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APPLICATION NOTE

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

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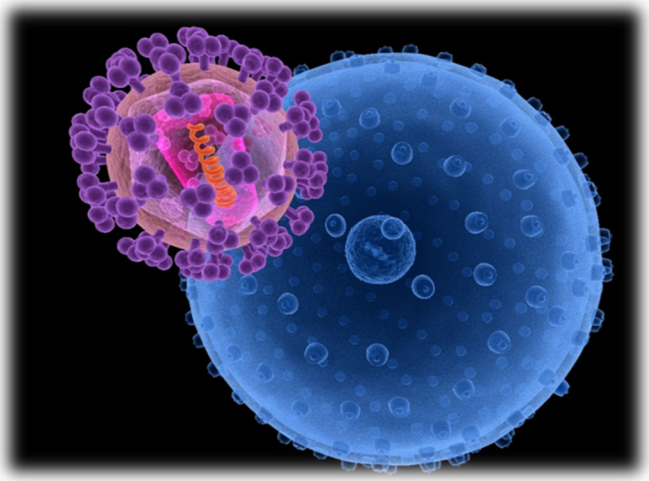
Summary

Lentivirus and gammaretrovirus are popular retroviral vectors under development for novel gene and cell therapies, with more than 700 products in clinical trials as of 2025.[1] As they enter 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 (MALS) and DLS 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 (total titer) and stability. FFF combined with multiple downstream detectors, including MALS and DLS, provides high-resolution size distributions and particle concentration along with in-depth assessment of aggregation and impurities. The details revealed by FFF-MALS 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

Lentiviral vectors (LVV) and gammaretroviral vectors (GRVV) are enveloped retroviruses ranging from ~25 nm to ~75 nm in radius (~50-150 nm diameter) that can deliver a genetic payload up to 11,000 bases and integrate it into the genome of target cells.[2] Though LVVs are preferred over GRVVs 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.[3–5] In addition, LVVs have been engineered to provide improved performance and minimize unwanted side-effects, such as integration into bystander cells, risk of mutagenesis, and induction of secondary malignancy.[4] Some successful strategies for LVV engineering include choosing appropriate viral envelopes to increase transduction efficiency and incorporating targeting moieties like single-chain variable fragments (scFv) and designed ankyrin repeat proteins (DARPin).[5]

Of particular interest is the use of LVV in chimeric antigen receptor (CAR) T cell therapy. To date, approved CAR T cell therapies are produced *ex vivo* from autologous T cells. This multiweek process requires harvesting patient T cells and transducing them with the appropriate gene before re-infusing into the patient. Meanwhile, the patient must undergo chemotherapy to deplete the immune system and promote the uptake of the CAR T cells. Altogether, the cost per patient can be up to $1.5 million and can pose significant safety and efficacy challenges.[4]

Recent advances in LVV engineering may enable *in vivo* CAR T cell therapy by delivering the gene editing vector directly to patients, effectively bypassing lengthy manufacturing process and harsh preparative therapies.[4,5] In fact, the first phase 1 clinical trial of *in vivo* Car T cell therapy was announced in 2025 for the treatment of

relapsed and refractory multiple myeloma.[6] The first-in-human results show the expansion of the desired CAR T cells within about two weeks of infusion, without the need for extracting and manufacturing T cells *ex vivo*, preparative chemotherapy, and lymphodepletion.[7] *In vivo* delivery of LVV or other gene vectors can, therefore, enable expansion of CAR T cell therapy beyond cancer to infectious diseases, and autoimmune diseases.[4]

Consistent LVV production under cGMP is still challenging, with excessive handling, consumables, and time required, as well as lack of suitable analytics for process development.[2,3] Multiple functional and biophysical characterization assays are currently employed at different stages of the purification process, including electron microscopy (EM) to determine empty/full capsid ratios, p24 ELISA to measure total viral titer, and qPCR or ddPCR to quantify packaged RNA.[2,10] However, 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 and significant cost.

