British Pharmacopoeia

Consultation

British Pharmacopoeia public consultation for draft guidance for Characterisation of the Capsid Particle Population in rAAV Products: Capsid Protein Characterisation.

Consultation period 18 December 2025 to 27 March 2026

Medicines & Healthcare products Regulatory Agency

British Pharmacopoeia

1. Patients, standards, and innovation

The quality of a medicine is critical to ensuring its safety and efficacy, and therefore the medicine's suitability for patients. Pharmacopoeial standards are part of an interlinked system, together with good practice guidelines and regulatory assessment, that form a foundation to ensuring medicines are of an acceptable quality. Additionally, standards have a place in supporting and enabling innovation through the availability of consistent and widely applicable quality requirements. Innovation in the field of medicines and healthcare has the potential to support patients throughout the world to live longer, healthier, and happier lives.

In recognition of the increasingly important role of biological medicines to healthcare worldwide, the Medicines and Healthcare products Regulatory Agency (MHRA) has developed and implemented a Strategy for pharmacopoeial public quality standards for biological medicines.[1] This strategy, adopted following consultation with stakeholders, laid out a vision of working collaboratively to explore and develop new standard setting approaches for biological medicines. It included a commitment to investigate and take forward standard setting opportunities for innovative Advanced Therapy Medicinal Products (ATMPs).

ATMPs have the potential to be transformative to patients and healthcare globally. However, development, characterisation, and production of these innovative medicines is challenging due to their high complexity, their product specificity, and the still-emerging technologies that support them. Publications such as the Advanced Therapies Manufacturing Taskforce Action Plan,[2] the Medicines Manufacturing Industry Partnership's Manufacturing Vision for UK Pharma[3] and stakeholder feedback have emphasised the important role that standards can have in the development of these medicines. This includes a focus on the value of widely applicable standards that could support knowledge building and facilitate analytics and characterisation.

This draft guidance was written by experts in the ATMP community to support those involved in the development of analytical methods throughout the product lifecycle, and therefore contribute to the quality assurance of innovative medicines for patients.

The MHRA and British Pharmacopoeia would like to recognise and thank the numerous experts in the BP's Working Party for ATMPs that have contributed to the development of this text. The work has been supported by a joint-staff secondment scheme between the BP and the UK's Cell and Gene Therapy Catapult.[4]

2. The draft document

As part of the MHRA strategy for the creation of pharmacopoeial public quality standards for biological medicines, the British Pharmacopoeia Expert Advisory Group for ATMPs, established in March 2020, has engaged with groups across the cell and gene therapy

[1] https://www.gov.uk/government/consultations/strategy-for-pharmacopoeial-public-quality-standards-for-biological-medicines

[2] http://www.abpi.org.uk/publications/advanced-therapies-manufacturing-action-plan/

[3] https://www.abpi.org.uk/publications/manufacturing-vision-for-uk-pharma-future-proofing-the-uk-through-an-aligned-technology-and-innovation-road-map/

[4] https://ct.catapult.org.uk/

community to develop non-mandatory guidance for key analytical technologies to ensure quality throughout the product lifecycle. The working party has developed guidance to support ATMP development across a wide range of organisations, laboratory settings, and therapy types. As such, guidance is product-agnostic and does not provide a step-by-step protocol, nor constitute a prerequisite for product acceptance, but instead offers measures to ensure the production of robust, comparable, and reproducible data within and across organisations.

The ATMP industry continues to grow rapidly worldwide, with increasingly sophisticated scientific discoveries being translated into therapies. There are a variety of challenges in characterising these experimental living medicines. Any CGT product must be characterised in terms of identity, purity and potency and the choice of, and route to, validation of these assays largely lies with the developer and manufacturer. Establishing robust potency assays grows in importance throughout the development of a CGT product and becomes critical in the later clinical stages. As products move towards pivotal clinical trials and licensure, establishing the mechanism of action of the product becomes critical. This requires that potency assays yield rich data which informs the interpretation of outcomes in vivo, whether in models or in early human trials.

This document builds upon the previous British Pharmacopoeia document, ‘Characterisation of the Capsid Particle Population in rAAV Products’, which provided comprehensive recommendations for assessing and quantifying the various rAAV particle species present in therapeutic products, including full, empty, partially filled/intermediate, and overpackaged capsids. The present document provides recommendations for a characterisation framework for analysis of capsid proteins, encompassing their identity, purity, structure, PTMs, and interactions. The guidance is organised according to the principal analytical methodologies used to assess these attributes.

3. How to contribute

The draft guidance for Characterisation of the Capsid Particle Population in rAAV Products: Capsid Protein Characterisation will be posted online for public consultation for a period of two months. During this time, we are asking stakeholders to complete and return the response document, available on our website, to BiolStandards@mhra.gov.uk.

When reviewing the guidance, you may want to consider the following points:

- Do you agree with the technical recommendations made in the document?
- Are the key methods for particle characterisation covered?
- Are there any aspects which you think are missing from the document?
- Is there any terminology within the document that you think needs to be more clearly defined?
- Is the document understandable and are recommendations clear and unambiguous?
- Could the format/style of the guidance be improved?

In addition to the request for technical comments, the response form includes more general questions around the value of the guidelines and other work within the area of ATMPs where standards and standardisation could add value. This information will be used to help the BP to understand and prioritise future work related to ATMPs.

4. Confidentiality and Freedom of Information

Information we receive, including personal information, may be published, or disclosed in accordance with the access to information regimes (primarily the Freedom of Information Act 2000 (FOIA), the Data Protection Act 1998 (DPA) and the Environmental Information Regulations 2004).

Please let us know if you would like any information you provide to be treated in confidence, and please indicate any commercial sensitivities. We will maintain that confidence and resist disclosure under the access to information regimes where possible and in compliance with our legal obligations. We will also consult you and seek your views before any information you provided is disclosed.

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Annex 1 - Draft guidance: Characterisation of the Capsid Particle Population in rAAV Products Capsid Protein Characterisation

Medicines & Healthcare products Regulatory Agency

Annex 1 Capsid Protein Characterisation guidance

Contents

Abbreviations

Acronym	Meaning
AAA	Amino Acid Analysis
AAV	Adeno-Associated Virus
AFM	Atomic Force Microscopy
AGE	Agarose Gel Electrophoresis
ALCOA	Attributable, Legible, Contemporaneous, Original, Accurate (data integrity principle)
ATP	Analytical Target Profile
BGE	Background Electrolyte
CAPEX	Capital Expenditure (context-dependent)
CDMS	Clinical Data Management System
CE	Capillary Electrophoresis
CEMS	Capillary Electrophoresis Mass Spectrometry
CGE	Capillary Gel Electrophoresis
CGT	Cell and Gene Therapy
CQA	Critical Quality Attribute
CZE	Capillary Zone Electrophoresis
DDA	Data-Dependent Acquisition (MS technique)
DDM	n-Dodecyl β-D-maltoside (detergent for membrane proteins)
DIA	Data-Independent Acquisition (MS technique)
DNA	Deoxyribonucleic Acid
DSP	Downstream Processing
DTT	Dithiothreitol (reducing agent)
ELISA	Enzyme-Linked Immunosorbent Assay

ESI	Electrospray Ionization (MS ionization method)
FLR	Fluorescence (detection method)
GMP	Good Manufacturing Practice
HCP	Host Cell Proteins (impurity in biologics)
HPLC	High-Performance Liquid Chromatography
HRAM	High-Resolution Accurate Mass (MS technique)
ICH	International Council for Harmonisation
IEX	Ion Exchange Chromatography
LC	Liquid Chromatography
MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
PAGE	Polyacrylamide Gel Electrophoresis
PTM	Post Translational Modification
QbD	Quality by Design
QC	Quality Control
RNA	Ribonucleic Acid
RP	Reverse Phase (chromatography technique)
SDS	Sodium Dodecyl Sulphate (for protein denaturation)
SEC	Size-Exclusion Chromatography
SPR	Surface Plasmon Resonance (binding assay)
TEM	Transmission Electron Microscopy
UHPLC	Ultra-High-Performance Liquid Chromatography
UV	Ultraviolet (spectroscopy)
USP	Upstream Processing

VP	Viral Particle
VPS	Virus Particle Size (context-dependent)

Terminology

The terminology here is intended to be a glossary of terms for readers to use as a reference tool and also to have a clearer definition of repeated concepts.

