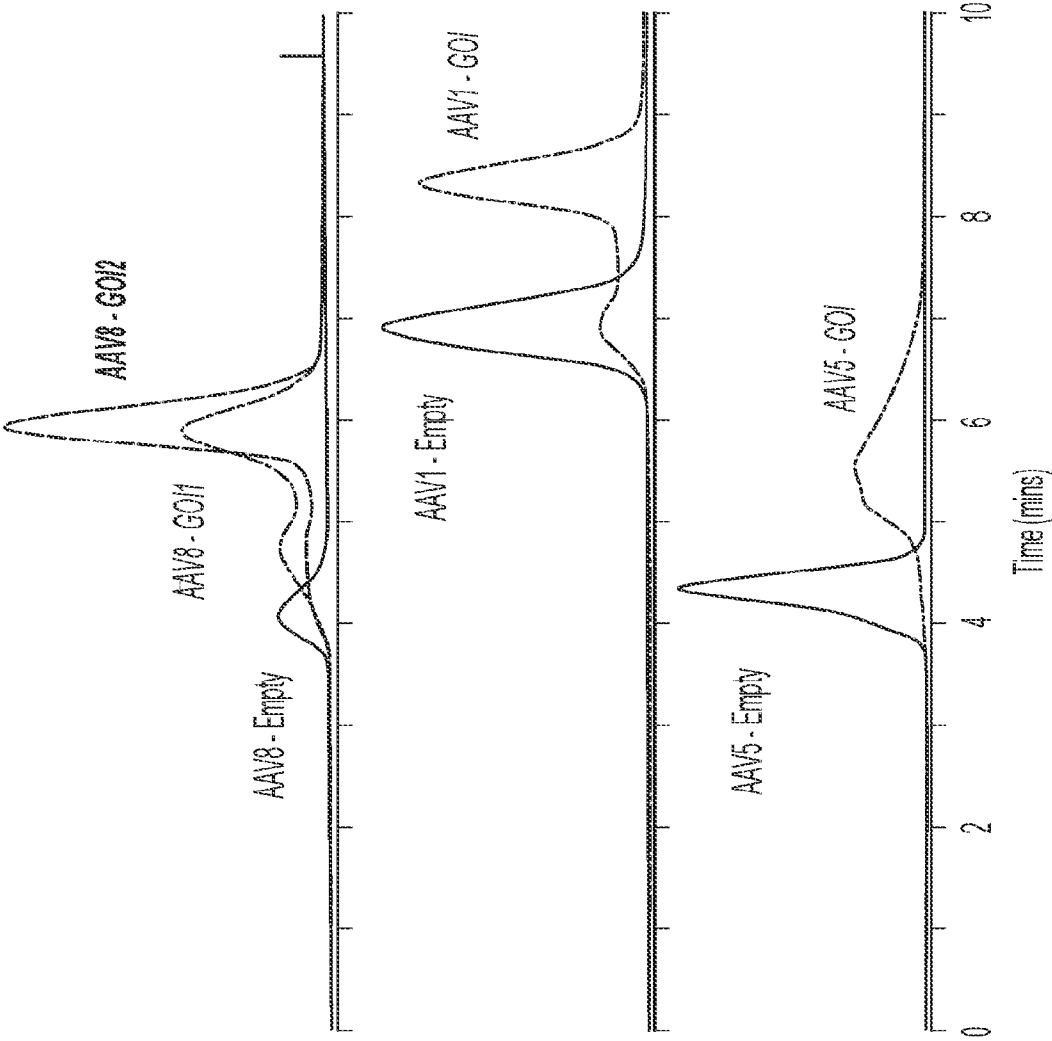


FIG. 3B

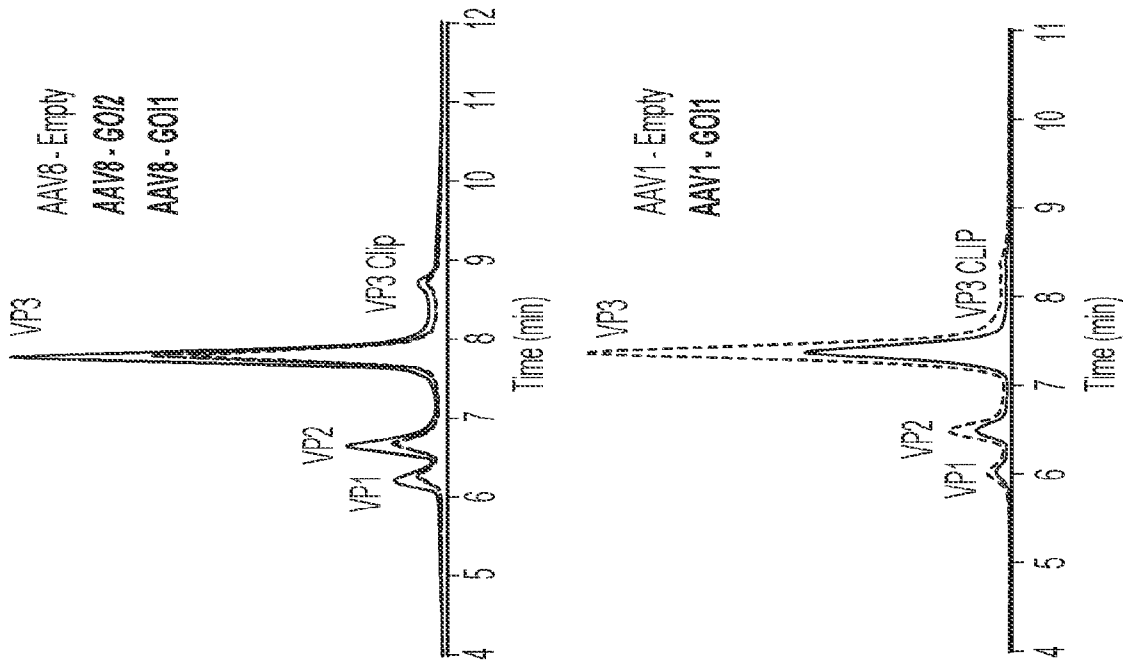


FIG. 3C