Recently, size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) has been proposed as a rapid, robust method for quantifying concentration, identity, and purity.[2] This reagent-free protocol can assess formulation stability, particle size and polydispersity, particle concentration, impurity profiling, and batch-to-batch reproducibility. Quantifying multiple product attributes in a single automated measurement greatly facilitates process understanding, leading to rapid and thorough process optimization. However, the size and polydispersity of many retroviral vectors can make them unsuitable for column separation. Moreover, the column stationary phase may interact undesirably with the viral particles and may shear or remove part of the sample.

In this study, we present a simple and robust workflow for biophysical characterization of retroviral vectors (RVV) using both batch dynamic light scattering (DLS) and field-flow fractionation (FFF) coupled with MALS and online DLS. Both techniques are rapid and nondestructive and can provide critical information about the size distribution and total virus particle concentration of RVV products throughout the downstream purification process.

Field-flow fractionation (FFF) separation technique

In FFF, sample separation takes place in an open channel with no stationary phase. Fractionation is achieved during longitudinal flow along the channel and derives from a field applied perpendicular to channel flow. In the case of asymmetric-flow field-flow fractionation (AF4), that field is the cross flow of the solvent (mobile phase) through a semi-permeable membrane. As illustrated in Figure 2, 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) farther from the membrane, while larger particles stay closer to the membrane. The parabolic profile of the channel flow leads to differential longitudinal solvent velocities and therefore different elution times 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).

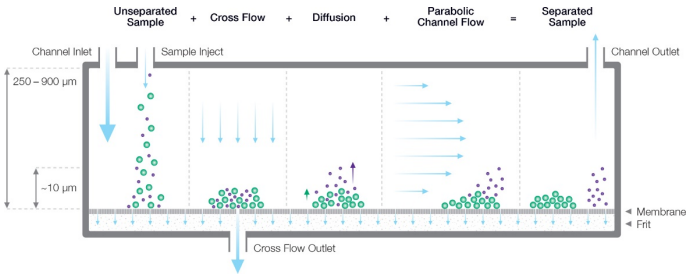


Figure 1. Cross-section of an FFF channel illustrating the principles of asymmetric-flow field-flow fractionation (AF4)

Downstream of FFF separation using the Eclipse™ field flow fractionation (FFF) system, a DAWN™ MALS photometer, and set of concentration detectors is used to provide in-depth quantitative measurements of molar mass, radius, particle concentration, and other biophysical properties.[11] The Eclipse FFF system's dilution control module (DCM) increases sample concentration at the detector providing enhanced sensitivity with negligible loss of resolution.

In this application note, we use FFF-MALS to demonstrate critical differences among LVV and GRVV samples that were not apparent by batch DLS alone. In addition, we highlight how the combination of FFF with simultaneous MALS and DLS detection may characterize trends in morphology of different virus preparations.

Figure 2. FFF-MALS system comprising autosampler and pump, Eclipse FFF system and separation channel, DAWN MALS photometer, Optilab refractometer, and UV detector.

Materials and Methods

GRVV samples 1-3 were prescreened by batch dynamic and static light scattering (DLS/SLS) in a high-throughput manner using a DynaPro™ Plate Reader. Results were analyzed using DYNAMICS™ software.

GRVV samples 1-3 were analyzed with both FFF-MALS and SEC-MALS for detailed assessment of particle size distribution and particle concentration. LVV samples with and without transgene payload (samples 4 and 5, respectively) were analyzed by FFF-MALS-DLS to investigate differences in molar mass and morphology with RNA loading.

For all samples, 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 FFF system. Size, molar mass, and particle concentration were measured using the DAWN MALS photometer with embedded WyattQELS™ DLS module, and an Optilab™ differential refractive index refractometer. FFF operation was controlled with VISION™ software, and analysis was performed in ASTRA™ software.

In addition to analysis by FFF, GRVV samples 1-3 were evaluated by size-exclusion chromatography (SEC). SEC-MALS analysis was carried out on the same system, wherein the Eclipse FFF system 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

Batch dynamic light scattering (DLS) provides a quick estimate of particle size distribution and particle concentration (physical titer), typically within 30 seconds or less. The DLS measurement 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*.

