Ultrahigh Sensitivity Analysis of Adeno-associated Virus (AAV) Capsid Proteins by Sodium Dodecyl Sulphate Capillary Gel Electrophoresis

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Adeno-associated virus (AAV) is currently one of the most widely used delivery vehicles for gene therapy, featuring long-term expression of the transgene and excellent disease correction history. During vector production, several quality control (QC) parameters should be closely monitored to comply with clinical safety and efficacy requirements. Among them, purity analysis of the AAV viral proteins is important for quality assurance and safety of the products. This paper introduces two sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE) workflows for adeno-associated virus capsid protein analysis. One is utilising UV detection for the intact forms and the other one with fluorophore tagging for enhanced sensitivity laser induced fluorescence detection.

1. Introduction

AAVs are members of the parvovirus family, being among the smallest DNA viruses with a diameter of approximately 20 nm. Importantly, they are non-enveloped, replication-defective viruses. AAVs are composed of three capsid proteins surrounding the DNA genome of approximately 4.8 kilobases (kb) [1]. The three viral capsid proteins are 87kD (VP1), 73kD (VP2) and 61kD (VP3) in size and assembled in an approximately 1 : 1 : 10 molar ratio, respectively [2, 3]. Sixty of these subunits form a perfect icosahedral structure [4, 5]. During AAV manufacturing and release, impurities should be subject to rapid and high resolution analysis, including residual host cell and helper virus related proteins from the culture medium and purification process [6].

Various serotype AAVs have become attractive therapeutic vehicles for gene therapy development due to features such as long-term transgene expression and excellent disease treatment capability [7]. Pseudo-serotyping of adeno-associated viruses showed novel tropism and biology on individual genome/capsid configurations, therefore, greatly improving the applicability and versatility of the system. Currently, thirteen human AAV serotypes have been identified increasing the applicability of this particular vector in gene therapy due to the distinct tropism of the different serotypes for various organs and tissues [8]. Cellular attachment of most AAV serotypes utilises binding to the carbohydrate moieties of various cell surface glycoproteins, an important first step towards successful transduction. AAV serotypes can be grouped by considering their receptor recognition as heparan sulphate proteoglycan (AAV2, AAV3, AAV6, and AAV13) as well as for N-linked oligosaccharides: terminal sialic acid (AAV1, AAV4, AAV5, and AAV6) and terminal galactose (AAV9) types [9]. Therefore, qualitative and quantitative analysis of the virus capsid proteins and their distribution ratio is of high importance.

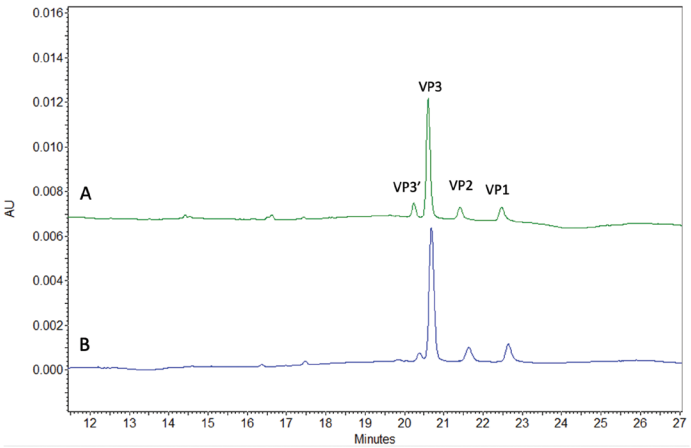

Figure 1. SDS-CGE-UV separation of the AAV8 (trace A, 1 x10^{13} GC/mL) and AAV2 (trace B, 0.5 x10^{13} GC/mL) capsid protein samples. Conditions: SDS-MW gel buffer system, 20 cm effective capillary length (30 cm total), 50 µm ID; UV detection at 214 nm; Separation voltage: 15 kV, temperature: 25°C. Injection: water pre-injection for 0.4 min at 20 psi followed by sample injection for 1 min at 5 kV. Peaks: VP1, VP2 and VP3: virus capsid proteins, VP3': virus capsid protein 3 with altered PTM.

