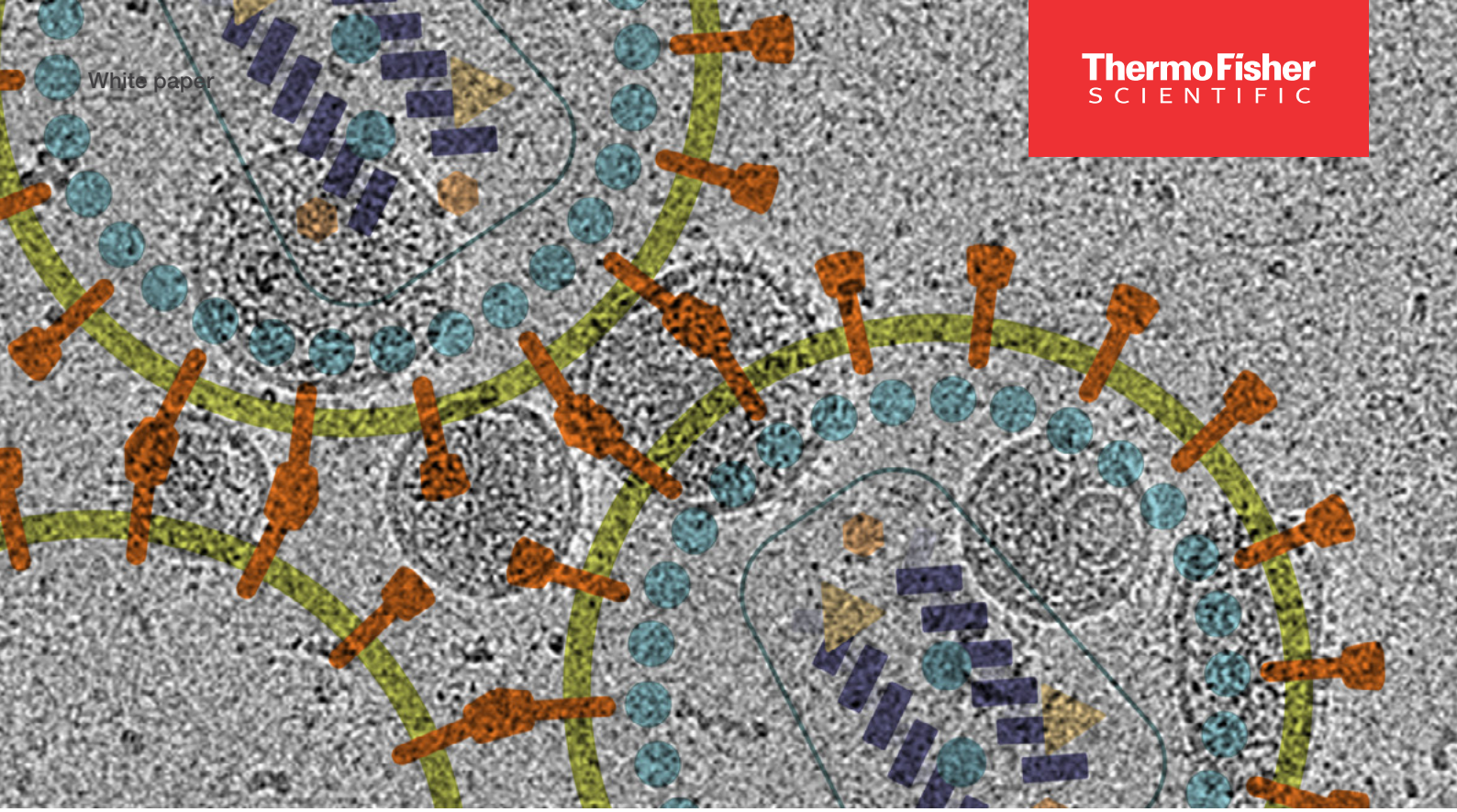
White paper



Advancing lentiviral vector-based gene therapy

Utilizing cryo-TEM and automated image analysis for improved lentiviral vector characterization

Authors

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Keywords

Cryo-TEM, lentiviral vector, gene therapy, lentiviral characterization, CQA, EPU Software, Amira Software

Introduction

The rapid advancement of cell and gene therapy (CGT) has led to an unprecedented demand for viral-based gene delivery vectors. In 2024 alone, there were 2,042 clinical trials, with 57% of gene therapies relying on *ex vivo* genetic modifications.[1] Lentiviral vectors (LVVs) have emerged as a critical component in this field, accounting for 48% of global gene therapy and cell-based immuno-oncology trials in 2022.[2-3] The therapeutic potential of LVVs is underscored by recent FDA approvals of products like Kymriah, Carvytki, Zynteglo, Lyfgenia, and Aucatzyl.[4–8] As developers of lentiviral-vector-based CGTs continue to innovate, the demand for high-quality LVV products is expected to rise significantly.[9] This growing need necessitates advanced characterization techniques that can enable process development, providing improved performance, efficacy, and safety.

