Sensitive AAV Capsid Protein Impurity Analysis by CE Using Easy to Label Fluorescent Chromeo Dye P503

Marcia Santos; SCIEX, Brea, California

Introduction

AAV or Adeno associated virus (Figure 1) is a popular class of gene therapy delivery vehicle used mostly due to non-pathogenicity and high stability. Structurally, AAVs are composed of icosahedral proteic shell called Capsid or Viral Protein (VP) which contains a viral genome. So far, AAV exists in some 13 human and primate serotypes which in combination with the primary sequence differences mediate the AAV cell and tissue specificity. For example, AAV 8 or serotype 8 is efficient in transducing hepatocytes. This structure has the ability to carry up to 5 Kb of pay-load of single stranded DNA molecule. The capsid or viral proteins are translated from the same mRNA encoding overlapping sequences of three capsid proteins, VP1, VP2 and VP3 with approximately 87, 72 and 62 kDa, respectively resulting in a total of 60 monomers. In addition, in vivo, these viral proteins are synthetized roughly in a ratio of 1:1:10, respectively.[1, 2]

Figure 1. Pictorial representation of an adeno-associated virus.

As AAV take center stage in gene therapy treatment of many genetic conditions, reliable and quantitative assays are critical for the proper characterization of these molecules as well as the quantitation of impurities.

Typically, AAV concentration used in gene therapy is in the order of $1x10^{10}$ GC/mL (GC=genomic copies), which equates to 50 ng/mL and thus falls well below limit of detection or quantitation of any UV absorbance based assays.

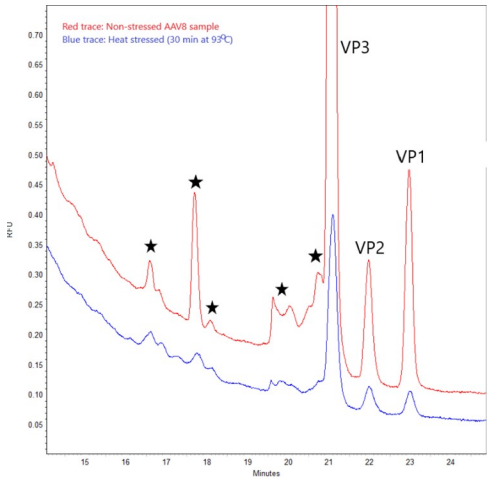

Figure 2 Overlay of Non-Stressed (red trace) and Heat-Stressed (blue trace) Both AAV8 Samples at $1.1x10^{9}$ GC/mL. Stars indicate regions on the electropherogram sensitive to heat treatment.

In this technical note, an easy sample preparation and labeling scheme is described using commercially available fluorescent tag (Figure 3) which doesn't require buffer exchange nor dye clean-up for the low level detection of AAV8. Capillary Electrophoresis (CE) with Laser Induced Fluorescence (LIF) detection using a commercially SDS-MW chemistry kit and pre-assembled bare fused silica cartridge was successfully used to separate the impurities and characterize the AAV capsid proteins.

The fluorescent tag used in this work is a pyrilium type of dye named Chromeo P503, which is reactive to primary amines. Chromeo P503 has very weak fluorescence as free dye; less than 1% quantum yield in solution. However, upon conjugation, not only does the fluorescence quantum yield rise to 50%, it also undergoes a substantial bathochromic shift of 100 nm.[3]

Key Features

- Easy two step denature and label sample preparation.
- No buffer exchange or free-dye clean up required.
- Sensitive detection of impurities at the $1x10^{9}$ GC/mL or 5 ng/mL range, typical of therapeutic products, using Capillary Electrophoresis with LIF detection and Stacking techniques.
- Use CE-SDS MW kit with no modifications to the chemistry.

