Count, size and quant your LNPs with Stunner

Introduction

Three of the most important characteristics about lipid nanoparticle (LNP) samples are how many you've got, what size they are and how much RNA you have – and there can be a lot of obstacles to gathering that data.

Nanoparticle concentration is a critical parameter to know about every batch of particles, but current methods can't keep up with high throughput, require dilutions that impact accuracy, or are just plain slow. Keeping particles the right size is everyone's goal for LNPs, but picking up on all the aggregates in a sample just doesn't happen when you're measuring at only one angle. Even just gathering size data on every sample is a challenge when many dynamic light scattering (DLS) instruments are stuck measuring samples one at a time.

Different assays are used to quantify RNA, DNA, protein, or small molecule payloads, but most rely on complicated disruption workflows, costly dyes, and wasteful standard curves. UV/Vis absorbance has been around forever for RNA, DNA, protein and small molecule quantification in purified samples, but the high turbidity of nanoparticles makes samples too cloudy for most UV/Vis spectrophotometers and keeps them from getting accurate readings.

Stunner is the only platform that pairs up high throughput UV/Vis with rotating angle dynamic light scattering (RADLS) to give you a total RNA readout, nanoparticle concentration, size and polydispersity, on a single 2 µL sample, without the need for dilutions, standards, or fluorescent dyes (Figure 1). All of Stunner's RADLS and UV/Vis data is done in a plate-based format, under 2 minutes per sample for up to 96 samples at a time.

This app note checks out how Stunner can size and count up LNPs with RADLS, and quantify RNA payloads with UV/Vis on two preparations of LNPs. For a deep dive into the details of RADLS, see the Tech Note "Dive deep into Stunner's light scattering."

Figure 1: Stunner is the only system that pulls together UV/Vis, RADLS and MALS data on the same 2 µL sample.

Results

Size

A RADLS read on Stunner reports out three different size distributions: intensity, mass, and number. What makes each of these unique with RADLS is that they're not normalized values – they're real readouts on the light scattering intensity, mass concentration, and number of particles measured in a sample.

The intensity distribution (Figure 2A) shows how much light scattering is coming from each particle size, so you see those rare, large aggregates that scatter tons of light but only have a few floating around. Scattering intensity is calculated at a 0° scattering angle (θ = 0°), which means it's angle-independent and automatically adjusts for Rayleigh or Mie light scattering behavior. As the name says, the mass distribution (Figure 2B) takes the intensity distribution and converts it to a mass concentration vs. size graph. The number distribution (Figure 2C) extends the same approach all the way to a particle concentration vs. size graph. If you've got two samples that have a different number of LNPs per mL, then this is where you'll see peak height start to vary.

Two batches of LNPs were prepared for characterization on Stunner. Small LNPs containing the ionic lipid SM102 and PolyA RNA were compared against large LNPs with the cationic lipid DDAB and purified calf liver RNA. The large LNP sample was analyzed unfiltered, and after sequential filtering using 0.45 µm and 0.2 µm filters.

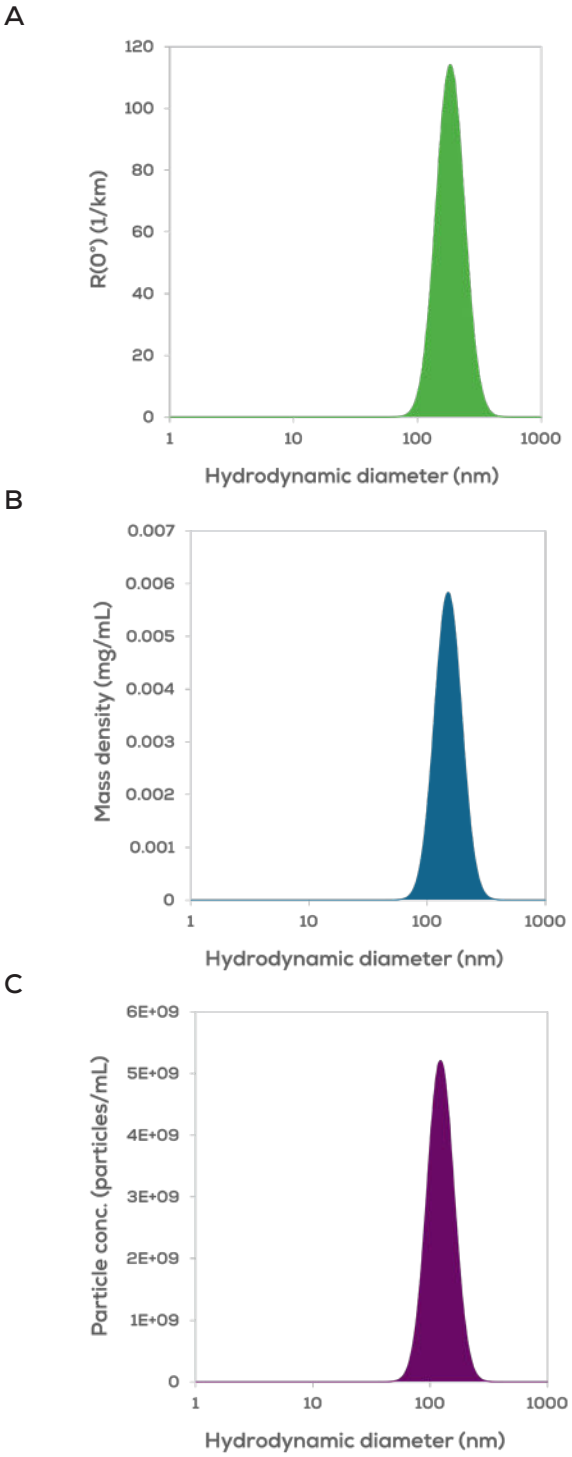

Figure 2: Intensity (**A**), mass (**B**), and number (**C**) distributions for a homogeneous LNP.

