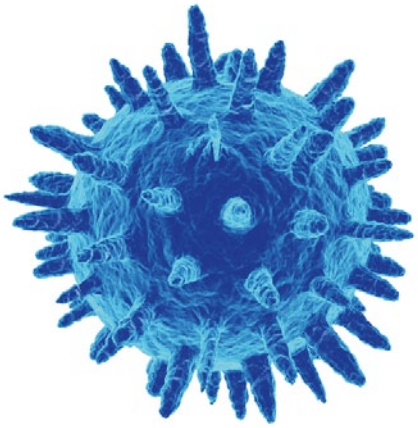
Separation and Characterization of Viruses and Antibodies Using Multi-Detector Field-Flow Fractionation

Introduction



Viruses are the biggest story of 2020 with the global coronavirus pandemic thrusting viruses to the forefront of almost every person's consciousness around the world. Development of antibodies to specific viruses form the core of future immune response.
Characterization of viruses, the development of vaccines, and the quantification of antibodies, have become the most important scientific pursuits for many labs.

Vaccines can be complex and may contain many species which span a large size range from antibodies, virus fragments such as proteins and nucleic acids, polysaccharide-protein complexes, and up to large aggregates often over 100 nm in diameter. This means these samples are challenging to separate and characterize by column-based chromatography techniques such as size exclusion chromatography (SEC). Asymmetrical Flow Field-Flow Fractionation (AF4) can help researchers in this field as it is a stationary phase-free separation technique for characterization of polydisperse samples from about 1 nanometer to 1 micron in diameter [1,2] and has already shown its merits in the characterization of viruses and virus-like particles [3,4] as well as antibodies and antigens and their aggregates and agglomerates [5,6].

A schematic for the AF4 channel is shown in Figure 1. The combination of cross flow and channel flow cause size separation over the course of the analysis, with smaller particles eluting to connected detectors before larger particles, including aggregates. In an alternative configuration, an electric current can be applied between the channel top and bottom to enable the measurement of surface charge and Zeta potential. This sub-technique is called Electrical-AF4 (EAF4).

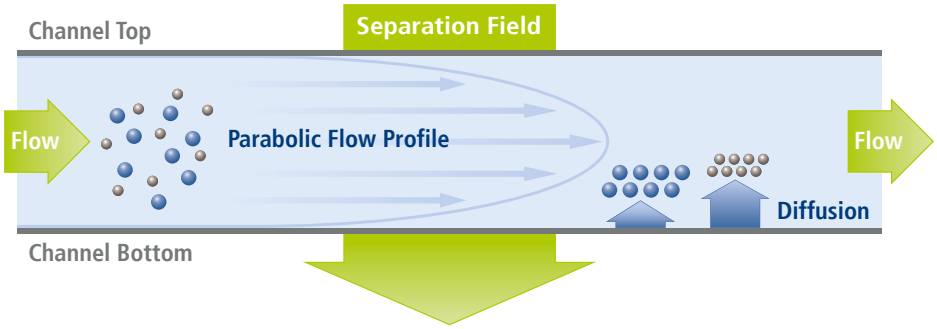


Figure 1. AF4 channel schematic.

For characterization of the separated particles or molecules a variety of detectors can be connected to the AF4, including dynamic light scattering (DLS) and multi-angle light scattering (MALS) for size measurement, with the latter also providing data on molecular weight when combined with a concentration detector such as UV/Vis or refractive index.

In this Whitepaper, AF4 and EAF4 coupled with multiple detectors were used to separate and characterize:

- Adeno-associated virus (AAV) monomers and their fragments/aggregates
- A mixture of two AAV serotypes after exposure to heat stress
- Separation of monoclonal antibodies (mAb) and quantification of mAb aggregates

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Characterization of Stressed-AAV Fragments and Aggregates with a Single Method Using AF4-MALS

AAVs are increasingly used for gene therapy due to their versatility and safety. They can be loaded with DNA or RNA and delivered to a specific cell type, with the goal being to treat or cure disease [7]. One of the biggest concerns for producing a uniform AAV sample is the purity of the virus monomer in solution, as both fragments and aggregates can be present in significant amounts.