SDS-CGE is one of the popular AAV capsid protein analysis methods in the cell and gene therapy sector. SDS-CGE offers automated protein separation with excellent resolution and quantitation capabilities [10]. The method can utilise both UV and laser induced fluorescent (LIF) detectors. The former does not require pre-separation labelling but has lower sensitivity. LIF detection, on the other hand, necessitates fluorophore labelling of the capsid proteins but features several order of magnitude higher sensitivities. In this paper two SDS-CGE workflows are introduced using 1) UV detection for unlabelled capsid proteins and 2) LIF detection after covalent dye labelling.

Experimental

Materials: Sodium dodecyl sulphate, methanol, N-ethylmaleimide (NEM) and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Amicon Ultra-0.5 Centrifugal Filters with 10,000 and 30,000 NMWL were purchased from EMD Millipore (Billerica, MA, USA). The ATTO-TA FQ (3-2-(furoyl quinoline-2-carboxaldehyde) Amine-Derivatisation Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA). The SDS-MW Analysis Kit was from SCIEX (Framingham, MA, USA) including the SDS-MW gel buffer and the SDS-MW sample buffer of 100 mM Tris-HCl (pH 9.0) with 1% SDS. Packaged AAV2 of pAV-CMV-GFP (titre 2.24 x10^{13} GC/mL, (genome copies per millilitre), AAV8 of pAV-CMV-GFP (titre 3.99 x10^{13} GC/mL) and packaged AAV8 of pAV-CMV-GFP (titre 1.57 X10^{14} GC/mL) were purchased from Vigene Biosciences (Rockville, MD, USA). The three samples were kept in Phosphate Buffered Saline (PBS, pH 7.5) with 0.001% pluronic F68 storage solution and were diluted in the same storage buffer to the desired concentrations. This latter was added to minimise sticking of AAV to hydrophobic plastic surfaces.

Sample Preparation: For UV absorbance detection, 5 µL of AAV solution (salt concentration of < 40 mM) was mixed with 5 µL of 1% SDS and 1.5 µL of 2-mercaptoethanol and incubated for 10 min at 50 °C to fully denature the sample proteins. After the denaturation step, 90 µL of DI water was added to the sample mixture. Buffer exchange was necessary if the salt concentration in the AAV sample was higher than that of 40 mM. For FQ labelling and LIF detection, 10 µL of AAV sample solution was mixed with 1.2 µL of 4% SDS in 150 mM NEM solution in a microfuge tube and incubated for 5 minutes at 70 °C followed by mixing with 1.5 µL of 2.5 mM FQ dye working solution and 1 µL of 30 mM KCN solution. The labelling reaction mixture was incubated for 10 minutes at 70°C. The reaction was quenched by the addition of 28 µL of 1% SDS, kept for an additional 5 min at 70°C. After the quenching step, 20 µL of DI water was added to the diluted reaction mixture. The labelled samples were immediately used for SDS-CGE-LIF separation.

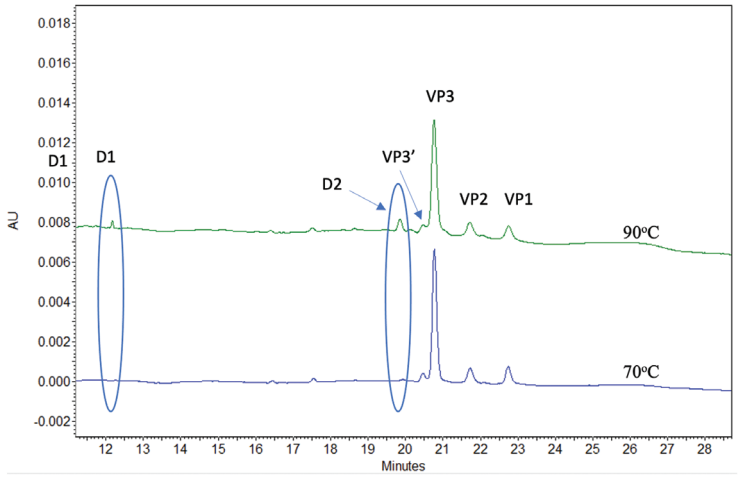

Figure 2. Effect of the denaturation temperature on the stability of the AAV8 capsid proteins. Separation conditions were the same as in Figure 1.