The z-average hydrodynamic radius (R_h) and polydispersity index (PDI) for the three GRVV samples are shown in Table 1. The results suggest slightly different mean sizes for all three samples. In addition, GRVV #2 appears to be relatively monodisperse whereas GRVV #1 appears to contain a broader distribution of sizes. These results not only render some useful initial information about these RVV samples but also aid the FFF method development that follows. In addition, the combination of dynamic light scattering and static light scattering enables an initial estimate of the total particle concentration.

Table 1: Hydrodynamic radius (R_h), polydispersity (PDI) and total particle concentration measured by batch DLS/SLS

Sample	R_h (nm)	PDI	Particle Concentration (particles/mL)
GRVV #1	65.4 ± 0.5	0.28 ± 0.02	(37.0 ± 1.5) × 10^9
GRVV #2	79.2 ± 0.2	0.08 ± 0.01	(7.39 ± 0.18) × 10^9
GRVV #3	85.2 ± 1.1	0.18 ± 0.02	(2.07 ± 0.02) × 10^9

For each sample, values are average and standard deviation of three measurements of a single well.

FFF-MALS:
High-resolution separation and quantification

The three GRVV samples were separated by FFF and characterized by online MALS and DLS. The 90° angle static light scattering traces and radii measured by MALS are overlaid in Figure 3. For all three samples, the main lentivirus population elutes around 50 minutes and consists of a fairly polydisperse population with sizes spanning 50 nm to 150 nm. This qualitatively agrees with DLS showing z-average radius ranging from 65 nm to 85 nm.

Understanding the difference in low-molar-mass species can provide useful information about sample purity and stability and can help select appropriate orthogonal techniques for additional sample characterization. Here, Sample 1 exhibits an additional peak, eluting around 26 minutes. The particle size of this second population ranges from several nanometers to ~30 nm, consistent with proteins and other cell culture remnants. These impurities have been effectively eliminated in Samples 2 and 3.

Multi-angle light scattering also enables the calculation of total particle concentration as a function of viral particle size and has been successfully applied to a variety of viruses.[12,13] The RVV titer calculated using the ASTRA software *Particle Size & Concentration* module varies from ~2 x 10^9 to ~2 x 10^{10} particles/mL in these three samples, in good agreement with the batch DLS/SLS results. The detailed particle size distribution measured by FFF-MALS is shown Figure 3 (bottom) with the total titer summarized in Table 2.

Through these GRVV samples, FFF-MALS is seen to provide clear differentiation of RVVs in terms of size, titer, and impurities. As expected from the batch DLS experiment, Sample 3 has the least amount of formulation protein and impurities from cell culture but also the lowest concentration of viral particles by a factor of two or more. The overall agreement between the FFF and batch DLS methods suggests good mass recovery of the FFF method with minimal retention of the RVV or membrane interactions.

The detailed size distribution from FFF-MALS provides key insights missed by batch DLS. In particular, FFF-MALS clearly shows the main population of lentivirus is the same size across all three samples with a mean ~65 nm. This population accounts for 70-80% of the total number of particles. A second larger population of aggregates with radius >80 nm is also apparent in all three samples and likely represents aggregates of the first population. The z-average radius of this second population is between 85 nm and 95 nm, depending on the sample, and accounts for 20-30% of the total number of particles.

