APPLICATION NOTE

AN9010: Characterizing lentiviral and gammaretroviral vectors by batch DLS and FFF-MALS

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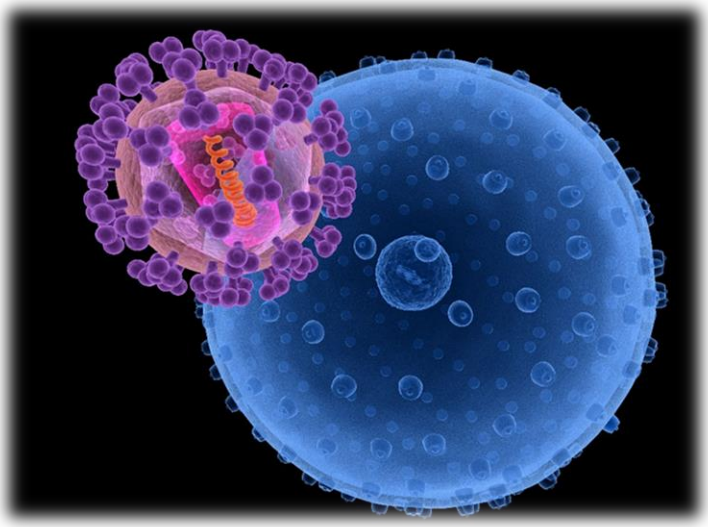

Summary

Lentivirus and gammaretrovirus are the most popular retroviral vectors under development for novel gene and cell therapies, with more than 300 products in clinical trials as of 2021.[1] As they enter into late development stages, quantifying the quality attributes and other properties of these products is of increasing importance. This note demonstrates the use of batch dynamic light scattering (DLS) and online multi-angle light scattering and dynamic light scattering in combination with field-flow fractionation (FFF-MALS-DLS) for characterizing these large viral vectors. DLS in batch mode rapidly screens particle size distribution, concentration/titer and stability. FFF-MALS-DLS, on the other hand, provides high-resolution size distributions and particle concentration along with an in-depth assessment of aggregation and impurities. The details revealed by FFF-MALS-DLS help scientists and process engineers understand lot-to-lot variations and critical manufacturing parameters, enabling production of these novel therapeutic products with consistently high quality.

Introduction

In addition to adeno-associated virus (AAV) and adenovirus, lentivirus (LV) is another virus type commonly used in gene therapy and genetically modified cell therapy. Lentiviruses belong to the retrovirus family; another member that is also used in these new therapies is gammaretrovirus. Retroviruses are enveloped spheres with diameters between 50-150 nm[1].

Though lentiviral vectors (LVV) are preferred over gammaretroviral vectors (GRVV) because of their reduced immunogenicity and genotoxicity, both are used in laboratories around the world for *in vivo* and *ex vivo* gene delivery as well as in clinical and preclinical investigations. However, consistent production under cGMP is still challenging, with excessive handling, consumables and time required as well as lack of suitable analytics for process development.[2]

Filtration, ultracentrifugation and liquid chromatography have been used for downstream purification in order to achieve high-quality vectors.[3,4] The impact of critical manufacturing parameters on vector quality at each of the upstream and downstream process stages is assessed with multiple biological and analytical assays. These include:

- SDS-PAGE to assess capsid purity and investigate protein contaminants;
- negative-stain electron microscopy (EM) to determine empty/full capsid ratios;
- p24 ELISA to measure viral titer;
- RT-qPCR to quantify genome containing particle;
- gene specific functional assays; and

- endpoint infectious assay by qPCR to quantify proviral DNA.[5]

Many of these assays suffer from poor repeatability, extensive hands-on effort and long analysis time. They also require additional validated reagents which may introduce variability as well as significant cost.

In this study, we discuss a simple and robust workflow for biophysical characterization of retroviral vectors (RVV) at different stages of the manufacturing process. It incorporates batch DLS-based methods and field-flow fractionation with multi-angle and dynamic light scattering (FFF-MALS-DLS). This reagentless, automated protocol assesses formulation stability, particle size, polydispersity, particle concentration, impurity profiling and batch-to-batch reproducibility. Quantifying multiple product attributes in a single, short run greatly facilitates process understanding, leading to rapid and thorough process optimization.