An AAV sample was divided into two aliquots, one of which was stressed using a confidential method to induce fragmentation and aggregation. Two samples were analyzed, "non-stressed" and "stressed" AAVs. To separate by size and characterize the AAVs and their fragments/aggregates, an AF4 system (Postnova AF2000) was used with two detectors monitoring the eluent. Firstly, a Postnova 21-angle multi-angle light scattering detector (MALS, PN3621) for measuring the radius of gyration (R_g); and secondly a UV/Vis detector (PN3211) which is sensitive to virus concentration and can be used to calculate the relative amount of fragment/aggregate present compared to AAV monomer. A carrier solution of 1x phosphate buffered saline was used in the experiments.

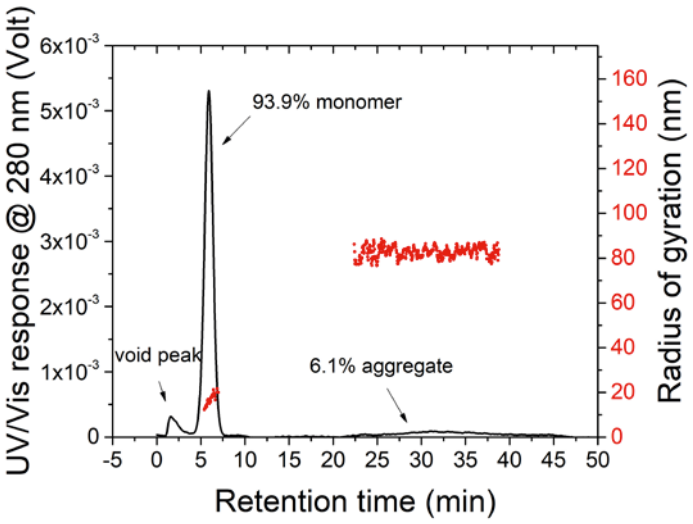

Figure 2. Separation of a non-stressed AAV sample: UV-based fractogram (black traces) overlaid with R_g values (red dots).

In Figure 2, the UV/Vis response is plotted as the red trace, with R_g calculated at each time point from MALS data (not shown) and plotted as red dots. The void peak (analogous to the solvent peak in chromatography) elutes first, around 2 minutes. The UV/Vis detector is sensitive to concentration, and the relative amount of each species is calculated by integrating the area under the peaks, excluding the void peak. The monomer elutes starting around 5 minutes, and has a measured Rg consistent with most AAVs, around 12-15 nm in radius. The large aggregates in the non-stressed AAV sample are monodisperse, with an R_g around 80 nm.

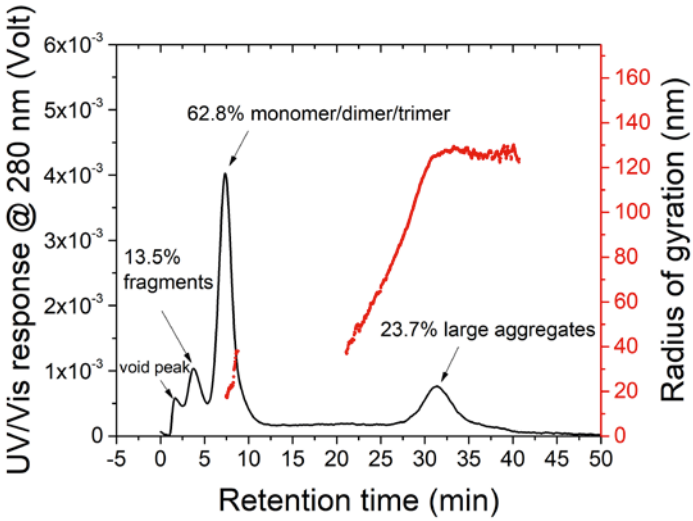

Figure 3. Separation of a stressed AAV sample: UV-based fractogram (black traces) overlaid with R_g values (red dots).