Performed in collaboration with CSL Behring, this study explores the use of cryo-transmission electron microscopy (cryo-TEM) for the detailed characterization of lentivirus-based vectors, aiming to enhance our understanding and optimization of these critical therapeutic tools.

Significance of lentiviral vector quality

LVVs are enveloped, spherical, single-stranded RNA viruses with diameters ranging from 80 to 120 nm; they are characterized by their complex structure.[10] Lentivirus-based vectors are preferentially used due to their ability to infect a broad range of cells, deliver large therapeutic genes, and ensure long-term stable expression in dividing cells. LVVs consist of a p24 protein capsid (containing the therapeutic gene of interest) surrounded by a lipid bilayer with envelope proteins, such as vesicular stomatitis virus glycoprotein G (VSV-G).[11]

There are, however, challenges in LVV development and utilization. The envelope's heterogeneity and fragility complicate large-scale purification and characterization, as cells produce numerous non-infectious particles alongside the infectious ones. Stability issues, influenced by various factors such as temperature, freeze-thaw cycles, ionic strength, adsorption, pH, and shear stress during manufacturing, often lead to product and infectivity loss.[12] Additionally, contaminants and impurities, such as LVV aggregates, inactive LVVs (i.e., with truncated RNA cargo or non-functional VSV-G), and process-related impurities, pose efficacy, consistency, and safety concerns.[10,13] Precise and early characterization is crucial for the development of safe and effective gene therapies.

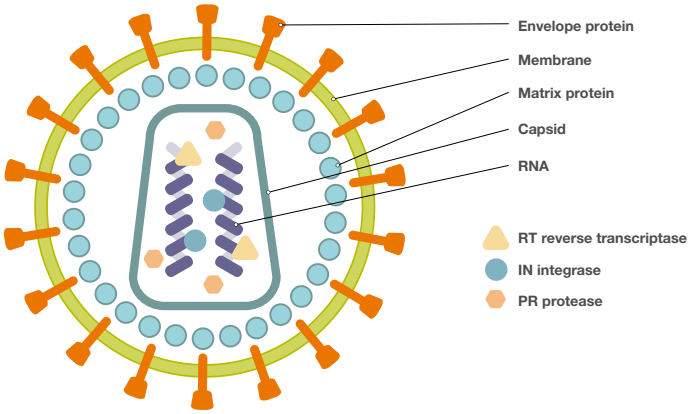


Figure 1. Simplified illustration of a lentivirus particle.

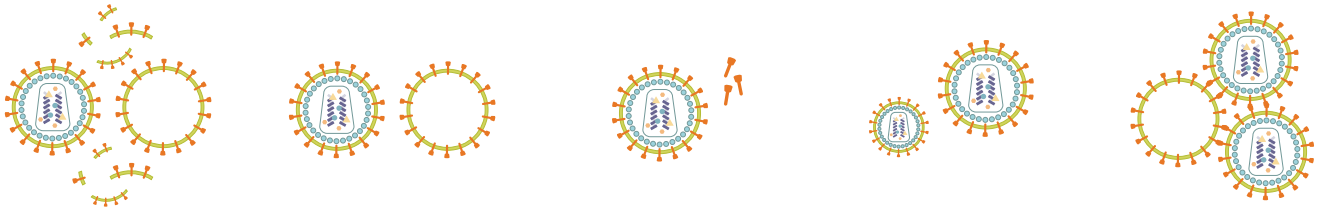
Classical approaches that utilize light scattering (e.g., dynamic light scattering (DLS) or other light-optical techniques) are commonly used to characterize the size distribution of lipid-based nanoparticles. These methods rely on idealized assumptions within their mathematical models and work best with homogeneous samples that have well-defined compositions. However, their effectiveness diminishes with heterogeneous samples such as LVVs, limiting their utility.[14]

Regulatory guidelines for the quality of lentiviral vectors

Regulatory authorities, including the US FDA[15,16] and the European Medicines Agency,[17,18] emphasize the analysis of key morphological features as a way to ensure the efficacy and quality of nanoparticle and viral-vector based therapeutics. This includes particle size distribution, shape, and morphology, as well as the ratio of infectious/non-infectious and empty/genome-containing particles.