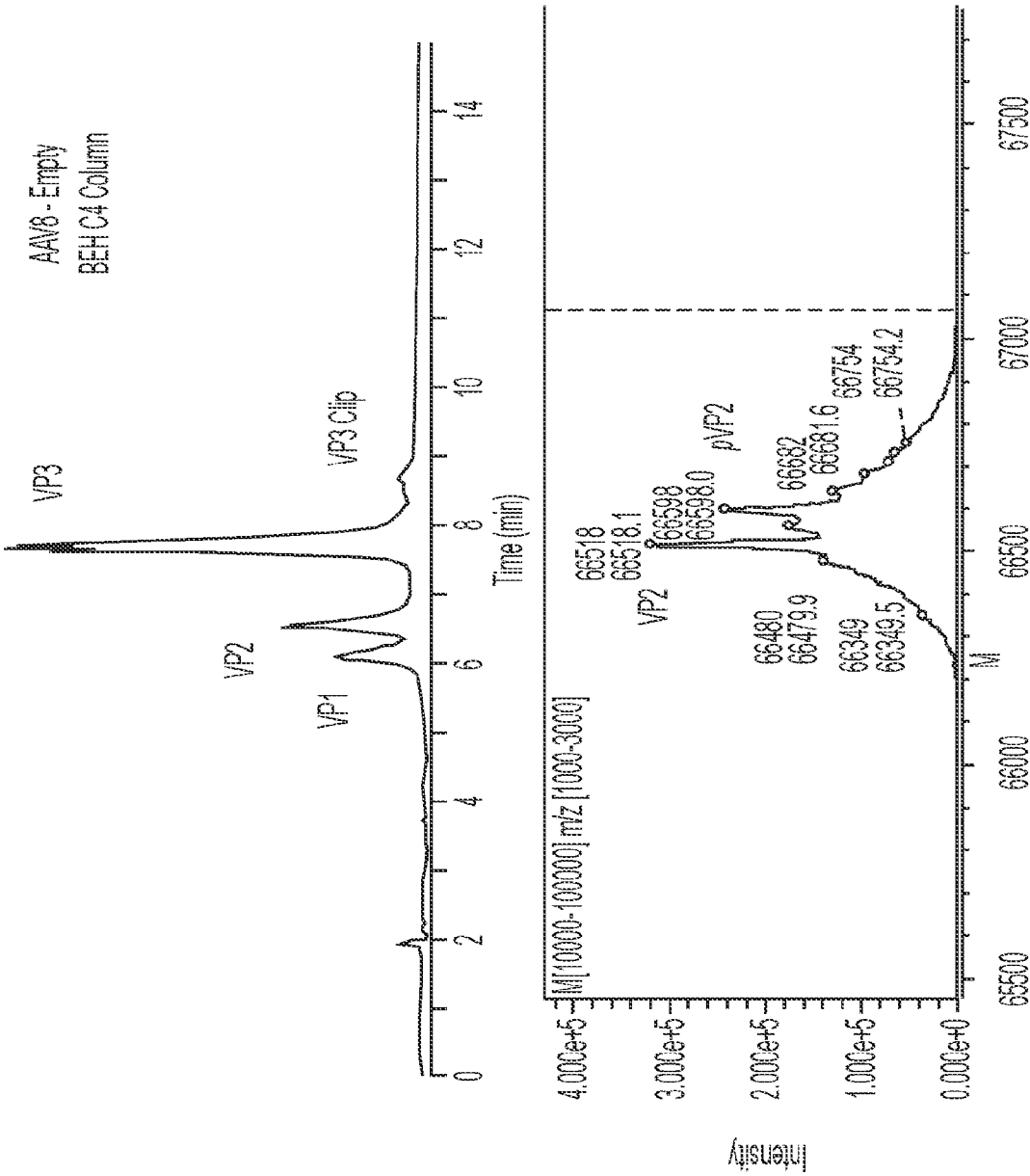
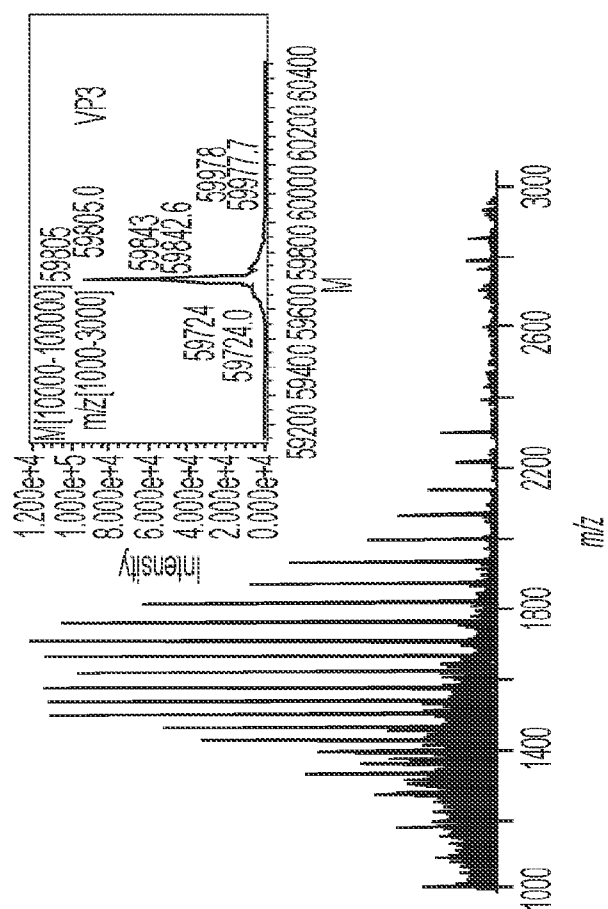
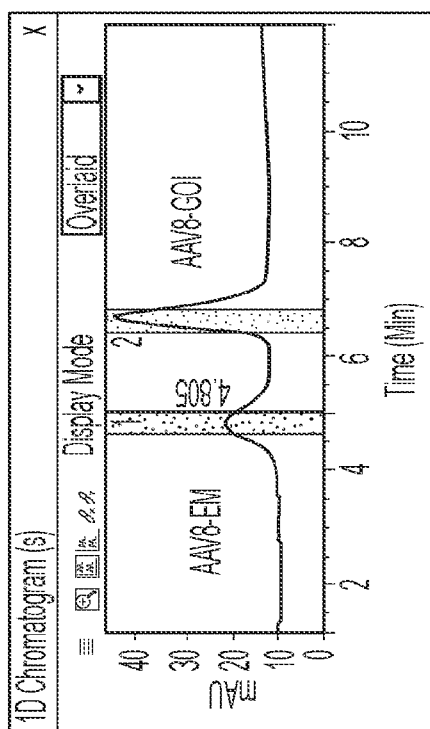