Capillary Electrophoresis

The PA 800 Plus Pharmaceutical Analysis system was equipped with UV and LIF detectors. For all separations a 500 V/cm electric field strength was applied. The EZ-CE pre-assembled capillary cartridge (bare fused-silica, 50 µm I.D., 30 cm total length, 20 cm effective length, SCIEX) was filled with the polymer based SDS-MW gel-buffer system. Detection: UV: 214 nm, LIF: 488 nm excitation wavelength with a 600 nm / 80 nm emission bandpass filter. The separation temperature was set to 25°C. Stacking injection: water pre-injection for 0.4-0.6 min at 20 psi followed by sample injection for 1 min at 5-10 kV. Data acquisition, processing and analysis were performed employing the 32 Karat Software 10 package.

Results and Discussion

Various serotype adeno-associated viruses are recently becoming one of the most frequently utilised gene delivery vehicles. Therefore, their purity analysis is of high importance, both from manufacturing and regulatory points of views. The sodium dodecyl sulphate capillary gel electrophoresis workflows introduced in this paper for high sensitivity purity analysis of the capsid proteins utilise both UV and laser induced fluorescent detection, this latter requiring covalent fluorophore tagging.

SDS-CGE-UV detection workflow

First, the AAV8 and AAV2 serotypes were analysed by SDS-CGE using UV detection in the 10^{12} - 10^{14} GC/mL concentration range. The upper (A) and lower (B) traces in Figure 1 depict the separation of the AAV8 and AAV2 serotypes, respectively. As one can observe, baseline separation of all three capsid proteins was obtained for both sample types. The VP3 : VP2 : VP1 ratio for the AAV8 sample was approximately 8 : 1 : 1, while for the AAV2 sample it was 7 : 1 : 1. Albeit, the theoretical ratio is 10 : 1 : 1, in practice the ratios do not necessarily match to that exactly. Multiple factors may affect the VP ratios, such as the recombination design of the viral proteins for different tropism, the production process conditions, etc. [11, 12]. The slight differences observed in the ratio measurements could influence the shape of the two viruses examined.

In both traces a small peak is visible in front of the VP3 peak (depicted as VP3'), which is probably a small portion of the VP3 capsid protein with altered post translational modification as suggested in [13]. The migration time and relative peak area reproducibility values were less than 0.34% RSD and 0.75% RSD (n=8), respectively.

One of the important aspects of SDS-CGE sample preparation is the incubation temperature during the denaturation step. This process is usually accomplished at 90°C for 3-5 minutes to ensure full denaturation of the proteins. However, the temperature should be reduced for sensitive proteins to avoid possible decomposition during the denaturation step. Figure 2 compares the SDS-CGE-UV traces of the AAV8 serotype sample after 10 minutes of 90°C (upper trace) and 70°C (lower trace) incubation. As can be seen, the higher temperature incubation resulted in the appearance of some decomposition products denoted as D1 and D2 in the upper electropherogram. These decomposition products may have originated from the VP3 protein since that peak appeared to decrease between the

two traces, however, this assumption needs to be verified with further experiments. In addition, the higher temperature denaturation step also caused an apparent resolution decrease between the VP3 and VP3' peaks, possibly due to decomposition related processes.

The limit of detection (LOD) and limit of quantitation (LOQ) values were assessed for the AAV8 sample with UV detection using the industry standard dilution series method [14]. The LOD was 1 x10^{12} GC/mL (peak to noise ratio 5) and the LOQ was 5 x10^{12} GC/mL (peak to noise ratio 15), with the linear detection response of r^2 =0.9991 in the concentration range of 5 x10^{11} – 1 x10^{14} GC/mL.