Advantages of cryo-TEM in lentivirus characterization

In the pharmaceutical R&D landscape, particularly in gene therapy, precise characterization of viral vectors at the single-particle level is highly desirable. Cryo-TEM is a direct visualization method that enables this kind of characterization, and has previously been used to accurately quantify the ratio of genome-containing to empty AAV viral-vector particles.[19] Unlike other techniques such as DLS, analytical ultracentrifugation, and size exclusion chromatography, cryo-TEM can provide enhanced insights into the morphology, integrity, and composition of viral vectors.



Purity	Particle quantification	Particle concentration	Size distribution	LVV aggregation
Cellular and subcellular byproducts in the final formulation can impact toxicity and biological activity. This also influences the maximal achievable transduction, and the dosing required for effective cell transduction.	Accurate quantification of both genome-containing and empty, non-infectious particles is essential for optimizing the vector production process and the dosing for cell transduction. Reliable methods for quantifying non-infectious particles are lacking, and advanced techniques, such as Cryo-EM, are needed to address this gap.	Accurate measurement of LVV particle concentration is crucial for optimizing dosing and transduction efficiency. Approaches such as the p24 ELISA method, however, do not specifically quantify intact LVV particles.	LVV particle size can be influenced by viral strain, production methods, and purification processes. Characterizing their size distribution is essential for understanding the physical properties of LVVs and optimizing their use in gene transfer and therapeutic applications.	LVVs, similar to other biotherapeutics such as biologics and proteins, are prone to aggregation and instability, which can impact their effectiveness and safety.

Table 1. Challenges in lentiviral vector characterization

Cryo-TEM offers several advantages:

1. **Estimation of multiple CQAs.** Quantifies multiple critical quality attributes (CQAs), such as shape, size, morphology, and spike density, from a single dataset.
2. **Minimal sample requirements.** Requires only small sample volumes, which also allows for rapid image analysis.
3. **Single-particle precision**. Provides precise, single-particle level characterization, which is unaffected by proteins, debris, and aggregation.
4. **Preservation of native states.** The rapid vitrification used in cryo-TEM preserves biological samples in their near-native hydrated state for analysis.
5. **Ability to distinguish structural features.** Can distinguish lentivirus particles from other membrane structures due to the prominent spikes on their surface.
6. **Impurity detection.** Helps identify impurities such as cellular debris and proteins, which can have a detrimental effect during transduction.
7. **Detailed visualization of internal structures.** Allows for the visualization of internal structures, providing insights into integrity and packaging.

The cryo-TEM workflow

The cryo-TEM workflow typically begins with sample preparation of a purified protein or complex in solution (Figure 1). The sample solution is placed onto a specialized grid and rapidly frozen by plunging it into a cryogenic fluid such as liquid ethane. This process transforms the water into amorphous ice, vitrifying the sample and preserving the native structure of the specimen. Once vitrified, the sample is kept at cryogenic temperatures throughout the imaging process to prevent structural alterations. The vitrified sample is then loaded into a cryo-TEM for imaging, which captures high-resolution images of the sample, providing detailed insights into the morphology, integrity, and composition of the sample, allowing for precise characterization.

Integrating cryo-TEM imaging with automated particle characterization

One of the unique advantages of cryo-TEM imaging is that it not only provides qualitative sample assessment but also enables the quantification of quality attributes. By utilizing a quantitative image analysis solution, such as Thermo Scientific™ Amira™ 3D Software, the composition of LVV formulations can be directly assessed. This quantitative approach facilitates:

- Better understanding and optimization of lentiviral production, purification, and formulation
- Stability testing through the monitoring of critical morphological characteristics
- Classification and quantification of morphological features that inform the efficacy, safety, and potency of LVV formulations

Materials and methods

Sample preparation

Sterilized, filtered, purified, and concentrated lentivirus particles were first suspended in a suitable buffer. The LVVs samples were then prepared for cryo-TEM analysis on Quantifoil R 1.2/1.3 300-mesh copper EM grids, supported by a 2 nm carbon film. Grids were initially glow discharged for 15 seconds at 5 mA. 4 µL aliquots of LVV sample, with a concentration of approximately 500,000 ng p24/mL, were then applied to the grids. After a wait time of 1 minute in a horizontal position, the grids were blotted for 3 seconds (0 blot force, 100% humidity, 4°C) and then plunge-frozen (vitrified) with pre-cooled liquid ethane on a Thermo Scientific™ Vitrobot™ Mark IV System.