Empty capsids: DNA or Genome-less particles

Serotype: wild type clades

Automation: the use or introduction of automatic equipment in a manufacturing or other process or facility.

Reference standard: A reference standard is a highly purified compound with a known and verified potency, used as a benchmark for analytical testing in various fields, particularly in pharmaceuticals. It is essential for assessing the quality, safety, and potency of pharmaceutical products throughout their development and market life

PTMs: Post translational modifications (see reference table)

PTMs Commonly Observed in AAV Capsid Proteins		
PTM Type	**Impact**	**Relevance**
Deamidation	Alters charge, affects capsid stability.	Occurs during storage or at suboptimal pH.
Oxidation	Reduces stability, increases immunogenicity.	Introduced during purification or storage.
Phosphorylation	Affects capsid assembly and receptor interactions.	May occur naturally in host cell systems.
Acetylation	Impacts protein interactions and immune response.	Minor but can occur in mammalian expression.
Glycosylation	Alters receptor binding and immunogenicity.	May occur if glycosylation-prone sequences exist.
Proteolysis	Degrades capsid proteins, reducing integrity.	Indicates protease activity in the process.

Capsid: The protein shell of a virus, including AAV, that encloses the viral genome. Capsid integrity, purity, and identity are critical quality attributes (CQAs).

Critical Quality Attribute: A physical, chemical, biological, or microbiological property or characteristic that must be controlled to ensure product quality.

Capsid Protein ratios (VP1-2-3 variable ratios seen differently across serotypes)

Snapback genome: A snapback genome in the context of AAV (adeno-associated virus) refers to an aberrant or unintended DNA structure that can form during AAV vector genome replication or packaging. It's often considered a type of impurity or defective particle and is particularly relevant when assessing vector genome integrity during analytical characterisation - The genome "snaps back" into a hairpin-like or duplexed form, mimicking a double-stranded configuration even though it’s incomplete

Migration of gel systems where proteins migrate under and electric charge differential based on mass.

Analytical Target profile: was not originally coined in the existing ICH guidelines (e.g., ICH Q2(R1)), but instead, emerged from the Quality by Design (QbD) framework for analytical method development — particularly driven by regulatory thought leaders and industry working groups. The ATP concept gained traction during the drafting of ICH Q14 and the revision of ICH Q2(R1).

Introduction

Advanced Therapy Medicinal Products (ATMPs) represent a rapidly evolving class of biotherapeutics that includes gene therapy medicinal products (GTMPs), somatic cell therapies, and tissue-engineered products. Among these, recombinant adeno-associated virus (rAAV) vectors have emerged as one of the most widely used platforms for in vivo gene delivery due to their favourable safety profile, durable gene expression, and broad tissue tropism. The increasing number of AAV-based therapies reaching clinical and commercial stages has created an urgent need for robust, standardized, and scientifically justified approaches to vector characterization, quality control, and regulatory compliance.

The inherent complexity of AAV-based gene therapies—owing to their biological variability, intricate manufacturing processes, and heterogeneity in final drug product—presents unique challenges that differ significantly from those encountered in the production of conventional biologics. Consequently, new analytical paradigms are required to comprehensively assess identity, purity, potency, genome integrity, capsid integrity, and content (empty/full ratios), as well as to control product- and process-related impurities such as host cell proteins (HCPs), residual host cell DNA, helper virus sequences, and packaging-related byproducts.

Modern AAV characterization requires a platform of orthogonal, phase-appropriate analytical technologies, including but not limited to:

- Capillary gel electrophoresis (CGE) for capsid protein profiling
- Liquid chromatography with UV or fluorescence detection (LC-UV/FLR) for impurity and degradation analysis
- Quantitative PCR or droplet digital PCR (qPCR/ddPCR) for genome titre
- Analytical ultracentrifugation (AUC), charge detection mass spectrometry (CDMS), and cryo-electron microscopy (cryo-EM) for particle composition
- Mass photometry and interferometric techniques for single-particle analysis
- LC-MS-based peptide mapping and PTM profiling for detailed capsid characterisation

As the industry moves toward lifecycle-based method development and embraces principles such as the Analytical Target Profile (ATP) and Quality by Design (QbD), regulatory authorities including the FDA, EMA, and NMPA are increasingly expecting data packages that demonstrate analytical robustness, specificity, and suitability for purpose, aligned with ICH Q6B, Q2(R2), Q14, and USP <1220> & <1225> guidelines.

This chapter aims to provide a harmonized, scientifically justified framework for the pharmacopoeia characterization of AAV-based gene therapy products, intended to support developers, manufacturers, and regulators in ensuring the safety, identity, strength, purity, and quality (SISPQ) of AAV vectors at all stages of development. The guidance encompasses method selection, validation principles, system suitability requirements, and emerging technologies that address current challenges in the characterization of these complex modalities.

More specifically, AAV vectors capsid proteins play a crucial role in gene therapy. These proteins form the capsid, that encases the viral genome, ensuring its stability and facilitating its delivery to target cells. The capsid is composed of three viral proteins: VP1, VP2, and VP3, which assemble into an icosahedral structure (reference). The capsid's surface interacts with cellular receptors, determining the vector's affinity for specific cell types, also called tropism (reference), this tropism is typically linked to the capsid serotype numbering Note: There is a wide range of cross reactivity between tissue type and serotype.

Modifications to capsid proteins can significantly impact the efficiency and specificity of the gene therapy product delivery. Post-Translational Modifications (PTMs) such as phosphorylation, deamidation, and glycosylation can affect the capsid's stability, immunogenicity, and interaction with cellular receptors (reference, reference).

Methods for analysing capsid protein characterisation

Comprehensive characterization of AAV capsid proteins—including the major subunits VP1, VP2, and VP3, as well as their post-translational modifications (PTMs), degradation products, and stoichiometry across the many serotypes of AAV *which can all display unique characteristics*—is a cornerstone of quality assessment for recombinant AAV-based gene therapy products. The ability to reliably identify and quantify these components supports the establishment of critical quality attributes (CQAs) tied to product safety, efficacy, and consistency, in alignment with global expectations outlined in ICH Q6B, Q2, Q14, and emerging guidance for Advanced Therapy Medicinal Products (ATMPs).

Looking ahead, the landscape of capsid protein characterization is evolving in response to regulatory and environmental drivers, such as the European Union's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) framework, which is expected to phase out or restrict certain dyes, detergents, and heavy-metal-based stains historically used in electrophoresis and protein visualization. As these reagents are withdrawn, analytical laboratories are adopting greener, REACH-compliant alternatives—including non-toxic fluorescent stains, biodegradable surfactants, and label-free optical detection techniques—to future-proof their workflows.

Simultaneously, the field is experiencing a shift toward next-generation analytical technologies capable of higher sensitivity and reduced sample consumption. These include label-free mass photometry, single-molecule interferometric techniques, and advanced LC-MS workflows with intact mass and PTM profiling, which are poised to complement or replace some legacy approaches, particularly those with high reagent burdens or limited resolution.