FFF separation technique

Because the size of RVV ranges from 50 to 150 nm in diameter, which is generally too large for size-exclusion chromatography (SEC), the separation technique of choice is asymmetric-flow FFF (AF4). Using Wyatt Technology's Eclipse™ FFF system combined with its DAWN® MALS instrument and other online detectors, we readily achieve extended characterization of retroviruses.

In FFF, sample separation takes place in an open channel with no stationary phase, where separation is achieved during longitudinal flow along the channel. Separation derives from a field applied perpendicular to channel flow. In the case of AF4, that field is the cross-flow of the solvent/mobile phase through a semipermeable membrane.

Before separation, the entire sample is focused in a narrow band near the inlet of the channel and concentrates at the membrane. As illustrated in Figure 1, the balance of forces between cross flow and diffusion results in each component of the sample achieving a different average height in the channel, based on its hydrodynamic volume: smaller particles diffuse faster and end up (on average) higher above the membrane, while larger particles stay closer to the membrane. The parabolic profile of the channel flow leads to a differential longitudinal solvent velocities and therefore different elution speeds for each size (Figure 1). Hence, akin to SEC, particles are separated based on hydrodynamic size, but in the case of FFF, smaller particles elute first (opposite to SEC).

Downstream of FFF separation, a set of light scattering and concentration detectors is used to determine the distributions of molar mass and radius, particle concentration and other biophysical properties of the sample.[6]

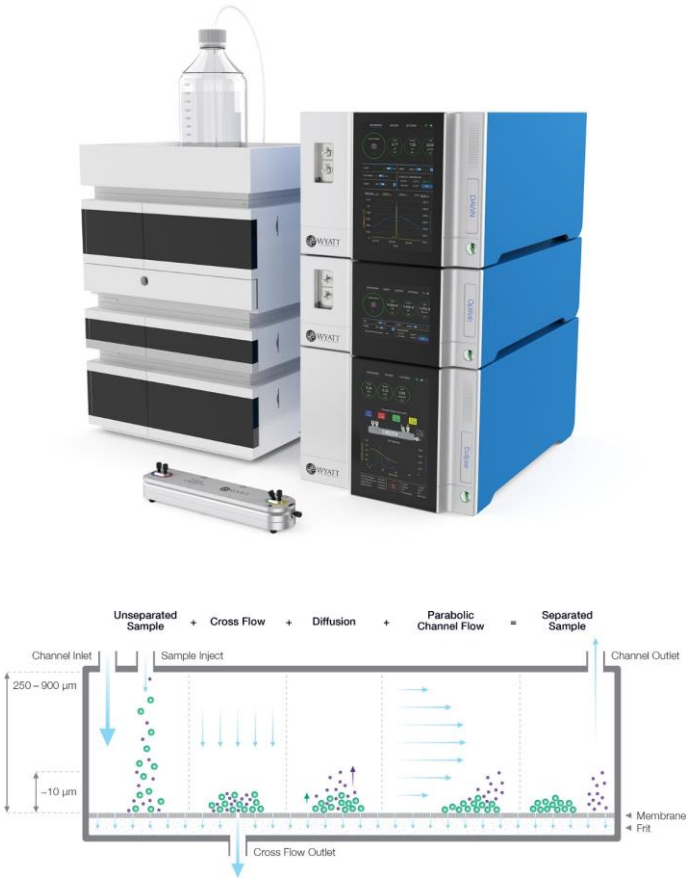

Figure 1. Top: a Wyatt FFF-MALS system comprising autosampler and pump, Eclipse FFF instrument and separation channel (foreground), DAWN MALS detector, Optilab RI detector and UV detector. Bottom: Cross-section of an FFF channel illustrating the principles of AF4.

