Outlining the current state of AAVs and evaluating albumins potential role in AAV stability with use of varying analytical techniques

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Abstract

Adeno-associated viral vectors are pivotal to the advancement of gene therapy, offering a promising vehicle for delivering genetic material with high efficiency and low pathogenicity. This literature review provides an analysis of the role of AAVs in gene therapy, highlighting recent developments and therapeutic outcomes across various genetic disorders. Current obstacles in AAV-based therapies such as physical stability and vector size limitations are also discussed. A significant focus is placed on the strategy of coupling AAVs with albumin to enhance vector performance and extend the expression duration of therapeutic genes. The review also outlines implications of this conjugation on the data derived from analytical techniques. It examines how this modification influences the accuracy and reliability of methods such as ELISA, qPCR, SEC-MALS and more. Findings from multiple studies provides insight into how albumin integration into AAV vectors might revolutionise gene delivery methods, potentially overcoming some of the current limitations in gene therapy research and application.

1. Introduction

Adeno-Associated Viruses are becoming increasingly significant in the biopharmaceutical field, particularly in gene therapy and vaccine production due to their low pathogenic profile, stable gene delivery capabilities and high specificity due to their multiple serotypes. This literature review aims to explore the multiple roles and challenges associated with AAVs within their uses. Outlined is the basic virology of AAVs and their application in delivering genetic material to host cells, highlighting their potential in treating genetic disorders such as retinal dystrophy (Buch et al., 2008). This review then discusses the use of AAVs in vaccine development, underscored by recent advancements in vaccine development for the treatment of HIV (Nahmad et al., 2022).

However, the use of AAVs is not without challenges. This paper examines key issues such as immune response elicitation, vector size limitations and concerns over the storage of these therapies, which complicate their clinical and therapeutic applications. Furthermore, this review delves into the various analytical techniques employed to measure and characterize AAV vectors, from qPCR for genome quantification to ELISAs for capsid protein evaluation and multiple other methods, testing a range of issues like aggregation and durability of expression.

A novel area of investigation is the role of albumin in enhancing the efficacy and stability of AAV vectors. Existing research details how albumin integration can modify AAV therapy outcomes, potentially reducing required dosages and mitigating immune responses. Moreover, this review discusses how albumin's presence might influence the analytical data obtained, potentially affecting the interpretation of vector purity and potential aggregation.

Through this comprehensive analysis, this paper aim to consolidate current knowledge and identify grounds for future research, thereby contributing to the optimization of AAV-based therapies.

2. What are AAVs?

Adeno-associated viruses are small, non-enveloped viruses that belong to the Parvoviridae family, characterised by a single stranded genome enclosed by its protein capsid. AAVs exhibit a broad range of serotypes, which are variations in the viral capsid proteins that determine tissue tropism and immune recognition. The varying affinity of serotypes creates very specific gene delivery to desired tissue types and due to AAVs having to depend on co-infection with a helper virus, such as adenovirus, for replication, make them very safe and efficient in gene therapy.

AAVs are widely used in gene therapy due to their ability to deliver genes to both dividing and non-dividing cells, while maintaining long-term expression with little integration into the host genome. Their applications range from treating genetic and neurodegenerative disorders like hemophilia, retinal dystrophy and spinal muscular atrophy to therapies for cancers. Without a helper virus, AAVs are safe for human use and non-pathogenic. Moreover, their low immunogenicity minimizes immune responses, which is crucial for the effectiveness and safety of gene therapies. This safety profile, combined with their versatile delivery, accounts for the growing interest and investment in AAV-based therapies within the biopharmaceutical industry.

2.1 .Structural characteristics

As mentioned, AAVs are non-enveloped viruses containing single-stranded genome. The structural characteristics of AAV are key to its ability to deliver genetic material into host cells. The size of the AAV capsid measures around 20-25 nanometres in diameter, which is significantly smaller compared to many other viral vectors used in gene therapy. This small size facilitates the diffusion and distribution of the virus in the tissue matrix, enhancing its therapeutic potential (Shaza et al., 2023).

Due to the small size of this capsid, AAV has a limited packaging capacity of about 4.7 kilobases, which is sufficient to carry small genes or therapeutic RNA sequences but limits its use with larger genetic payloads. The limitations of AAVs size will be discussed later in this paper in regard to its use in gene therapy (Nayak & Herzog, 2010). The capsid of AAV is composed of three primary proteins: VP1, VP2, and VP3. These proteins are not only crucial for the formation of the viral capsid but also play significant roles in cell entry into host cells. VP3 is the most abundant, constituting about 80-90% of the capsid protein mass, whereas VP1

and VP2 are present in smaller amounts with a typical ratio of 1:1:10 relative to VP3 (Shaza et al., 2023).

2.2. Serotypes

Serotypes are variations within a species of microorganisms or viruses. They are classified based on the antigens they express on their surface. Classification helps in understanding the diversity of organisms and their interaction with the immune system. In terms of AAVs, this serotype classification also helps with its use in gene therapy as each serotype has varying tropism.

AAVs have multiple serotypes, each differing in their capsid protein compositions, which affects their tissue specificity. Currently, there are more than a dozen identified AAV serotypes with the most commonly referenced and researched serotypes being AAV1 through AAV9. Researchers continue to explore and characterize additional serotypes, which could potentially expand this list and provide more options for targeted gene therapy applications (Gao et al., 2005).

The ability of different AAV serotypes to recognize specific tissue types is a critical feature for their use in gene therapy. Each AAV serotype has a unique configuration of proteins in its capsid, which determines its ability to bind to specific cell surface receptors. For example, AAV2, one of the most studied serotypes, uses heparan sulphate proteoglycan as its primary receptor (Shaza et al., 2023). AAV2 recognises this receptor and enters the host cell via receptor-mediated endocytosis.

2.3. AAV vector construction

The baculovirus expression vector system is used in gene therapy has for the production of recombinant adeno-associated viruses that house desired therapeutic DNA. The gene of interest that encodes the protein to be expressed is cloned into a baculovirus transfer vector. This vector contains the necessary regulatory elements to ensure that the gene of interest is expressed inside the host cells. The vector is then used to transfect insect cells, where it recombines with a baculovirus DNA backbone, creating a recombinant baculovirus that includes the gene of interest.

The BEVS production of AAVs requires the co-infection of insect cells with three different recombinant baculoviruses Bac-Rep that expresses the major AAV replication enzymes (Rep78 and Rep52); Bac-Cap that expresses the AAV virion coat proteins; and Bac-GOI that expresses the gene of interest flanked by the AAV inverted terminal repeat elements that are required for the rescue, replication and packaging of the gene (Rachael S. Felberbaum). Once the gene of interest is incorporated into the baculovirus genome, the recombinant virus is used to infect insect cell cultures like Sf9 or Sf21 cells. The infected cells will then express the protein encoded by gene. The expressed protein can be harvested from the insect cells.

2.4. Serotype application in gene therapy

Gene therapy using AAV2 is an example of a promising approach for treating genetic disorders. AAV2 is a popular choice due to its ability to deliver genes effectively and safely into human cells. It is also non-pathogenic, which reduces the risk of causing diseases.

One notable example of gene therapy using AAV2 is the treatment for Lebers Congenital Amaurosis. LCA is a rare genetic disorder that leads to blindness or severe visual impairment at an early age. The therapy, known by the commercial name Luxturna, involves using AAV2

to deliver a normal copy of the RPE65 gene directly into retinal cells. This gene is crucial for normal vision, and its malfunction is a common cause of LCA (Buch et al., 2008).

The use of AAV2 in these treatments is critical because the virus serves as an effective vector to deliver the correct version of the gene directly into retinal cells. This approach can restore the function of the mutated gene, slowing the progression of the disease or even restoring some level of vision. The success of Luxturna being one of the first to demonstrate the potential for gene therapy to restore vision in patients with inherited retinal disease has paved the way for further research and development of gene therapies for other types of inherited retinal dystrophies as well.

2.5. Use in vaccine development

AAVs are also used in vaccine development, primarily as delivery vehicles. This approach has gained interest because it uses the non-pathogenic nature of AAVs, along with their ability to elicit a durable immune response. AAV vectors are engineered to carry genes that encode antigens specific to a pathogen. These antigens are usually key proteins of the pathogen that, when presented to the immune system, can trigger an immune response without causing the disease.

Once the AAVs deliver the genetic material into the host cells, they begin producing the pathogen's antigens encoded by the introduced genes. The expression of these antigens stimulates the immune system, triggering both B-cell and T-cell responses. The immune system recognizes these antigens as foreign, launching an attack that involves the production of specific antibodies and activation of T-cells. This exposure trains the immune system to recognize these antigens in the future, equipping the body with a memory response. If the actual

pathogen ever invades, the immune system can rapidly activate and attack it, often preventing the onset of the disease or significantly reducing its severity (Ronzitti, Gross and Mingozzi, 2020)

Researchers have been investigating AAV vectors as a tool for HIV vaccine development. These studies focus on using AAVs to deliver genes encoding HIV antigens to host cells. The cells then express these antigens, initiating an immune response that could protect against future HIV infections (Nahmad et al., 2022).

3. Issues and limitations with AAVs

The stability of AAVs is a critical factor that can significantly impact their clinical efficiency and safety. Instabilities such as aggregation and degradation of the viral capsids negatively affect the vector's ability to transduce target cells and may lead to a diminished therapeutic effect or increased immunogenic responses in patients.

Aggregation of AAVs is a significant concern as it can lead to the formation of large, non-functional molecules. This not only reduces the overall bioavailability of functional viral molecules but can also provoke immune responses that degrade these aggregates, potentially causing adverse effects in patients. Aggregation typically arises from suboptimal formulation conditions, variations in storage temperatures, or mechanical stresses during production and administration.

Stability of the viral genome within the capsid is also crucial. Any genomic instability could result in the loss of gene expression once the gene is delivered to the host cells. Research continues to focus on developing robust production and formulation methods that minimize these risks, ensuring that the viral vectors maintain their integrity from production through to administration.

To address these stability issues, analytical techniques are employed to characterize and quantify the physical and chemical stability of AAV vectors. Techniques such as dynamic light scattering and size exclusion chromatography with multi-angle light scattering are crucial for determining the size distribution, aggregation state, and purity of AAV preparations. These methods help in understanding how different formulations affect AAV stability and guide improvements in vector design and manufacturing processes (Cole et al., 2021).

3.1. Physical stability and storage related issues

Long-term storage of AAV vectors can lead to gradual loss of potency. Optimal storage conditions need to be maintained to ensure the longevity and efficiency of the vectors. As a result of suboptimal storage conditions, AAV vectors can also experience physical instabilities like aggregation and degradation of the viral capsids. Aggregated AAV particles reduce the efficiency of the gene delivery process because they are not able to efficiently enter target cells. Moreover, the presence of aggregates can trigger immune responses, increasing the risk of adverse reactions in patients.

Aggregates of viral vectors can be more immunogenic than their monomeric counterparts. When AAV particles aggregate, they can change their physical and chemical properties, presenting a different profile to the immune system than individual, non-aggregated particles. Aggregates are also more likely to be recognized and phagocytosed by macrophages. This uptake facilitates the presentation of viral antigens to T cells, enhancing the adaptive immune response. The presence of aggregates can therefore increase both the breadth and depth of immune activation against the vector. (Ronzitti et al., 2020)

Examples of a storge related issues are the AAVs being subjected to a suboptimal temperature and physical stress. Temperature can have a large effect on protein stability, with fluctuations

during manufacturing, storage, or shipping causing protein unfolding and denaturation, which further promotes aggregation. Mechanical forces such as stirring and shaking during the storage and shipping of these vectors can introduce physical stress that leads to capsid aggregation. (Srivastava et al., 2020).

Addressing the aggregation issue is critical not only to ensure the clinical efficacy and safety of AAV-based therapies but also to comply with regulatory standards for therapeutic products. Ongoing research in the field is focused on better understanding the mechanisms of AAV aggregation and developing strategies to mitigate this issue. This paper will later detail just how this aggregation is measured and how it effects the data gathered from various analytical techniques.

3.2. Size related limitations

The packaging capacity of AAV is a critical consideration for its use as a gene therapy vector. Its limited capacity of approximately 4.7 kilobases can only accommodate a relatively small amount of genetic material. The size restricts the type of genes or genetic elements, that can be packaged into the viral genome without compromising its structural integrity or its ability to efficiently deliver and express the gene inside host cells.

Duchenne muscular dystrophy is caused by mutations in the dystrophin gene. The full length of dystrophin gene is 2.6 mb. Full-length dystrophin cannot be packaged into AAV due to its size. Researchers have had to develop miniaturized versions of the gene, known as microdystrophins, that retain essential functions but can fit within AAV's limited capacity. However, these microdystrophins do not fully replicate the functionality of the full-length protein (Duan, 2018).

The cDNA of the CFTR gene, which is defective in cystic fibrosis, is about 4.5 kilobases long. While this size fits within the forementioned capacity, practical constraints related to the inclusion of regulatory elements necessary for proper gene expression often make packaging and delivery via AAV challenging. Alternative approaches using smaller functional domains of the CFTR gene or optimizing vector designs are necessary to make AAV a viable option for cystic fibrosis gene therapy (Flotte et al., 2013).

3.3. Durability of Expression

Durability of expression in gene therapy refers to the longevity of therapeutic gene expression after it has been delivered into the patient. AAV vectors are popular in gene therapy due to their ability to mediate long-term gene expression, non-pathogenicity, and broad tropism for various cell types. AAV's durability of expression can vary depending on the type of cells it targets. For example, in dividing cells the expression tends to diminish over time as the episomal AAV genomes are diluted or lost during cell division.

Immune responses against AAV vectors or the transgene product can influence expression durability. Some patients might develop neutralizing antibodies against the AAV capsid or an immune reaction against the transgene product, both of which can reduce the efficacy and longevity of the treatment. An example of an AAV based therapy with a lack of expression durability is the treatment of haemophilia. Early attempts to develop an AAV vector-based gene therapy for haemophilia B demonstrated that it was possible to express clotting factors in the human liver at therapeutically relevant levels, although expression was short-lived due to the development of a cytotoxic immune response directed against the vector-transduced hepatocytes (Monahan et al., 2021). The main of obstacle to sustain expression in AAV gene therapy is to mitigate the immune response to the viral capsid. Many individuals have pre-existing neutralizing antibodies against AAV due to natural infections with wild-type AAVs,

which can neutralize the therapeutic vector before it can deliver the gene. Even if the initial administration is successful, the introduction of the viral vector can stimulate an immune response, leading to the elimination of transduced cells over time.

Durability is crucial for the therapeutic efficacy of gene therapies. A durable response means that patients may require fewer treatments over their lifetime, reducing overall therapy costs and improving quality of life. However, the need for re-administration due to reduced expression or cell turnover must be balanced against potential immune responses. Advances in AAV vector engineering, understanding of immune modulation, and improvements in delivery methods are ongoing to enhance the durability and safety of AAV-mediated gene therapy. This includes developing new AAV serotypes with decreased immunogenicity and increased efficiency, as well as strategies for immunosuppression during treatment to enhance performance. Measuring the durability of gene expression in AAV-mediated gene therapy involves several methods and metrics that track how long and how effectively the therapeutic gene continues to function in the treated organism. An example of these techniques is the ELISA test, which will be mentioned later in the paper with regard to AAVs.