Cryo-TEM image acquisition

Images of vitrified samples were collected on a Thermo Scientific™ Glacios™ Cryo-TEM at an accelerating voltage of 200 kV with a Thermo Scientific™ Falcon™ 4i Direct Electron Detector operated in counting mode. Each image was captured with an exposure time corresponding to a total electron dose of approximately 15 e/Å^2 and a calibrated pixel size of 0.27 nm. Grid mapping and image acquisition were performed with Thermo Scientific EPU Software.

Figure 2. LVV sample preparation, cryo-TEM imaging, and analysis.

Automated cryo-TEM image analysis with Amira 3D Software

Cryo-TEM image analysis was performed with Amira 3D Software. The workflow was automated with a recipe, which is a predefined sequence of processing steps or operations, commonly known as macros (Figure 3). The recipe includes all necessary parameters and instructions for tasks such as image enhancement, segmentation, and analysis. First, a denoising step using Noise2Void was performed to enhance the signal-to-noise ratio.[20] This was followed by segmentation of the membrane with a VGG19 neural network.[21] The deep learning model was pre-trained on a set of 20 grayscale and corresponding label images, which were created semi-manually. A series of non-linear filtering-based morphological operations enabled the reconstructions of the full particles from the membrane segments. The separate identification of each particle allowed for automatic, individual measurements. This type of particle identification is the final goal of many similar recipes, though some of the intermediate steps may vary depending on imaging conditions, image compression, artifacts, etc.

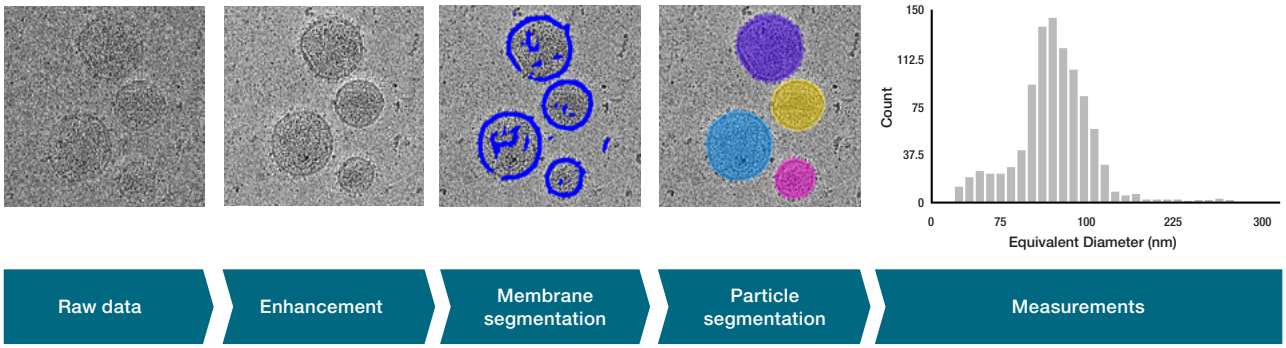


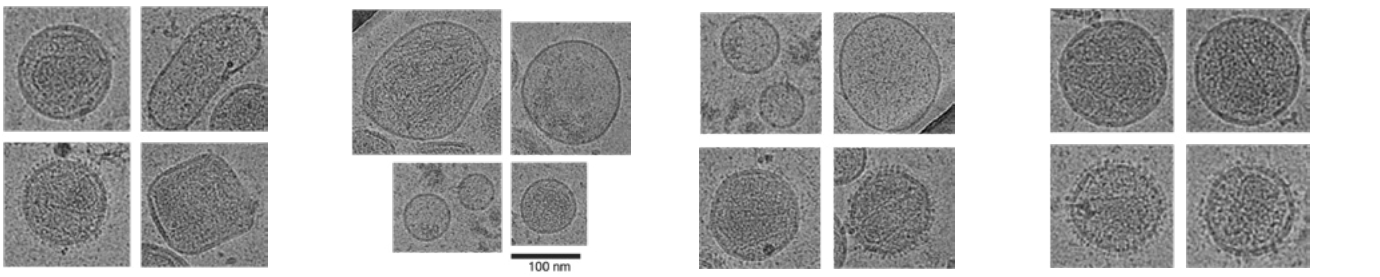
Figure 3. Particle picking and analysis workflow in Amira 3D Software.