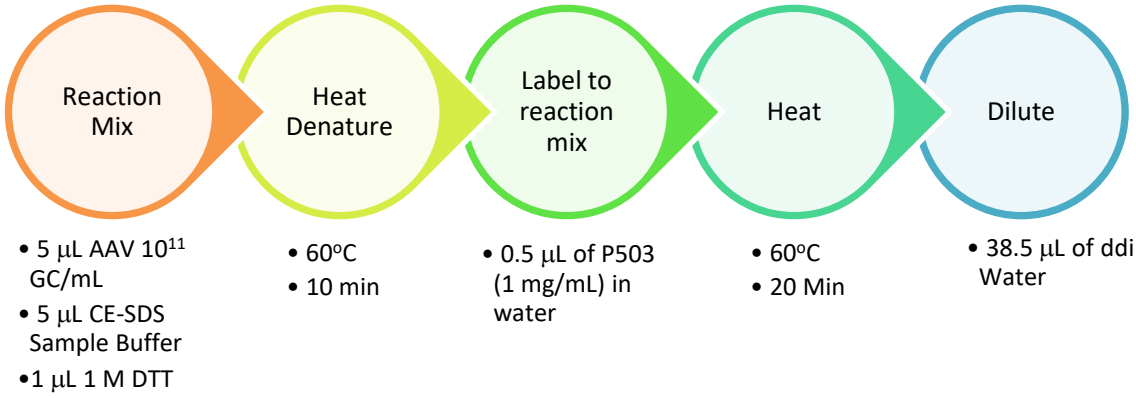

Figure 3: Simple Two Step "Denature and Label" Sample Preparation Scheme of AAV.

Materials, Instrument and Methods

Materials: The CE-SDS MW kit (PN 390953) and LIF Performance Test Mix (PN: 726022) were from SCIEX, Framingham, MA. Packaged AAV8 of pAV-CMV-GFP with titer at $1.10x10^{13}$ GC/mL (titer as supplied by vendor) was purchased from Vigene Biosciences (Rockville, MD, U.S.A.). AAV formulation buffer (1X PBS with 0.001% Pluronic F68) was also from Vigene Biosciences. Chromeo P503 catalog # 15106, was purchased from Active Motif (Carlsbad, CA). Phosphate Buffered Saline Bioreagent Suitable for Cell Culture 10X, Sigma-Aldrich, PN P5493-1L was used as base for CE-SDS custom sample buffer. Sodium Dodecyl Sulfate, J. T. Baker, PN 4095-04.

Sample storage: Upon arrival, 5 µL aliquot of AAV8 sample were stored at -80 °C freezer to avoid multiple freeze-thaw cycles.

Instrument and software: A PA 800 Plus Pharmaceutical Analysis System equipped with LIF detector and solid-state laser with excitation wavelength at 488 nm were from SCIEX (Framingham, MA) and a 600 nm band pass emission filter from Edmund Optics (Barrington, NJ). Data acquisition and analysis were performed using 32 Karat software™ V10. The separation method used in this work has already been described by Li.[4] Briefly, the CE separation method takes advantage of stacking technique by introducing a plug of water (20 psi/0.6 min) prior to the sample injections (-10 kV/60 seconds).

LIF Calibration: To ensure consistent response of LIF detector throughout this study, the LIF detector was calibrated using LIF Calibration Wizard and Performance Test Mix (PN: 726022).

Preparation of Chromeo P503 Working Solution: A vial of Chromeo P503 dye comes in 1 mg of lyophilized powder. The lyophilized powder was reconstituted by adding 1 mL of methanol. Make 10 µL aliquots to prevent contamination due to over-handling. After reconstitution, the dye label can be stored at 2-8 °C for six months according to the manufacturer's instructions.

Sample Dilution Procedure Prior to Labeling: One 5 µL aliquot of AAV8 sample was taken out of the freezer and diluted as follows: To prepare $1.10x10^{11}$ GC/mL: 1 microliter of AAV8 $1.10x10^{13}$ GC/mL was added to a 99 µL of 50 mM phosphate buffer solution pH 8. To prepare $1.10x10^{10}$ GC/mL: 1 microliter of AAV8 $1.10x10^{13}$ GC/mL was added to a 9 µL of 50 mM phosphate buffer solution pH 8.

Sample Denaturing Procedure Prior to Labeling: 5 µL of AAV8 diluted as in previous session were mixed with 5 µL of Tris sample buffer and 1µL of 1M DTT. Both reaction mixes were briefly vortexed for proper mixing and heated to 60 °C for 10 minutes. After, the reaction tubes were allowed to cool down to room temperature.

Sample Labeling Procedure:. In this protocol, the least amount of sample possible is used, which may lead to challenges in pipetting very small volume. Scaling up the sample prep is possible and should be used if sample volume is not an issue. 0.5 µL of 1 mg/mL of Chromeo P503 Labeling Working Solution was added to each reaction tube and briefly vortexed. Once again, both tubes were heated to 60 °C for 20 minutes. Afterwards, both tubes were first allowed to cool to room temperature and then 38.5 µL of DDI water was added to either reaction tube. After labeling and dilution, the final concentrations are AAV8 $1.1X10^{10}$ and $1.1X10^{9}$ GC/mL respectively.

Results and Discussions

AAV Process and Product Related Impurities[5]: Similar to a therapeutic monoclonal antibody production, there are stringent manufacturing requirements for the removal of product and process related impurities. Furthermore, sensitive analytical techniques for the detection and quantitation of impurities for product safety. This work is focused on the capsid impurities.