4. Analytical techniques in the analysis of AAVs

Analytical techniques are crucial for testing the aggregation of AAVs during the development of AAV-based therapies. Aggregation can significantly impact the safety, efficiency, and stability. Dynamic Light Scattering and Size-Exclusion Chromatography provide essential data on the size distribution of the viral molecules, identifying the presence of unwanted aggregates that may induce immune responses or reduce the biological activity of the therapy. Analytical Ultracentrifugation offers detailed insights into the sedimentation properties of molecules, distinguishing aggregates from monodisperse preparations. Transmission Electron Microscopy visually confirms the physical state and integrity of the viral vectors. Together, these methods

ensure that AAVs are free from aggregates that could compromise their intended function or lead to adverse effects in patients.

4.1. Size-exclusion chromatography with multi-angle light scattering

Size Exclusion Chromatography coupled with Multi-Angle Light Scattering is a powerful analytical technique used to determine the molecular weight and size of macromolecules in a solution. In the context of analysing AAVs, SEC-MALS offers critical insights into the properties of these viral vectors.

In SEC, a solution containing the molecules of interest are passed through a column packed with porous beads. These beads allow smaller molecules to enter their pores, delaying their travel through the column, while larger molecules bypass these pores and pass through the column faster. This process separates the molecules based on their hydrodynamic size.

As the separated molecules exit the SEC column, they enter the MALS detector. A laser beam is directed at the sample, and the light scattered by the molecules is measured at multiple angles. The intensity of the scattered light at these angles is used to calculate the molecular weight and size of the molecules (Kenrick et al., 2021).

This method provides accurate determination of the viral capsid molecular weights, which is crucial for understanding their biophysical properties and ensuring batch-to-batch consistency. AAVs can also form aggregates, which may influence their efficacy and safety as gene delivery systems. SEC-MALS can identify and quantify these aggregates by providing insights into the size distribution and molecular weight of all molecules present in the sample. This method of analytical technique can also be used to measure the stability of AAVs under various conditions during storage, handling, and administration. This is important as mentioned before, AAVs

stability can be compromised by these varying environmental factors. It is important to know but how much when evaluating the effectiveness of the therapy (Cole et al.,).

4.2. Dynamic light scattering

Dynamic Light Scattering is another technique used to measure the size distribution of small molecules in suspension, such as AAVs. In DLS, the laser is directed at a sample containing the AAVs. Upon interaction with the laser, they scatter the light in all directions. This scattering pattern changes over time as the particles move randomly due to Brownian motion. The scattered light is collected and analysed over time. The fluctuations in the intensity of the scattered light are due to the random movement of the molecules. The temporal fluctuations in the scattered light intensity are autocorrelated to produce a correlation function. This describes how quickly the scattering pattern changes, which is related to the speed of the molecules movement. From the decay rate of the autocorrelation function, the diffusion coefficients of the molecules are determined. The Stokes-Einstein equation is then used to convert these diffusion coefficients into hydrodynamic radii, providing the size of the particles (Wyatt Technology, n.d.).

The method of DLS is important in the analysis of AAVs as it gives insight into size distribution, which ensures that the AAVs can reliably entre target cells. Measuring the size distribution also allows for quality control during AAV production. It is crucial to ensure that the preparation is free from aggregates, which can affect the safety and efficacy of the therapy. Aggregation of viral vectors like AAVs can trigger immune responses and lead to reduced efficacy. DLS is sensitive in detecting aggregates, allowing for early identification, and troubleshooting of issues during the manufacturing process. Aggregated particles can also lead to incorrect dosing, impacting therapeutic outcomes and not providing a reliable batch-to-batch

consistency, with is crucial for the development and application of the therapy (Wagner et al., 2022).

By providing detailed insights into the physical characteristics of AAVs, DLS plays a fundamental role in the development, production, and clinical application of these gene delivery tools. This ensures that AAV therapies are not only effective but also safe for patients.

4.3. Transmission Electron Microscopy

Transmission Electron Microscopy is a powerful imaging tool used primarily to observe the internal structure and composition of specimens at high resolutions. TEM can visualize the arrangement and size of particles and materials. It provides detailed images of the crystal structure, lattice defects, and interfaces within materials.

TEM is particularly useful for measuring and analysing AAVs due to its high-resolution imaging capabilities. TEM can directly visualize the capsid structure of AAVs. This is crucial for ensuring the integrity and consistency of the viral capsids, which can affect their ability to deliver genetic material effectively. Through TEM imaging, researchers can measure the size of AAV particles and assess their morphology. This is important for quality control, as variations in size and shape can impact the efficacy and safety of the viral vectors (Dobnik et al., 2019).

TEM can be paired with AUC to gain an understanding of the AAV capsid composition. TEM provides direct visual confirmation of the structural integrity and state of the capsids, while AUC offers quantitative data about the relative amounts of full and empty capsids. In the TEM images, full capsids appear darker due to the genetic material inside them. Empty capsids appear lighter or more translucent. During AUC, full capsids, being denser due to the presence

of nucleic acids, will sediment differently than empty capsids. From the creation of a sedimentation profile the capsid percentages can be distinguished to determine the relative quantities of full versus empty capsids (Wang et al., 2019).

The use of both of these analytical techniques is important to the development of AAV based therapies as the higher the proportion of full capsids, the greater the potential for successful gene therapy and knowing the percentage of full capsids helps ensure that the administered dose contains sufficient genetic material to achieve the desired therapeutic effect. Empty capsids may also trigger immune responses without contributing to therapeutic effects so reducing the number of empty capsids can lower the risk of adverse reactions.

4.4. Analytical ultracentrifugation

Analytical ultracentrifugation on its own works by spinning a sample contained within a cell at high speeds in an ultracentrifuge. This process creates a centrifugal force that causes molecules to migrate through the solution at rates dependent on their mass and shape. By observing this migration, properties such as size distribution and state of aggregation can be inferred.

AUC operates on the principle of sedimentation velocity and sedimentation equilibrium. To measure sedimentation velocity the centrifuge runs at high speed and the sedimentation of molecules is monitored in real-time. As molecules sediment according to their size and mass, data is collected on how quickly they move through the solution. This information helps in determining the molecular weight and shape of the particles. In the measurement of sedimentation equilibrium, the sample is spun at a lower speed where sedimentation is balanced out by diffusion. This equilibrium state allows for precise measurements of molecular weights

and is useful for studying interactions like self-association or binding. The optical system within the AUC measures the concentration of particles across the sample cell as they sediment. This data is used to create a sedimentation profile, which is analysed to derive information about the size distribution, molecular weight, and shape of the Molecules.

The properties measured by this instrument can be useful in the analysis of AAVs in the way that it can detect and quantify the extent of aggregation in AAV preparations, which is critical for assessing the quality and efficacy of AAV vectors in gene therapy. It can also provide a purity analysis by differentiating between AAV particles and other components such as proteins, AUC can provide a detailed profile of the sample purity. (Maruno et al., 2021)

5. Analytical techniques for post-therapy development and administration

Using a variety of analytical techniques in the analysis AAVs is critical in the development and application of gene therapies. These techniques ensure that AAV vectors are safe and complete the desired effect. Measuring factors such as concentration, purity and capsid integrity, researchers can determine the optimal dosage and confirm that the vector is free of harmful contaminants and structurally sound. This is essential for efficient delivery and expression of the therapeutic gene. Furthermore, assessing genome integrity ensures that the genetic material within the vector remains intact and without mutation. Techniques that evaluate transduction efficiency, potency, and specificity allow for the optimization of the vector to target specific cells. Additionally, testing for toxicity and immunogenicity is vital to ensure that the therapy does not elicit harmful immune responses in patients.

5.1. qPCR

Quantitative Polymerase Chain Reaction is a laboratory technique used in molecular biology to amplify and simultaneously quantify a targeted DNA molecule. qPCR allows researchers to measure the amount of a specific DNA sequence in a sample, making it a powerful tool for a wide range of applications, including medical diagnostics and genetic research. This method allows the researcher to analyse if the AAV is carrying the gene of interest before its use in gene therapy.

Since AAVs contain single-stranded DNA and qPCR is more efficient with double-stranded DNA, a conversion step is needed. This involves synthesizing a complementary DNA strand using specific enzymes like reverse transcriptase or other DNA polymerases that can generate a double-stranded DNA template from the single-stranded viral DNA.

The qPCR mixture to test includes the double-stranded DNA template, primers, DNA polymerase enzyme, dNTPs and a fluorescent dye or probe that will allow for the quantification of DNA during the amplification process. During qPCR, the DNA template is amplified through repeated cycles of heating and cooling, which allow the DNA to denature, anneal and extend. The key component of qPCR is the fluorescent marker, which increases in fluorescence intensity proportionally to the amount of DNA amplified. The cycle number at which the fluorescence surpasses a background level, known as the Ct value, is inversely proportional to the amount of viral DNA in the sample. Lower Ct values indicate a higher initial amount of target DNA (Shmidt and Egorova, 2021).

By comparing the Ct values from the sample with those of a standard curve, the amount of AAV DNA in the original sample can be quantified. This is crucial for assessing viral concentration, which is important in the development of a gene therapy, helping in does control for patients.

5.2. In situ Hybridization

In situ hybridization is used in molecular biology and medical diagnostics to detect specific nucleic acid sequences within a preserved tissue section or cell sample. This method allows researchers to visualize the precise location of particular genes or RNA transcripts in tissue samples, helping to understand gene expression.

A labelled nucleic acid probe is designed to specifically bind to the target sequence of DNA or RNA. The probe can be labelled with radioactive isotopes, fluorescent dyes, or enzymes that produce a colorimetric signal. Tissue are fixed to preserve structure and nucleic acids, then embedded in a medium like paraffin. The prepared sample is treated to make the DNA or RNA accessible to the probe, followed by the application of the probe to the sample under conditions that allow for specific binding to the target sequence. Depending on the probe label, the location of hybridization is visualized using radiographic film for radioactive labels, fluorescence microscopy for fluorescent labels, or colorimetric methods for enzyme labels (Harun et al., 2023).

In AAV gene therapy, in situ hybridization can be used to evaluate the distribution and persistence of the therapeutic gene within tissue samples. This is crucial for clinical monitoring, providing insights into the vector's efficiency and safety. ISH can be used to visualize where in the tissue the AAV vectors are located after administration. Understanding the distribution pattern helps researchers ensure that the vector is targeting the correct tissues. ISH can be employed to observe the expression levels of the inserted therapeutic gene. This allows researchers to assess whether the therapeutic gene is being actively transcribed in the target tissues and to what extent. This assessment also ensures that the vector does not integrate into the genome in undesirable locations, potentially causing undesirable effects in the patient. This

method is used in research to image and evaluate the longevity of serotypes of AAV when paired with a long term expression promoter LAP2 (Maturana and Engel, 2024).

For therapeutic applications, it's important to know the therapeutic genes durability of expression, giving researchers information of the need for a potential re-dose of the therapy. ISH can be used in longitudinal studies to monitor the stability and persistence of gene expression over time. This post-therapy monitoring is important to confirm that the therapy reaches the intended target and functions as expected, helping assess therapeutic outcomes and potential side effects related to gene expression.

5.3. Neutralizing Antibody Assay

Enzyme-Linked Immunosorbent Assay is a commonly used laboratory technique designed to detect and quantify substances such as proteins, antibodies, and hormones in biological samples. The method is highly sensitive, specific, and can be easily scaled up for high-throughput screening, making it a staple in both research and diagnostic settings.

In this method of neutralizing antibody assay the microplate is coated with the antigen, binding it to the surface of the plate. All unbound sites on the plate are blocked with a non-specific protein, such as bovine serum albumin to prevent false positive results. a primary antibody specific to the antigen is added to the plate for It to bind to the antigen. The plate is then washed to remove any unbound antibody. A secondary antibody, which is linked to an enzyme, is added to bind to the primary antibody. After another wash to remove unbound secondary antibodies, a substrate specific to the enzyme is added to the plate. The enzyme acts on the substrate to produce a detectable signal which is typically a change in colour (Alhajj et al., 2023).

This method is highly versatile, specific and can be adapted for different targets, making it essential for various applications in science and medicine, such as diagnosing diseases, ensuring vaccine quality, and measuring biological responses to various substances.

ELISA can be used to detect and quantify the capsid proteins of AAVs. These proteins are essential for the virus's ability to infect cells and are a key marker of virus presence and concentration in a sample. ELISA allows for the precise quantification of AAV molecules, which is crucial for dose determination in therapeutic applications. Knowing the exact number of viral molecules ensures consistency and efficacy in gene therapy treatments where AAVs are commonly used as vectors to deliver genetic material. ELISA can also detect antibodies against AAVs in patient samples, which is important for evaluating pre-existing immunity in individuals. This immunity can impact the effectiveness of AAV-based therapies.

Research has been done to test the effectiveness of an ELISA-like method named virus receptor-linked immunosorbent assay by adopting fusion with a streptavidin-binding peptide (SBP). It was demonstrated that optimized VIRELISA assays exhibited satisfactory performance for the titering of AAV2 (Cui et al., 2019). VIRELISA tests are specifically designed to detect the immune response to viruses. This makes them crucial in the diagnosis of viral infections such as HIV, hepatitis, and the presents of viral vectors such as AAV.

6. Albumin use in AAVs and how it affects the use of analytical techniques

Albumin is a protein commonly found in blood plasma and plays a significant role in the stability and efficacy of vaccines. It is used in vaccine formulation to stabilize live or attenuated viruses, proteins, and other biological components against degradation or denaturation during storage and handling. Albumin can bind to and stabilize the active components of vaccines, preventing them from adhering to container surfaces or aggregating, which can lead to loss of

efficacy. In lyophilized vaccine formulations, albumin serves as a protectant that guards the vaccine components against damage caused by the freeze-drying process and subsequent storage. It also helps maintain the pH and osmolarity of vaccine solutions, which is crucial for maintaining the integrity and biological activity of the vaccine ingredients (Zsófia Edit Pápay et al., 2021). These storage stability effects can also benefit AAV therapies with the coupling of albumin. Albumin in the formulation helps protect AAVs against environmental stresses, such as temperature fluctuations and mechanical stress. Moreover, albumin can act as a non-immunogenic carrier or filler in these formulations, which can further help in reducing any potential immune responses against the vector or the therapeutic proteins it carries. By molecular shielding, albumin surrounds the active components like viral molecules or proteins preventing the active molecules from coming into direct contact with each other or with the surfaces of the container, thereby reducing the likelihood of aggregation. This is particularly important for delicate structures like viruses or complex proteins, where aggregation can lead to loss of biological activity or efficacy. Albumin has a long half-life in the bloodstream due to its interaction with the FcRn which recycles it back into the bloodstream instead of allowing it to be degraded by lysosomes. When therapeutic molecules are bound to albumin, they can benefit from this recycling mechanism and remain active in circulation for extended periods. This extended circulation time increases the likelihood of the vectors reaching their target tissues, thereby improving the efficiency of gene delivery (Kuten Pella et al., 2022).

As mentioned in this paper AAV vectors have the issue of potentially inducing an immune response. This can lead to rapid clearance from the body and reduced efficacy. Albumin is a naturally occurring protein in the human body and is generally non-immunogenic. Coupling AAV vectors with albumin can help mask the viral components from the immune system, potentially reducing the vector's immunogenicity and enhancing patient tolerance. This coupling can also increase the cellular uptake of AAV vectors by enhancing their interaction

with cellular receptors. Albumin is naturally taken up by cells via specific receptors, such as the albondin receptor. When AAV vectors are bound to albumin, this complex can interact with these albumin receptors on the surface of cells. This receptor-mediated endocytosis can then facilitate the uptake of the albumin-bound AAV vectors into cells (De Caneva et al., 2019). These properties make albumin a valuable component for enhancing the delivery and effectiveness of AAV-based gene therapies, enabling more successful treatments for a variety of genetic disorders

Depending on the method and the specific setup of the experiment, albumin might interfere with certain analytical assays. Researchers need to carefully design their experiments and select appropriate analytical methods to accurately assess how albumin-bound AAVs behave in biological systems and how they influence the outcomes of gene therapy applications. Adjustments in the experimental protocols might be necessary to account for the physical and biological properties introduced by coupling AAVs with albumin.