Results

Rapid qualitative assessment of lentivirus samples with cryo-TEM imaging

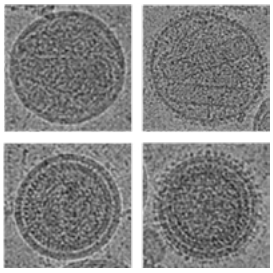
Cryo-TEM imaging provides both qualitative and quantitative assessments of CQAs for lentivirus samples. A quick review of the cryo-TEM dataset can immediately reveal qualitative aspects of the sample without the need for extensive and time-consuming bioanalytics (Table 2).

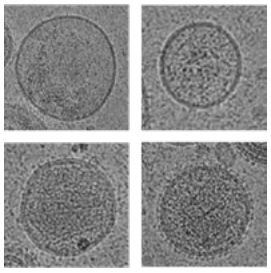
The true advantage of cryo-TEM, however, is in its ability to eliminate blind spots. It allows for the quantification and sorting of similarly sized particles into distinct groups (e.g., viruses, vesicles, etc.) and the analysis of surface features at the individual virus level. By analyzing statistics across multiple particles, cryo-TEM can reveal different populations (if they exist) rather than providing a mere average of CQAs.

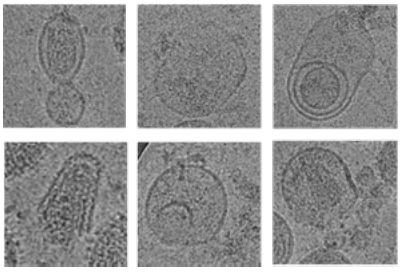


Shape analysis	Size distribution	Content analysis	Surface features
Compare regular round particles to those with irregular shapes.	Evaluate product heterogeneity by analyzing viral vector size distribution and aggregation. This attribute is important for the assessment of batch-to-batch variability, formulation development, as well as process development and validation.	Evaluate the ratio of empty vs. genome-filled particles, which impacts product quality as well as transduction efficiency and efficacy. **Packaging analysis** Differentiate LVV particles from other membrane-enclosed structures.	Assess the presence or absence of envelope protein (spikes) and differentiate between smooth and spiky particles, which reveals functional lentivirus particles.

Table 2. Critical quality attributes revealed with cryo-TEM







Morphological assessment	Surface spike density	Impurities and aggregates
Evaluate the morphology and internal architecture of viral particles.	Directly assess the distribution and density of spike proteins per virion	Identify harmful impurities, including cellular debris, broken particles, host cell proteins, and DNA, along with other product- and process-related contaminants.

Table 2. Critical quality attributes revealed with cryo-TEM

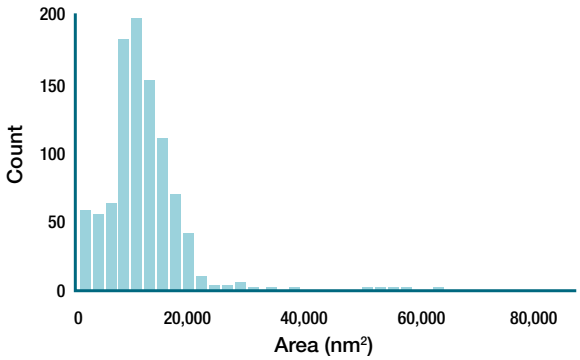
Correlation of particle morphology and transduction efficiency

The correlation of structural elements with transduction efficiency enhances product comparisons across manufacturing stages and helps assess the impact of process changes during development. The transduction efficiency of LVVs is influenced by the number of mature, functional particles, which feature glycoprotein-decorated membranes and capsids containing viral genomes. Non-functional particles can compete with these functional particles for cellular uptake, ultimately reducing overall transduction efficiency.