The following sections outline the principles, strengths, limitations, and regulatory considerations for each major method used in capsid protein characterization today,

while also highlighting the emerging trends and innovations shaping the future of AAV analytics.

1. LC-Mass Spectrometry

Method description

Liquid Chromatography-Mass Spectrometry (LC-MS) is an analytical technique used to characterise Adeno-Associated Virus vector (AAV) capsid proteins. The technique involves separating the VPs using generally reverse-phased or hydrophilic liquid chromatography and then identifying and quantifying them using mass spectrometry.

This method allows for the measurement of intact proteins and resulting peptides, providing detailed information about the capsid proteins primary sequences and stoichiometry (reference, reference, reference). For the analysis of intact proteins, Top-down mass spectrometry (MS) is used.

To deeper characterize the VPs, the peptide mapping, which is a well-known technique in the biopharma industry, is used in the identification and quantification of VPs' PTMs and the characterization of the amino acid sequences. It involves the proteolytic digestion of VPs followed by LC-MS analysis. The technique can achieve a high level of sequence coverage (>90% of VPs' sequence characterised) (reference, reference, reference).

Advantages

An advantage of LC-MS is its ability to analyse different AAV serotypes, engineered variants and truncated species (impurities). It offers rapid analysis, making it suitable for high throughput needs. Top-down MS allows for detection of degradation products and sequence variants and map PTM's with greater precision that can be a challenge using bottom-up approaches. With sample preparation requiring fewer steps than bottom-up approaches (such as not requiring reduction and alkylation), the number of experimental artefacts is reduced as no chemical modification is required. This can help avoid modifications introduced during sample preparation in comparison to enzymatic digestion.

Limitations

The sample preparation for peptide mapping can be complex and time-consuming and may result in sample loss and causing high variability for quantification. About the acquisition time, the gradient may take up to 90 minutes, resulting in a long analysis time. Another challenge to perform peptide mapping as a routine analysis is material availability because about 100µL of material at 1E+13 VP/mL is required per analysis.

For intact protein analysis, the signal from low abundant proteins may be masked from the signal of high abundant proteins within a sample. Therefore, larger sample

quantities, enrichment techniques, (protein capture methods, immunoprecipitation) and high-resolution mass spectrometers are required. Efficient protein separation is required due to co-elution of proteoforms and complex mass spectra with overlapping peaks, which can make analysis difficult.

Additionally, two other factors may limit access to this technology: the high capital expenditure (CAPEX) required for utilities, equipment, and software licenses, and the expertise needed to develop and run LC-MS-based assays. Furthermore, the range of LC mobile phase compositions is limited, as the ionization process necessary for MS analysis is sensitive to non-volatile salts (e.g. sodium, Tris, …) and adducts, which can affect sensitivity due to ion suppression.

From a method performance assessment perspective, the evaluation of the accuracy of the method is challenging because of the lack of standards and orthogonal techniques. This makes the relative quantification challenging.

Sample preparation

Two types of sample preparation are considered depending on whether the LC-MS is meant to analyse intact VPs or generated peptides of VPs.

Intact mass analysis

Intact mass protein sample preparation is simplified as no proteolytic digestion is required for breaking down proteins into peptide fragments. This reduces mixture complexity. Protein extraction and purification is required.

Proteins are first extracted using a lysis buffer. To prevent protein degradation and protein modifications, appropriate lysis buffers are required (protease/phosphatase inhibitors). Keeping samples at low temperatures prevents post translational modifications during extraction. Then, following protein extraction, protein solubilization is performed by dissolving the extracted proteins into a suitable buffer with high salt contents or detergents such as sodium dodecyl sulphate (SDS) or DDM.

Many buffers are not MS compatible, so a buffer exchange step is required to remove interfering components and remove volatile salts using ultrafiltration and centrifugation for an MS compatible sample. Immunoprecipitation can be used to isolate target proteins, particularly useful for a targeted approach.

Finally, protein separation and purification separate the target protein either through liquid chromatography (LC) or by SDS-PAGE.

Peptide mapping

The first step involves denaturing the capsid to expose VPs' peptide bonds, making them accessible to proteases. This is typically achieved using

denaturing agents like urea or guanidine hydrochloride, followed by incubation at an appropriate temperature, such as 37°C, for 30 minutes to 2 hours.

Following denaturation, a reducing agent like dithiothreitol (DTT) or 2-mercaptoethanol is added to break disulfide bonds, preventing protein aggregation and promoting complete digestion. The sample is then incubated again at the denaturation temperature. To prevent the reformation of disulfide bonds, the free thiol groups of cysteine residues are alkylated using an alkylating agent like iodoacetamide.
Next, the sample undergoes buffer exchange, generally by using desalting cartridge, into a buffer compatible with the selected protease and subsequent chromatographic steps. A common buffer is ammonium bicarbonate. Finally, enzymatic digestion is performed using a protease that cleaves the protein at specific amino acid residues. Trypsin is a popular choice due to its specificity for lysine and arginine residues. Nevertheless, the use of other proteases such as chymotrypsin or pepsin has been reported in the literature (reference, reference).The protease-to-substrate ratio is optimized to ensure complete digestion while minimizing enzyme autolysis.

Method set up and execution

The method consists of the trapping and the separation of compounds of interests (VPs or peptides) using a gradient of eluting phase. Eluting compounds are then ionised and their mass/charge ratio measured.

Intact mass analysis

Intact proteins are fragmented into the MS, typically by ESI for efficient fragmentation. The intact mass of the protein is firstly analysed by MS1. Following identification of the intact protein, the protein is then fragmented in the mass spectrometer using MS/MS to create product ions. The product ions generated are compared to databases and used for protein identification and characterisation.

For specific parameters, a high resolution such as 15,000 at 200 m/z is required for accurate identification of fragment ions and a scan range that is appropriate for the intact mass of the proteins between a few hundred to a few thousand m/z, which can be further narrowed down for specific fragments. Sample amount usually required is 1-10 µl injection volume at a concentration range usually around 0.5-1 µg.

Peptide mapping

Ions, corresponding to the eluted peptides, are guided into the mass spectrometer and ultimately analysed, giving a mass-to-charge ratio for each ion. One peptide may result in several ions. To detect as many ions of interest (ionised peptides) as possible, the mass-to-charge ratio range is typically from

200 to 2000 m/z, which may be modified based on the expected masses of the peptides after digestion using in silico digestion. Ion isolation and fragmentation, and analysis of the resulting fragments (MS/MS analysis) are recommended to improve confidence of the level and identification of peptide. High resolution mass spectrometers can reach more than 60,000 (at 200 m/z) resolution in full scan mode ("MS only"). However, in MS/MS analysis, it is recommended to limit the resolution to 15,000 m/z, for example (at 200m/z) to limit the duty cycle of the instrument and so to ensure MS/MS analysis speed scan.

Data acquisition and analysis

Two different approaches (Data Dependent Acquisition (DDA) and Data Independent Acquisition (DIA) exist to select the parent ion that will be fragmented for further analysis.

Usually, data acquisition and processing are performed using two different software packages. Besides, processing software capable of processing raw data generated from different types of instruments are now deployed in the biopharmaceutical industry making the choice of software broad (reference, reference).

The first step in the data processing is the deconvolution of the mass spectra (raw data) into a list of masses. Those masses are then confronted with theoretical masses of compounds archived in a database. The matching of experimental masses and theoretical masses results in the identification of the VPs or the peptides, in the case of peptide mapping experiments. A mass tolerance and, in some cases a false discovery rate, is set to prevent identification errors. The identification relies on the matching of theoretical and experimental masses. Additionally, to confirm protein identity, fragmentation and matching of the fragment masses may be considered.