Albumin coupling to AAVs can significantly influence the data obtained from analytical techniques like DLS, SEC-MALS and AUC. DLS measures the size distribution of particles in a solution by analysing the light scattered from a laser beam as it passes through the sample.

When albumin is coupled to AAVs, the overall hydrodynamic radius of the particles increases. This increase in size will be detected by DLS, potentially shifting the apparent size distribution to larger diameters. The increased size might also affect the polydispersity index, indicating a broader size distribution if heterogeneous coupling occurs.

SEC-MALS combines size exclusion chromatography to separate particles based on size with light scattering measurements to determine the molecular weight and size of these particles. Coupling albumin to AAVs will affect both the elution profile and the light scattering data. The albumin-attached AAVs might elute at a different volume compared to uncoupled AAVs due

to their larger size. Additionally, the light scattering data will show an increase in molecular weight and potentially different angular dependence of the scattered light, which can be used to infer changes in particle shape or structure.

AUC separates particles under high-speed rotation, allowing determination of their mass, density, and shape properties. Sedimentation behaviour of the AAV particles will be affected by this coupling and will likely increase due to the larger mass and potentially altered shape of the albumin-AAV complexes. AUC can provide detailed insights into the heterogeneity of the sample, showing whether the albumin is uniformly attached to the AAVs or if multiple populations exist (Borzova et al., 2016).

Coating AAV vectors with albumin also affects the data obtained from ELISA. The albumin coating helps to mask the AAV vectors from the immune system, which reduces the binding of neutralizing antibodies as detected by ELISA. This can lead to higher apparent transduction efficiencies because the immune system is less likely to recognize and neutralize the vectors. However, the presence of albumin does not interfere with the quantification of vector genomes by quantitative PCR, ensuring accurate measurements of vector quantities in samples. This approach can enhance the delivery and efficacy of gene therapies, especially in patients with pre-existing immunity to AAVs (Wright, 2008).

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Comparing separation resolution of multiple SEC columns and evaluating AAV-albumin interactions using varying analytical techniques

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Table of Contents

Abstract

This study compares analytical approaches for assessing the stability of adeno-associated virus (AAV) formulations. We begin by identifying the most effective size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) column for characterizing AAV samples. Subsequently, additional techniques, including dynamic light scattering (DLS) and analytical ultracentrifugation (AUC), were employed to investigate AAV stability under varying conditions. The stability was evaluated in the presence and absence of albumin across multiple buffer. A key finding was that buffer with higher ionic strength appears to enhance AAV stability, suggesting potential benefits for long-term storage and therapeutic efficacy. These results provide valuable insights for optimizing AAV formulations for clinical and research applications.

Aims

Stability of viral vector formulation is a challenge in current pharmaceutical industry. The primary aim of this work is the identification of the most efficient column for viral vector formulation characterisation by carrying out SECMALs based characterisation of albumin based viral vector formulation, to then compare these results with other hydrodynamic methods.

During this study a range of columns were tested on SECMALs to evaluate the column which give us the most efficient resolution and accurate molecular weight for our samples. We tested, neat AAV (as provided by the supplier), diluted AAV and a control AAV, as well as both serotypes accompanied with albumin.

Selecting the most efficient column for SEC-MALS analysis is greatly important for formulation development. Efficient columns help in achieving the best separation of molecules based on their size, which is essential for accurate molecular weight

determination. Poor resolution can result in overlapping peaks, making it difficult to distinguish between different molecular species. Inaccuracies can be a result of the sample interacting with the column matrix, which can alter the elution volume. This can make molecules appear larger or smaller than they actually are, leading to incorrect Mw determinations. Different columns are optimized for various types of solvents and samples. Choosing the right column ensures compatibility with the samples chemical properties, providing accurate results for future therapy development and stability.

Once defined, part B involved further testing of these samples subjected to stress. Testing environmental stresses are greatly beneficial as they directly translate to how the samples will behave under the transport and storage conditions when the therapeutic is developed and used in industry.

Second objective was of this study was to compare the results obtain by SECMALs with alternative biophysical methods such as Dynamic Light Scattering (DLS) and Analytical Ultra Centrifuge (AUC).

Overall, it was aimed to identify the conditions that are most suitable for viral vector stability in the presence of the albumin.

Introduction

In the rapidly growing field of gene therapy, adeno-associated viruses have emerged as promising vectors due to their safety profile and efficiency in delivering genetic material to target cells. The therapeutic potential of AAVs hinges on their ability to maintain structural integrity and functionality under various conditions, both in vivo and in storage (Srivastava et al., 2020). One potential positive influence on AAV stability is its interaction with human serum albumin. Albumin's role in modulating

AAV stability is not yet fully understood, making it a significant area of investigation for enhancing gene delivery efficacy.

This study employs a combination of analytical techniques—Size Exclusion Chromatography coupled with Multi-Angle Light Scattering (SEC-MALS), Dynamic Light Scattering (DLS), and Analytical Ultracentrifugation (AUC) to detail the impact of albumin on AAV stability. Each method provides unique insights into the size distribution, aggregation behaviour, and molecular interactions of AAV complexes, offering a comprehensive understanding of their properties.

The integration of multiple analytical approaches is crucial in gene therapy research as it allows for a more complete analysis of viral vector characteristics, ensuring the reliability and reproducibility of results. By highlighting the relationship between AAV stability and albumin, research can contribute to the optimization of gene therapy protocols and enhance therapeutic outcomes.

Introduction to SEC-MALS

Size Exclusion Chromatography coupled with Multi-Angle Light Scattering (SEC-MALS) is a powerful analytical technique used to determine the molecular weight, size, and structure of macromolecules in solution. SEC is composed of spherical, porous particles, with precisely controlled pore sizes and pore size distributions, which allows the differentiation of molecules based on their molecular size. SEC separates species based on their hydrodynamic radius. As the sample flows through the column, large molecules travel faster through the column as they do not penetrate the pores. On the contrary, small molecules take longer to elute from the column, as they spend more time inside the pores. (Valentina D' Atri et al., 2024). SEC-MALS is crucial in fields such as biochemistry, polymer science, and pharmaceuticals, where understanding the

precise molecular structure of substances is essential for applications such as drug formulation.

Choice of column is crucial as it directly affects the resolution, separation efficiency, and suitability for analysing specific types of samples. The columns in SEC-MALS play a pivotal role in the overall effectiveness of the method in analysing macromolecules like proteins, polymers, and nucleic acids. The pore size of the beads in the column must be appropriate for the size range of the sample molecules. Using a column with the wrong pore size can lead to poor separation or sample degradation (Podzimek, 2021).

The chemical composition of the column also needs to be taken into consideration as to prevent any reaction that could alter the sample or damage the column. To minimize the possible interactions between the molecule of interest and the SEC column, all components must be inert. (Valentina D' Atri et al., 2024). This is particularly important for sensitive biomolecules like proteins, which might denature or aggregate if interactions are too strong. Columns designed for high-resolution separations are critical when analysing complex mixtures of macromolecules to ensure that peaks are well-resolved and distinct. The efficiency of a column, often determined by the particle size and shape, affects the sharpness of the peaks and the accuracy of the MALS analysis.

After the molecules are separated by SEC, they then pass through a MALS detector. MALS involves the detection of light scattered by species as a function of concentration and size in solution. ASTRA software then uses the angle of scattered light to quantify physical attributes of the scattering species (McIntosh et al., 2021). When the laser is passed through a sample solution containing molecules, the light is scattered in various directions. In MALS, the scattering intensity is measured at multiple angles relative to the direction of the incoming laser beam. Typically, detectors are arranged

around the sample at different angles, ranging from small angles to wide angles. The intensity of scattered light depends on the molecular weight and concentration of the molecules in the sample. By analysing the intensity at zero angle the absolute molecular weight of the molecules can be determined. The angular variation in scattering intensity helps in understanding the shape of the molecules; spherical, rod-like, or have some other complex structure.

MALS is an absolute technique that uses a collimated beam from a laser source to determine the exact mass in solution of proteins, lipids, detergents, nucleic acids, sugars, or heterologous complexes and to evaluate their gyration radius (Velours et al., 2022). When combined with Size Exclusion Chromatography, MALS provides a powerful way to characterize molecules as they are separated by size. SEC-MALS allows for the determination of molecular weights and structural data for each molecule eluted from the SEC column, giving insights into the polydispersity and molecular structure distribution within the sample.

Methods: Column selection

Sample buffer preparation

2L of SEC-MALS Sample buffer (ABX buffer A), containing 150mM of component A, 25mM of component B and 25mM of component C was prepared. pH level was measured with pH probe and adjusted with sodium hydroxide to fit a 6.8pH. Buffer volume was made up to final 2L volume using deionised water and filtered with 0.1µm vacuum filtration unit. Buffer B mirrors this formulation with added 1mM of component D.

Table 1: Albumin dilution: 200mg/ml--> 10mg/ml in sample buffer

	mg/ml	Final volume (ml)	Albumin stock volume (ml)	Buffer volume (ml)
200	50	1	0.25	0.75
200	20	1	0.1	0.9
200	10	5	0.25	4.75
200	5	1	0.025	0.975
200	2.5	1	0.0125	0.9875
200	1.25	1	0.00625	0.99375
200	0.75	1	0.00375	0.99625
10	2.5	1	0.25	0.75
10	1.25	1	0.125	0.875
10	0.75	1	0.075	0.925

Table 2: AAV and control AAV sample preparation for SEC-MALS with running buffer and albumin. Two varying brands on AAV were used in the analysis (supplier 1 and 2).

Sample	Conc GC/mL	Required conc GC/mL	Volume (ul)	Stock to take (ul)	ABX- BA	buffer with Albumin	Albumin(10mg/ml)	Water (µl)
AAV	1.30E+13	1.00E+12	300	23.08	60	N/A	N/A	216.92
					N/A	60	30	186.92
Control AAV	2.48E+13	1.00E+12	300	12.08	60	N/A	N/A	227.92
					N/A	60	30	197.92

Running buffer preparation

For preparation of 2L of SEC-MALS running buffer, ABX running buffer was prepared by using 150mM component A, 50mM component B, 25mM component C and 100mM of component D. All components . were dissolved in 1.6L of deionised water. pH level was measured with pH probe and adjusted with sodium hydroxide to attain 6.8pH. Buffer volume was made up to final 2L volume using deionised water and filtered with 0.1µm vacuum filtration unit.

Standards

Albumin 1mg/mol and 10mg/mol were also run alongside samples to provide reference points of Mw and detailed performance quality of columns (table 3) subjected to test. BEH protein standard containing Thyroglobulin, IgG, BSA, Myoglobin and Tryptophan was also run to test separation efficiency.

Table 3: Detailing name and characteristics of each column along with flow rate used in each experiment.

Column	Packing particle size (μm)	Pore size (Å)	Flow rate (ml/min)
TSK3K (7.8 x 300mm)	5	250	0.5
TSK4K (7.8 x 300mm) (Old)	8	450	0.5
TSK4K (7.8 x 300mm) (New)	8	450	0.5
Agilent (7.8 x 300mm)	5	1000	0.5
Water X-bridge (7.8 x 300mm)	2.5	450	0.5

SEC-MALS

Samples were run across four different 7.8 x 300mm SEC-MALS columns and guard columns (TSK3K, TSK4K, Agilent & Waters X-bridge), each comprised of varying packing particle and pore sizes for the separation of samples. The column was equilibrated with running buffer for 15 hours The flow rate was slowly elevated to 0.5 mL/min over 2 h before loading 50 μL samples onto the column.

The stationary and mobile phases were contained within DAWN consisting of an automated, thermally controlled vial sampler at 4 °C and binary pump. UV absorbance of column eluates at 260 nm and 280 nm was detected by a Optilab dRi detector. Vision run was used for controlling the HPLC system and analysing UV absorbance data. All steps post-injection were performed at 25 °C.

ASTRA

Data was exported and analysed via Astra, with baselines defined, the berry model was used with fit degree 2 across all samples as the best fit. Elution profile plots were also created in Astra.

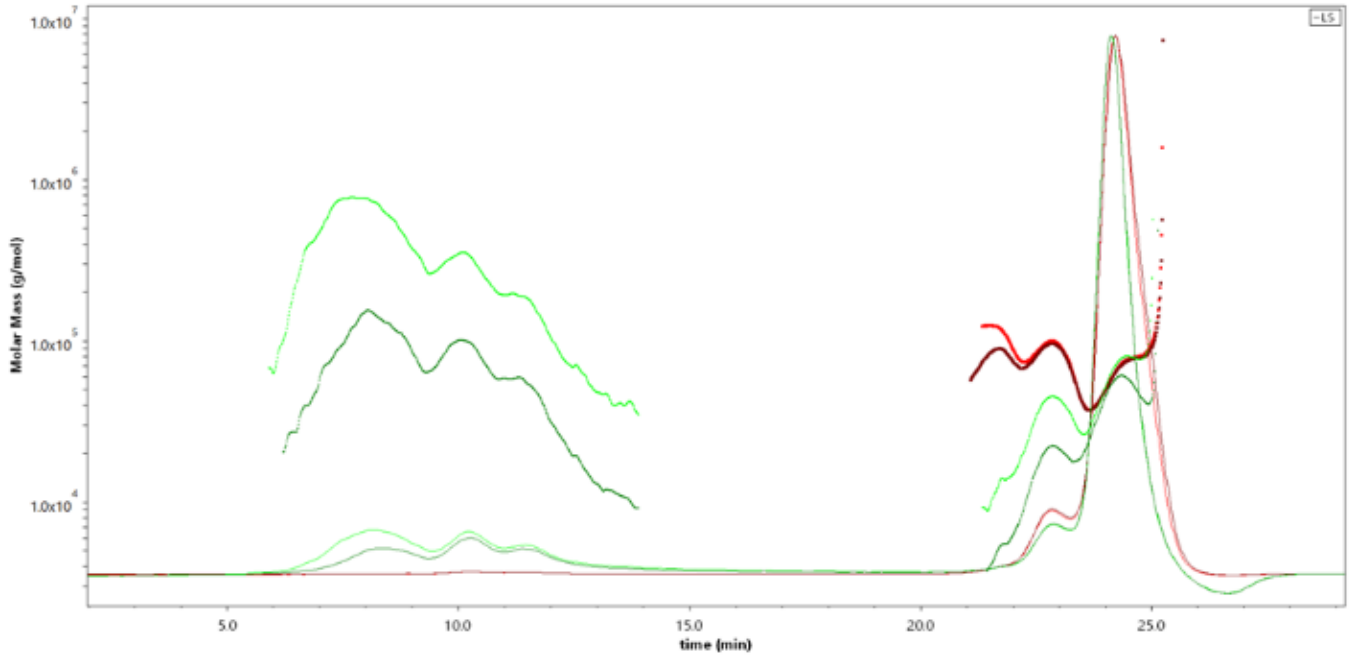
Results and discussion

In this study, we have undertaken an evaluation of various columns (Table 3) used in SEC-MALS to compare their performance and overall efficacy in separating Molecules. The primary objective was to identify the optimal column that not only ensures precise molecular weight determination but also enhances resolution and reproducibility. Through the testing of each column under standardised parameters such as suspension buffer and flow rate, we assessed parameters such as peak resolution and molecular weight data which are critical for accurate macromolecular characterisation. This discussion will analyse data of each column type, highlighting the factors that contribute to superior performance, such as resolution and accurate Mw readings to possibly define which column is best for the separation of our samples.