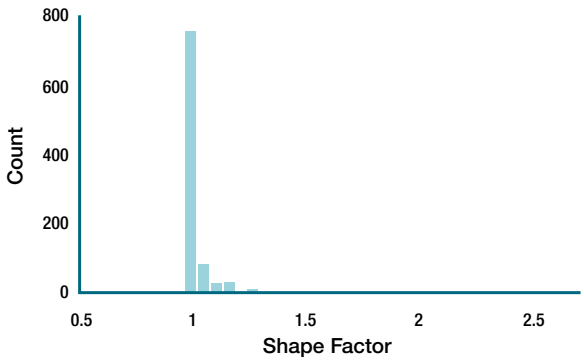
Precise quantification of lentivirus CQAs via automated image analysis

Quantitative analysis of cryo-TEM data can provide more detailed and precise evaluation. In this study, approximately 240 images were selected and analyzed using the automated workflow described in Figure 3. Through this process, around 959 particles were examined for CQAs such as shape, size, surface area, and particle elongation (Figure 4). This automated image analysis provides accurate and reproducible measurements, facilitating a comprehensive understanding of the physical properties and consistency of the lentivirus samples.

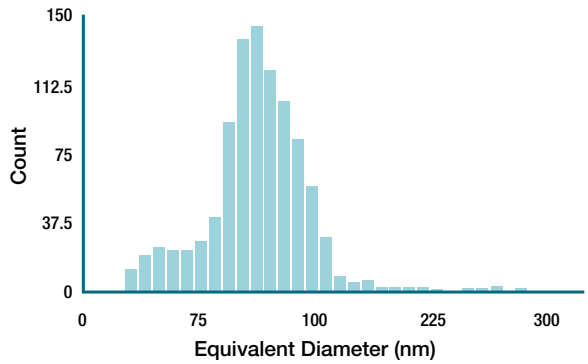
By leveraging both the qualitative and quantitative capabilities of cryo-TEM imaging, researchers can help ensure the high quality and efficacy of lentivirus-based gene therapies.



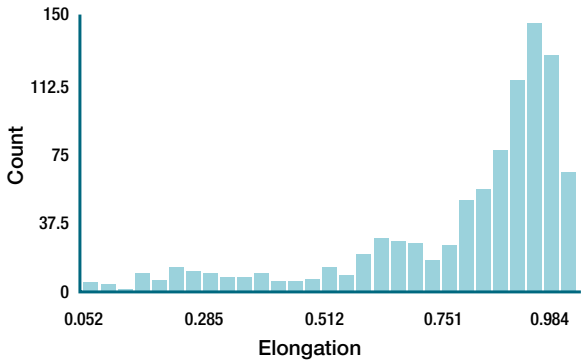
The surface area of the particle.



Equal to 1 for a perfect circular shape, with higher values indicating objects that are less circular.



Defined as the diameter of a disk with the same area as the particle.



Calculated as the ratio of the major and minor axis of a fitted ellipse in the eigenvalue space after decomposition. Values close to 1 indicate circularity and values close to 0 indicate more elongated forms.

Figure 4. Quantitative characterization into selected CQAs for the lentivirus samples

Discussion and way forward

The characterization of LVV products, when manufactured at a pharmaceutical scale, presents significant challenges due to the presence of particulate impurities of heterogeneous sizes. Multiple complementary analytical techniques are often required to thoroughly characterize LVV particles and, specifically, particulate impurities, which are frequently overlooked by biochemical methods.[10] However, these techniques necessitate relatively clean LVV samples and may not provide accurate estimations of size and titer for complex LVV samples.

Cryo-TEM offers exceptional precision in the characterization of LVVs. It overcomes common limitations of traditional light scattering techniques and provides advanced insights into LVV formulations. Such precise characterization is crucial for LVV process development and for ensuring the stability of gene therapy products. Additionally, detailed morphological analysis is necessary for adherence to regulatory guidelines, which help to ensure that high standards of quality and efficacy are maintained for LVV-mediated therapies.

While cryo-TEM offers significant advantages in the characterization of LVVs, it is not without its challenges. The vitrification process used for sample preparation can require iteration for each sample to optimize image quality. Additionally, high-quality cryo-TEM equipment, computational resources, and experienced analysts are often necessary and are not widely accessible in an industrial setting, though large networks of academic imaging centers can be found around the globe. Although cryo-TEM provides numerous benefits in particle characterization, advancements in accessibility and automation will be critical for widespread and standardized adoption in drug development.