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The UV response and R_g measurements for the stressed AAV sample are shown in Figure 3. Fragments generated during stressing the sample comprised 15.0% of peak area. These fragments were too small for their R_g to be measured by MALS (< 8 nm radius.) The peak between 5 and 10 minutes contains the AAV monomer, which was also observed in this retention time range in the non-stressed sample. However, in the stressed sample, a population of larger R_g values is observed eluting later in this peak, likely due to dimers and trimers having formed during the stressing protocol. For the non-stressed sample, the aggregate was present at 6.1% (Figure 2), and for the stressed sample the aggregate was present at 23.7% (Figure 3). For the stressed AAV, there is substantial polydispersity in the aggregate size, ranging from about 40 nm to 120 nm in radius.

In addition to quantification of a wide size range from small virus fragments to large virus aggregates, fractions can be collected at any point in the fractogram. This could be done to enable further off-line analysis of nucleic acids or proteins contained in the fragments.

Studying Stability of Two AAV Serotypes Under Heat Stress Using AF4-MALS

One of the critical quality attributes that needs to be quantified for a safe and effective AAV product is its stability. In this application, AF4-MALS with a UV/Vis diode array detector was utilized to study the aggregation of two AAV serotypes when they are stressed by heat.

Two AAV serotypes, AAV1 and AAV5, with stock concentrations of $2x10^{13}$ particles/mL, were used for this study. The samples were diluted 5x with the formulation buffer (phosphate buffered saline (PBS) containing 0.001% (v/v) 10% Pluronic F-68) prior to analysis. The samples were stressed at 60°C and 75°C for 35 minutes. An AF4 system (Postnova AF2000) system interfaced with a MALS detector (Postnova PN3621) and a diode array UV/Vis detector (Postnova PN3241) were utilized to analyze the AAV samples using the formulation buffer as the AF4 carrier solution. For each analytical run a 50 µL injection of the prepared sample solutions was used.

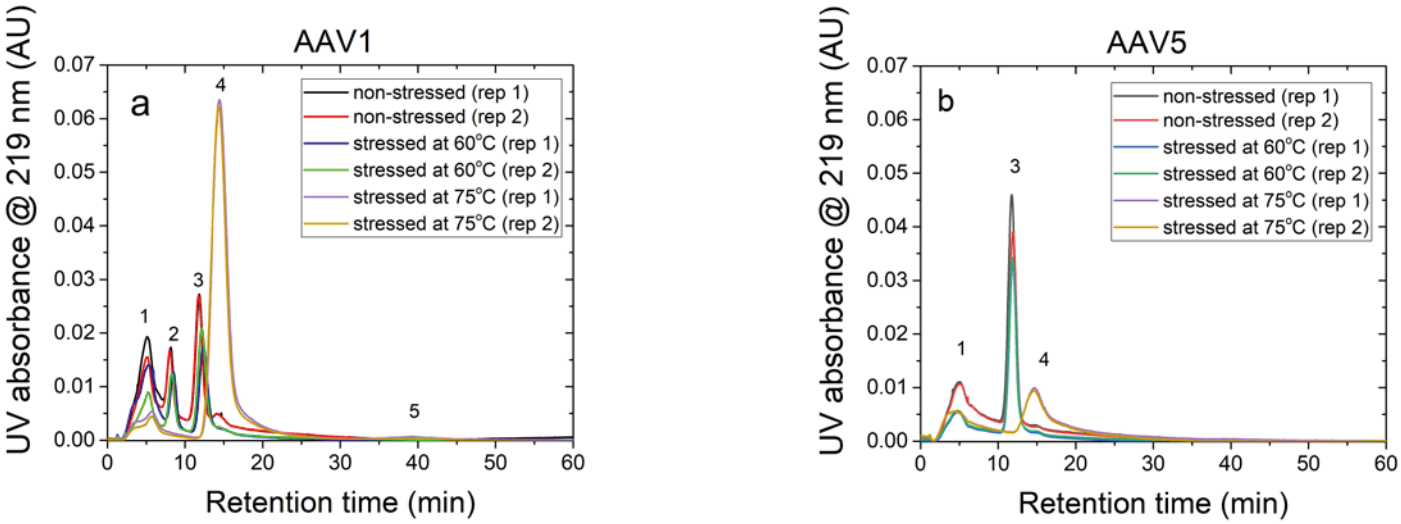

Figure 4 a and b: UV absorbance at l = 219 nm versus retention time of two serotype AAV samples at room temperature and stressed at 60°C and 75°C, a) AAV1; b) AAV5, inserted numbers represent different components, 1 and 2: fragments, 3: monomer, 4: oligomers, 5: larger aggregates.