SDS-CGE-LIF detection workflow

While UV detection during the SDS-CGE separation of intact adeno-associated virus capsid proteins provided an adequate quantification limit (as shown above), in-process control during manufacturing would require higher sensitivity, preferably ≤1 x10^{10} GC/mL (~50 ng/mL), which is the typical AAV concentration in gene therapy. To increase detection sensitivity, laser induced fluorescent detection was used after fluorophore labelling of the capsid proteins with the 3-2-(furoyl quinoline-2-carboxaldehyde) (FQ) dye. The sample preparation procedure including the FQ labelling reaction and the quenching step took less than one hour. The resulting electropherograms for the AAV8 and AAV2 samples are shown in Figure 3. Again, baseline separation of all three viral capsid proteins was obtained with the VP3 : VP2 : VP1 ratio of 8 : 1 : 1 and 7 : 1 : 1, respectively, i.e., the fluorophore labelling did not change the overall peak distribution profiles.

The detection and quantitation linearities were evaluated as in the UV detection method, but in the 1 x10^{10} – 1.6 x10^{14} GC/mL range. The linear detection response result was r^2 = 0.9989 with the LOD and LOQ values of 1 x10^{10} GC/mL and 3 x10^{10} GC/mL for the VP3 peak.

Conclusions

As adeno-associated viruses are more and more extensively considered by the biopharmaceutical industry as delivery vehicles for gene therapy, reliable and quantitative assays are critical for their proper characterisation and impurity quantification. In this paper, sodium dodecyl sulphate capillary gel electrophoresis was employed for ultrahigh sensitivity AAV capsid protein analysis using UV detection for intact and LIF for fluorophore labelled forms. Both approaches resulted in excellent size separation of the VP1, VP2 and VP3 proteins along with the resolution of a small VP3 impurity peak (VP3'). The migration time reproducibility for both methods was <0.34% RSD, while the corrected peak area reproducibility was <0.75% RSD for SDS-CGE-UV and < 5% RSD for the SDS-CGE-LIF analysis of the fluorophore labelled capsid proteins. The linearity of detection ranged over two orders of magnitude for UV detection (LOD = 1 x10^{12} GC/mL) and four orders of magnitude with LIF detection (LOD =1 x10^{10} GC/mL).

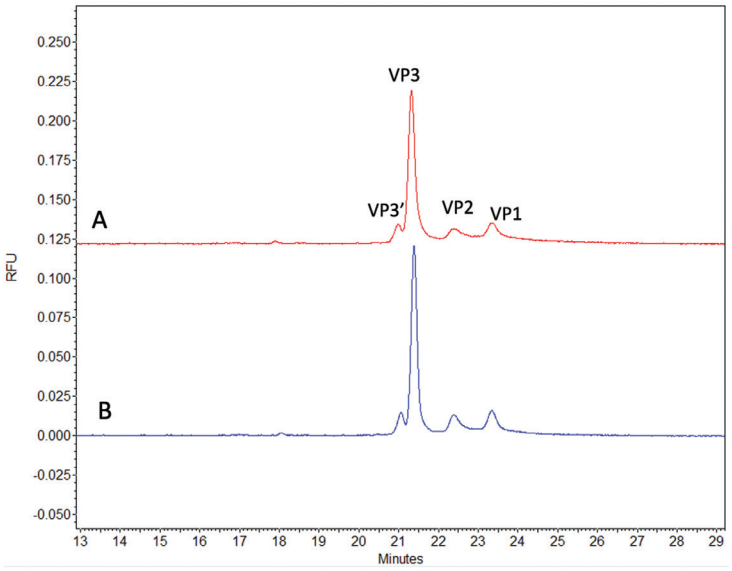

Figure 3: SDS-CGE-LIF separation of the AAV2 (trace A, 1 x10^{10} GC/mL) and AAV8 (trace B, 1 x10^{10} GC/mL) capsid protein samples labelled with the FQ dye. Conditions were the same as in Figure 1, but with LIF detection (488 nm excitation, 600 nm emission filter).

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