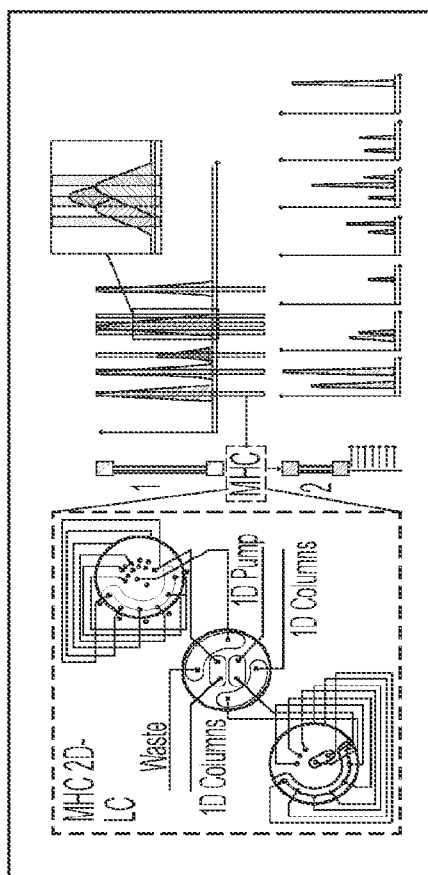
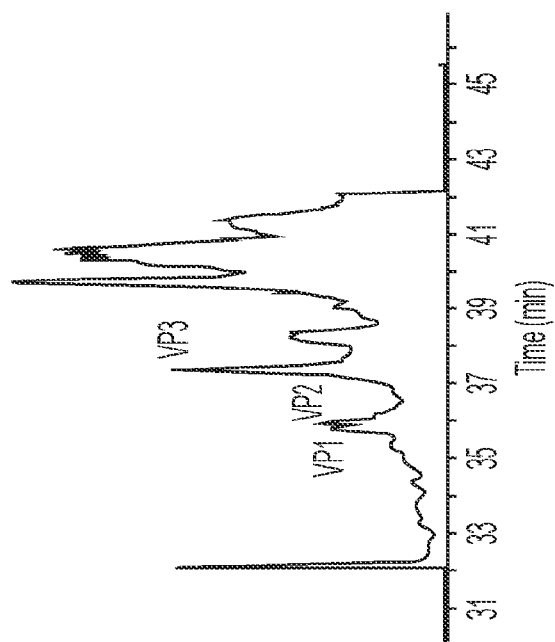