Table 4: Data gathered from most prominent peak as detailed on figure 2 comparing RS10 and RS1 run on varying columns

Column	Sample	M_n (kDa)	± (%)	M_w (kDa)	± (%)	Polydispersity (Mw/Mn)	± (%)	r_w (nm)	± (%)
Agilent	RS1	61	0.4	63	0.4	1.027	0.6	26	5.9
	RS10	62	0.3	64	0.4	1.035	0.5	24	5.4
	RS10	61	0.5	64	0.5	1.034	0.7	24	7.8
	RS1	14	10.9	38	0.5	2.753	10.9	27	19.3
TSK4K	RS10	87	0.4	88	0.4	1.015	0.58	23	5.9
	RS10	82	0.4	83	0.4	1.014	0.6	23	6.4
	RS1	22	6.6	29	2.8	1.329	7.18	34	29.2
	RS1	26	4.2	53	2.1	2.005	4.67	79	2
TSK3K	RS10	127	0.6	133	0.7	1.048	0.93	27	8.8
	RS10	126	0.3	131	0.3	1.038	0.37	22	5.3
	RS1	0	0	0	0	0	0	0	0
	RS1	0	0	0	0	0	0	0	0
X-bridge	RS10	99	0.8	115	0.7	1.161	1.11	18	13.6
	RS10	116	1.7	144	1.6	1.244	2.31	35	16.2
	RS1	0	0	0	0	0	0	0	0
	RS1	85	6.2	94	5.1	1.106	8.02	0	0

A)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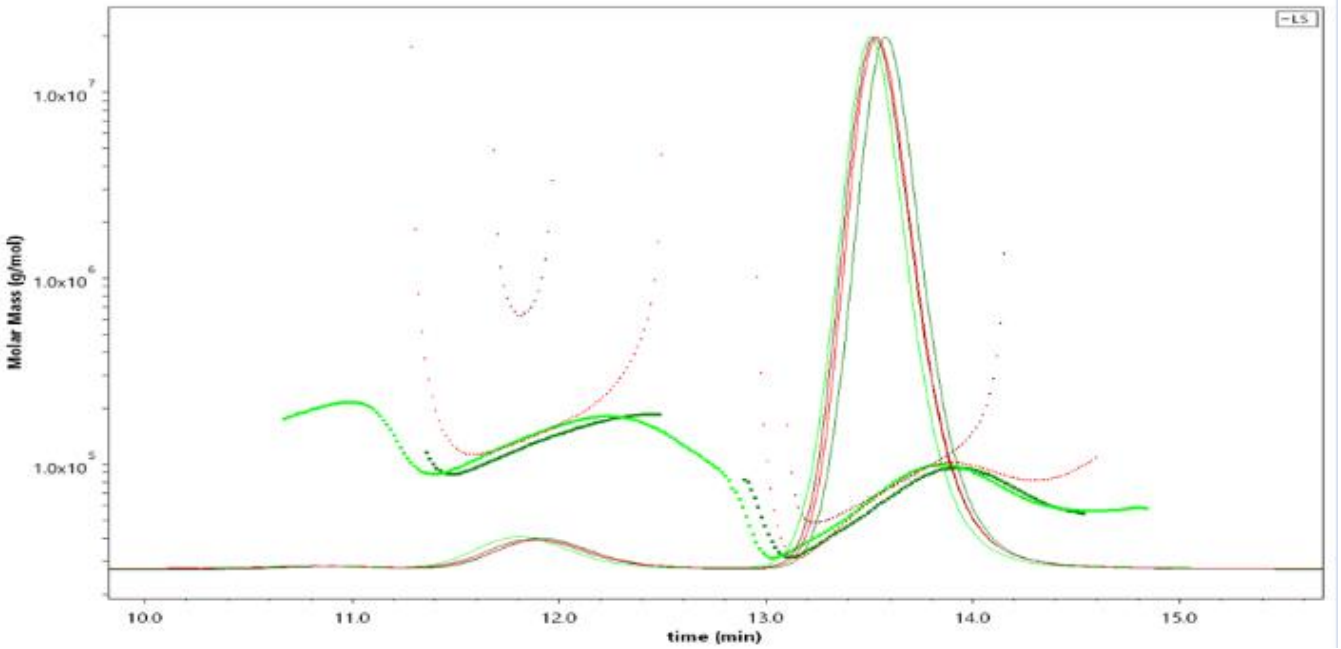
20.0
25.0
time (min)

B)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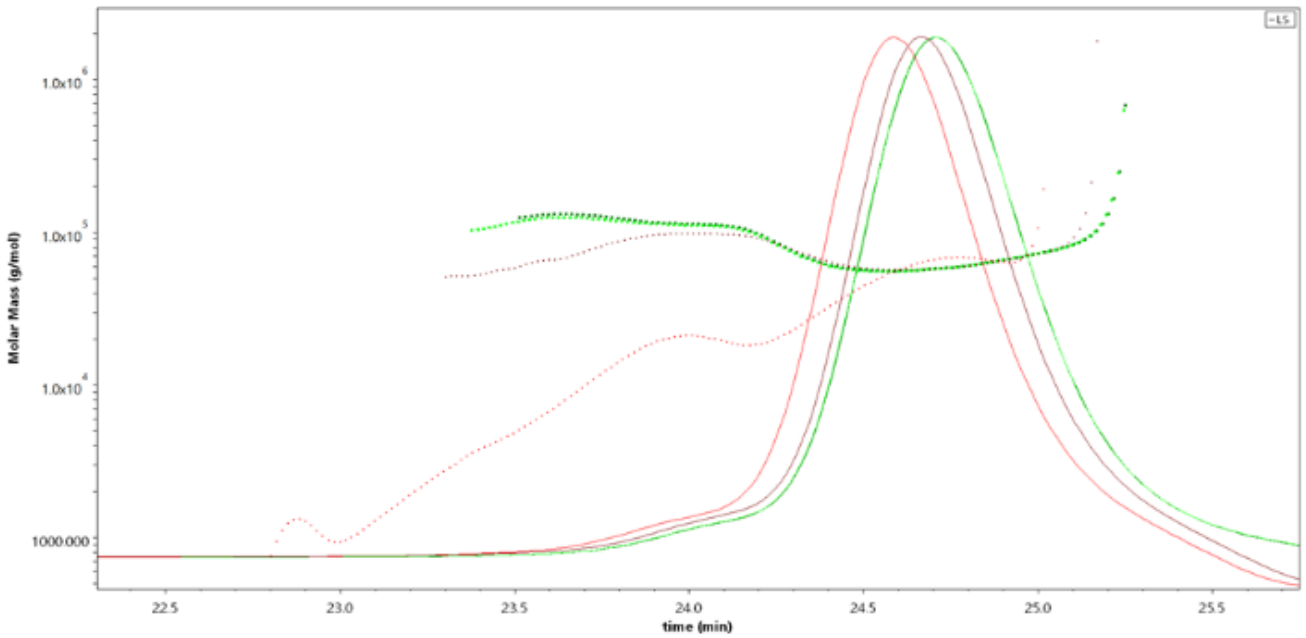
time (min)

C)



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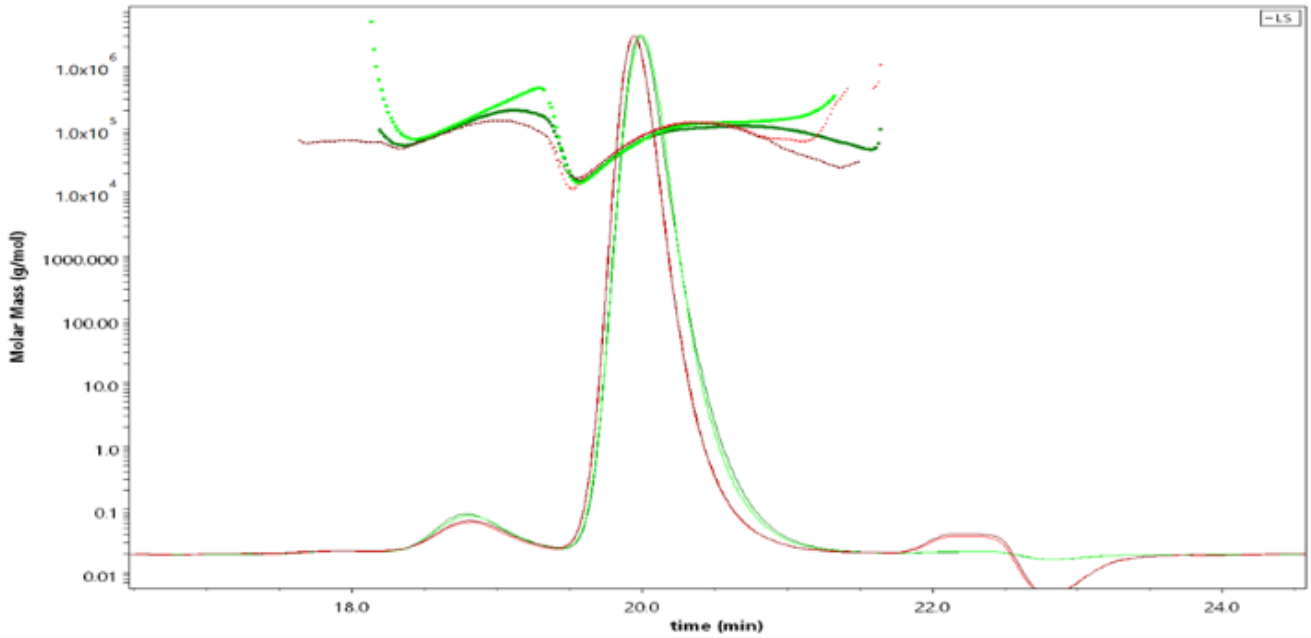


Figure 1: Astra elution profiles detailing RS1 (Green) and RS10 (Red) across multiple columns: A-TSK3K, B-TSK4K, C- Agilent & D- X-bridge) at 0.5ml/min at a fixed temperature of 25^{o}c

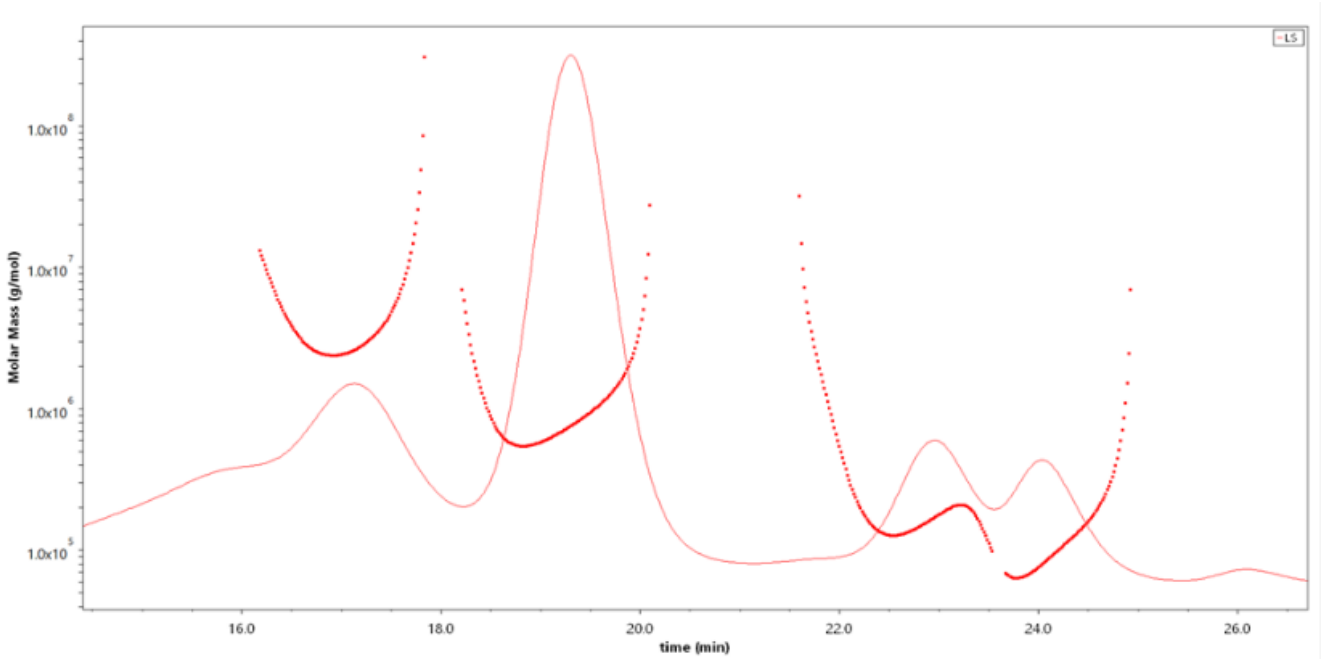
Table 4 notably shows the molecular weight values for our albumin standards RS1 and RS10. The molecular weight of albumin is 66.5 kDa, and so the column that presents

Mw data closest to this value goes a long way to indicate the most effective column. Within this context, the Agilent column poses as the best as its Mw values of 63.6, 64.3 and 64.1 kDa are closest to that of albumin, with exception of the second value of RS1 being 38.8 kDa. However, figure 1 may lend to a different interpretation of column quality as the elution profile of the Agilent column shows a very broad peak with shouldering, suggesting poor resolution capability of the column.

Table 5: Data gathered in astra detailing the Mw of each protein present in Waters BEH450 SEC Protein Standard Mix across multiple columns (TSK4K, Agilent & X-bridge).