System selection and suitability

Each mass spectrometer manufacturer has developed high resolution analysers to meet scientific expectations. There is no exclusiveness on a specific type of analyser. However, that guidance is applicable for high resolution mass spectrometry equipped with an electrospray ionisation source.

Method development, qualification, and validation

Method development is required for sample preparation, separation, MS Parameters and data analysis.

Sample preparation method development requires purification of proteins to improve sample complexity which will help to improve signal to noise ratio.

For separation optimisation of LC is required for efficient separation including column type selection, mobile phase and flow rate. The sample needs to be compatible for

ionization and fragmentation techniques. This requires the selection of the correct solvent. For example, for ESI, common solvent examples include methanol, acetonitrile and water, alone or in combination.

Validation is required to distinguish between different proteins and modifications. This is important for specificity, which can impact misidentification. The method also needs to be repeatable across different experiments and reproducible in different laboratories, where applicable.

2. Liquid chromatography-UV/FLR

Method description

Liquid Chromatography (LC) is widely used in gene therapy for the separation, identification, and quantification of components in rAAV samples. Two types of detection are commonly used: UV and fluorescence.

UV detection is commonly used to monitor the presence of nucleic acids and proteins in rAAV preparations. It provides an accessible and reliable method for quantifying these components based on their absorbance at specific wavelengths (280nm for protein and 260nm for nucleic acids). In certain applications, fluorescence detection offers higher sensitivity compared to UV detection. For example, it is particularly useful for detecting certain proteins by exciting aromatic amino acid residues (e.g. tryptophan). Therefore, it is recommended to know the excitation properties of the proteins of interest prior to selecting the method.

For characterising viral vector proteins, two main column stationary phases may be considered: reversed phase, which separates the capsid proteins based on their hydrophobicity. Alternatively, hydrophilic interaction can resolve capsid protein structures based on their hydrophilic properties. These two column chemistries are orthogonal, and the selection should be assessed on the serotypes of interest and amino acid sequences, which give information on capsid proteins hydrophobicity and pI.. The LC-UV/FLR technique may be used to elaborate an identity testing as each vector protein's physicochemical properties, given by the composition of amino acid residues, result in a specific retention time of each vector protein. Usually, VP3 protein is well separated from the two other VPs. However, separating VP1 and VP2 proteins may be challenging due to the intrinsic property of AAV where alternative splicing of the *cap (VP1 protein)* gene meaning VP2 and VP3 are created from the same transcript, as such resulting in a higher sequence homology and so, and a higher similarity of their physicochemical properties, it is important to control for this during LC testing.

Method development activities are expected for any new engineered capsid. In reversed phased separation, various mobile phase solutions such as solutions of trifluoroacetic acid in water and acetonitrile (*e.g.* 0.1% volume/volume). Although hydrophilic-based methods require greater development on mobile phases composition: buffer salts concentration in the aqueous phase (*e.g.* from 5 to 100mM) and pH range (*e.g.* from 2 to 7.5).

Advantages

LC technology is widely used in biopharma industries and well-established in quality control laboratories. Development work is still required but the method implementation from development to routine testing laboratories is accessible and rapid.

Limitations

Due to the overlapping homologous regions over the three capsid proteins (VP1, VP2 and VP3), a coelution of VP1 and VP2 may occur. This phenomenon should be controlled on a case-by-case scenario as it depends on VP's amino acids sequence, from serotype to serotype.

Because LC-UV and LC-Fluo rely on VP's elution times, it is required to characterise all the peaks observed during the development by an additional technique like LC-MS (see Section 1). This will ensure that any new peak observed in testing will have to be investigated using the same relative methodology.

Sample preparation

The sample preparation is limited to sample dilution if needed and the denaturation of the rAAV capsid by using a chemical chaotropic reagent, temperature denaturation (artefactual modification may be generated) or any unfolding mean to make VPs available prior to their separation.

Method set up and execution

No specific recommendations are needed compared to classic proteins separation assay. Pure and UHPLC-grade solutions are recommended.

Data acquisition and analysis

Most of the chromatography data systems enable the acquisition, processing, and reporting of LC data. The analysis of data relies usually on peak integration and quantification based on peak area, system selection and suitability.

To achieve optimal separation, it is recommended to use UHPLC systems. Additionally, the optical cell length should meet UHPLC specifications to avoid any peak broadening that could compromise resolution. Those requirements are common for proteins separation methods in general, no further recommendations for rAAV VPs are expected.

Method development, qualification, and validation

The main activity in development is to identify the best chromatographic conditions to separate the three VPS and other potential subspecies by screening column

chemistries, determining mobile phases compositions and defining the chromatographic gradient.

3. Antibody-based techniques: ELISA

Method description

Enzyme-Linked Immunosorbent Assay (ELISA) is a plate-based immunological method used for the detection and quantification of proteins, antigens, or antibodies. In AAV analysis, ELISA is commonly employed to quantify AAV capsid proteins. ELISA relies on the specific binding between an antigen and an antibody, with detection achieved through enzyme-mediated signal generation, typically using colorimetric, fluorescent, or chemiluminescent readouts.

The sandwich ELISA is the most common type of ELISA utilised in AAV analysis, owing to the high degree of specificity imparted by its methodology. Typically, capture antibodies targeted against the antigen of interest are precoated onto a plate. Samples, containing the antigen of interest, are then added to the plate and incubated to allow binding before a wash step to remove unbound antigen. A detection enzyme-conjugated or fluorophore-conjugated antibody targeted against the antigen is added, in effect forming a sandwich. After a further wash, the subsequent steps are dependent on the readout method.

For colorimetric readout, the substrate is converted by the enzyme leading to a quantifiable colour change. For fluorometric based assay it can be read directly, whilst for luminescence-based assay a substrate is catalysed by an enzyme to generate light which can be quantified using a plate reader.

Advantages

With high sensitivity, ELISA methods can detect even low concentrations of target analytes, making it ideal for impurity and potency assays in CGT. It is very versatile, it can be adapted for a wide range of analytes, including total capsid proteins, VP subunits, or neutralizing antibodies. There are many commercially available ELISA kits streamline implementation for common AAV serotypes, making lab to lab standardisation easier and more robust. ELISAs have been utilised historically to measure protein concentrations and are a well-established methodology, hence their wide adoption as a means of measuring AAVs.

Limitations

High dependency on well characterised or high-quality antibodies. Requires highly specific antibodies, which may not always be available for novel AAV serotypes, these can be developed but may extend the drug development lifecycle. A common challenge is that certain sample components/impurities/artefacts can interfere with antibody binding and/or readout, leading to variability, which may require a minimal sample dilution factor for accurate quantification.

From the therapy producer's perspective, consideration must be paid to the kit itself, specifically the comparability of the AAV serotype standard provided and how similar/not this native particle will be to their own particles as there could be differences. A related point is the capacity of the kit itself to recognise the correct conformation of the target antigen when it is intact and present on the AAV capsid itself rather than as free antigen.

Special attention must be paid to the type of detection method, with colorimetric and luminescence protocols available. Colorimetric detection tends to have a narrow quantification range, whilst luminescence (inclusive of fluorescence) although having a broader range does not display a strong dose response.

Sample preparation

Prepare samples by diluting in the assay buffer to fall within the assay's dynamic range. Prepare a standard curve using a known concentration of the target analyte. It is important to ensure consistent sample preparation to minimise variability. Consider additional clarification where appropriate, consider using a depth filter or centrifuge samples to remove particulates that might interfere or cause matrix inhibition with the assay.