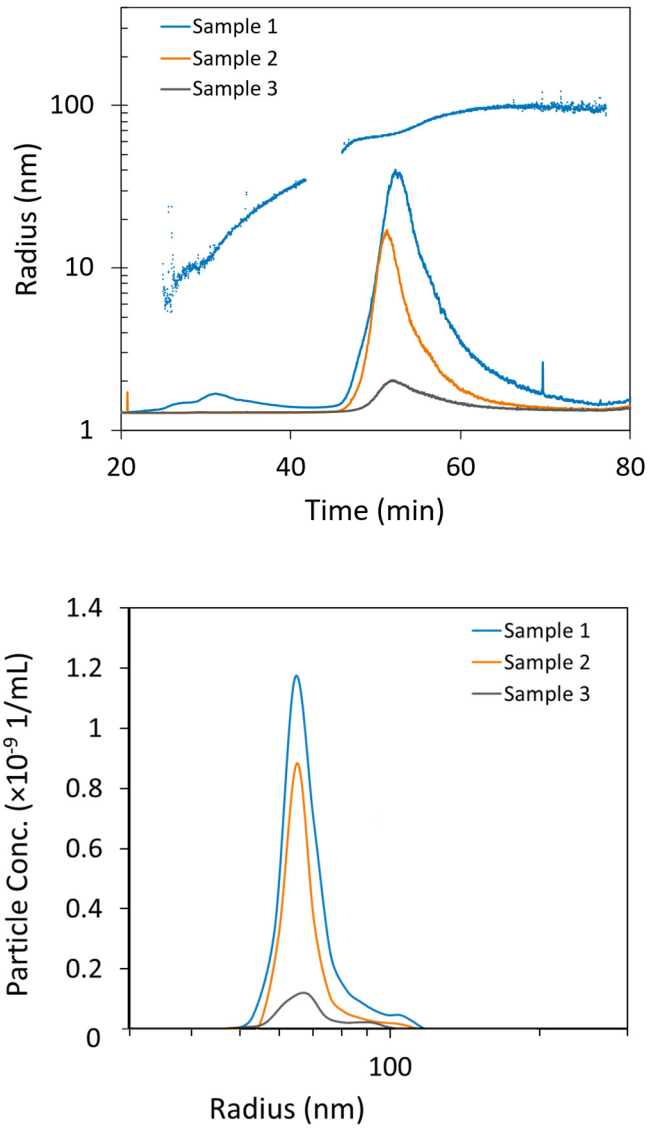


Figure 3. Top: LS fractograms overlaid with radius measured by MALS for three GRVV samples. Bottom: Particle concentration as a function of radius measured by MALS.

Table 2. Size and particle concentration (total titer) for three GRVV samples measured via FFF-MALS and SEC-MALS.

	FFF-MALS[1]		SEC-MALS[2]	
Sample	R_z (nm)[3]	Particle Concentration (1/mL)	R_z (nm)[3]	Particle Concentration (1/mL)
GRVV #1	81.6 ± 2.3	(18.2 ± 1.2) × 10^9	70.7 ± 1.9	(3.88 ± 0.51) × 10^9
GRVV #2	78.9 ± 1.2	(10.5 ± 0.6) × 10^9	75.5	3.35 × 10^9
GRVV #3	82.3 ± 2.8	(2.32 ± 0.57) × 10^9	74.0	1.64 × 10^9

1. Average and standard deviation of four 50-µL injections for Sample 1 and two 50-µL injections for Samples 2 and 3
2. Average and span of two 50-µL injections for Sample 1 and value from a single 50-µL injection for Samples 2 and 3
3. Z-average geometric radius assuming lentivirus are spherically shaped

FFF-MALS vs. SEC-MALS:
Retroviral vector separation and quantification

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 RVV. 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 SEC separation of GRVV are overlaid in Figure 4. The particle size distributions are shown in Figure 5 with data summarized in Table 2.

Differences in recovery and size distribution between SEC-MALS and FFF-MALS are immediately apparent. The average size measured by SEC-MALS is up to 13% smaller than that measured by FFF-MALS, and the particle concentration measured by SEC-MALS is 30-80% less than that measured by FFF-MALS. For all three samples, SEC appears to remove particles with radius greater than ~90 nm, as shown in the particle size distributions in Figure 5. However, additional details about the type and severity of the difference depend on the sample.