FIG. 3D



4A
G
L



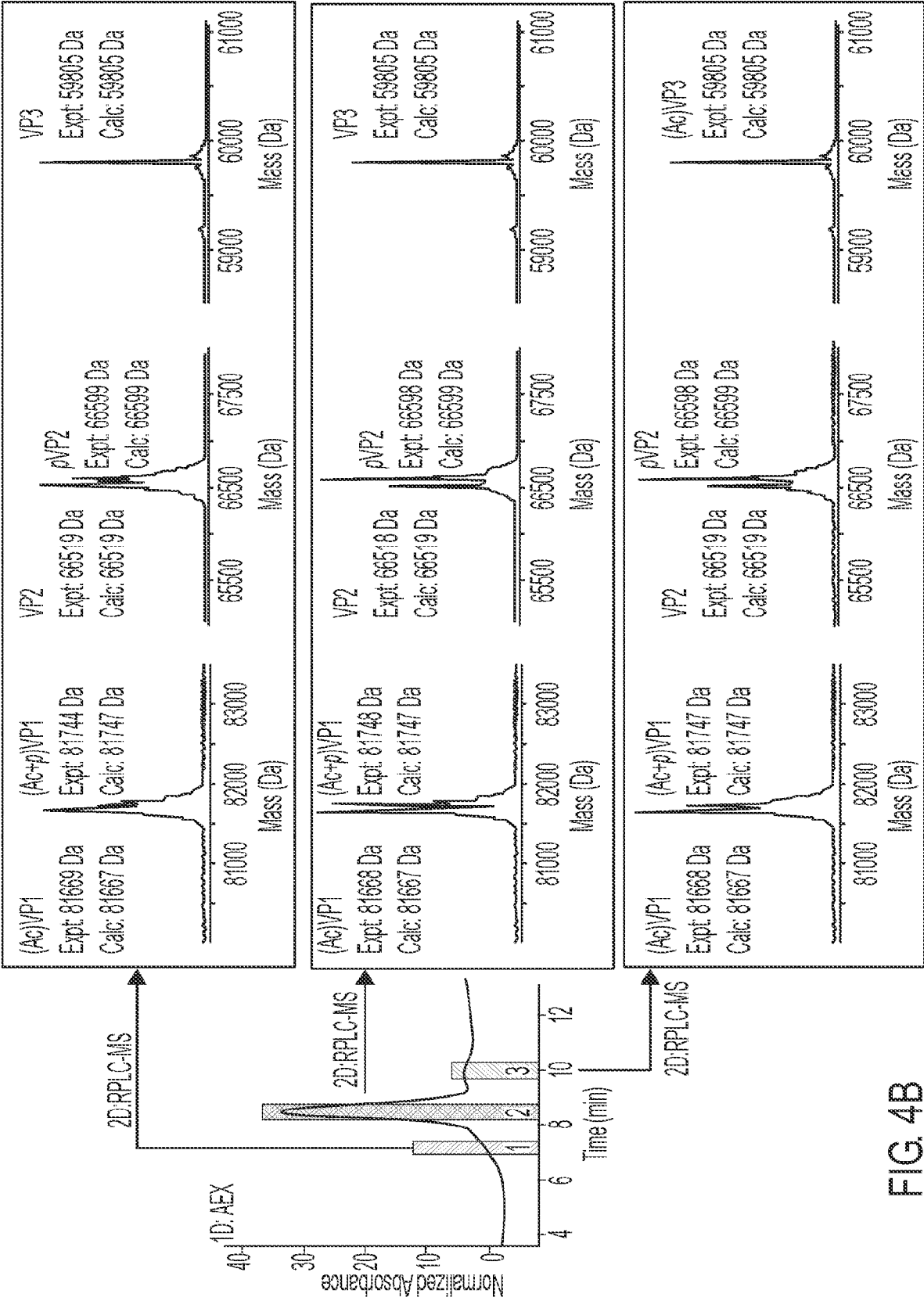
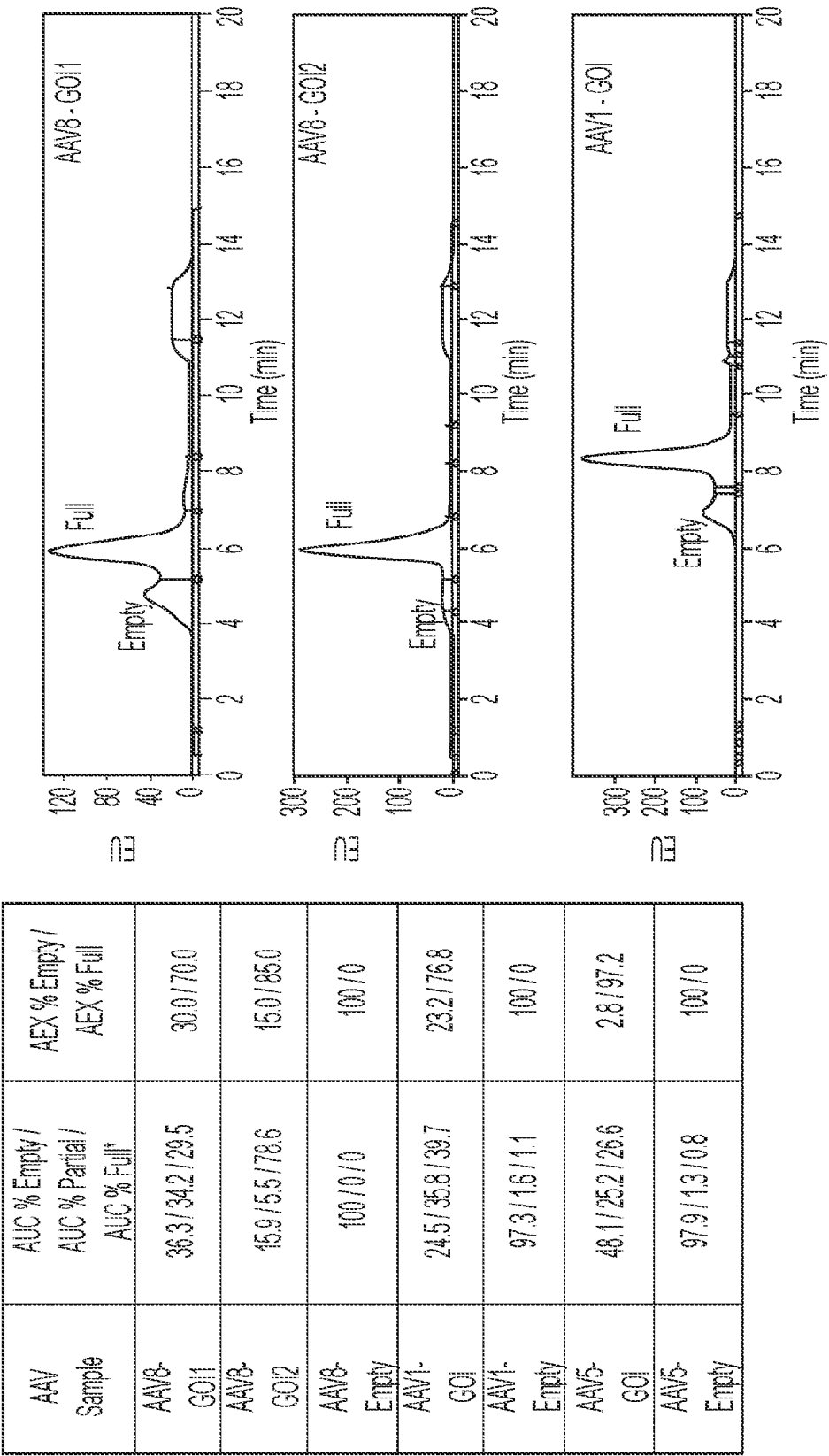