Method set up and execution

Typically, manufacturer's will provide their own protocols which come with their kits, but the processes tend not to deviate from kit to kit and the level of automation. The key element will be the generation of a standard curve using the appropriate standard material. The protocol between different ELISA kits rarely differs in the steps but rather with the incubation times and reagent dilutions, with capture antibody pre-coated plates a common feature.

Briefly, a capture and conjugated detection antibody are used which can recognise the specific AAV serotype capsid proteins, preferably in the particle conformation i.e. specific to protein on capsids rather than free capsid proteins. Subsequent steps will follow the standard sandwich ELISA procedure dependent on the specific readout method (section above, *Method description*).

Data acquisition and analysis

A standard curve is usually performed in every run, from which the data from the samples are interpolated from. Plot a standard curve based on known concentrations of the analyte and corresponding signals, from this you can calculate sample concentrations by interpolating from the standard curve. Automated rather than manual calculations can be less error prone.

System selection and suitability

When choosing a system, if you are using engineered or non-standard capsids, special attention will need to be paid to ensure the feasibility of implementing a method compatible with custom capsid antibodies.

Microplate reader capable of measuring the required signal (e.g., absorbance, fluorescence, or luminescence). Plate washer for consistent washing steps. For

ensuring an acceptable dynamic range, ensure the assay covers the expected analyte concentration range by performing representative pre-studies and better understanding the sample/analyte. It is important to consider the level of automation associated with the protocol/kit, for example an automated plate washer would enable more consistent washing steps, while end-to-end automation solutions exist from some vendors.

Method development, qualification, and validation

Identification of the correct ELISA kit or generate and quantify a standard which is relevant to your analyte. Optimize antibody concentrations, incubation times, and washing steps for maximum sensitivity and specificity, and robustness against temperature and incubation time. Demonstrate linearity, precision, and accuracy using representative samples. Assess the compatibility of the assay to an orthogonal method with different sample matrices. Note: Some off the shelf antibody kits may not be suitable for engineered AAV variants due to lack of validated epitope development. To develop an ELISA method for non-standard capsid variants, this may require the generation and incorporation of custom antibodies specific for the capsid variant.

4. Affinity-based techniques: SPR and interferometry

Method description

Affinity-based technologies such as SPR and BLI are powerful, label-free analytical tools for studying molecular interactions. They are increasingly applied in AAV characterization, including:

- Capsid–receptor binding antibody binding and neutralization assays
- Potency correlation and stability studies
- Capsid ligand screening (e.g., heparin, HSPG, TfR interactions)

Both techniques measure binding kinetics in real time and are widely used in nonclinical development, comparability, and QC studies, especially for characterizing AAV–host receptor or antibody interactions.

Affinity-based methods such as SPR and BLI are invaluable tools in the analytical toolkit for AAV characterization. They provide a functional measure of capsid–ligand interactions, which correlates with biological activity, transduction potential, and batch-to-batch consistency.

Advantages

SPR and BLI offer real-time binding analysis without the need for fluorescent or radioactive labels, preserving native biological interactions. They are capable of measuring the strength and kinetics of AAV capsid interactions with receptors, antibodies, or other ligands, providing critical insight into biological function and

product consistency. SPR, in particular, delivers high-resolution kinetic measurements and is considered the gold standard for characterizing complex binding events. BLI, while somewhat less sensitive, offers greater throughput and operational simplicity, making it highly suitable for comparability or batch testing in QC environments.

These methods are especially helpful for understanding AAV–receptor interactions and monitoring capsid integrity and potency through ligand binding, which is directly linked to in vivo transduction.

Limitations

Despite their utility, both techniques have inherent limitations. One of the key challenges is sample matrix interference—buffer composition, serum components, or excipients in AAV formulations can affect signal stability or lead to non-specific binding. Moreover, immobilization of the ligand (e.g., antibody or receptor protein) on the sensor surface must be optimized to avoid orientation artifacts, which could distort binding profiles.

SPR systems typically require more complex instrument handling, buffer stringency, and regeneration conditions compared to BLI, making them less ideal for routine, high-throughput QC. On the other hand, BLI's lower sensitivity can limit its ability to detect weak or transient interactions, and sensor variability can affect inter-laboratory reproducibility if not carefully controlled.

Neither method provides information about structural integrity or heterogeneity of the capsid itself—unlike orthogonal methods such as mass spectrometry or electron microscopy. Instead, they provide functional binding data, which complements but does not replace structural characterization.

Sample preparation

For optimal performance, AAV vector samples should be well-purified and free from interfering substances such as detergents, high concentrations of salts, or serum proteins. Ideally, samples are formulated in a neutral buffer such as PBS or HEPES. Prior to analysis, they should be filtered (0.22 µm) and buffer-exchanged if needed to match the assay conditions.

Ligands, such as purified antibodies or recombinant receptor fragments, are used either as immobilized capture reagents (e.g., anti-capsid antibodies bound to a chip or sensor) or as analytes in solution that interact with immobilized rAAV particles.

Method set up and execution

In SPR, a ligand is typically immobilized onto a gold-coated sensor chip via amine coupling or biotin-streptavidin interactions. The AAV sample is then injected over the chip surface under controlled flow, allowing measurement of association and

dissociation phases. The same surface may be regenerated multiple times using acidic or salt-based buffers, enabling repeated sample injections.

BLI operates by dipping a ligand-loaded biosensor (such as Protein A or Ni-NTA-coated tips) into wells containing AAV samples. Binding interactions are monitored in real time by tracking shifts in light interference patterns. Assay setup is compatible with 96- or 384-well plates for increased throughput.

Data acquisition and analysis

Both SPR and BLI generate sensorgrams that depict binding in real time format. From these, kinetic parameters such as the association rate (ka), dissociation rate (kd), and the equilibrium binding affinity (KD) can be calculated. These values provide insight into the strength and duration of interactions, which is particularly useful when characterizing receptor tropism, assessing changes due to capsid mutations, or evaluating the impact of stress or formulation on AAV potency.

In comparability settings, overlaying sensorgrams from different batches or serotypes allows developers to assess consistency in binding behaviour—a critical attribute to measure.

System selection and suitability

System suitability is essential, particularly in a GMP setting. It typically involves running controls to verify baseline stability, regeneration efficiency, reproducibility of binding curves, and sensor integrity. For SPR, this may include confirming chip activity after multiple regeneration cycles. For BLI, sensor re-use and tip-to-tip variability must be managed carefully, with calibration standards used to maintain consistency.

These methods must demonstrate adequate specificity (e.g., distinguishing between related capsid variants), and reproducibility/robustness (e.g., consistent performance across temperature, buffer, and operator changes).

Method development, qualification, and validation

For regulatory use, method development should define critical assay parameters such as ligand concentration, immobilization density, regeneration conditions, and analyte injection profiles.

Validated SPR or BLI assays can be used in potency assessments, stability testing, serotype comparability, and functional lot release testing for gene therapy vectors.

5. SDS-PAGE

Method description

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a widely used electrophoretic technique for the separation of proteins based on their molecular weight. Proteins are denatured and coated with SDS, a detergent that imparts a uniform negative charge, allowing separation solely by size as they migrate through a polyacrylamide gel under an electric field. SDS-PAGE is commonly used for qualitative and semi-quantitative analyses of protein composition, including capsid proteins (VP1, VP2, and VP3) in AAVs. SDS-PAGE is a versatile, cost-effective, and accessible method for analysing AAV capsid proteins.