Materials and Methods

Samples used for this application note consisted of LVV and GRVV from different stages of the manufacturing process. All samples were prescreened by batch DLS in a high-throughput manner using a DynaPro® Plate Reader DLS/SLS instrument. Results were analyzed using DYNAMICS® software.

The FFF setup included an Agilent® 1260 Infinity™ HPLC pump, autosampler, and DAD UV detector. Phosphate-buffered saline (PBS) was the mobile phase. Separation

was achieved on the Eclipse short channel with 275 µm spacer thickness and a 30 kDa MWCO regenerated cellulose membrane. FFF flow rates (channel flow and cross flow) were controlled by the Eclipse. Size, molar mass, and particle concentration were measured using the DAWN MALS detector with embedded DLS module, and an Optilab® differential refractive index detector. FFF operation was orchestrated with the VISION™ FFF program, and analysis was performed in ASTRA® software.

In addition to analysis by FFF-MALS-DLS, measurement by size-exclusion chromatography with MALS (SEC-MALS) was also performed. SEC-MALS analysis was carried out on the same system, wherein the Eclipse can switch automatically between SEC and FFF modes. SEC separation made use of two columns in tandem, Shodex OHpak SB-807 and SB-805. In SEC mode the mobile phase, PBS, was flowed at 0.5 mL/min.

Results and Discussion

Batch DLS: Rapid sample screening

The characterization workflow, presented here, starts with batch dynamic light scattering (DLS) measurements. Batch DLS provides a quick estimate of particle size distribution and particle concentration (physical titer), with each sample generally requiring 30 seconds of measurement time. The DLS measurement, performed in either the cuvette-based DynaPro NanoStar® or the plate-based DynaPro Plate Reader, is an important step towards successful sample characterization because the results help us understand whether the sample concentration and purity are appropriate for the subsequent SEC-MALS and/or FFF-MALS analysis. Batch DLS measurements do not alter the sample and require very few sample constants or parameters to be known *a priori*.

In Figure 2, the DLS-determined size distributions of a RVV produced under three varied purification conditions are overlaid. A broad distribution was seen for all three samples, and a population of smaller species was also observed in one sample. These results not only render some useful initial information about these RVV samples but also aid the FFF method development that follows as the next step in the workflow.

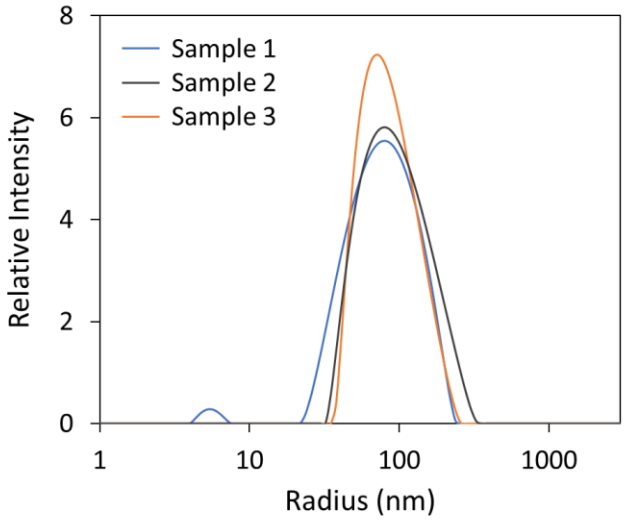

Figure 2. Size distributions of a retroviral vector purified under three different conditions.

FFF: High-resolution separation

To ensure the safety and efficacy of the final products, viral vectors must be produced consistently with high quality. Due to RVV's innate heterogeneity and manufacturing complexity, achieving high quality requires a thorough understanding of the manufacturing parameters and the ability to differentiate small lot-to-lot variations. Though DLS provides a quick—yet valuable—analysis of RVV size distributions, its resolution is rather low. FFF provides reproducible, high-resolution separations, thus meeting key characterization requirements.

After confirming that the three aforementioned RVV samples had adequate purity and concentration, they were then separated by FFF and characterized by online MALS and DLS. The 90°-angle static light scattering traces and hydrodynamic radii distributions measured by the online DLS are overlaid in Figure 3.

