FlowCam®

Flow Imaging Microscopy to Monitor Lipid Nanoparticle Aggregation

OVERVIEW

Lipid nanoparticles (LNPs) are gaining attention as effective drug delivery vehicles, especially since their successful use in the COVID-19 vaccines. Both Moderna and Pfizer SARS-CoV-2 vaccines depend on LNPs to deliver messenger RNA (mRNA), a delicate nucleic acid molecule, to the cytosol of target cells. LNPs are a spherical assembly of lipid and/or lipid-like molecules that encapsulate and protect the therapeutic nucleic acid payload in the patient's body. Whether the active pharmaceutical ingredient (API) is mRNA or some other nucleic acid, the application of LNP-based vaccines and gene therapies for cellular delivery is of high general and scientific interest.

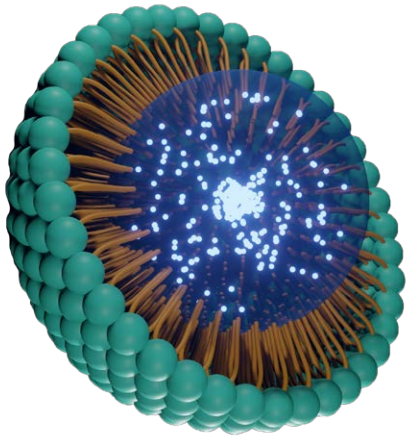

Figure 1. A schematic view of a lipid nanoparticle with enclosed active ingredient

As a newer biotherapeutic, strategies to improve LNP stability are actively being researched as established APIs like proteins have been previously and continue to be researched. Like protein-based biotherapeutics, LNPs are subject to aggregation and other forms of degradation during storage periods and following stresses like freeze-thaw cycles or agitation due to sample (mis)handling. At present, LNP formulations must be shipped and stored refrigerated, if not frozen, to maintain stability. While these low-temperature storage conditions are effective as observed with the COVID-19 vaccines, they complicate shipping and storage logistics and can reduce access to these vaccines for patients that live in areas lacking sufficient cold-chain resources – especially in warmer climates. Designing LNP formulations that are stable when stored using standard refrigeration, or ideally at room temperature, is an open and important challenge for the field.

Improving the stability of LNP formulations may reduce the cold-chain shipping and storage requirements these treatments require and allow more patients to access these powerful therapies easily and affordably.

Due to the safety risks that API aggregates and other particles pose, good manufacturing practice (GMP) requirements are in place to set quality standards. These standards ensure that pharmaceutical manufacturers do not release batches of any parenteral drug products with significantly elevated numbers of particles. Of particular note is USP <788> ("Particulate Matter in Injections") and similar regulations which restrict the number of subvisible particles (i.e. particles 2-100 µm in diameter) that can be present in commercialized parenterals. It is important to check how changes in LNP and formulation design influence the particle content of these drugs to minimize the regulatory and potential product safety risk posed by particles in these formulations.

Flow imaging microscopy (FIM) instruments are commonly used to count, size, and image API aggregates and other particles in biotherapeutics and are well-suited for analyzing particles in LNP formulations. FIM instruments use a combination of light microscopy and microfluidics to capture images of these particles in a high-throughput fashion. These measurements yield particle images that can be counted to obtain particle concentrations and analyzed with image analysis software to obtain particle size and shape information. This information can then be used to optimize formulations to mitigate aggregation and improve stability as well as to track subvisible particle content in drug product batches during manufacturing.

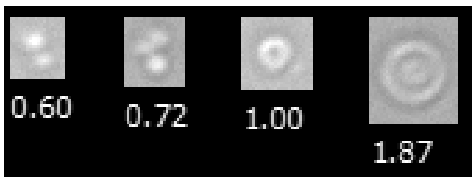

Figure 2. Flow imaging microscopy images of LNPs, captured by FlowCam Nano. Values below each image are the diameter (equivalent sphereical diameter) for the particle in µm.

FlowCam-series FIM instruments like FlowCam 8100, FlowCam LO, and FlowCam Nano capture high-resolution particle images that are

effective for analyzing particles in LNP formulations. FlowCam 8100 and FlowCam LO yield images of subvisible particles including those subject to USP regulations. FlowCam LO additionally performs light obscuration (LO) measurements on samples, the technique required for monitoring particle counts and sizes via USP <788>. This combination of LO and FIM is recommended by USP <1788> ("Methods of Determination of Subvisible Particulate Matter") to validate the performance of the compendial LO measurements on translucent particles commonly found in LNP formulations and other biotherapeutics.