CE-SDS has been used as the gold standard for the characterization of therapeutic proteins from manufacturing to lot release. Combining the reproducibility and specificity typical of CE-SDS with sensitivity of LIF detection and easy to label sample preparation, AAV capsid protein purity can be easily determined.

Figure 4 shows a typical profile of P503 labeled AAV8 at the target concentration (10^{10} GC/mL) of clinical formulation compared to an absorbance UV. The labeling drastically improves sensitivity.

Formulation buffer and the impact on assay sensitivity: The type of injection typically used in CE-SDS assays is electrokinetic, where a low voltage is applied to drive charged ions into the capillary. However, there is an inherit bias towards highly charged small ions in electrokinetic injection. The combination of a large protein molecule of interest, such as capsid proteins, present in formulation buffer with high salt content will decrease the sensitivity of the assay, due to the dramatic difference in mobilities. Smaller ions move faster during electrokinetic injection than their larger protein counterparts. Figure 5 shows a comparison of the electropherogram of AAV8 at the limit of detection (1.1 10^9 GC/mL) in 2 different formulation buffers. Red trace is AAV8 in 1X PBS which has a much higher salt content thus lower peak height, compared to AAV8 in 50 mM phosphate buffer. Additionally, the higher pH of phosphate buffer promotes the labeling reaction. In this figure the signal to noise ratio were: 31, 3 and 7 for VP3, VP2 and VP1, respectively. This is within the acceptable S/N of 3 for limit of detection.

Additionally, the relative proportions between the capsids VP3, 2 and 1 were 10.8:1:1.8 respectively. While we demonstrated a separation of trace amounts of AAV with good signal to noise, this was accomplished with an increased amount of dye to protein ratio than recommended by the manufacturer.[3] We found this necessary to promote labeling. However, we observed a significant tailing effect (Figure 5). This condition may be mitigated with buffer exchange and concentration of the initial AAV sample to 10^{11} or higher GC/mL.

When working with labeled protein molecules, it is important to run a blank to ensure proper peak assignment. Even though

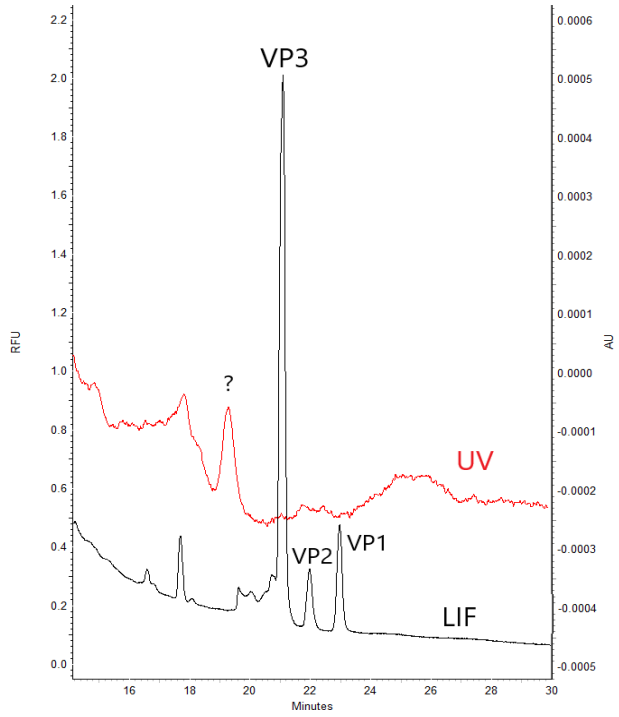

Figure 4. Comparison Between AAV8 1.1x10[10] Using UV Absorption (red trace) and LIF (black trace). Left Y axis shows RFU units and Right Y axis show Absorbance. ? denotes an unknown peak.

P503 dye used in this work has very low quantum yield when unbound (<1%)[3], it is reactive towards primary amino groups and CE-SDS separation gel in a 100 mM Tris sample buffer. For this reason, a blank composed of AAV diluent buffer, formulation buffer and CE-SDS sample buffer was used. Figure 6 shows an overlay of the electropherograms from Blank and AAV8 at 1.1x10^9 and 10^{10} sample analysis.