Advantages

There are Versatile Staining Options, such as being compatible with Coomassie Blue, silver staining, or fluorescence-based dyes for visualisation. It is inexpensive compared to other advanced techniques like CGE and has a very broad application.

Limitations

This method can be lower resolution and more semi quantitative, for example protein quantitation is less precise due to variability in operator staining and loading styles/training. It is a very manual process and can be time consuming compared to more automated techniques. Due to this variability, therefore the method often requires significant qualification rounds and validation efforts to meet regulatory QC standards.

Sample preparation

Sample prep can be divided into 3 stages: (1) Protein Denaturation, (2) Loading preparation, (3) Loading control.

For stage 1, typically mix the sample with SDS-containing loading buffer and a reducing agent (e.g., beta-mercaptoethanol or DTT) and then heat the sample at 95°C for 5–10 minutes to fully denature proteins.

For stage 2, Centrifuge samples briefly to remove debris or bubbles. Load equal amounts of protein per lane to ensure relative comparability.

For stage 3, it is important to select and include a protein ladder (molecular weight marker) to estimate protein sizes, making sure to match the right ladder with the sample/analyte composition for easier analysis.

Method set up and execution

Method setup involves 4 stages:

Stage 1: Gel preparation: You can either cast your own or get premade gels. Typically, a user would cast a polyacrylamide gel with a stacking gel (low %) and a resolving gel (higher %). The gel percentage depends on the size range of the proteins (e.g., 12% gel for typical AAV capsid proteins).

Stage 2: Sample loading

Working in a clean environment, a user loads the prepared samples and molecular weight marker into the wells of the gel, repeat any samples that may have leaked out of the well to ensure sample equivalence.

Stage 3: Electrophoresis (running the gel). Submerge the gel in running buffer and apply an electric field (e.g., 100–200 V). Run until the dye front reaches the end of the gel, ensuring adequate separation which can be easily visualised.

Stage 4: Staining/Destaining. Stain the gel with Coomassie Blue or an alternative dye to visualise proteins. Destain to remove background staining for clear resolution of protein bands.

Data acquisition and analysis

After staining, capture gel images using an appropriate gel camera and documentation system. Identify protein bands based on their migration relative to the molecular weight marker. Then you can estimate stoichiometry of VP1, VP2, and VP3 capsid proteins by comparing band intensities, typically the ratio should be 1:1:10 for VP1:VP2:VP3. You can use densitometry software to analyse band intensities and estimate protein amounts.

System selection and suitability

Standard SDS-PAGE setup including a gel casting system, electrophoresis chamber, and power supply. Gel imaging system (e.g., UV transilluminator or fluorescence scanner). When assessing suitability, try to ensure proper polymerization of the polyacrylamide gel (absence of air bubbles or inconsistencies).

Method development, qualification, and validation

The VP proteins have been shown to be variable across serotypes, so it is important to ensure the VP stoichiometry matches the intrinsic properties of the serotype. In other words, not every capsid follows the 1:1:10 ratio, especially under variable manufacturing process conditions.

Optimise denaturation conditions, gel matrix composition, capillary dimensions, and voltage for maximum resolution. Confirm the suitability of the method for detecting VP proteins and impurities across process/batch variations.

Validate fluorescent labelling if required for sensitivity enhancement.

6. Capillary Gel electrophoresis UV/FLR

Method description

Capillary Gel Electrophoresis (CGE) with UV and Fluorescence (FLR) detection is a robust and automated technique used for the separation, analysis and quantification of AAV capsid proteins, VP1, VP2, and VP3.

Proteins are denatured under reducing conditions, separated by molecular weight using a gel-with a thin capillary//lane allowing migration through the matrix/lattice, and following migration can be detected either by UV absorbance or fluorescence emission after labelling.

The method provides high-resolution data for determining V1-VP2 VP3 capsid protein stoichiometry and can be used to detect impurities or viral degradation products. It is more sensitive than conventional AGE and is capable of detecting small differences in molecular weight or charge heterogeneity while AGE is more for Suitable for assessing plasmid topology, genomic DNA size, or RNA integrity in gene therapy workflows.

Advantages

The method provides high resolution and unlike AGE based methods, can offer simultaneous quantitation and detection of impurities or degradation products. It also permits higher reproducibility and reduced manual handling/steps. Depending on the type of detection/florescence it can enhance sensitivity for low-abundance proteins

Limitations

Performance depends on the quality of the gel matrix used. Similarly, if the sample has an unusual number of impurities, these impurities may interfere with accurate quantitation. Some samples may require some manipulation on voltage and temperature to obtain best resolution.

Sample preparation

Normally involves, preparing a denaturation buffer containing SDS, a reducing agent (e.g., dithiothreitol), and additives such as a suitable fluorescent label/dye for enhanced sensitivity in FLR detection mode. The sample/analyte is typically clarified/centrifuged to remove insoluble components and minimise bubble formation prior to injection.

Method set up and execution

The major steps for method set up and execution are as follows:

(1) Capillary Conditioning

(2) Sample Loading
(3) Electrophoretic Separation
(4) Detection

Proper capillary conditioning is essential to ensure reproducible and high-quality separations in CE, especially when analysing proteins, which are sensitive to surface interactions and buffer inconsistencies. Conditioning prepares the inner surface of the fused-silica capillary, removes residual contaminants, and stabilises the electroosmotic flow (EOF), which is critical for consistent migration times and peak shapes. Maintain separation at a constant temperature (e.g., 25°C) to ensure reproducibility. Apply a high electric field (e.g., 15–20 kV)

Between runs, a short rinse with background electrolytes (typically 1–2 minutes) is performed. If protein adsorption or peak distortion is observed, an intermediate rinse with NaOH and water may be added.

At the end of the run, the capillary is flushed with NaOH, water, and stored in background electrolytes or water depending on the system recommendation to prevent drying and preserve capillary integrity.

Data acquisition and analysis

CE systems are usually connected to software capable of acquiring data and processing them. In a regulated environment, chromatographic data system is the best solution to comply with ALCOA++ principles/GMP.

The data processing is based on peaks integration and relative quantification of main (VPs) and subspecies (truncated or oligomeric VPs species).

System selection and suitability

The selection of capillary systems is limited. Different options exist and will depend on the required throughput mainly, as well as sample volume.

Method development, qualification, and validation

The first aspect to consider is the detection type (UV or Fluo) as sample tagging, for Fluo detection, may be required during the sample preparation.

Fused silica capillary is the most common used capillary type. The dimensions should be assessed even though ready-to-use ones are available on the market. To ensure consistency and reproducibility, it is our recommendation to use read-to-use pre-assembled capillaries instead of custom ones where appropriate. To reduce interactions between the analytes and the capillary wall, coated capillaries (*e.g.* polyacrylamide) are used, Ultimately, it improves reproducibility and peak shape.

Because CE is sensitive to sample buffer composition, buffer exchange may be required to ensure efficient separation and consistency across runs. Also, buffer compatibility should be assessed to maintain stable electroosmotic flow and consistent separation.

Optimisation of electrophoresis conditions is needed and will depend on rAAV samples as it is expected that VPs' charges differ across rAAV serotypes.

7. cIEF UV/FLR

Method description

Capillary Isoelectric Focusing (cIEF) is an electrophoretic separation technique that resolves proteins and protein complexes based on their isoelectric point (pI). In this method, a sample is loaded into a capillary filled with a pH gradient medium containing ampholytes. An electric field is applied, causing proteins to migrate and focus at the position where their net charge is zero (pI). Detection is performed by UV absorbance (typically 280 nm) or fluorescence (FLR) if proteins or ampholytes are labelled. For AAV characterization, cIEF is used to evaluate capsid charge heterogeneity, detect post-translational modifications (PTMs) such as deamidation or sialylation, assess product-related variants, and verify capsid stability and lot comparability.