FlowCam Nano uses a high-magnification immersion oil objective to image submicron particles (particles 0.3-2 µm in diameter). As this size range is somewhat larger than the size of a typical LNP monomer (50-300 nm), FlowCam Nano is capable of imaging small oligomers of LNPs. FlowCam Nano is therefore especially well-suited for identifying the early onset of LNP aggregation as it can detect the smallest aggregates that can form. While submicron particles and aggregates are not subject to GMP regulations like subvisible particles, monitoring and mitigating the smaller aggregates in a formulation will help prevent larger aggregates that are subject to GMP regulations from forming. Additionally, as a high-throughput, solution-based technique, FlowCam Nano can reveal small aggregate information much faster than other optical and electron microscopy techniques and with sufficient image resolution to inform process decisions.

This application note will discuss how FlowCam 8100 and FlowCam Nano can be applied to analyze aggregation and particle formation in an LNP formulation. The LNP formulations in this study were exposed to one of two accelerated stability conditions (freeze-thaw stress and heat stress) to induce aggregation and degradation. Particle concentrations, sizes, and images of the stressed samples were analyzed using FlowCam 8100 and FlowCam Nano. The results demonstrate the combined benefit of FlowCam instruments in LNP biopharmaceutical formulation monitoring and process improvement strategies.

METHODS

An LNP formulation in phosphate-buffered saline (PBS) was prepared and stressed to generate aggregates and other particles for analysis. To remove any large particles in the initial sample, 15 mL of LNP formulation was centrifuged at 6,000 RPM for 1 hour and the top 12 mL of sample were used in the analysis. One 5 mL aliquot was exposed to accelerated freeze-thaw stress in which the sample was frozen at -20 °C for 20 minutes and then thawed in a room temperature water bath for 5 minutes. This freeze-thaw cycle was repeated four times after which the sample was kept at room temperature for the remainder of the analysis. A separate 5 mL aliquot was exposed to accelerated heat stress in which the sample was kept in a 60 °C water bath for four hours. The sample was allowed to cool to room temperature and kept at room temperature throughout the analysis.

The stressed samples were analyzed via FlowCam 8100 and FlowCam Nano to identify subvisible and submicron particles, respectively. The FlowCam 8100 unit was configured with a grayscale camera, a 10X objective, and an FOV80 flow cell. FlowCam 8100 measurements were performed on three 200 µL aliquots per sample. The FlowCam Nano unit uses a 40x objective and FOV60 flow cell. FlowCam Nano measurements were performed on three 100 µL aliquots per sample. Images of air bubbles and other artifacts captured on both FlowCam instruments were excluded.

FlowCam 8100

Name	Count	Particles / ml
LNP Freeze-thaw (FlowCam 8100)	8629	5479.65
2022-03-22 LNP Freeze-Thaw 3 (Filtered)	3220	6122.17
2022-03-22 LNP Freeze-Thaw 2 (Filtered)	2846	5417.42
2022-03-22 LNP Freeze-Thaw 1 (Filtered)	2563	4896.50
LNP Heat (FlowCam 8100)	16024	10287.39
2022-03-22 LNP Heat 3 (Filtered)	5779	11226.53
2022-03-22 LNP Heat 2 (Redo) (Filtered)	5579	10850.97
2022-03-22 LNP Heat 1 (Filtered)	4666	8825.00

FlowCam Nano

Name	Count	Particles / ml
LNP Freeze-thaw (FlowCam Nano)	2935	207671.34
2022-03-22 LNP Freeze-Thaw (Nano) 3 (Filtered)	1001	212482.81
2022-03-22 LNP Freeze-Thaw (Nano) 2 (Filtered)	590	125237.14
2022-03-22 LNP Freeze-Thaw (Nano) 1 (Filtered)	1344	285297.24
LNP Heat (FlowCam Nano)	855	60496.31
2022-03-22 LNP Heat (Nano) 3 (Redo) (Filtered)	317	67288.43
2022-03-22 LNP Heat (Nano) 2 (Filtered)	257	54552.45
2022-03-22 LNP Heat (Nano) 1 (Filtered)	281	59648.02

Figure 3. Particle concentrations reported by FlowCam 8100 (top) and FlowCam Nano (bottom). Each table is broken into two sets of four rows delineated by color. Blue rows indicate measurements from the freeze-thaw sample and red rows indicate those from the heating sample. The top row of each set of four contains the average particle concentration (rightmost column) from three replicate measurements and the remaining rows indicate particle concentrations measured during each individual measurement.

Sizing and Counting: Figure 3 shows the particle counts and concentrations (particles/mL) measured by FlowCam 8100 and FlowCam Nano. FlowCam 8100 concentration measurements exhibited good reproducibility between replicates with the highest and lowest particle concentrations only differing by approximately 10% of the average value. While less consistent than FlowCam 8100 measurements, FlowCam Nano particle concentration measurements still exhibited reasonable agreement between replicates.