FIG. 4B



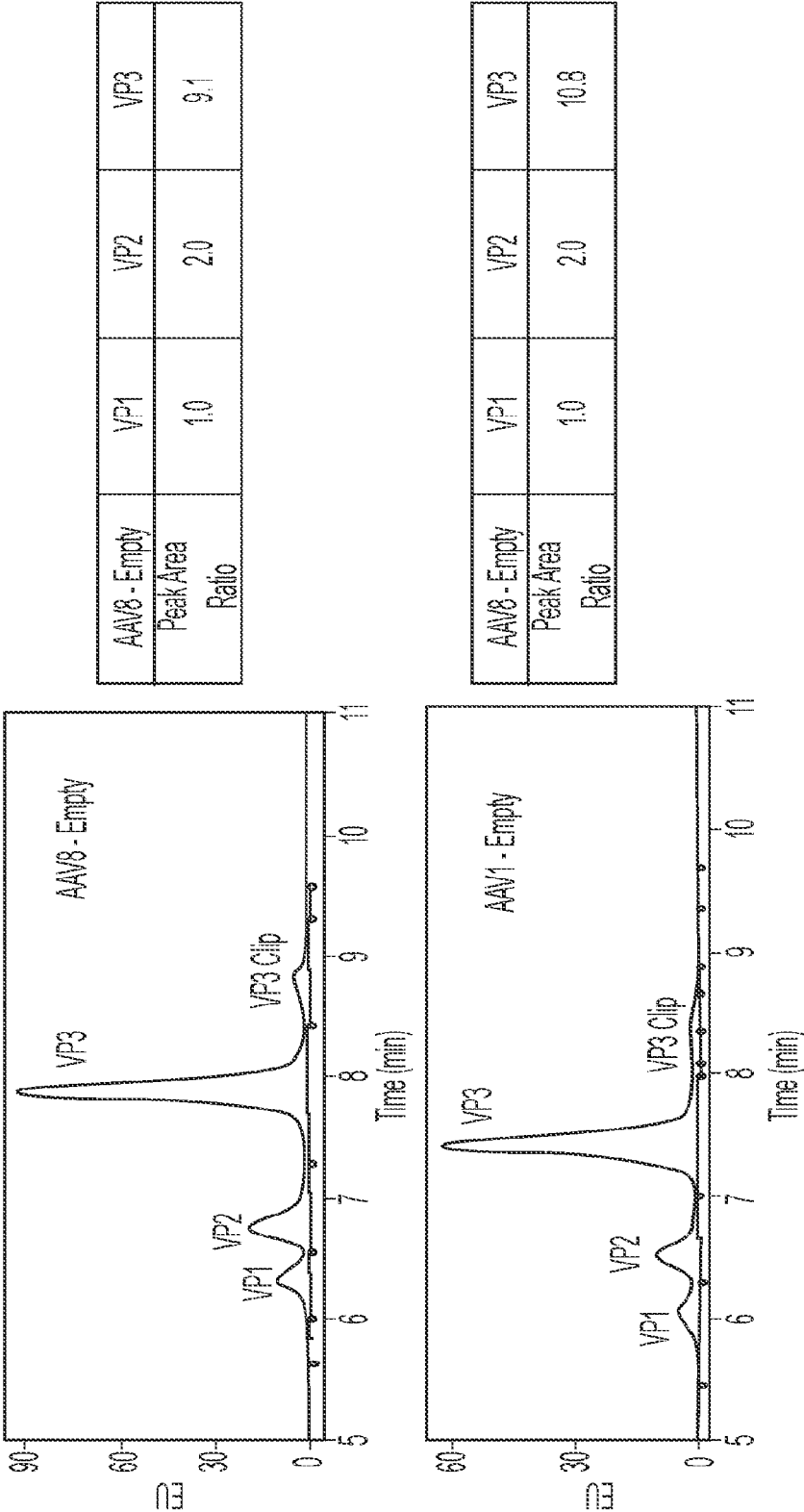


FIG. 6

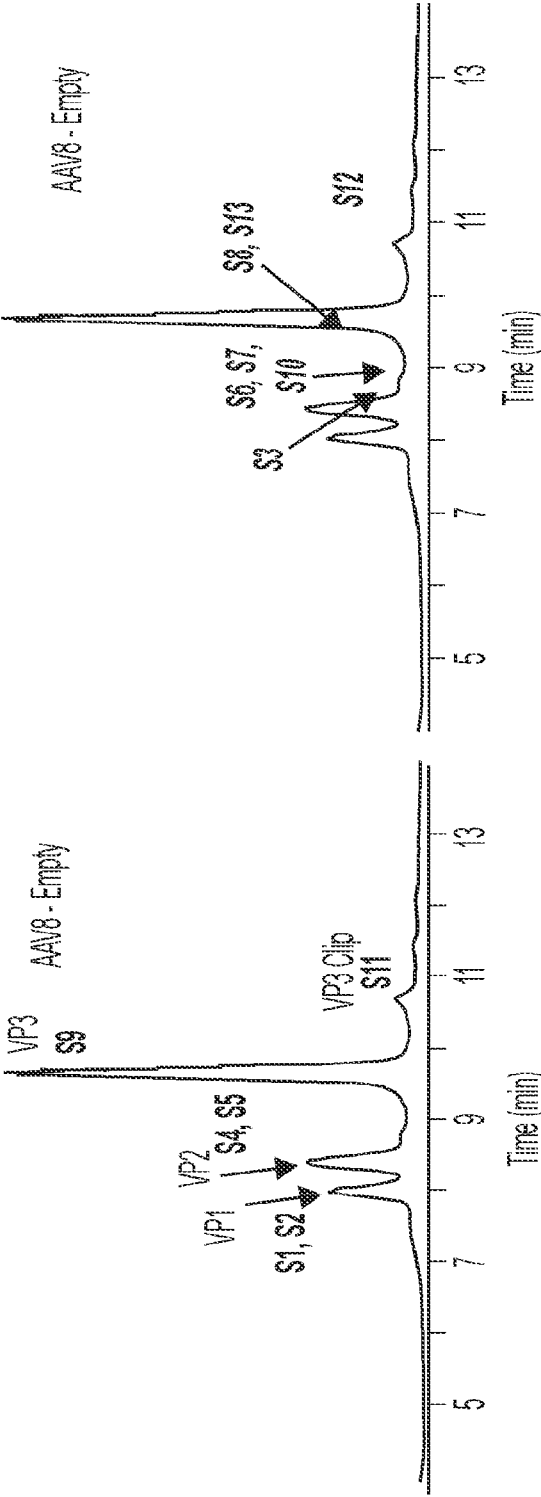


FIG. 7B

FIG. 7A

AAV8

Species #	Identity	Sequence	Observed Mass	Theoretical Mass
1	(Ac)VP1	2(Ac)-738	81667	81667
2	(Ac)VP1 + P	2(Ac)-738	81746	81747
3	(Ac)VP1 Clip (DP)	2(Ac)-659	72456	72455
4	VP2	139-738	66519	66519
5	VP2 + P	139-738	66598	66599
6	VP2 Clip (DP)	139-659	57307	57307
7	VP2 Clip + P (DP)	139-659	57386	57387
8	VP3	205-738	59762	59763
9	(Ac)VP3	205(Ac)-738	59805	59805
10	(Ac)VP3 + Ox	205(Ac)-738	59820	59821
11	(Ac)VP3 Clip (DP)	205(Ac)-659	50592	50593
12	(Ac)VP3 Clip 2 (DG)	205(Ac)-624	47333	47333
13	Clip Fragment (DP)	187-738	61562	61562

FIG. 7C

AAV1

Species #	Identity	Sequence	Observed Mass (Da)	Theoretical Mass (Da)
1	(Ac)VP1	2(Ac)-736	81286	81286
2	(Ac)VP1 + Ox	2(Ac)-736	81302	81302
3	(Ac)VP1 + P	2(Ac)-736	81365	81365
4	VP2	139-736	66093	66093
5	VP2 + Ox	139-736	66110	66109
6	VP2 + P	139-736	66173	66173
7	VP2 Clip (DP)	139-659	49846	49846
8	VP2 Clip + P (DP)	139-659	49926	49926
9	(Ac)VP3	204(Ac)-736	59516	59517
10	(Ac)VP3 + Ox	204(Ac)-736	59532	59533
11	(Ac)VP3 Clip 2 (DG)	204(Ac)-626	47197	47196
12	(Ac)VP3 Clip (DP)	204(Ac)-590	43270	43270
13	Clip Fragment (DG)	329-736	45311	45311
14	Clip Fragment (DP)	185-736	61288	61288