	M_n (kDa)	± (%)	M_w (kDa)	± (%)	Polydispersity (Mw/Mn)	± (%)	r_w (nm)	± (%)
Peak 1 Thyroglobulin Dimer								
X-bridge	0	0	0	0	0	0	0	0
TSK4K	1632	0.6	1648	0.6	1.01	0.9	34	7.9
Agilent	750	2.5	828	1.2	1.104	2.76	33	13.6
Peak 2 Thyroglobulin								
X-bridge	2932	0.4	3427	0.4	1.169	0.55	28	3.8
TSK4K	1073	0.7	1134	0.6	1.056	0.87	36	6.9
Agilent	862	0.3	901	0.3	1.046	0.44	28	3.5
Peak 3 IgG								
X-bridge	717	0.4	820	0.4	1.144	0.57	27	4.4
TSK4K	490	0.7	592	0.6	1.207	0.91	31	9.7
Agilent	421	0.3	524	0.3	1.244	0.44	27	3.7
Peak 4 BSA								
X-bridge	158	0.4	176	0.4	1.115	0.61	31	3.6
TSK4K	110	1.1	136	0.9	1.241	1.4	38	10
Agilent	107	0.3	112	0.3	1.044	0.44	26	4
Peak 5 Myoglobin								
X-bridge	81	0.5	92	0.6	1.132	0.78	33	3.8
TSK4K	51	1.1	66	1.1	1.302	1.57	39	10.6
Agilent	58	0.4	65	0.4	1.12	0.54	25	5.3
Peak 6 Tryptophan								
X-bridge	162	12.1	203	11.8	1.252	16.9	167	5.9
TSK4K	12	2.8	14	2.9	1.237	4.06	19	54.6
Agilent	0	0	0	0	0	0	0	0

A)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1.0x10^7
1.0x10^6
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Molar Mass (g/mol)
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18.0
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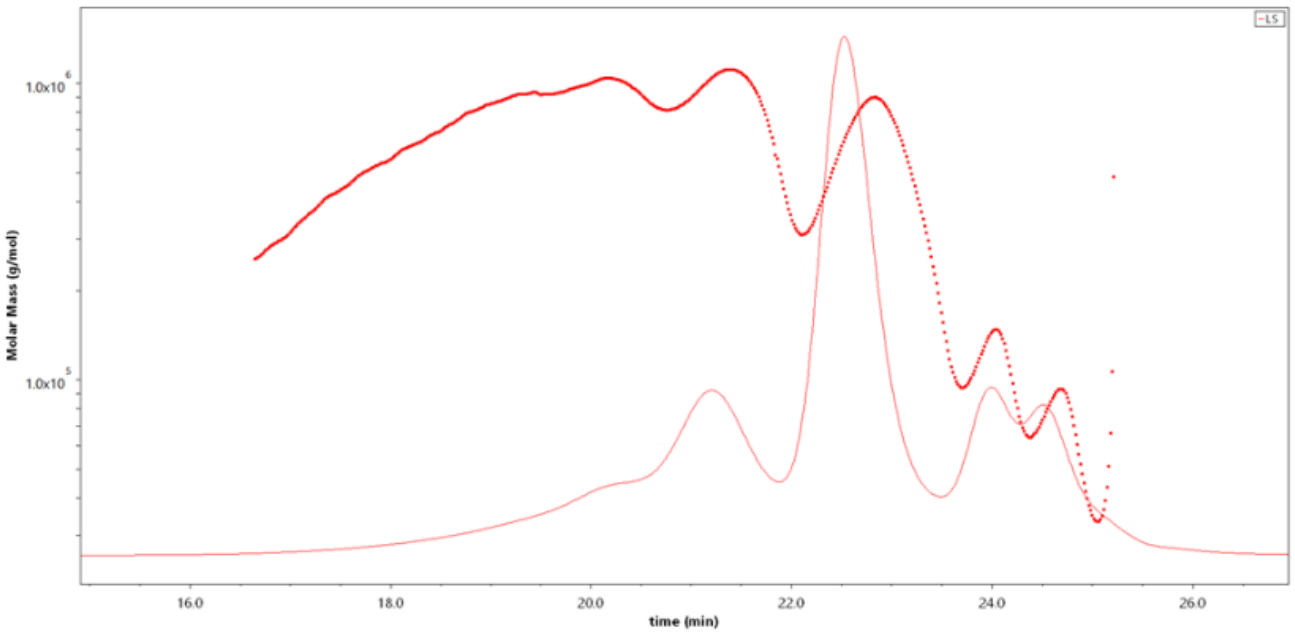
24.0
26.0
time (min)

B)


LS
1.0x10^6
1.0x10^5
Molar Mass (g/mol)
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24.0
26.0
time (min)

C)

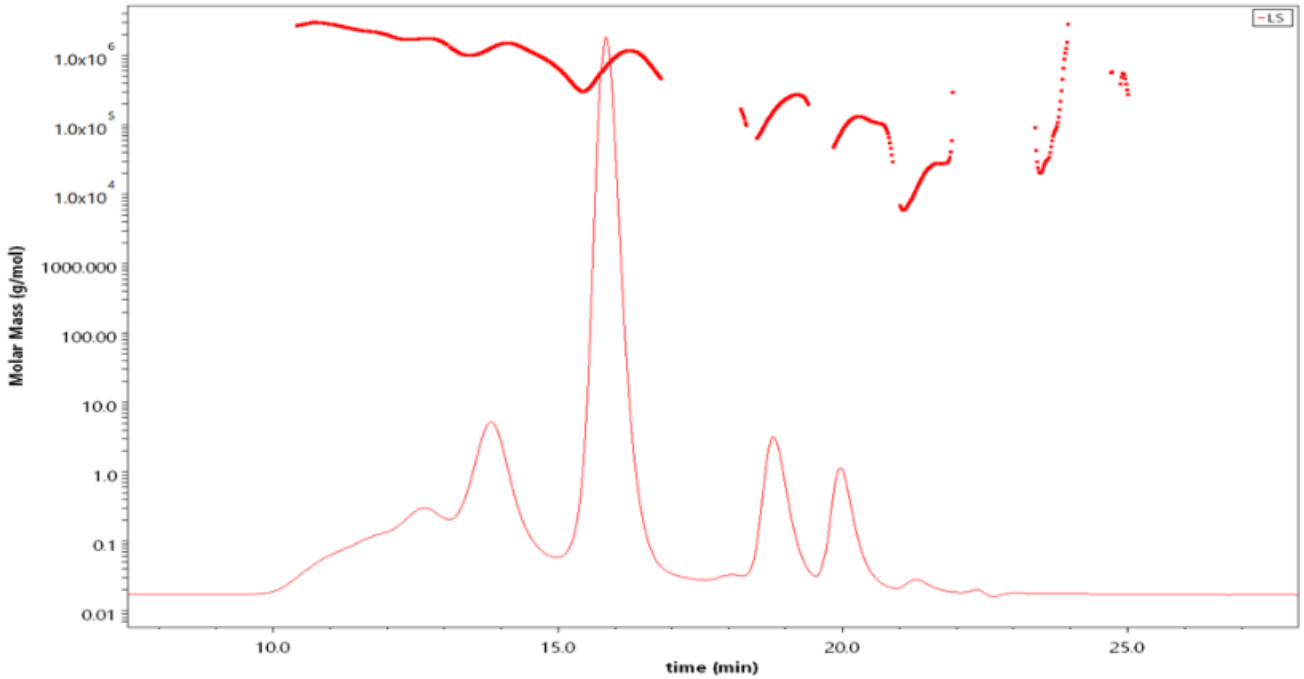

Figure 2: Astra elution profiles of Waters BEH450 SEC Protein Standard Mix run across multiple columns; A - TSK4K, B - Agilent & C - X-Bridge) at 0.5ml/min at a fixed temperature of 25°c

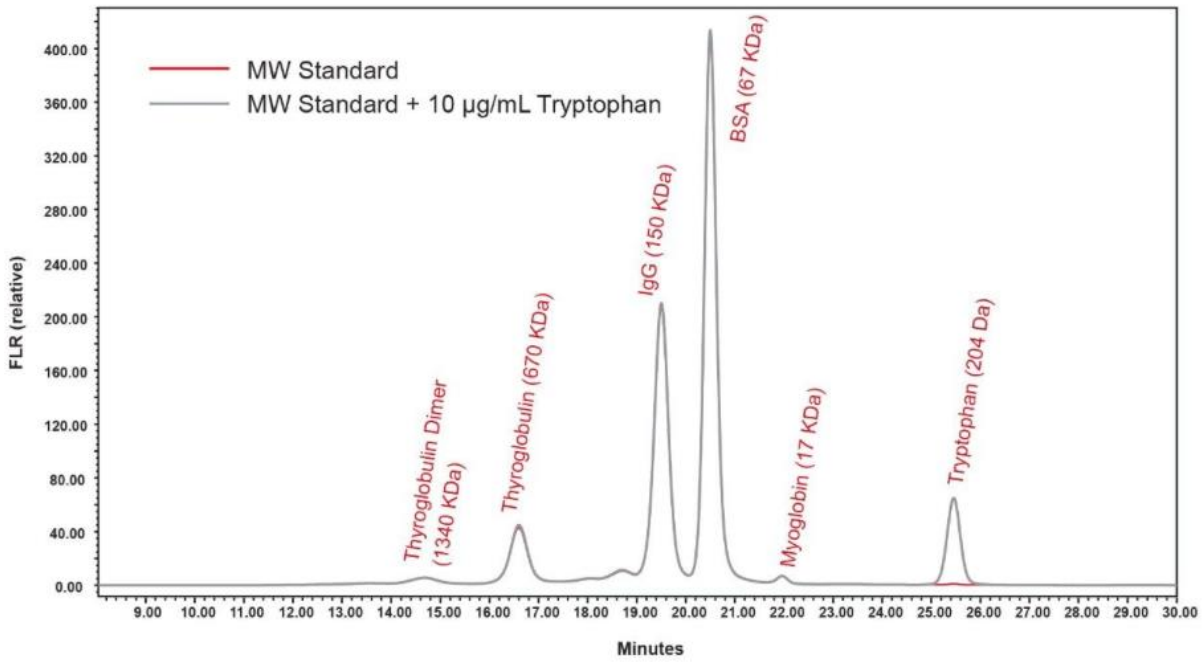

Figure 3: Taken from (Waters Corporation) to display M_w values of the same BEH protein standard used in this research.

Table 5 shows M_w data for the BEH protein standard and when making the comparison to Figure 3 (Waters Corporation) similar M_w data would support a claim to high column efficiency as data sets that match indicate more reliable findings than a single experiment.

However, looking at Figure 3 and table 5 M_w data does not mirror that of any column tested in this experiment, meaning M_w data alone does not help in the selection of the most efficient column. This inconclusive data reading may be down to the samples interactions with the columns. Molecules that interact with the gel or the matrix of the column will have altered elution profiles which will misrepresent their size and molecular weight (Liz Bevan, 2016).

Table 6: Data gathered in Astra detailing differences between two varying qualities of AAV when run through TSK4K column.

	AAV- Supplier 1	AAV- Supplier 2
M_w (kDa)	1651.4 (±0.6%)	4672.4 (±0.7%)
M_n (kDa)	693.7 (±6.0%)	4197.5(±0.8%)
r_w (nm)	75.6 (±0.8%)	35.0 (±4.6%)
Polydispersity Mw/Mn (g/mol)	2.381 (±6.037%)	1.113 (±1.048%)

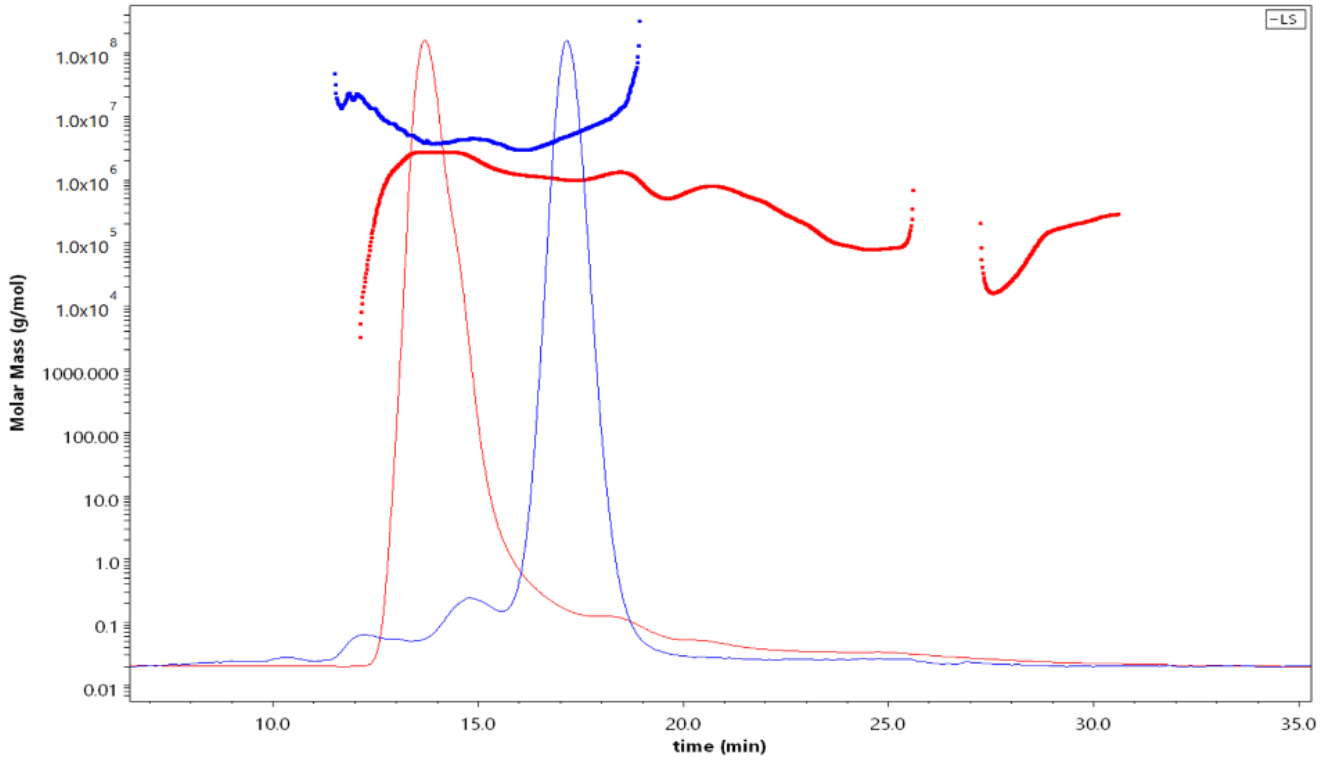

Figure 4: Elution profile created in Astra comparing quality of two varying brands of AAV supplier 1 (red) & supplier 2 (blue) both run through TSK4K column at 0.5ml/min at a fixed temperature of 25°c

Figure 4 shows initial comparison between two varying brands and suppliers of AAV. The neat sample was run on the TSK4K column at 0.5 ml/min. It was hypothesised that AAV from supplier 1 would be of a much higher quality than its counterpart from supplier 2. This would be demonstrated by better resolution and molecular weight data fitting that of AAV. Although both samples boast a varying elution time there is no clear indication as to what the higher quality sample is, due to both elution profiles having a shoulder. Shoulders on an elution profile indicate heterogeneity in the sample, meaning both samples may contain different species of slightly different sizes that elute close to each other but are not fully resolved. It may also indicate interactions between molecules within the sample such as aggregation, or an issue in the column separation efficiency.

Table 7: Data collected from first peak of AAV samples when run through varying columns.