AF4 is a well-suited separation technique to study the aggregation of AAVs. Due to its open-channel architecture, AF4 can separate aggregates much larger than what can be accomplished by column chromatography. Figures 4a and 4b show plots of the UV signals obtained at 219 nm absorbance wavelength versus retention time for non-stressed and stressed AAV1 and AAV5 serotypes. The numbered peaks in the graphs are the subpopulations present in the samples which have been separated by AF4 based on their hydrodynamic sizes.

Eluting first are the smaller viral fragments in peaks 1 and 2 followed by the virus monomer and oligomers in peaks 3 and 4 respectively. Peak 5 contains the larger virus aggregates that elute latest in the separation (35 to 60 minutes), which are detected only for the stressed AAV1 sample at 75°C. For non-stressed AAV1, monomeric and oligomeric viral particles constitute about 26% and 7% of the sample, respectively. The non-stressed AAV5 sample contained more monomer than AAV1, with 43% in the monomeric and 5% in the oligomeric forms.

The data indicates that both serotypes are reasonably stable up to 60°C. However, they exhibited a significant change in their size distributions when they were stressed at 75°C. In both stressed samples, the monomer (peak 3) vanished completely, and the oligomer peak (peak 4) grew substantially (66% for AAV1 and 26% for AAV5). This suggests that the virus particles form oligomers as they undergo heat stress.

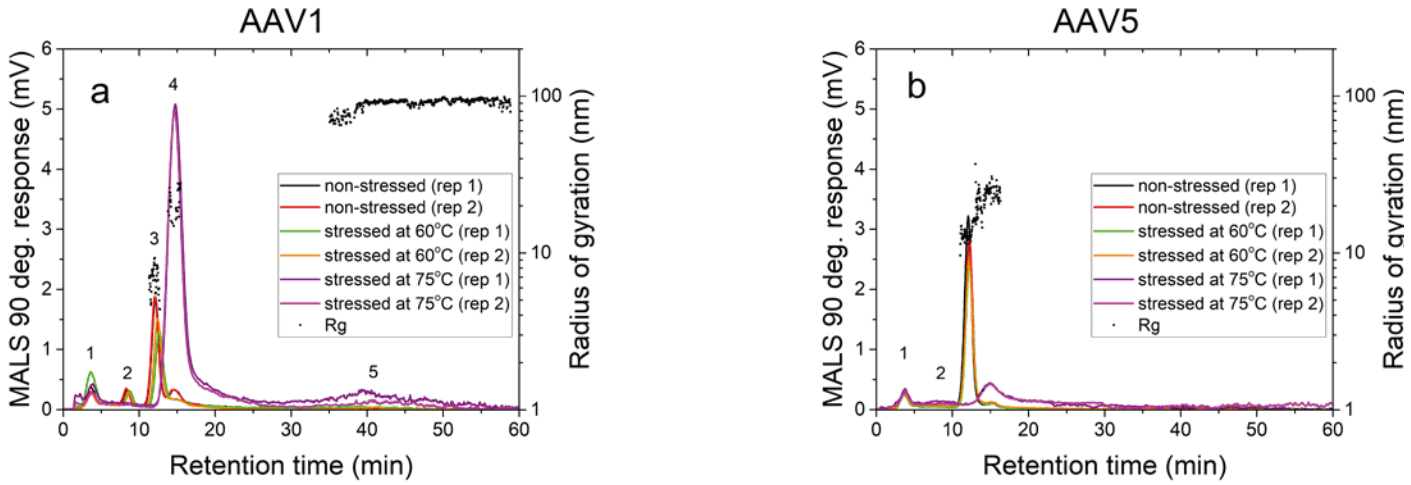

Figure 5 a and b: 90° light scattering signal-based fractograms and radius of gyration (R_g) profiles of two serotype AAV samples at room temperature and stressed at 60°C and 75°C, a) AAV1, b) AAV5, inserted numbers represent different components, 1 and 2: fragments, 3: monomer, 4: oligomers, 5: larger aggregates.