FIG. 7D

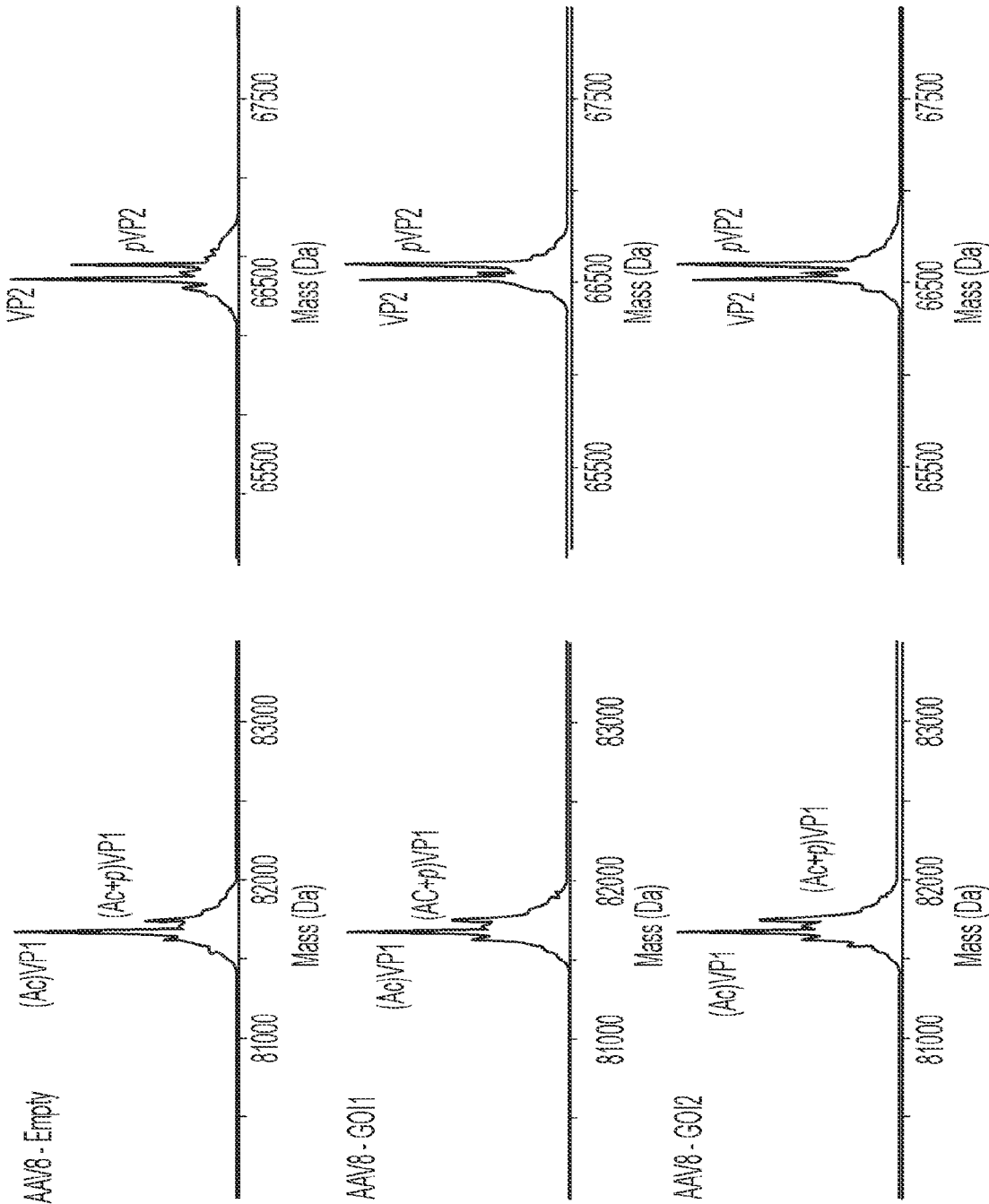


FIG. 8A

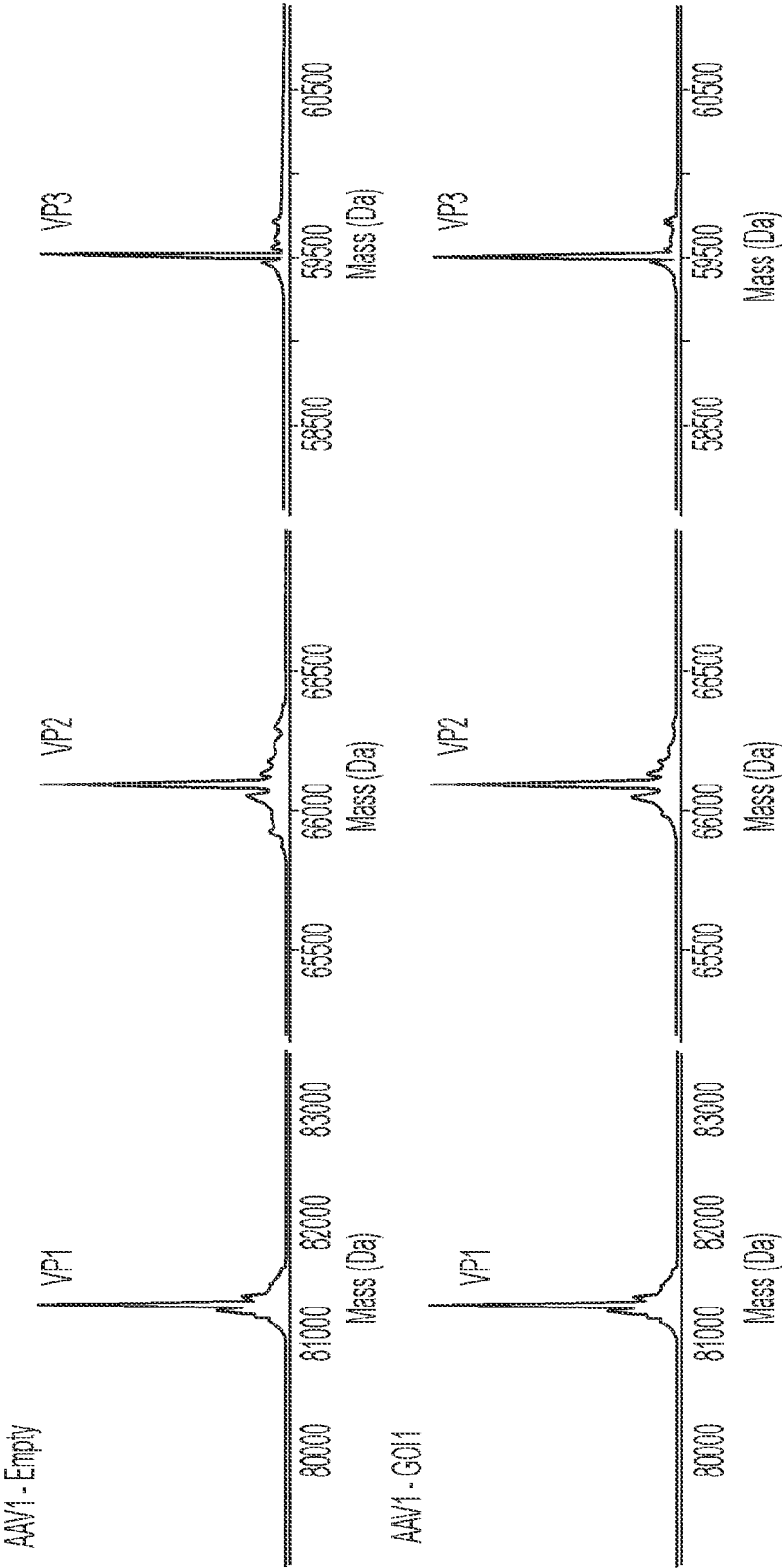