Interestingly, the particle counts reveal that the LNP sample exposed to heating stress contained more subvisible particles and fewer submicron particles than the sample exposed to freeze-thaw stress.

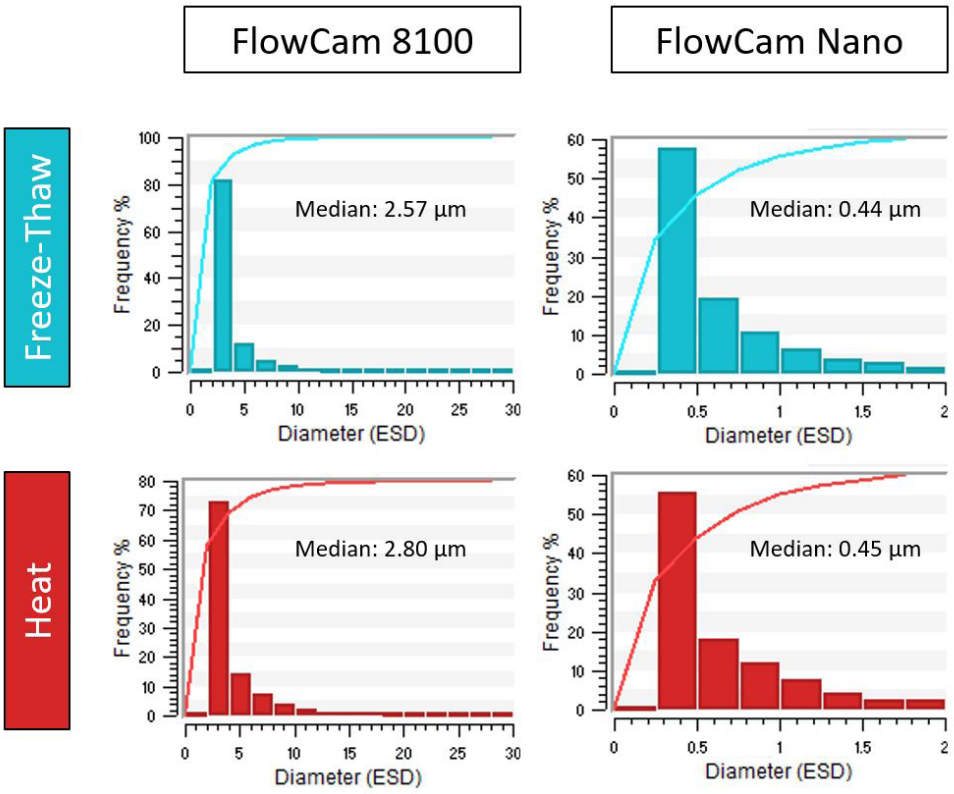

Figure 4. Particle size distributions measured by FlowCam 8100 (left column) and FlowCam Nano (right column) for the freeze-thaw- (top row) and heat-stressed (bottom row) samples. Median diameters are listed on each figure. The curve above the histogram represents the cumulative particle size distribution function for that measurement.

The particle size distributions in each size range as shown in Figure 4 are also consistent with this observation. While the differences in submicron particle size between samples were negligible, the subvisible particle size distribution generated by heating stress was shifted towards larger particle sizes relative to the distribution for particles generated by freeze-thaw stress. These results suggest that heating stress was more damaging to this LNP formulation than freeze-thaw stress. The former produced higher concentrations of large particles while the latter contained particles much closer to the size of smaller LNP particles.

Dynamic light scattering (DLS) measurements of these samples were also performed to confirm these trends. Figure 5 shows the resulting particle size distributions. The heat-stressed LNP formulation exhibited a much broader particle size distribution than either the freeze-thaw stress or the unstressed control and is skewed toward larger particle sizes. This shift in the heat-stressed sample is consistent with the elevated subvisible particle content but reduced submicron particle content observed via FIM for that sample.

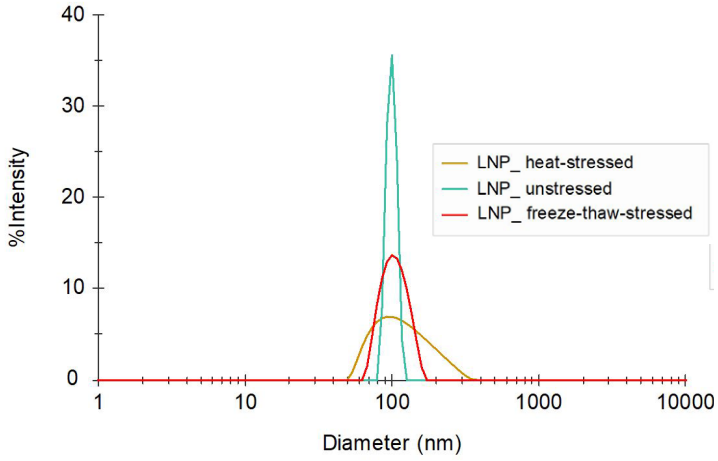

Figure 5. Particle size distributions for the unstressed and stressed LNP samples as measured via dynamic light scattering.