The detailed sample profiles obtained with FFF-DLS and batch DLS may be correlated against RVV purification conditions in order to understand the effect of purification parameters. A peak at around 26 minutes was seen in Sample 1 with a hydrodynamic radius of approximately 3.5 nm, which corresponds to a small protein in the formulation buffer. Additionally, Sample 1 has a significant impurity peak (27 to 40 min, with sizes of 10- 45 nm), likely from the cell culture.

Sample 3 has the least quantity of formulation protein and impurities from cell culture, but also the lowest

concentration of viral particles. The peaks eluting from 48 to 68 minutes have radii ranging from 50 to 150 nm, consistent with the expected size for RVV. The RVV titer, calculated from MALS data using ASTRA's *Number Density* module, varies from 2.0 x 10^9 to 1.6 x 10^{10} particles/mL in these three samples. The detailed results are tabulated in Table 1.

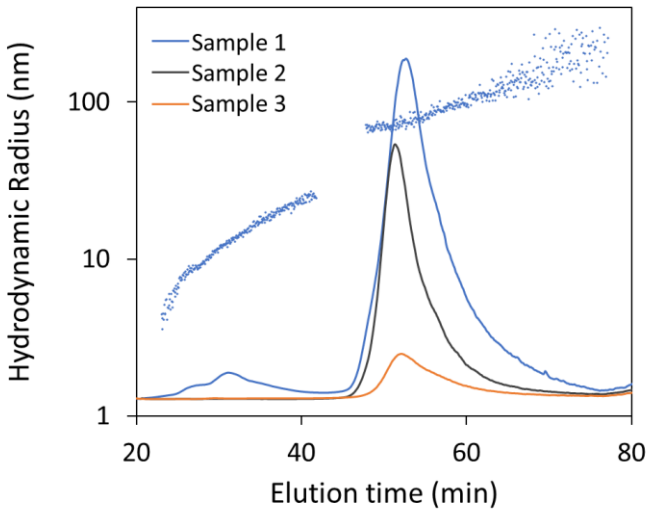

Figure 3. LS fractograms overlaid with hydrodynamic radius from online DLS for the same three LV samples as in Figure 2.

Table 1 presents viral particle size and concentration (physical titer) that were collected by both batch DLS and FFF-MALS. The agreement between FFF-MALS and batch DLS on particle concentration is quite good, showing the same concentration trend of these three samples. Fractionation based on size not only allows deeper sample characterization with much higher resolution compared to batch DLS, but also eliminates bias arising from impurities that are close in size to the sample of interest. Such impurities cannot be resolved by batch DLS measurement and therefore bias the DLS results.

Through these RVV samples, FFF is seen to provide clear differentiation of RVVs produced under different conditions, at different production stages, or from different lots in terms of size, titer and impurities. Understanding the difference in low-molar-mass impurities can provide useful information not only about sample purity and stability but also to help select appropriate orthogonal techniques for additional sample characterization. In summary FFF-MALS-DLS and batch DLS results help scientists and process engineers optimize the manufacturing process and product quality of these complex vectors.

Table 1. Characterization data for retroviruses that were collected using a DynaPro Plate Reader and an Eclipse/DAWN FFF-MALS system. PC = particle concentration, i.e. physical titer.

Sample	R_{hz} from FFF-DLS (nm)	PC from FFF-MALS (mL^{-1})	R_h from DLS (nm)	PC from DLS (mL^{-1})
1	99.2 ± 0.6	$(1.62 \pm 0.01) \cdot 10^{10}$	79.2 ± 0.8	$(1.3 \pm 0.2) \cdot 10^{10}$
2	94.9 ± 0.4	$(9.68 \pm 0.02) \cdot 10^{9}$	85.3 ± 1.1	$(5.1 \pm 0.1) \cdot 10^{9}$
3	114 ± 2	$(1.98 \pm 0.01) \cdot 10^{9}$	92.0 ± 0.9	$(1.5 \pm 0.1) \cdot 10^{9}$

FFF-MALS: In-depth sample characterization and quantification

One of the important parameters when it comes to LVV characterization is genome loading. Robust quantification methods using light scattering already exist for smaller viral vectors such as AAVs[7] but is more challenging for larger viruses. Here we present a method that provides qualitative differentiation of empty and filled LVVs. For this, we used empty and full lentiviral vectors that were purified by ultracentrifugation, kindly provided by Baylor College of Medicine Gene Vector Core.