Advantages

- High-resolution separation of charge variants, including PTM-induced isoforms.
- Provides pI values that can be tracked for comparability studies and stability.
- Low sample consumption (typically <10 µL per injection).
- Orthogonal to mass and size-based techniques (CGE, LC-MS), enhancing characterization depth.
- Automation-friendly for QC labs using commercial cIEF platforms.
- Sensitive to minor charge shifts that may indicate degradation or manufacturing variability.

Limitations

- Requires specialized instrumentation and trained analysts.
- Sample matrix must be free of particulates, salts, and non-volatile buffers; otherwise, focusing is impaired.
- PTMs altering charge (e.g., sialic acids, deamidation) may complicate interpretation if not confirmed by LC-MS.
- Ampholytes can introduce batch-to-batch variability and may be under regulatory scrutiny (REACH) for certain formulations.
- Not widely used as a lot release test due to complexity and data interpretation requirements (mainly for extended characterization).

Sample preparation

- Exchange AAV sample into a low-salt, volatile buffer (e.g., 10–20 mM ammonium acetate) via ultrafiltration or buffer exchange.
- Typical input: 1E10–1E11 VP (corresponding to 0.5–5 µg total protein) per injection.
- Mix with carrier ampholytes covering the expected pH range (e.g., pH 3–10 or 5–8).
- Optionally include fluorescent dye for FLR detection (must be validated and REACH-compliant).
- Degas or centrifuge briefly to remove bubbles and particulates.

Method set up and execution

1. Capillary Conditioning:
   Flush capillary with conditioning solution (e.g., NaOH, water, then ampholyte solution) to prepare surface and prevent carryover.
2. Sample Loading:
   Introduce sample-ampholyte mix via pressure or electrokinetic injection.
3. Focusing:
   Apply a stepwise or constant high-voltage field (typically 15–30 kV) to allow proteins to migrate and focus at their pI.
4. Mobilization and Detection:
   After focusing, proteins are mobilized (e.g., by chemical mobilization or pressure) and detected online via UV (280 nm) or FLR.
5. Calibration:
   Use pI markers for internal calibration and alignment across runs.

Data acquisition and analysis

Data is recorded as an electropherogram showing focused peaks at specific migration times/pH positions.

Assign pI values using calibration standards.

Integrate peaks to assess relative abundance of charge variants (main vs. acidic/basic species).

Compare profiles against reference standards for batch release or comparability.

Flag shifts in pI or emerging minor species as potential indicators of instability, PTM changes, or manufacturing variability.

System selection and suitability

- Instruments such as SCIEX PA 800 Plus or similar cIEF-capable platforms must be qualified (IQ/OQ/PQ).
- Suitability confirmed by:
- Resolution between adjacent pI markers (Rs ≥ 1.5).
- Repeatability of migration time or pI (≤2% RSD for main peak across replicates).
- Detection sensitivity meeting validated thresholds (S/N ≥ 10:1 for main VP peaks).
- Include system suitability samples (reference standard, pI ladder, and blank) in every run.

Method development, qualification, and validation

Development focuses on:

- Optimizing ampholyte range (broad vs. narrow pH).
- Determining voltage profile and mobilization strategy for best resolution.
- Confirming compatibility of fluorescent dyes and buffers (REACH-compliant where possible).

Validation per ICH Q2(R2) or USP <1225>, assessing:

- Specificity (ability to resolve major and minor species).
- Accuracy and precision (migration time/pI reproducibility, peak area quantification).
- Linearity (over the relevant VP/protein concentration range).
- Limit of Detection (LOD) and Quantitation (LOQ) for minor variants.
- Robustness, including evaluation of:
    - pH gradient stability,
    - ampholyte batch variation,
    - minor voltage or temperature fluctuations.

8. CZE-ESI-MS

Capillary electrophoresis encompasses such methodologies as capillary gel electrophoresis (CGE), capillary zone electrophoresis (CZE), and capillary isoelectric focusing (cIEF). The focus of this section will be CZE due to it being the most prevalent form of CE hyphenated with mass spectrometric detection.

Method description

CZE-ESI-MS allows for the separation and detection of proteins and peptides based on analyte electrophoretic mobility in a free-solution buffer under and electric field. The methodology is well established for the analysis of biotherapeutic products and is orthogonal to other commonly used separation methodologies such as reversed phase, HILIC, HIC, and Ion exchange chromatography. Coupling of the CE separation directly to a mass spectrometer allows for accurate mass identification of proteins or peptides and determination of post translational modifications present.

Advantages

CE-ESI-MS is both sensitive, fast and can provide good resolution between species bearing charge altering PTMs. Characterisation of intact protein and protease treated peptide mapping approaches are both possible. Relatively short run times allow for higher sample throughput and sensitivity fits in well with the small volumes of AAV material available for testing at multiple steps in the manufacturing process. The maturity of both CE and MS in application to biopharmaceutical characterisation means that a wealth of expertise is available. Multiple vendors have developed MS compatible CE systems compatible with a range of vendor platforms, including systems that comprise both spray device and automated sample handling functionality. Software is available for the analysis of the complex datasets generated, either from the vendors of the MS platform or third-party vendors aiming to cover multiple platforms.

The mechanism of separation lends itself to superior resolution of charge variant species, allowing for improved confidence of quantification and identification. Compared to the widely deployed RP-LC-MS approach, CE-MS does not suffer from the same issue regarding potential loss of small hydrophilic peptides.

Limitations

Though both capillary electrophoresis and mass spectrometry are mature techniques for biopharmaceutical characterisation, their hyphenation requires a sound technical understand of both. The hardware capital expense and range of platforms on offer may complicate selection of a system. Buffer composition must be compatible with the MS based detection which requires volatile components, typically meaning a change from background electrolytes usually deployed for CE separations. Quantification

based on MS detection can be influenced by ion efficiencies difference between analytes and matrix effects.

Coupling separation technologies and detection technologies from different vendors may present challenges in terms of hardware/software interfaces, maintenance, and system suitability assessments/qualifications.

Sample preparation

Typically, a buffer exchange step is required to remove salts, which can interfere with both the separation and ionisation of analytes. The presence of process related impurities, such as host cell protein, may obfuscate results. Affinity purification of process samples may be necessary for meaningful analysis.

Proteolytic digestion may be performed to enable a bottom up, peptide mapping, analysis.

Method set up and execution

Capillaries are selected to enable MS compatibility and to minimise protein adsorptive losses. Conditioning is performed prior to sample injection and between samples, in order to regenerate the capillary surfaces and minimise the effect of adsorbed protein on method reproducibility. BGE (background electrolyte) composition and pH should allow sample solubilisation and minimise degradation of capillary coating, organic solvents may be added to aid sample solubility and ionisation but may cause denaturation. Sample is injected and an electrical field applied to drive sample migration through the capillary and into the MS detector.

Data acquisition and analysis

Data acquisition takes place within the mass spectrometry data acquisition software; processing can be performed using either vendor produced analysis software or through third part options. Both top-down and bottom-up experiments can be analysed and provide data informing on protein/peptide masses and PTMs.

System selection and suitability

A key consideration when working with AAV is the requirement for high sensitivity due to low protein concentration and smaller batch sizes. Both sheath flow and sheathless systems are available with the latter operating at nanoflow rates and providing higher sensitivity and lower sample consumption. Due to the capital expense of CZE-MS systems it may be preferrable to choose a system based on currently available equipment i.e. select a CZE device or mass spectrometer based on existing setup.