The AAVs' radius of gyration (R_g) in non-stressed and stressed samples was obtained from evaluation of the MALS signal intensities at different angles. The R_g data points (black circles) of non-stressed and stressed AAV1 and AAV5 samples are shown in figures 5a and 5b, respectively. Each point represents a size measurement for a volume of sample passing through the MALS flow cell. On average, virus monomers (peak 3) are in the order of 8-10 nm in radius. The oligomer peak varies from 10-30 nm in radius. The large aggregates (peak 5), only present in the stressed AAV1 sample, are ~100 nm in radius and represent approximately 2% of the population.

Electrical Asymmetrical Flow FFF Analysis of the NIST Monoclonal Antibody Reference Material 8671

The U.S. National Institute of Standards and Technology (NIST) has produced a reference material monoclonal antibody (RM 8671 NIST mAb), intended for use in evaluating the performance of methods for determining physicochemical and biophysical attributes of mAbs [8]. It provides a representative test molecule for development of novel technology for therapeutic protein characterization. The NIST mAb is intended for a variety of uses that may include system suitability tests, establishing method or instrument performance and variability, comparing changing analytical test methods, and assisting in method qualification. In this work, the NIST mAb is used to compare separation, aggregation quantification, and recovery parameters for AF4 versus SEC, and to demonstrate the capabilities of the electrical AF4 (EAF4) module (Figure 6) to simultaneously measure size and surface charge characteristics of antibodies and proteins.

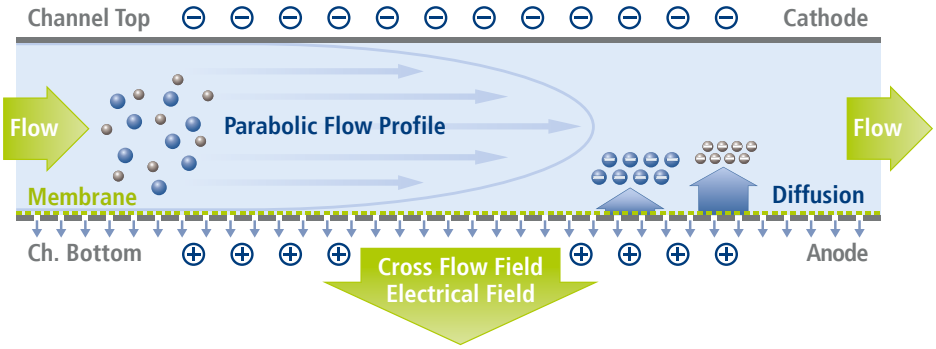

Figure 6. Schematic of the EAF4 channel. In addition to hydrodynamic size separation via the flow field, an electric field allows for further separation by a molecule's or particle's surface charge.

Experimental Details and Results

The NIST mAb was analyzed by AF4-UV-MALS (Figure 7) to evaluate separation of monomer from aggregates and determine the % of aggregates present. The analysis was performed using PBS as carrier. Aggregates measured by AF4 represented 9.4% of the total mass injected as measured by the area under the UV-Vis detector signal. Although the NIST mAb Report of Investigation [9] stated that aggregate was measured by SEC-UV to be present at 3.2%, the open-channel architecture of FFF likely allows better recovery of aggregates than column chromatography.