Two different protocols were used to produce LVVs with and without genetic content, confirmed by an RT-qPCR assay for vector RNA genome and by end point proviral DNA quantitation after infection of HEK293 cells. FFF separation followed by online MALS-DLS detection determined two distinct size-related parameters: root-mean-square radius, a.k.a radius of gyration (R_g) from MALS, and hydrodynamic radius (R_h) from DLS. The ratio R_g/R_h, otherwise termed the 'shape factor', is indicative of the particle's structure. Shape factor values for several common nanoparticle shapes are tabulated in WP2611: Characterization of nano-pharmaceuticals with field-flow fractionation and light scattering (FFF-MALS-DLS).

Two light scattering fractograms are shown in Figure 4, overlaid with the R_g/R_h ratio. A viral vector that contains genetic material tends to have more mass closer to the center of mass and thus has a smaller rms radius compared to an empty vector of the same physical diameter. On the other hand, the hydrodynamic radius, R_h, will be the same for empty and full LVV of the identical physical diameter because R_h is determined by the diffusion coefficient of the particle. The ratio of these two radii obtained from MALS and DLS distinguishes empty from

full vectors. Recalling that the elution time in FFF is dependent solely on the diffusion coefficient, we see that empty and loaded vectors eluting at the same time have the same diffusion coefficient, and therefore the same R_h, but different R_g values, and FFF-MALS determines this effect independently for each eluting size. The traditional approach to distinguishing empty and full viral vectors, cryo-EM imaging, is unable to adequately differentiate them (Figure 4, bottom panel).

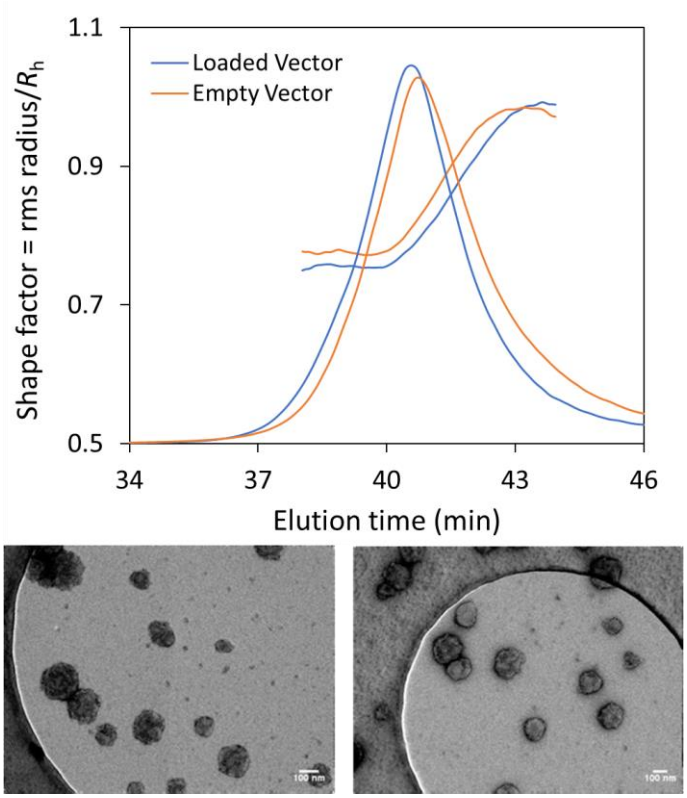

Figure 4. Top: Empty (red) and loaded (blue) LVV light scattering fractograms overlaid with the R_g/R_h ratio . Bottom: Cryo-EM images of empty (left) and full (right) lentiviral vectors.