System suitability should be assessed using appropriate model molecules, peptides or proteins depending on the objective of the method. These should assess performance of the CZE-MS hyphenated system in terms of the expected parameters used for analysis of AAV samples.

Method development, qualification, and validation

Method development should include assessment of sample pre-treatment e.g. denaturation of the AAV capsid into its constituent proteins for intact protein analysis or digestion of the AAV capsid into peptides for a peptide mapping analysis. Optimisation of background electrolyte composition and additives may be necessary to obtain satisfactory resolution of analytes. Non-volatile components, surfactants, and high buffer concentrations may lead to ion suppression, noisy background, and contamination of the mass spectrometer source.

Optimisation of MS settings should be performed to maximise ionisation, de-solvation, and adduct removal and for peptide mapping to optimise fragmentation of peptides. Depending on the system being used it may also be necessary to optimise the position of the ESI sprayer and spray stability.

9. Summary table comparing methods

Comparison of Analytical Methods for AAV Capsid Protein Characterization							
Criteria	**LC–Mass Spectrometry (LC-MS)**	**Liquid Chromatography – UV/FLR**	**Antibody-Based (ELISA)**	**Affinity-Based (SPR & Interferometry)**	**SDS-PAGE**	**Capillary Gel Electrophoresis (CGE, UV/FLR)**	**Capillary Zone Electrophoresis – ESI-MS (CZE-ESI-MS)**
Sample Volume	50–200 µL	50–200 µL	50–100 µL	10–100 µL (depends on chip or sensor)	10–30 µL	5–20 µL	5–20 µL
Sample Concentration (Range)	~1E10–1E12 VP (≥5 µg protein for peptide mapping)	~1E10–1E12 VP (~1–20 µg protein)	≥1E9–1E11 VP/mL	≥1E9 VP/mL (purified)	~1E10–1E12 VP (1–10 µg protein)	~1E10–1E11 VP (~0.5–5 µg protein)	~1E10–1E11 VP (~0.5–5 µg protein)
Time to Result	4–12 hours (prep + run)	1–3 hours	4–8 hours (batch)	1–4 hours (setup + run)	4–6 hours	1–2 hours (including prep)	2–6 hours (sample conditioning + MS run)
Expertise Required	High (MS specialist)	High (chromatography and GMP-trained analyst)	Low–moderate (standard QC skillset)	High (specialist for assay and data interpretation)	Low–moderate (common lab skill)	Moderate (trained analyst)	Very High (specialist in electrophoresis-MS interfacing)
Suitability as In-Process Control (IPC)	No (extended characterization only)	Yes (can monitor process impurities)	Yes (for capsid titre)	Potential (affinity kinetics during development)	Limited (not ideal for rapid IPC)	Yes (for VP ratios, identity, impurities)	No (mostly research-level, not routine QC yet)

Matrix Effects / Purity Requirements	Requires highly purified capsid protein (low salt/detergent)	Needs purified sample; crude feeds risk column fouling	Tolerates moderate impurities but binding inhibited by detergents/ proteins	Requires clean sample (buffered, low particulates)	Tolerates moderate impurities ; high detergent s can interfere	Requires clean, denatured protein; tolerates some impurities	High purity essential; non-volatile buffers incompatible
Complexity of Equipment Qualification (GMP)	Very High (MS calibration, contamination controls, IQ/OQ/PQ)	High (detector calibration, system suitability, column performance checks)	Medium (kit qualificatio n, plate reader validation)	High (sensor chip qualification, instrument PQ)	Low (basic validation ; gels reproduci ble)	Medium (capillary performance checks, polymer validation)	Very High (MS and CE interfaces both require extensive PQ)
Resolution	Very High (PTMs, sequence variants, truncations)	High (intact VP proteins, degradation products)	Low (bulk VP capsid detection, no subunit resolution)	Moderate (binding kinetics, epitope-specific)	Moderate (VP bands resolvabl e, low precision vs. CGE)	High (VP1/VP2/VP3 ratios, ~1 kDa resolution)	Very High (charge-based resolution + MS mass accuracy)
Detects Aggregation?	No (focus on peptides or intact proteins, not particles)	Indirect (peak broadening possible)	No (bulk readout only)	Indirect (binding response anomalies)	No (denaturi ng method)	No (denaturing method)	Indirect (can detect complexes if optimized)

983

10. Other methods worth mentioning

MALDI-MS

As an alternative to LC-MS, also known as Electrospray Ionization Mass Spectrometry (ESI-MS), Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-MS) has been utilised for decades in the field of protein characterisation, with both techniques serving as complementary methods. MALDI-MS can be applied for intact mass analysis of capsid proteins and for analysing peptides following protein digestion.

In both cases, no preliminary separation is necessary, resulting in a single mass spectrum that simplifies interpretation. Additionally, one significant advantage of MALDI-MS is its ability to generate single ion species in most cases, making data interpretation easier compared to LC-MS. The mass spectrum of the peptide mapping sample is called peptide mass fingerprint as it is unique to the proteins thanks to endopeptidase cleavage specificity.

Despite these advantages, MALDI-MS has several inconveniences, the first one being the absence of separation which results in analysing multiple proteins or peptides simultaneously. Therefore, ionisation competition among the analytes, ion suppression due to salts presence and abundant species may make the analysis not robust and not specific. Another drawback is that coupling MALDI-MS with a LC system is challenging and the MALDI-MS analysis requires ultimately several actions of the analyst (sample/matrix co-crystallisation, installation of the plate into the mass spectrometer, …). Those aspects make MALDI-MS less used in the biopharma industry; LC-MS being the gold standard for protein characterisation by MS.

CDMS

Charge detection mass spectrometry (CDMS) is a novel technology that allows for the direct determination of charge and mass through analysis of samples at the single ion level. Direct measurement of charge negates the need for complex deconvolution algorithms and their associated processing artefacts and greatly simplifies the analysis of complex samples where charge envelopes would otherwise overlap.

CDMS has been approached using different technologies by vendors and research groups and is currently referred to as both CDMS and direct mass technology.

Sill a relatively immature technology, there is a lack of widespread use and understanding as well as limited associated literature and software offerings for data acquisition optimisation and data analysis. Although this technology may be primarily suited towards empty: full type analysis, there is the potential to obtain capsid higher order structural information based on capsid charge state distributions.

Concluding remarks

This guidance addresses the development and manufacture of recombinant adeno-associated virus (rAAV) medicinal products, with a focus on ensuring the quality, safety, and efficacy of these therapies through robust capsid protein characterisation.

Achieving these goals requires:

- Comprehensive analysis of rAAV capsid proteins, including the identification and quantification of VP subunits, assessment of post-translational modifications (PTMs), and evaluation of capsid integrity and purity.
- Minimising the presence of product- and process-related impurities, such as host cell proteins, truncated or misassembled capsids, and other contaminants that may impact product safety or efficacy.
- Applying orthogonal and validated analytical methods to confirm the identity, composition, and consistency of capsid proteins across batches and throughout the product lifecycle.

The analytical approaches and best practices described in this guidance reflect the current state of technology and regulatory expectations. As the field evolves, new methods and insights will continue to shape best practice in capsid protein characterisation.

This document is intended to support developers and manufacturers of rAAV-based medicinal products in meeting regulatory requirements and ensuring product quality. However, ultimate responsibility for product quality and compliance remains with the developer, manufacturer, or sponsor. All decisions regarding analytical strategies and quality control should be based on sound scientific principles and risk-based approaches.

Feedback and collaboration from the scientific and regulatory community are encouraged to ensure this guidance remains relevant and effective as technologies and standards advance.