Column	Sample	M_n (kDa)	± (%)	M_w (kDa)	± (%)	Polydispersity (Mw/Mn)	± (%)	r_w (nm)	± (%)
TSK4K	AAV neat	366	1.10	696	0.5	1.902	1.22	52	1.6
	AAV 1e12	574	2.90	709	2.2	1.235	3.59	82	3.4
	AAV P1	18	0.90	21	1.0	1.151	1.35	49	2.9
Agilent	AAV neat	693	6.00	1651	0.6	2.381	6.04	75	0.8
	AAV 1e12	6098	0.80	21115	0.5	3.463	0.93	64	0.7
	AAV P1	426	2.2	554	1.5	1.300	2.6	56	4.7
X-bridge	AAV neat	423	0.40	429	0.4	1.016	0.57	49	2.3
	AAV 1e12	359	2.10	366	1.8	1.019	2.74	52	10.1
	AAV P1	1511	1.70	1756	1.9	1.162	2.56	50	5.3

A)

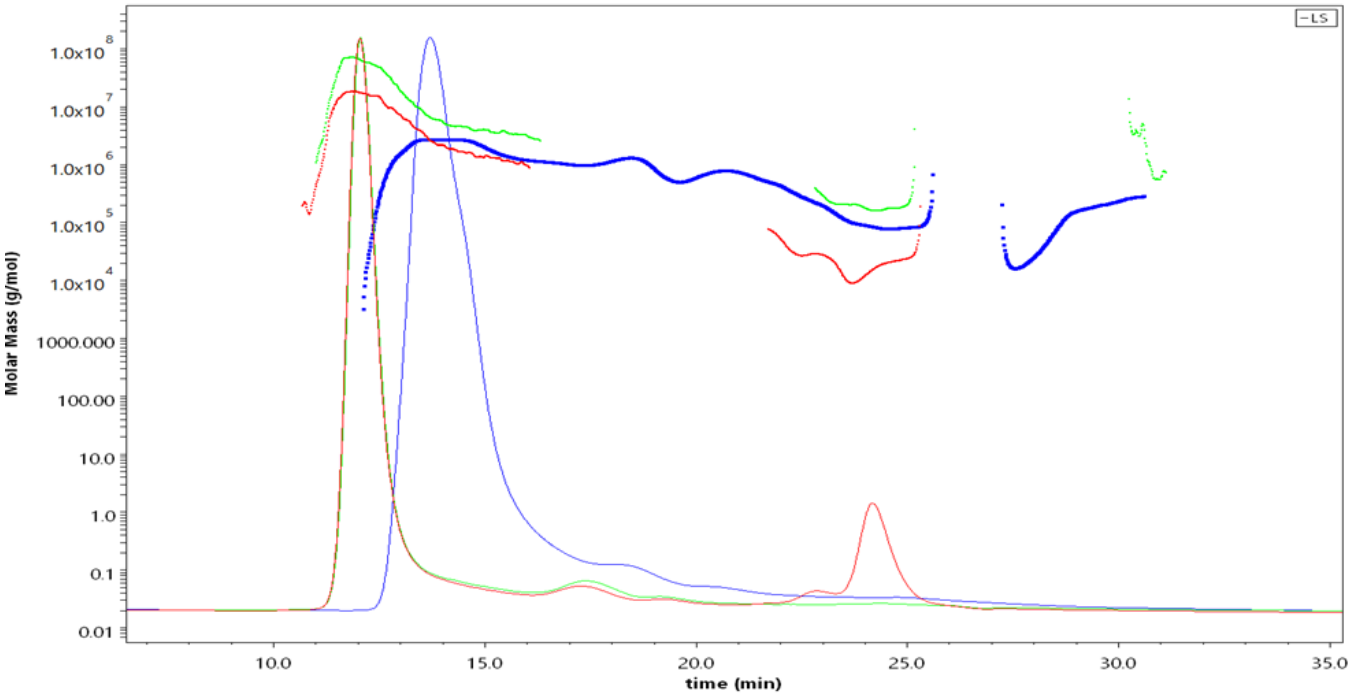

Figure 5: Astra Elution profiles of multiple samples of AAV, neat (Blue), diluted (Green) & with albumin (Red) across TSK4K column at 0.5ml/min at a fixed temperature of 25°c.

B)

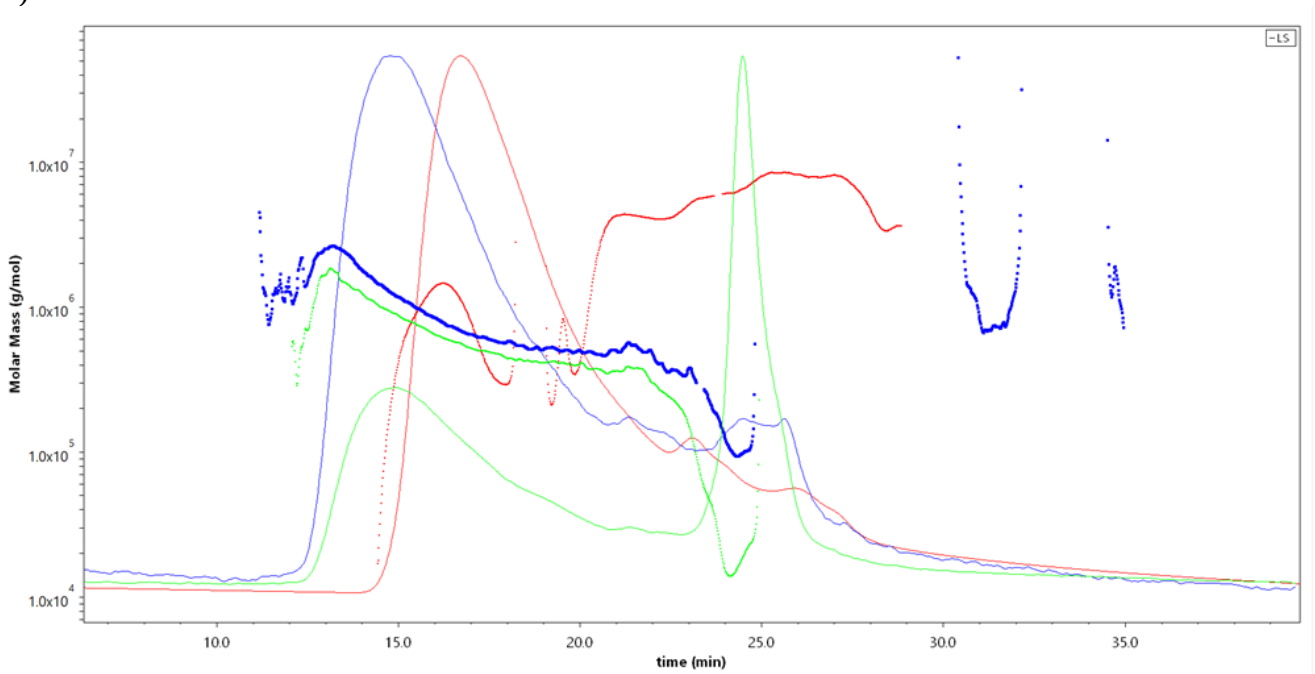

Figure 6: Astra Elution profiles of multiple samples of AAV, neat (red), diluted (Blue) & with albumin (Green) across Agilent column at 0.5ml/min at a fixed temperature of 25°c.

C)

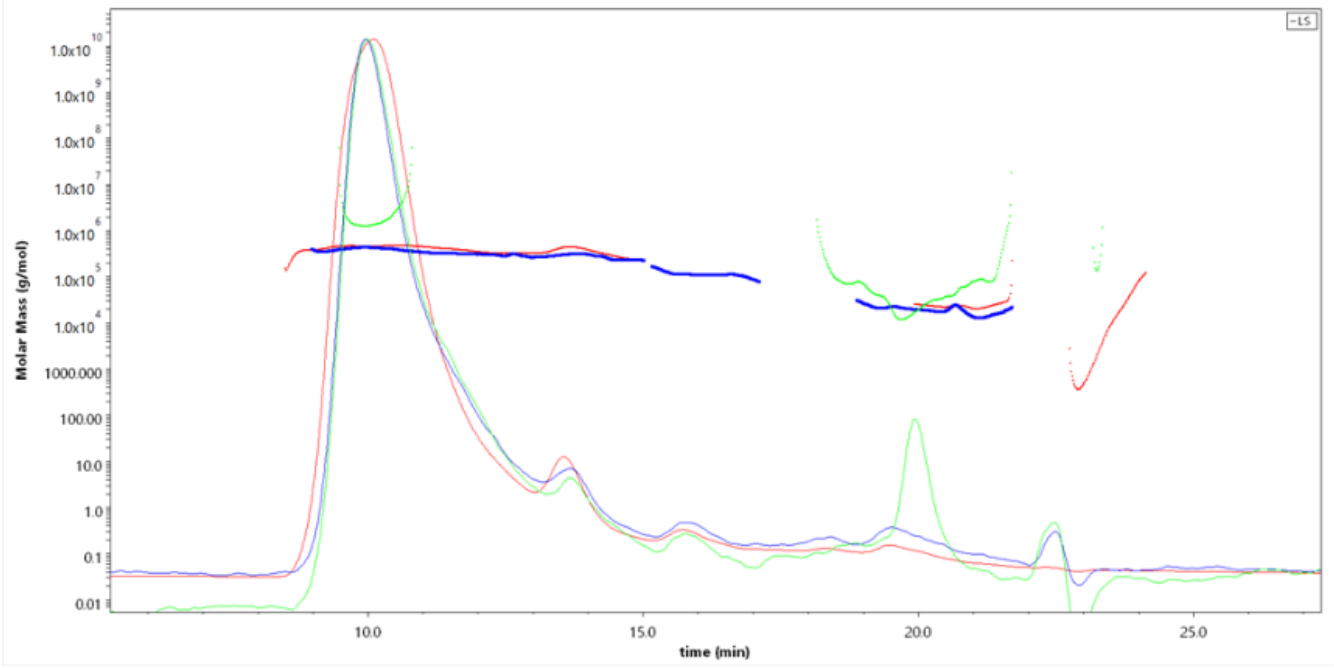

Figure 7: Astra Elution profiles of multiple samples of AAV, neat (red), diluted (Blue) & with albumin (Green) across X-bridge column at 0.5ml/min at a fixed temperature of 25°c.

Results from figures 5, 6 & 7 clearly indicate the Agilent column as the worst in terms of separation quality, due to the poor resolution and wide peaks. Compare this to the sharpness of the peaks presented on the TSK4K and X-bridge columns and we can see

a clear difference in quality. Sharp peaks in an elution profile indicate a high level of column efficiency, meaning the column can effectively separate molecules within a similar size profile.

The large shoulder on the Agilent column and to some extent the X-bridge column, indicate components within the sample that have a slightly different size or shape that elute close to each other, but are not fully resolved. Shoulders on an elution profile may also indicate poor column performance or degrading column health.

The TSK4K column also stands out in terms of separation quality as it has a well resolved albumin peak at an elution time of around 24 minutes. An albumin peak is also present on the X-bridge elution profile however, it has much more noise in comparison to the TSK4K elution profile.

Complementing data presented in table 7 does not detail a superior column, as there are many outliers across all columns tested.

Table 8: Data collected in Astra on varying samples of control AAV run across X-bridge column.

	M_n (kDa)	± (%)	M_w (kDa)	± (%)	Polydispersity (Mw/Mn) ± (%)		r_w (nm)	± (%)
Control AAVneat	883	1.1	949	1.0	1.075	1.52	53	3.1
Control AAV diluted	268	12	280	10.6	1.047	16.02	82	14.1
Control AAV with albumin	15	1.4	17	2.10	1.14	2.5	39	7.3

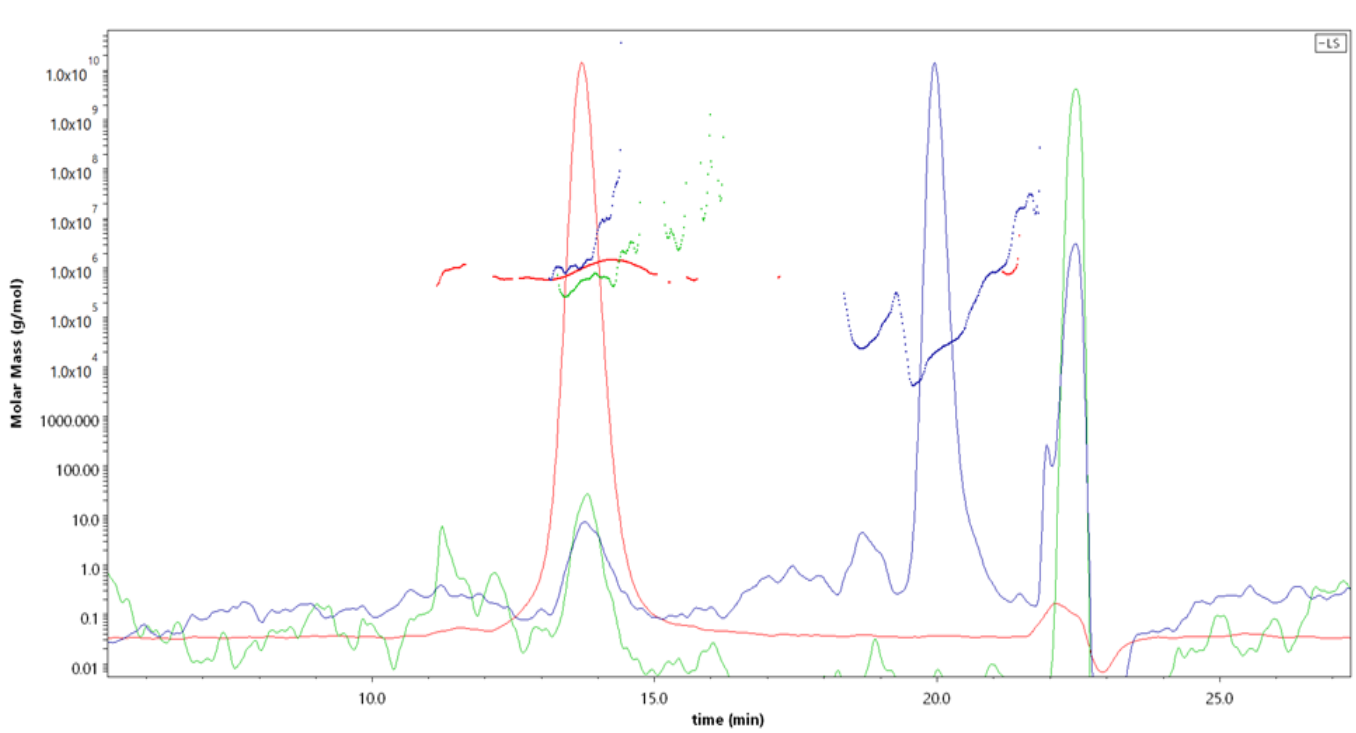

Figure 8: Astra Elution profiles of multiple samples of control AAV neat (Red), diluted (Green) & with albumin(Blue) across X-bridge column at 0.5ml/min at a fixed temperature of 25°c.

Table 9: Data collected in Astra on varying samples of control AAV all run across Agilent column.

	M_n (kDa)	± (%)	M_w (kDa)	± (%)	Polydispersity (Mw/Mn) ± (%)		r_w (nm)	± (%)
Control AAVneat	827	2.50	904	2.1	1.093	3	39.3	17.7
Control AAV diluted	0	0	0	0	0	0	0	0
Control AAV with albumin	9	13.4	33	36.1	3.541	38	0	0

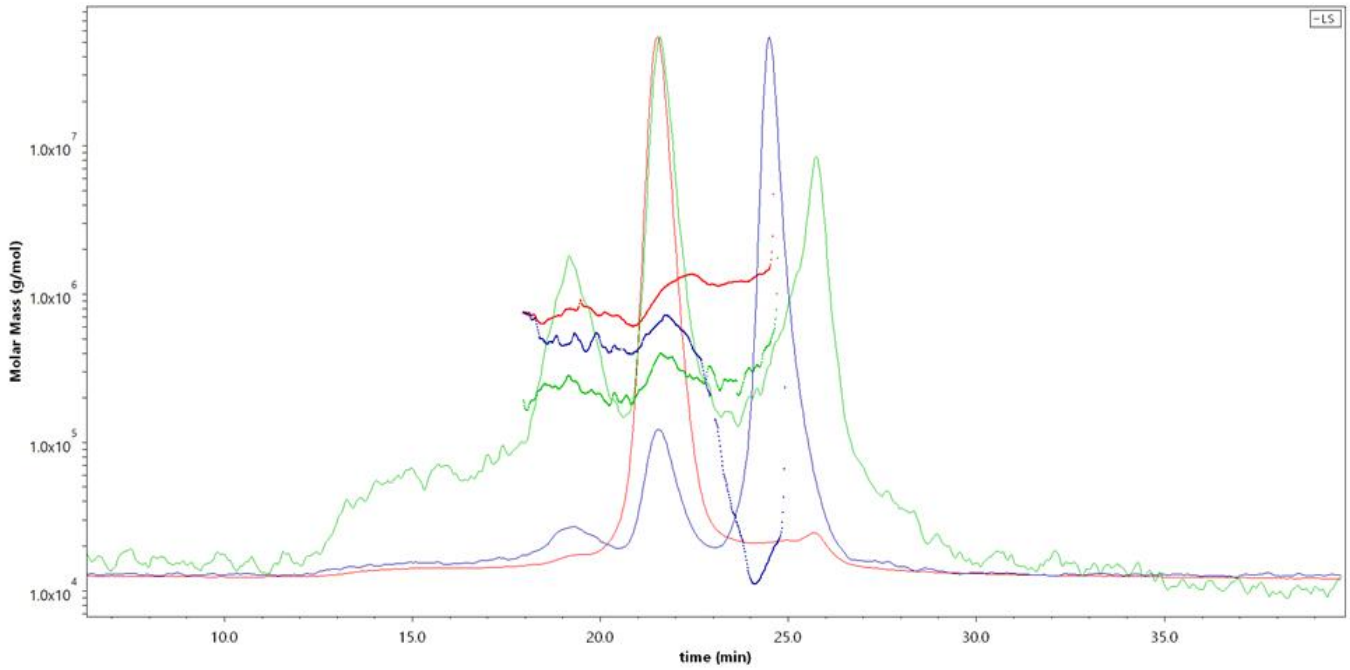

Figure 9: Astra Elution profiles of multiple samples of control AAV neat (Red), diluted (Green) & with albumin(Blue) across Agilent column at 0.5ml/min at a fixed temperature of 25°c.