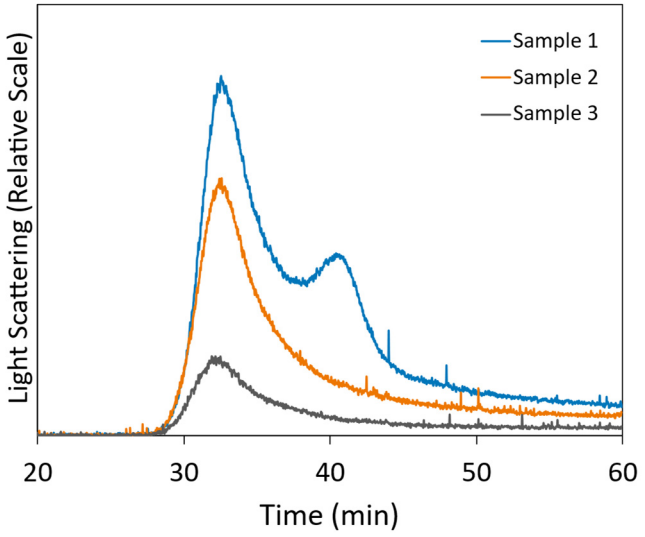


Figure 4. LS chromatograms overlaid for three GRVV samples analyzed by SEC-MALS.

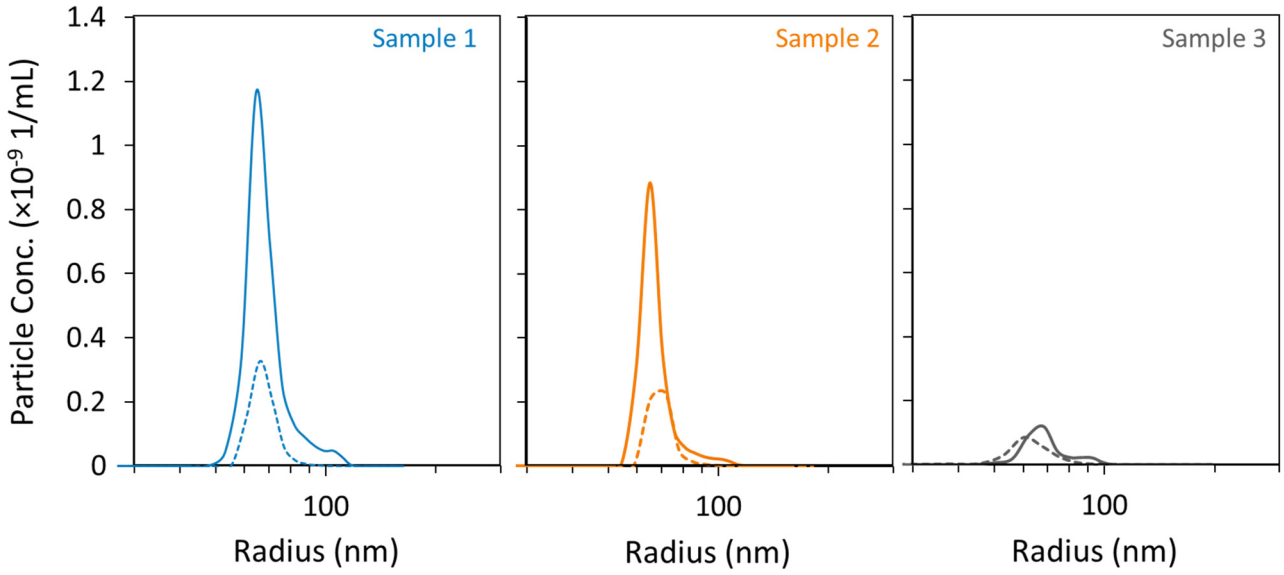


Figure 5: Particle size distribution for GRVV measured by FFF-MALS (solid lines) and SEC-MALS (dashed lines). FFF data correspond to fractograms shown in Figure 3; SEC data correspond to chromatograms in Figure 4.

By FFF, the virus fraction in Sample 1 eluted in a single broad peak well-resolved from the smaller species, but the SEC-MALS chromatogram shows significant coelution of these two populations. Based on the retention time, we may assume that the secondary peak eluting ~40 minutes is the smaller protein and other growth media impurities that were seen by FFF. However, the measured radius for this peak is ~40-50 nm, which is significantly higher than expected for these species. This suggests large virus particles and aggregates are being retained on the column and coeluting with these smaller species. In addition, the aggregate species ~80-150 nm in radius are not observed for this sample by SEC-MALS (Figure 5, left), and the total particle concentration is reduced nearly five-fold compared to FFF-MALS (Table 2).