FIG. 8B

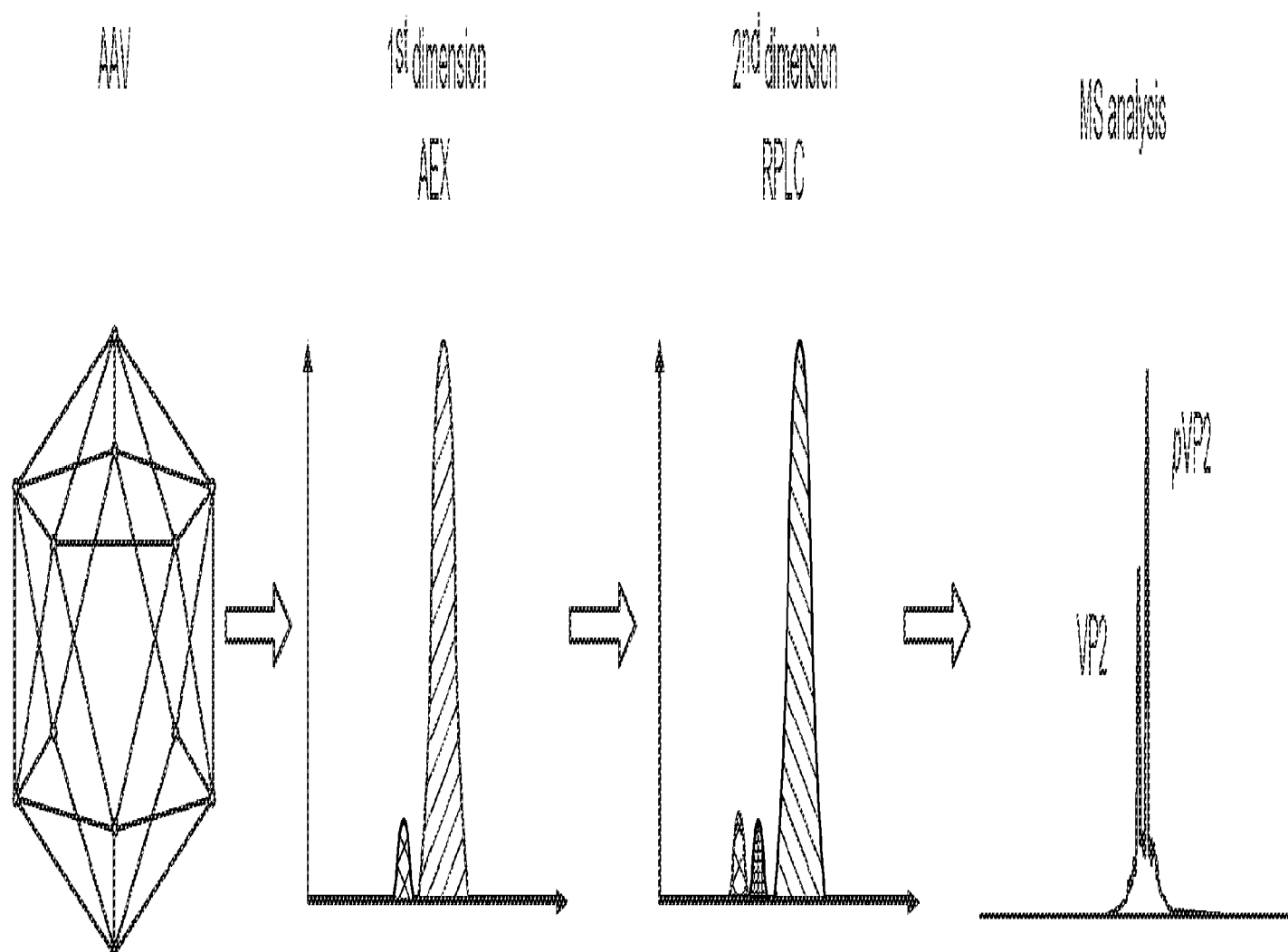


FIG. 2A