Automated image analysis, as performed with Amira Software, has proven essential for the quantitative characterization of lentiviral vectors. Amira Software offers an extensive array of analysis tools with the flexibility needed to develop tailored workflows for specific datasets. This method provides a robust and unbiased approach for the processing of large volumes of imaging data, enabling detailed statistical analysis of particle morphology and size.

To maximize the utility of morphological data, it is essential to establish clear correlations between LVV particle morphology, transduction efficiency, and safety-related parameters such as aggregation or impurity load. By systematically linking structural features observed via cryo-TEM with downstream biological performance and safety outcomes, critical quality attributes (CQAs) can be identified. These insights can then inform the optimization of manufacturing processes, allowing key morphological metrics to be used as analytical targets for in-process control and final product specification. This data-driven approach would support both process robustness and regulatory alignment, while ultimately improving clinical outcomes.

Moving forward, the integration of cryo-TEM imaging with automated image analysis will be instrumental in advancing the field of gene therapy. This combination not only enhances the accuracy of lentivirus sample characterization but also streamlines the process, making it more efficient and reliable. By leveraging these advanced technologies, researchers and manufacturers can help ensure that high-quality, effective gene therapy products are developed, which meet stringent regulatory standards.

References

1. Gene, Cell, & RNA Therapy Landscape Report, Q3 2024 Quarterly Data Report. ASGCT (2024).
2. Regenerative Medicine: The Pipeline Momentum Builds. Alliance for Regenerative Medicine (2022).
3. Cell and Gene Therapy Catapult, Annual Review 2022 CGT Catapult (2022).
4. FDA approval brings first gene therapy to the United States FDA News Releases (2017).
5. Cellular & Gene Therapy Products: Carvytki. US FDA (2022).
6. Cellular & Gene Therapy Products: Zynteglo. US FDA (2022).
7. Cellular & Gene Therapy Products: Lyfgenia. US FDA (2023).
8. Cellular & Gene Therapy Products: Aucatzyl. US FDA (2024).
9. Pamenter, G, et al. Lentiviral vector determinants of anion-exchange chromatography elution heterogeneity. Biotechnol Bioeng 121:9 (2024). doi: 10.1002/bit.28766
10. Stadler, D, et al. Challenges in the analysis of pharmaceutical lentiviral vector products by orthogonal and complementary physical (nano)particle characterization techniques. Eur J Pharm Biopharm 200 (2024). doi: 10.1016/j.ejpb.2024.114340
11. Gutierrez-Guerrero, A, et al. Pseudotypes: Precious Tools to Improve Gene Modification of Hematopoietic Cells for Research and Gene Therapy. Viruses 12:9 (2020). doi: 10.3390/v12091016
12. Perry, C, et al. Insights into product and process related challenges of lentiviral vector bioprocessing. Biotechnol Bioeng 121:8 (2023). doi: 10.1002/bit.28498
13. Segura, MM, et al. New developments in lentiviral vector design, production. Expert Opin Biol Ther 13:7 (2013) doi: 10.1517/14712598.2013.779249
14. LNP meets high-throughput Cryo-EM: Reliably quantifying LNP morphology. ATEM Structural Discovery (2024).
15. Guidance for Industry - Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs). US FDA (2020).
16. Guidance for Industry - Considerations for the Development of Chimeric Antigen Receptor (CAR) T Cell Products. US FDA (2024).
17. Guideline on development and manufacture of lentiviral vectors. European Medicines Agency (2005).
18. Guideline on the quality, non-clinical and clinical aspects of gene therapy medicinal products. European Medicines Agency (2018).
19. Nishiumi, H, et al. Combined 100 keV Cryo-Electron Microscopy and Image Analysis Methods to Characterize the Wider Adeno-Associated Viral Products. J Pharm Sci 113:7 (2024). doi: 10.1016/j.xphs.2024.03.026
20. Krull, A, et al. Noise2Void - Learning Denoising From Single Noisy Images. IEEE/CVF Conference on Computer Vision and Pattern Recognition (CVPR) (2019). doi: 10.1109/CVPR.2019.00223
21. Simonyan, K, et al. Very Deep Convolutional Networks for Large-Scale Image Recognition. ICLR (2015). doi: 10.48550/arXiv.1409.1556

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