Samples 2 and 3, on the other hand, showed lesser changes in peak shape compared to that FFF-MALS, but we still observed a decrease in overall size distribution (Figure 5) and drastic difference in total particle concentration. Although there was a minimal change in the z-average radius, the particle concentration decreased by 2-3×, suggesting significant retention of viral particles on the column (Table 2). Thus, while SEC-MALS may provide useful characterization of some RVVs, it is less suitable than FFF-MALS as a general method for retroviral vector quantification.

FFF-MALS-DLS: Investigating genetic payload

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,[14] but this quantification 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 (LVV samples 4 and 5, respectively), 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 endpoint proviral DNA quantitation after infection of HEK293 cells.

FFF separation followed by online MALS and DLS detection determined two distinct size-related parameters:

- Root-mean-square (RMS) radius, a.k.a. radius of gyration (R_g), measured by MALS
- Hydrodynamic radius (R_h) measured by online DLS

The ratio R_g/R_h, termed "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)."[15]

Two light scattering fractograms are shown in Figure 6, 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 and approximately be equal to the physical diameter. The ratio of these two radii obtained from MALS and DLS can help to confirm differences between empty and full vectors. The traditional approach to distinguishing empty and full viral vectors, cryo-EM imaging, is unable to adequately differentiate them (Figure 6, bottom panel).

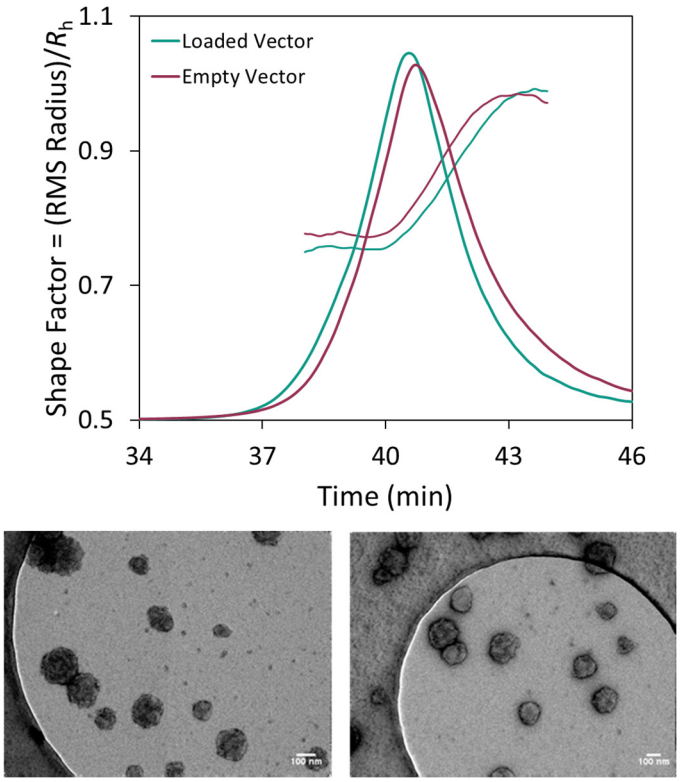


Figure 6. Top: Light scattering fractograms for loaded and empty LVV overlaid with the R_g/R_h ratio. Bottom: Cryo-EM images of empty (left) and full (right) lentiviral vectors.

Conclusion

Comprehensive and reliable characterization along with accurate quantitation are essential for ensuring the quality and efficacy of retroviral 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. 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. Moreover, the high resolution and in-depth characterization enabled by FFF-MALS and charge detection mass spectrometry (CDMS)[16] are well-suited to quantify LVV used for *in vivo* CAR-T therapy where the virus is considered the drug product.

As shown in this application note, light scattering solutions can help scientists and process engineers optimize the manufacturing process and product quality of these complex vectors. DLS provides a quick albeit low resolution screening of RVV size distributions. SEC-MALS can provide some fit-for-purpose measurements of size distribution and sample quality. Finally, FFF-MALS provides reproducible, high-resolution separation combined with in-depth analysis of multiple quality attributes simultaneously: sample purity, sample size, degree of aggregation, stability, physical titer, and more. This is done in a quick, automated manner with no requirement for reagents, thus meeting key characterization requirements.

Acknowledgements

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