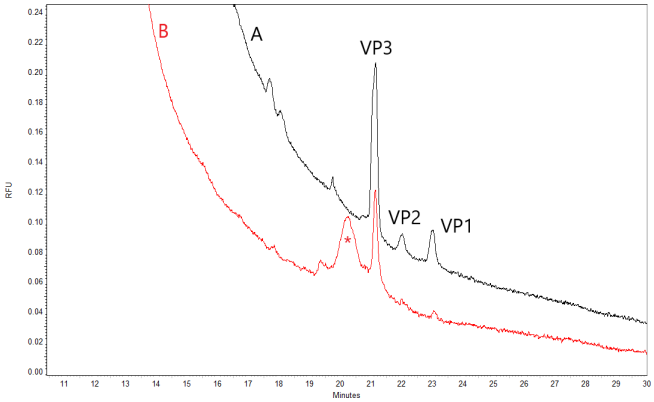

Figure 5. Comparison Between Different Salt Levels in Formulation Buffer of AAV8 at Low Trace Levels 1.1x10[9] GC/mL. (Red) 1X PBS; (Black trace) 50 mM Phosphate buffer pH 8. *marks an artifact from blank.

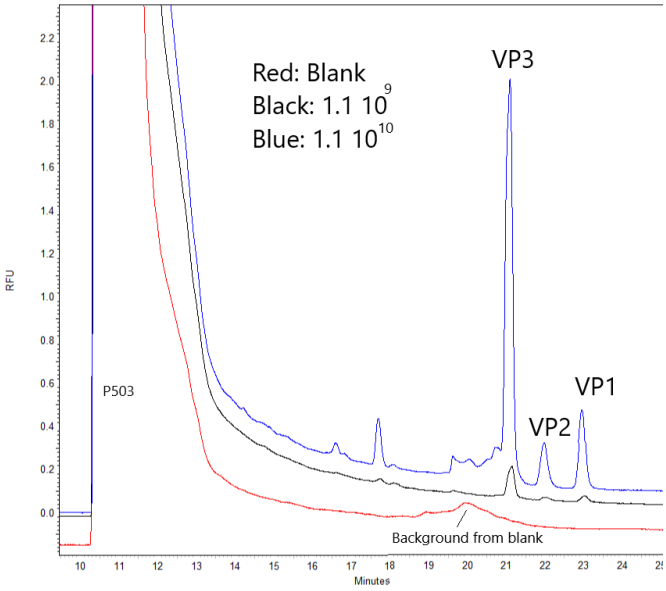

Figure 6. Overlay of Blank (red trace) and AAV8 at 1.1x10^9 (black trace) and 1.1x10^{10} (blue trace). Note the raise in the blank run signifying a labeled product that cannot be attributed to an impurity in the AAV.

Even though AAVs are stable, degradation is of great concern in any biopharma product. Figure 2 (see front page) showcases the comparison between heat stressed at 93 ^{0}C for 30 minutes and non-stressed AAV8 samples at the same concentration 1.1x10^{10} GC/mL. Generally, there is a significant decrease in the overall peak intensity for the heat stressed samples. It is worth mentioning the considerable change in the profile of the electropherogram in the heat stressed samples specially for the lower molecular weight peaks presumed attributed to possible impurities in the original sample, are sensitive to the heat. Demonstrating that the proposed labeling scheme is suitable for assessing purity of AAV8 capsid proteins.

Conclusions

- CE-SDS MW kit when combined with LIF detection can successfully achieve trace levels detection of AAV8 capsid proteins, successfully analyzing AAV8 at limit of detection at 1.1x10^9 GC/mL.
- Easy 2 step labeling with P503 dye does not require buffer exchange prior to analysis.
- By using formulation buffer with low salt concentration and slightly higher pH, while still keeping AAV8 stability, allows for better sensitivity due to favorable electrokinetic injection conditions and improved sensitivity due to advantageous labeling environment.

References

1. M.Agbandjie-McKenna *et al.; Molecular Therapy; Methods & clinical Development*, (2017), ***6***, 171-182.
2. B. Bothner *et al.; Journal of Virology,* (2013), ***87-24***, 13150-13160.
3. Chromeo P503 product insert, https://www.activemotif.com/documents/1641.pdf
4. Purity Analysis of Adeno-Associated Virus (AAV) Capsid Proteins using CE-LIF Technology; SCIEX Technical Note RUO-MKT-02-10086-A.
5. J. Fraser Wright, *Biomedicines* (2014), **2**, 80-97.

For Research Use Only. Not for use in Diagnostic Procedures. Trademarks and/or registered trademarks mentioned herein are the property of AB Sciex Pte. Ltd., or their respective owners, in the United States and/or certain other countries.

AB SCIEX™ is being used under license. © 2019 DH Tech. Dev. Pte. Ltd. RUO-MKT-02-10600-C

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
sciex.com

International Sales
For our office locations please call the division headquarters or refer to our website at
sciex.com/offices