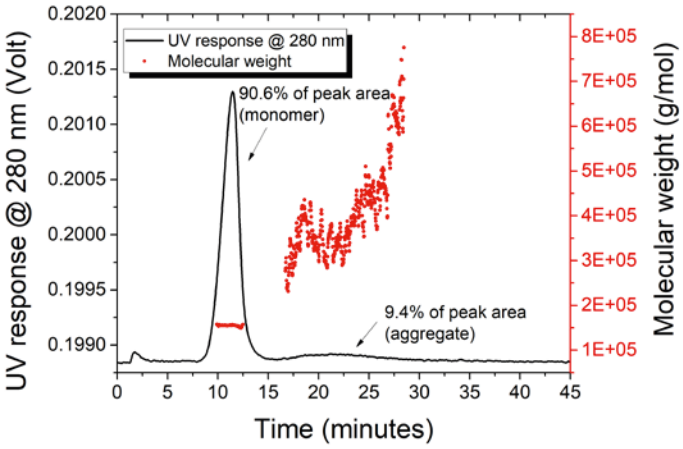

Figure 7. AF4-UV-MALS analysis of NIST mAb. UV fractogram shown in graph; molecular weight calculated from MALS and UV data.

The NIST mAb was analyzed with EAF4-UV-MALS in PBS using a control run (no electric field) and two varied electric field strengths (Figure 8). The retention time shifted toward earlier elution with greater negative charge on the channel bottom, indicating the NIST mAb is negatively charged in PBS. The shift in retention time can be related to the net drift velocity and plotted versus the applied electric field; the slope represents the electrophoretic mobility, which was measured to be -1.68 ± 0.05 µm cm V^{-1} s^{-1} under the experimental conditions in this study (Figure 9).

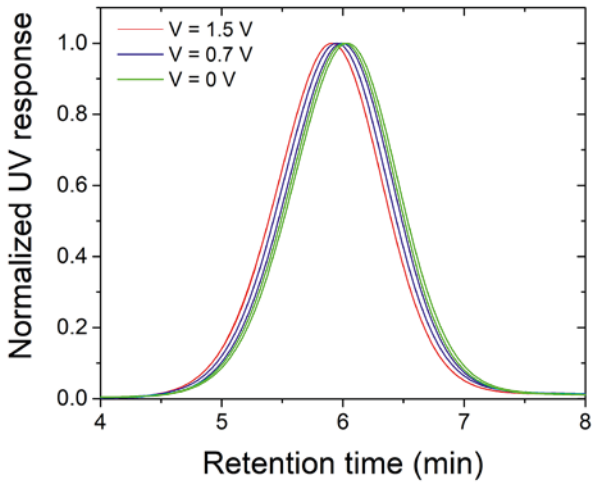

Figure 8. NIST mAb retention time shift with applied electric field.

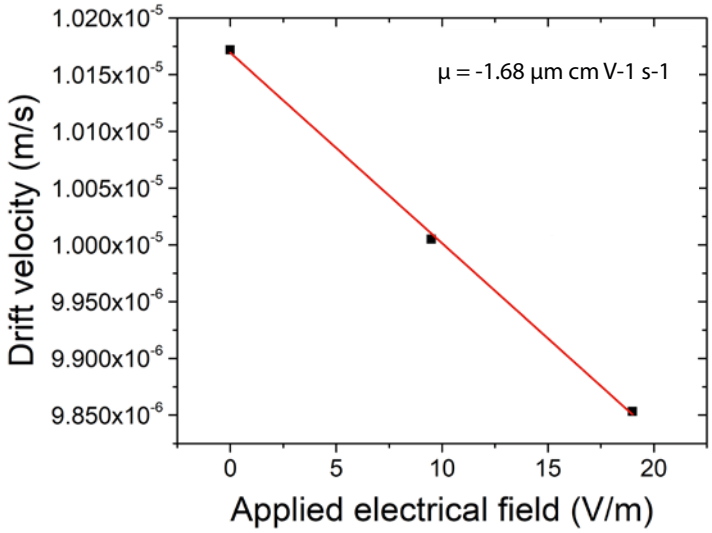

Figure 9. Measured electrophoretic mobility.

Conclusion

The open channel design of AF4/EAF4 has large advantages over chromatographic techniques as the absence of a stationary phase results in less sample or aggregate loss due to interaction or filtration in the column packing. From the data presented here, we see that AF4/EAF4 can separate a wide range of sizes within a single method, from virus fragments less than 10 nm in radius, to large aggregates above 100 nm in radius. It can be equally applied to any or all the components in virus and vaccine research from small antibodies of only a few nm in size, up to large protein-polysaccharide complexes or virus particles and aggregates of well over 100 nm.

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