FFF vs. SEC for viral vector separation

Since SEC is more readily available than FFF for most labs, it is of value to compare and understand the separation performance of these two methods for GRVV. In order to minimize the removal of large-sized viral particles in the sample, we chose SEC columns with appropriately large pore sizes. LS traces obtained from both SEC and FFF are overlaid together with R_g, the rms radius from MALS, in Figure 5. Even though the same volume of sample was injected (50 µL) for both SEC and FFF analysis, recovery of the GRVV sample from SEC is significantly lower than from FFF. The SEC columns also removed or degraded particles above 87 nm while the resolution for species less than 30 nm is absent. Because of its low recovery, poor resolution, and misleading results, we do not recommend using SEC for the separation of gammaretrovirus or lentivirus.

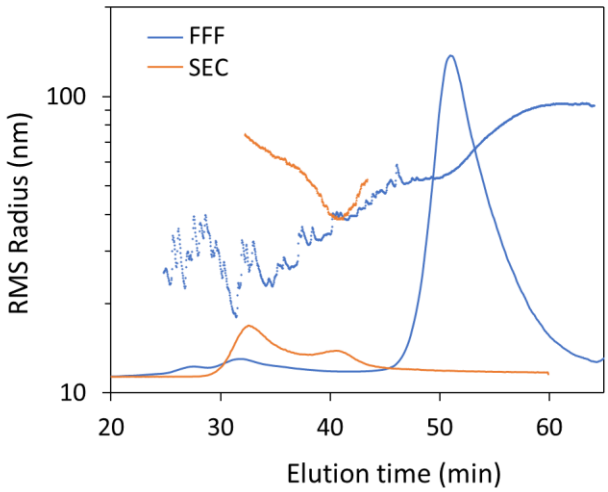

Figure 5. Overlay of radius and LS traces obtained from SEC-MALS (orange) and FFF-MALS (blue). SEC removed particles (through filtration and/or shearing) above 87 nm in radius.

Conclusions

Comprehensive and reliable characterization, along with accurate quantitation, are essential for ensuring the quality and efficacy of retrovirus-based vectors used in novel gene and cell therapies. However, the inherent complexity of these products leads to significant analytical challenges at every stage of development. By utilizing FFF-MALS-DLS, we were able to meet some of these challenges, determining in a single run multiple key attributes: sample purity, sample size, degree of aggregation, stability, physical titer and more. This is done in a fast, automated manner with no requirement for reagents thanks to the Eclipse FFF instrument and DAWN MALS detector with embedded DLS module. Due to their robustness, ease of use, and GMP compliance, both batch DLS and FFF-MALS-DLS measurements can be applied in every stage of the manufacturing process for screening, quantitation, extended characterization, process development and quality assurance.

Acknowledgements

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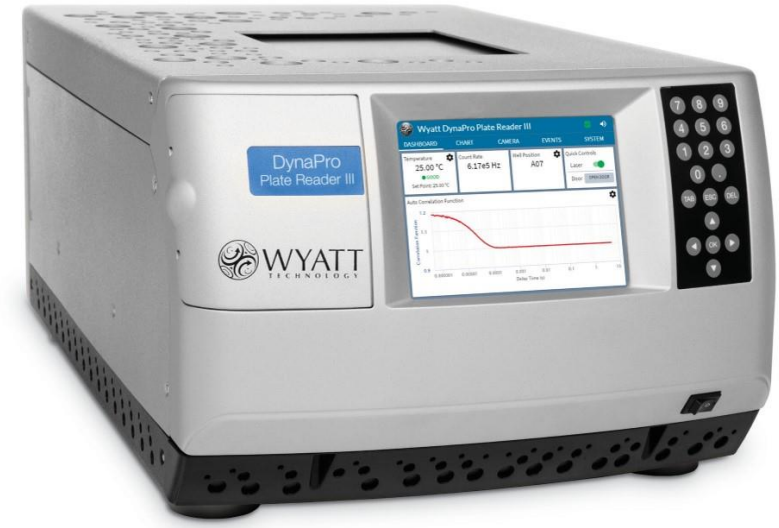

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