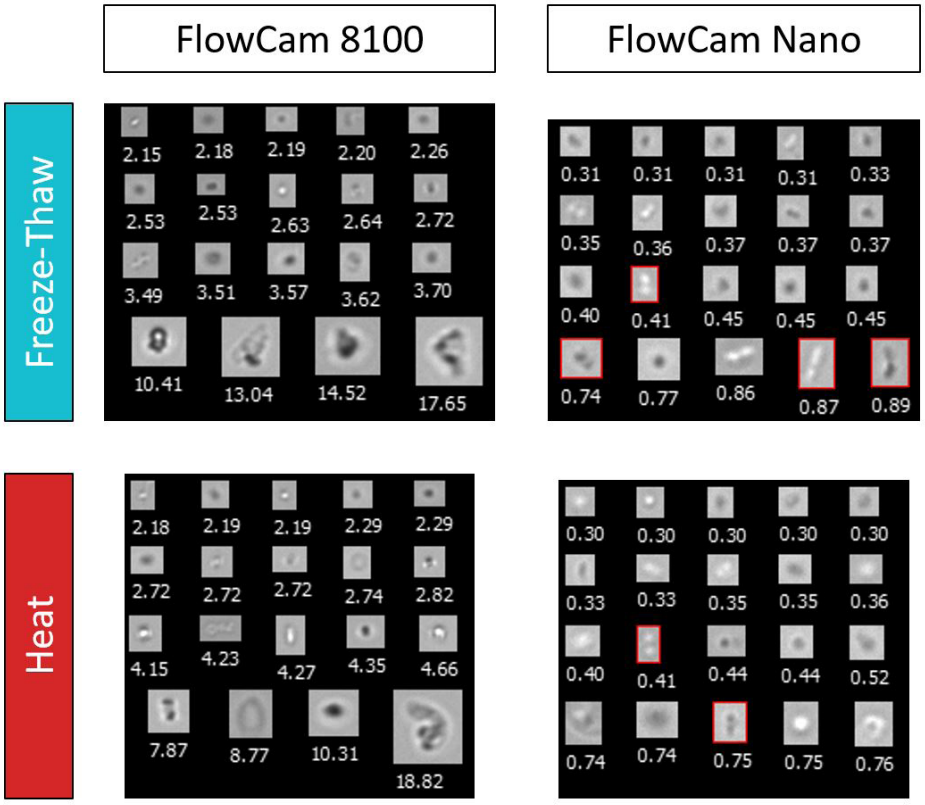

Figure 6. Sample particle images obtained from both samples using FlowCam 8100 (left) and FlowCam Nano (right). Values below each image are the diameter (equivalent sphereical diameter) for the particle in µm. Highlighted images from FlowCam Nano are referred to by the main text.

Image Analysis: Figure 6 shows examples of FlowCam 8100 and FlowCam Nano images obtained from freeze-thaw- and heat-stressed samples used to compute the sample statistics in Figures 3 and 4. The images show that the LNP particles generally exhibited the amorphous shapes that are expected of other types of API particles (e.g. protein aggregates). It is important to highlight the boxed FlowCam Nano images for both stresses. Each of these images appears to contain two or more distinct but attached particles, suggesting that these particles could be dimeric LNP structures. This observation suggests that FlowCam Nano can be used to monitor LNP aggregation even when only oligomeric LNP aggregates have been generated. The particle image information available from FlowCam Nano and FlowCam 8100 lets users monitor the stability of LNPs in their formulation as well as check for other common sources of formulation instability like container-closure compatibility – information that can be valuable when selecting or optimizing LNP formulations to maximize stability.

CONCLUSIONS

FlowCam is an excellent tool for monitoring particle content and aggregation in LNP formulations as it is for many other biopharmaceutical APIs. FlowCam 8100 and FlowCam Nano measurements provide users not only with useful particle counts and sizing data in the submicron and subvisible size range, but also images that can be used to track common sources of formulation instability. FlowCam Nano is especially useful for this purpose as the high magnification it offers allows for early detection of LNP aggregation and possibly other mechanisms of degradation to be monitored in a high-throughput fashion. The information available from FlowCam is invaluable to researchers working with LNP formulations, allowing researchers to monitor and improve the stability of their formulations and maximize the positive impact of these emerging therapies on patients.