On both runs of control AAV across the Agilent and X-bridge column, we did not collect molecular weight data to fit the range of control AAV as defined by (Fu et al., 2023). They defined the Mw of control AAV to 3689 kDa, which is much closer to the consensus of the Mw of this serotype of AAV. To compare between the elution profile in figure 8 and 9, figure 8 shows a much higher degree of peak resolution, meaning the column used in figure 8 is much more efficient at separating the molecular weight of the molecules in the sample tested.

Table 8 and 9 collate findings of Mw data for a neat sample of control AAV, of which are drastically far away from others research. This may be due to multiple factors such as a difference in methodology of my experiment to theirs, such as varying buffers and analytical techniques used to gain results.

Conclusion

The selection of appropriate chromatography columns is pivotal both in scientific research and industrial formulations, influencing the accuracy, efficiency, and outcomes of chemical analyses. In research, the correct column choice is essential for achieving high resolution and sensitivity, enabling scientists to evaluate differences and interactions within complex mixtures. In industrial contexts, particularly in the development and manufacture of pharmaceuticals and other therapeutic products, the choice of column affects not only the purity and quality of the final products but also the scalability of production processes.

To conclude on the best selection of column for the separation of our sample, it was decided early on in the experimental procedure that the Agilent column was worst in terms of separation efficiency for our sample. This is displayed throughout as poor peak resolution and shoulders on figures 9, 6, 2 and 1. Data gathered on molecular weight of samples across the multiple columns was inconclusive as per the variables listed in results for varying elution times and thus an inaccurate reading of molecular weight. The X-bridge column was selected as the best and most efficient column for the separation and analysis of our samples. Figures 8, 7, 4, 2 and 1 all show the X-bridge as the best in terms of peak resolution, as well as showing minimum shouldering, meaning the column is in good condition and has appropriate and effective column packing for the samples used in the experiment.

Introduction to DLS

Dynamic light scattering is an analytical technique used to measure the size of particles in a suspension or solution. DLS is widely used for characterizing the size of nanoparticles, colloids, proteins, polymers, and other macromolecules, providing critical information in research and industrial applications such as pharmaceuticals and material science.

The principle of DLS revolves around analysing the fluctuations in light scattering caused by particles as they undergo random Brownian motion. The instrument sends a laser through a sample to test. As the laser light hits the particles, it scatters in various directions. The nature of this scattering depends on the size of the particles and their refractive index relative to the surrounding medium. Molecules suspended in a fluid undergo Brownian motion, causing the scattered light to fluctuate in intensity over time. The scattered light is collected at a specific angle and the fluctuations in its intensity are measured over time. The intensity fluctuations are analysed using an autocorrelation function, which provides a correlation of the signal with itself over different time intervals. This function helps in determining how quickly the scattering pattern changes, which is directly related to the speed of particle motion and thus the size of the molecule as the smaller the molecule the higher their Brownian activity (Chemistry LibreTexts, 2016).

Brownian motion details the random, erratic movement of molecules suspended in solution. Molecules continuously changing direction as they collide with the molecules of the surrounding fluid. The rate in which the molecules move is driven by thermal motion, the higher the temperature the more vigorously the molecules move, leading to more collisions. Smaller particles are influenced more dramatically by thermal collisions compared to larger particles as they are less influenced by the viscosity of

the medium and more heavily influenced by collisions, leading to more pronounced Brownian motion.

From the rate of fluctuation of the scattered light, calculations can be made of the diffusion coefficient of the molecules. Using the Stokes-Einstein equation, the diffusion coefficient can then be used to calculate the hydrodynamic radius of the particles.

$$D = \frac{kBT}{6\pi\eta r}$$

Where D is the diffusion coefficient of the molecule.

Using the same equation, the hydrodynamic radius of the molecule can be calculated by rearranging to express *r*.

$$r = \frac{k\ BT}{6\pi\eta D}$$

Static Light Scattering and Dynamic Light Scattering are both analytical techniques used to analyse properties of molecules in solution, such as their size. They operate based on different principles and are suited for different applications.

Static Light Scattering measures the intensity of light scattered from a sample at various angles but at a fixed time point. This technique is used to determine the molecular weight and radius of gyration of molecules. SLS is effective for analysing large particles and provides averaged information over the entire sample (Stetefeld, McKenna and Patel, 2016). Larger particles scatter light at smaller angles more intensely, and the angular distribution of scattered light can provide detailed information about particle size and shape. SLS utilizes this data to calculate the radius of gyration and molecular weight of particles.

Dynamic Light Scattering focuses on the fluctuations in the intensity of scattered light over time. This method is particularly useful for determining the size distribution of small particles in a sample through the analysis of how quickly the particles undergo Brownian motion. DLS is ideal for samples where particles are uniformly dispersed and are small enough for Brownian motion to be significant. Smaller particles move more rapidly due to Brownian motion, causing more rapid fluctuations in light scattering. DLS analyses these fluctuations to calculate particle size based on the diffusion coefficients, which are inversely proportional to particle size (Mauer et al., 2017).

Method

Sample preparation

Sample preparation followed the methods of our prior SEC-MALS experiment in the formulation of buffer, AAV neat, diluted and with albumin across the two varying suppliers of AAV. This ensures consistent concentration and provides a more accurate comparison of results.

DLS

30µl of each sample was injected into DLS 380 well flat bottom plate to the order of Table 10, 11 and 12 to then be sealed with sealing tape and read on Wyatt plate reader 3. Each well was scanned twice across three radius regions: 1-10nm, 10-100nm and 100-1000nm at a fixed temperature of 25^{o}c.

Results & Discussion

During this experiment, Dynamic Light Scattering was used to explore the hydrodynamic characteristics of AAV molecules in suspension, both in the presence and absence of albumin. The experiments were conducted using two different buffer formulations characterized by low and high salt concentrations, aiming to ascertain the effects of ionic strength on molecules size and stability. Particular attention was paid to how the presence of albumin and varying salt conditions influence its colloidal properties. To establish a comparative framework, a control AAV was selected due to its high stability and comparable biological properties to our AAV to test.

Table 10: Sample data from DLS Plate A showing readings across varying radius (0.1-10, 10-100, 100-1000). Comparing multiple suppliers of AAV to control AAV

Sample	Radius (nm)	%PD	Radius (0.1-10nm)	Radius (10-100nm)	Radius (100-1000nm)
Buffer A	0.545	25.4		12.85	263.81
Buffer B	0.4	38.795	4.03	24.905	
Albumin in buffer A	2.935	31.845	3.795		
Albumin in buffer B	3.915	19.575	3.54	83.83	183.14
Neat AAV supplier 1	222.525	Multimodal	7.255	21.125	251.97
AAV supplier 1 diluted buffer A	64.675	45.755	7.17	37.525	124.95
AAV supplier 1 with albumin buffer A	68.54	43.785	5.63	27.075	280.62
AAV supplier 1 diluted buffer B	158.59	Multimodal	7.39	26.15	295.825
AAV supplier 1 with albumin buffer B	154.96	Multimodal	7.755	36.995	260.465
Neat AAV supplier 2	16.63	22.075		15.325	248.285
AAV supplier 2 diluted	161.04	Multimodal	6.5	78.635	101.42
AAV supplier 2 with albumin	5.83	Multimodal	2.01	60.91	119.005

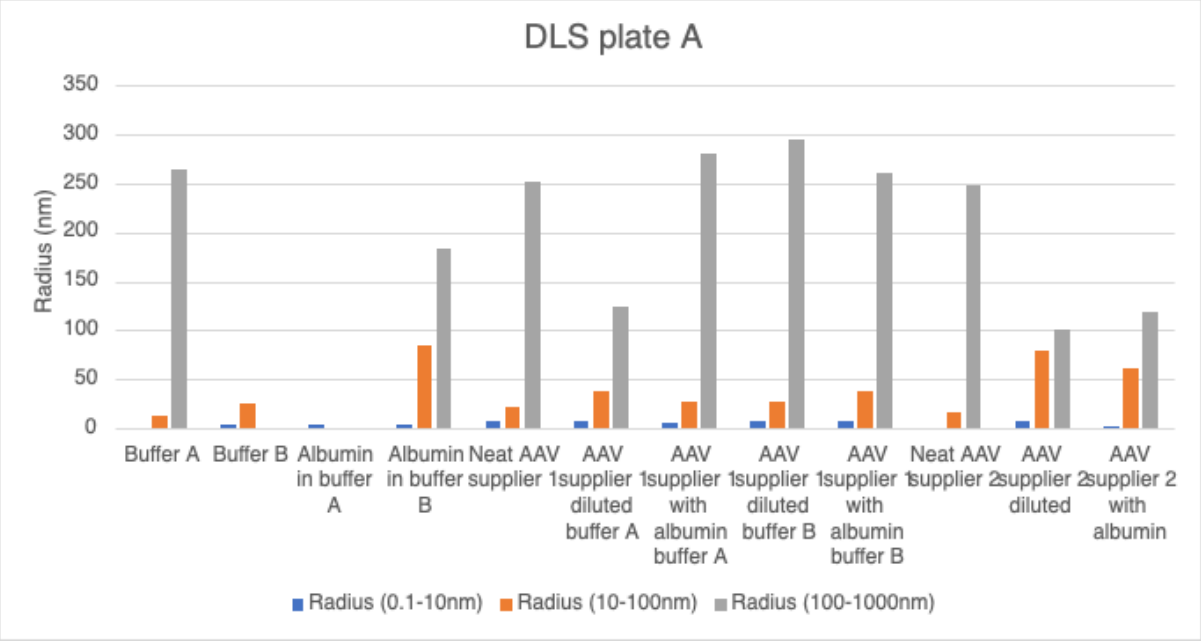

Figure 10: Plotted data from DLS Plate A showing readings across varying radius (0.1-10, 10-100, 100-1000). Comparing multiple suppliers of AAV to control AAV

Table 11: Sample data from DLS Plate B showing readings across varying radius (0.1-10, 10-100, 100-1000). Comparing multiple suppliers of AAV to control AAV.

Sample	Radius (nm)	%PD	Radius (0.1-10nm)	Radius (10-100nm)	Radius (100-1000nm)
Neat AAV supplier 1	15.235	11.51		15.255	232.365
AAV supplier 1 diluted buffer A	167.315	Multimodal	8.33	33.855	128.565
AAV supplier 1 with albumin buffer A	7.08	Multimodal	2.34	72.035	153.605
AAV supplier 1 diluted buffer B	13.86	32.07		13.5	
AAV supplier 1 with albumin buffer B	5.295	41.31	4.335	14.545	
Neat AAV supplier 2	17.095	29.77		14.925	140.56
AAV supplier 2 diluted	128.6	Multimodal	6.405	31.535	189.205
AAV supplier 2 with albumin	8.24	Multimodal	2.12	54.405	133.41
Control AAV	14.375	9.11		13.805	
Control AAV diluted	28.7	Multimodal	8.29	18.675	
Control AAV with albumin	4.455	43.615	3.595	24.035	157.9
Buffer A	0.415	28.2	2.32	69.33	131.48
Buffer B	0.4	19.21	8	57.26	479.03
Albumin in buffer A	3.45	33.155	2.915	40.16	203.68
Albumin in buffer B	4.64	30.27	5.33		120.02

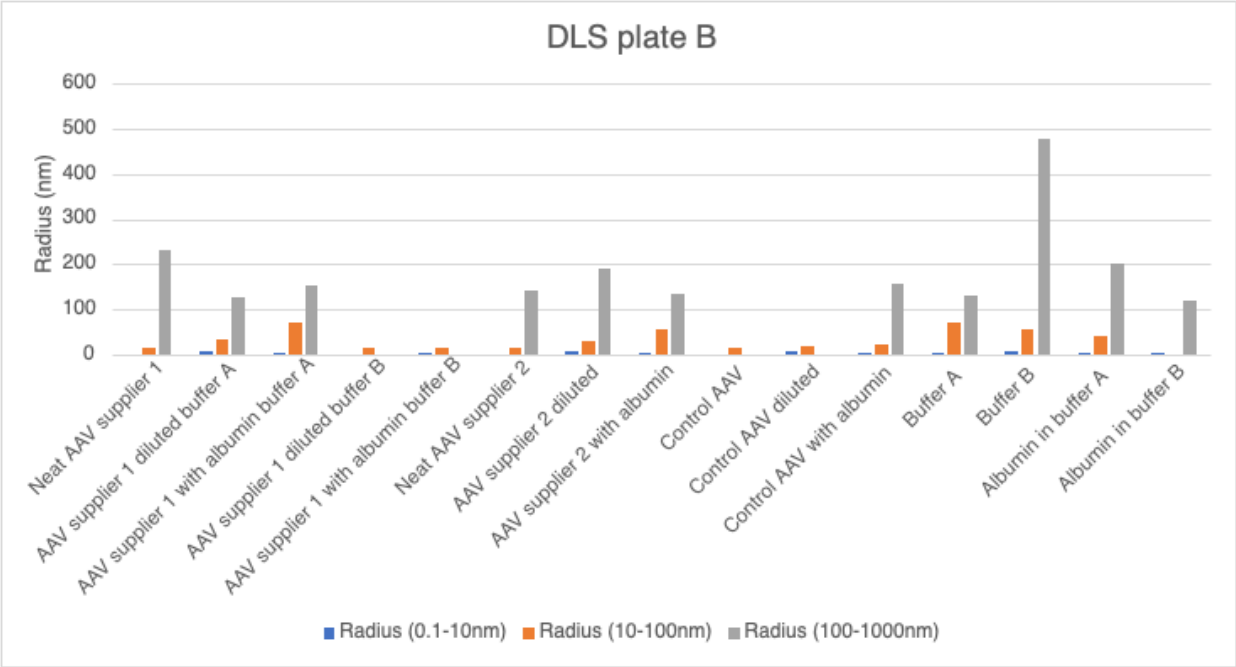

Figure 11: Plotted data from DLS Plate A showing readings across varying radius (0.1-10, 10-100, 100-1000). Comparing multiple suppliers of AAV to control AAV

As defined by (Xujin Zhang et al., 2021) the radius of AAV falls in a range of 14.7 to 15.6nm. This is supported by the reading of neat AAV from supplier 1 on plate B being 15.255nm and neat AAV from supplier 2 being 15.325nm. The control AAV also shows data within this range at 13.805nm on plate B.

The varying buffers had an effect of the sizes displayed across plate A and B. AAV from supplier 1 read 33.855nm in buffer A and 13.5nm in buffer B. The different buffers also had a similar effect when albumin was introduced to the samples of AAV. AAV from supplier 1 with albumin in buffer B read 14.545nm, whereas AAV from supplier 1 in buffer A read 72.035nm.

This may be due to the higher ionic strength in buffer B. higher ionic strength plays a crucial role in shielding electrostatic charges on proteins and reducing their tendency to aggregate. This occurs as the increased ions in the buffer form a layer around protein

surface charges, shielding these charges. Shielding weakens the electrostatic interactions that can lead to aggregation. The presence of more ions enhances the hydration of the ions themselves rather than the proteins, which decreases the proteins' effective charge through a process known as charge screening. Consequently, this stabilization keeps proteins dispersed, maintaining their solubility and preventing the formation of larger aggregates, which is particularly important in applications like drug formulation (Saito et al., 2013).

Introduction to AUC

Analytical ultracentrifugation is a powerful and versatile technique widely utilized for the study of proteins, nucleic acids, and complex biological assemblies. The technique involves subjecting a sample to high-speed centrifugation, which separates the components based on their size, shape, and density. Under the influence of centrifugal force, particles in a solution move at a rate that reflects their molecular mass and conformation. By observing this movement, researchers can infer the sedimentation coefficients, molecular weights, and interaction properties of the solutes (Edwards et al., 2020).

In SV experiments, the sample is spun at high speeds, and the rate at which particles move under centrifugal force is measured using optical systems, such as UV-visible absorbance or interference optical detection. This method allows for the observation of the sedimentation process in real time, yielding information on the size distribution, shape, and heterogeneity of the molecules. SV is particularly useful for studying the size and conformation of particles, and it can distinguish between different molecular species, such as monomers, dimers, and higher oligomers. SE experiments are

conducted at lower speeds, allowing the sample to reach an equilibrium where the sedimentation force is balanced by the diffusion of particles back up the concentration gradient. This technique is highly sensitive to molecular weight and is used to determine the mass, stoichiometry, and association (Zhao et al., 2013).

AUC is highly sensitive and can accurately detect and quantify aggregates of AAV molecules. This ability is vital because even low levels of aggregation can impact the safety and efficacy of the viral vectors used in gene therapy. The degree of aggregation can indicate the stability of the viral preparation. Factors that cause aggregation are pH imbalances, ionic strength variations and thermal instability. Once identified they can be adjusted to optimize stability. Aggregation of AAV vectors can potentially elicit an immune response in patients, which is undesirable in gene therapy as it can reduce the efficiency of gene delivery and possibly lead to adverse reactions and so, stability is crucial for patient safety (Maruno et al., 2023).

Method

Sample preparation

Table 12: sample preparation for AUC run. Buffer and albumin preparation mirror samples used in prior experiments.

	AAV vol to get 1^12GC/ml	Albumin (µl) from 200 mg/ml	Buffer A 5X→1X (µl)	Buffer B 5X→1X (µl)	water (µl)
AAV diluted in buffer A	105.26	0.00	400		1494.74
AAV with albumin in buffer A		10		400	1484.74
AAV diluted in buffer B		0.00	400		1494.74
AAV with albumin in buffer B		10		400	1484.74

PBS preparation

One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25^{0}C with 0.001% Poloxamer

Table 13: showing sample list and cell location for AUC run.

Cell A	Albumin in buffer A
Cell B	Albumin in buffer B
Cell C	AAV in PBS buffer
Cell D	AAV in buffer A
Cell E	AAV with albumin in buffer A
Cell F	AAV in buffer B
Cell G	AAV with albumin in buffer B
Cell H	Counterweight

AUC

8 hole rotor Sedimentation velocity experiment was performed at 20.0°C using the Optima XL-I analytical ultracentrifuge (Beckman, Palo Alto, USA) equipped with Rayleigh interference optics. 400 µl solution and solvent were injected into 12 mm XLI blue carbon filled cells and centrifuged at an initial speed of 13,000 and ramped to 40,000k, scanning every 20 minutes. Sedimentation coefficient distributions c(s) vs s, where s is the sedimentation coefficient (in Svedberg units, S = 10–13 sec) were obtained using the "least squares g(s)" method in SEDFIT (Dam and Schuck, 2004). Partial specific volume for cells 1 and 2 for 13,500 rpm data 0.733ml/g and 0.709ml/g for all other cells in this data set and 40,000 rpm data. Buffer density and viscosity for three varying buffers calculated in SEDNTERP. plotted using corrected S20,w

Results and discussion

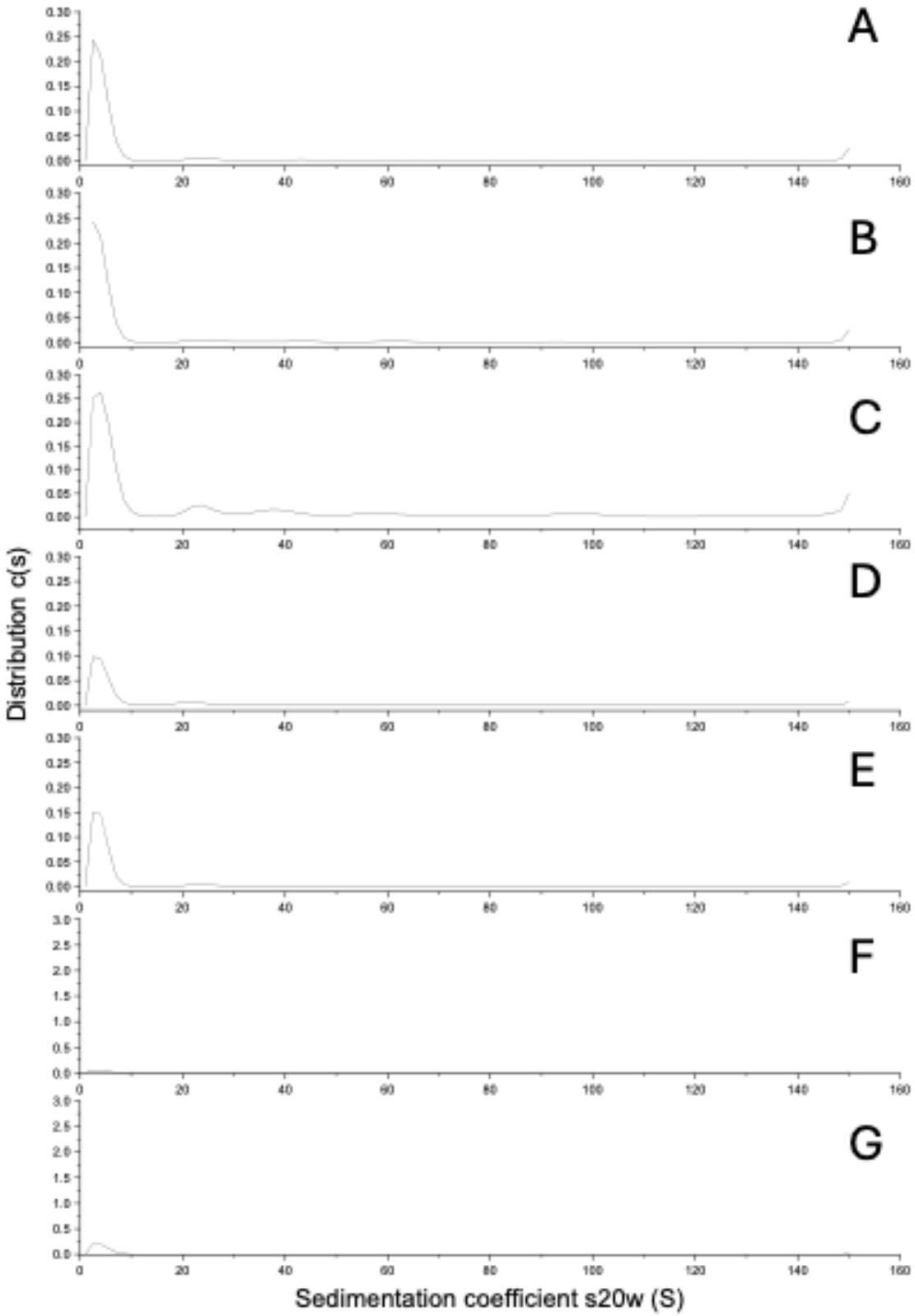

Figure 12: 13.5k rpm sedimentation coefficient distribution of varying samples of AAV of which order is detailed in table 13.

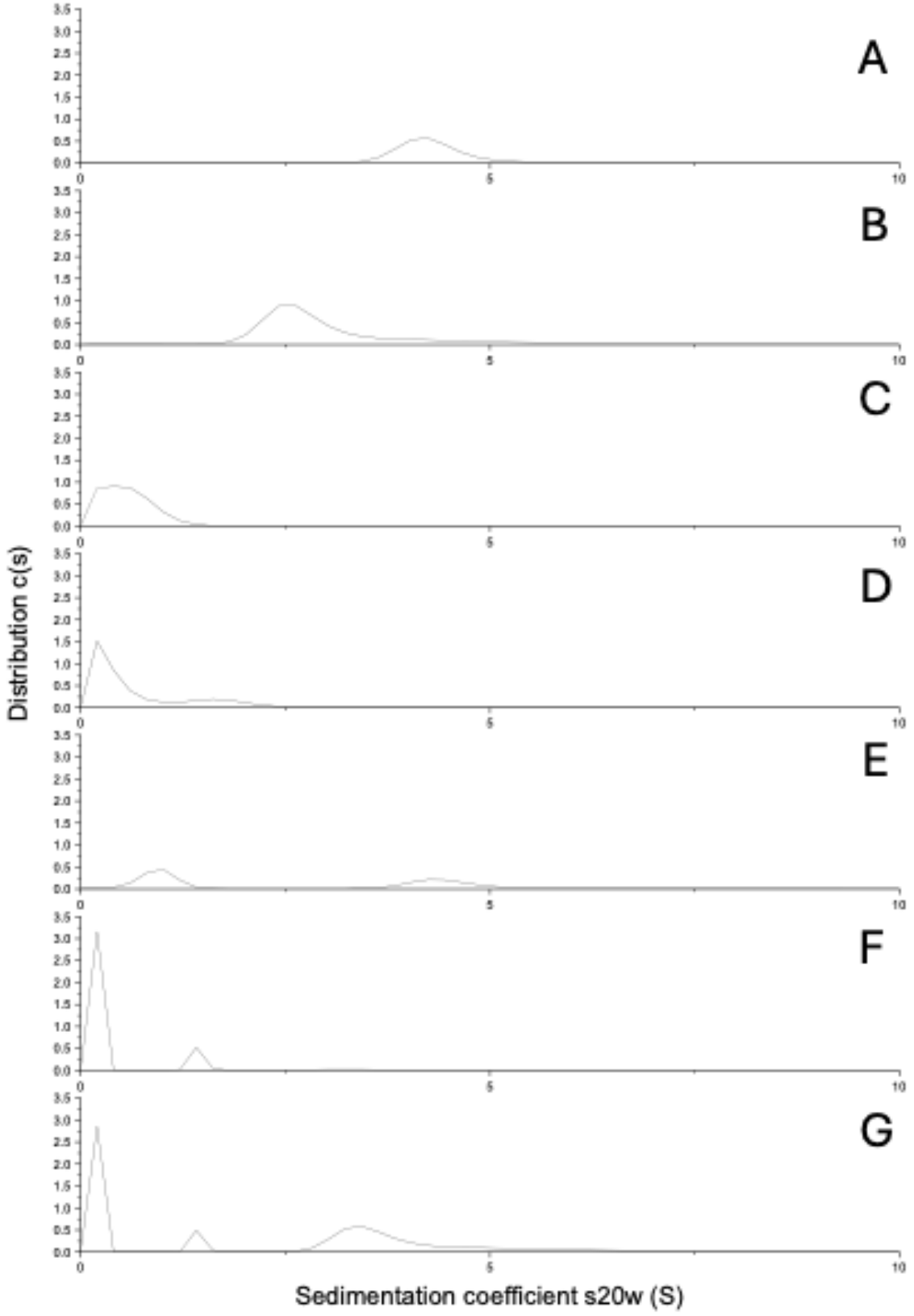

Figure 13: 40k rpm sedimentation coefficient distribution of varying samples of AAV of which order is detailed in table 13.

AAV vectors must maintain their structural integrity under physiological conditions to ensure successful gene delivery and expression. AUC provides a sophisticated method to evaluate the physical stability and aggregation propensity of these vectors by observing their behaviour in solution. AAV samples were subjected to AUC analysis under various ionic strengths in the buffers used. This approach was chosen to mimic different biological environments, allowing us to evaluate the structural resilience and aggregation of AAV capsids. By varying the ionic strengths, we aimed to delineate the conditions that either stabilize or destabilize the capsids.

From prior work we know the S values and the Mw in which AAV and albumin fall into. The S value of HSA should be around 4-5, with a Mw of 66.5 kDa. Figure 13 shows peaks at around this S value across all samples, providing inconclusive data as albumin is only present in cells A, B, E and G as detailed in table 12. AAV has a much higher Mw than HSA at around 4,700 kDa. Figure 12 shows high Mw peaks around this value but much like the low Mw data, peaks appear across all cells. This again presents inconclusive data as cells A and B do not contain AAV, yet they contain high Mw peaks. This may be due to a misinterpretation of data as peaks line up across varying samples, meaning the expressions are more likely to be background noise. This is further supported by the low concentration of each high Mw peak.

Sample A and B on figure 13 present different s reading, with sample B being slightly lower. Sample A and B is albumin in a varying buffer as shown in table 13, sample B containing a buffer with a ionic strength. The lower s value may indicate shielding electrostatic charges on albumin and reducing its tendency to aggregate as a lower s values indicate a smaller molecule. Increased ions in the buffer form a layer around protein surface charges, shielding these charges. Shielding weakens the electrostatic interactions that can lead to aggregation (Saito et al., 2013).

Conclusion and Further work

Across both AUC and DLS, data may suggest that buffer B, containing a higher ionic strength led to less aggregation and a higher stability in samples. To reaffirm this further work could be done around subjecting these samples to added stresses, both environmental and storage. This area of research would benefit the production of gene therapies as stress testing an accurate way of testing how their potency and efficiency lasts both in vivo and in long storage conditions (Ronzitti et al., 2020).

Appendix

Troubleshooting

The waters GTxResolve Premier BEH SEC column started to leak when running buffer at 0.5mL/min, with leak continuing when flow rate was reduced to 0.3mL/min. The guard column was removed, and the column was run again at a gradually increasing flow rate of buffer. At 0.3mL/min the injector valve started to leak, subsequently the instrument was set back to a 0.1mL/min flow rate and a tube guard with a higher tolerance was fitted to the injector valve. The system then didn't present any leaks when slowly ramped back to 0.5mL/min. We then ran into issues when running a comparison between two varying brands of AAV. The same Waters x-bridge column, now with guard column attached, was used to compare the quality AAVs from supplier 1 & 2, to which the quart pump started to leak during testing. It was hypothesised that the membrane in the quart pump degraded during the nitic acid cleaning, and so was replaced. During initial testing of the two varying brands of AAV it was hypothesised that AAV from supplier 1 is a clear leader in terms of quality. This was not clearly demonstrated on the elution profile across the TSK4K column on the run comparing the two brands of AAV. Upon further inspection of the sample, supplier 1 AAV was unpurified, concluded by its cloudy appearance. Subsequently a new sample of AAV

was ordered to be tested against supplier 1 AAV lysate and AAV from supplier 2. This test was to be run on a brand new TSK4K column, as upon viewing the TSK4k data it was hypothesised that the column may be faltering due to the age of the instrument.

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