Replicates of the small LNPs were measured using RADLS to demonstrate the repeatability of Stunner's self-optimizing optics. Stunner optimizes light scattering overlap for each sample so that replicates can lie right on top of each other (Figure 3). The mass distribution mean diameter of the large LNPs was 159 nm with a 4% CV when measured in quadruplicate on five instruments, while the small LNPs measured 82 nm and 5% CV.

An overlay of the number distribution of the small and large LNPs shows the difference in number mean diameters: 47.8 nm vs. 127 nm for the two reads shown (Figure 4). The area under the curve for these distributions indicates the particle concentration present in both samples.

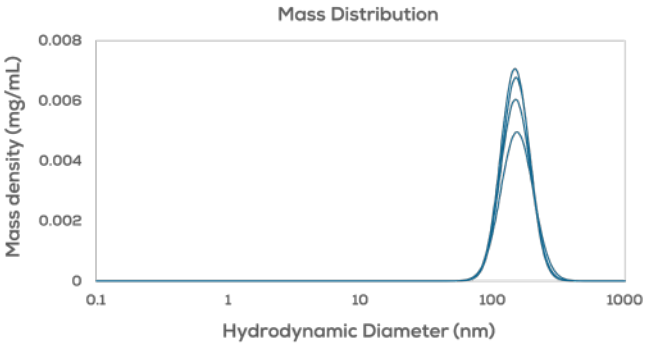

Figure 3: Overlay of the mass distribution for four replicates of a large LNP.

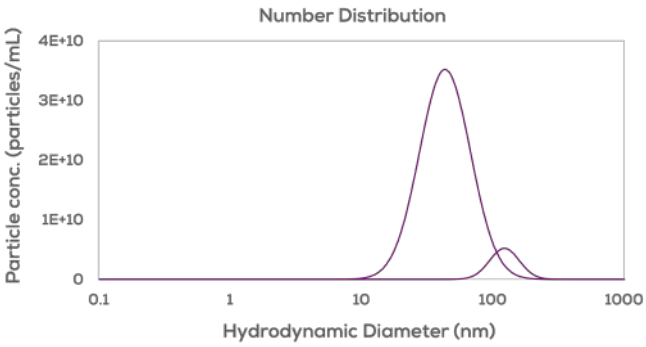

Figure 4: Comparison of the number distributions for a small LNP (left peak) and a large LNP (right peak). The area under the curve is the particle concentration for each sample.

The mass or number distributions are a good way to visualize the most common particle sizes in a sample, while Z-average size is a common metric to use when concerned about aggregation since it is more sensitive to the presence of aggregates. For a sample with aggregation, the Z-average size will creep upwards before mass or number distributions begin to show changes. RADLS calculates the Z-average diameter for a sample at $\theta = 0°$, making it an angle-independent size metric that is uniquely sensitive to large particles missed by not measuring enough angles.

Table 1 shows how the Z-average diameter decreases as the largest particles were remove from the large LNP sample by successive filtration steps. As expected, the Z-average diameter decreases as smaller filter pore sizes are used. In addition the variability around the Z-average diameter decreases as larger aggregates are removed. As the table shows, Z-average diameter will usually report a larger average size than the main peak of the number distribution since it is sensitive to large aggregates. Number mean diameter is a better representation of the most common particle sizes present in a sample.

Count

When the number of LNPs present in a sample increases, it's easy to predict that the intensity of light scattering will also increase – but light scattering intensity also increases when LNPs get larger, and scattering intensity will change depending on the measurement angle for each instrument.

Careful multi-angle light scattering (MALS) intensity measurements made as part of RADLS measurements calculate angle-independent size and intensity to get a more accurate nanoparticle concentration.

Particle concentration measurements were made in the same read used to measure the sizes of the small and large LNP samples from above. Stunner's Estimated Particle Concentration metric was used to measure the particle concentration coming from the expected LNP peak in the number distribution. The small (SM102) LNPs had an average particle concentration of 7.5e11 particles/mL with a 30% CV (Figure 5A) while the large (DDAB) LNPs had an average particle concentration of 8.5e10 particles/mL with a 28% CV. The ~8.8x difference in particle concentration can also be visualized by the area under the curve for the number distributions shown in Figure 4.

The intermediate precision of measuring particle concentration was evaluated across five instruments (Figure 5B). The means of all instruments were within 30% of the average of all measurements.

Particle size homogeneity plays a role in the measurement precision of particle concentration. The large LNP sample was filtered through a 0.2 µm filter, but the same sample was also tested unfiltered and after filtering through a 0.45 µm filter. Removing larger particles had a strong effect towards improving the precision of measuring particle concentration.

Sample	Average of Z-Avg Dia. (0°) (nm)	%CV	Number Mean Dia (nm)	%CV
Unfiltered	206	27%	136	5%
0.45 µm filtered	189	7%	129	4%
0.2 µm filtered	161	5%	124	4%

Table 1: Mean diameters and precision data for Z-average diameter at 0° compared to the number mean diameter for the large LNP same measured unfiltered, after filtration with a 0.45 µm filter, and after filtration with a 0.2 µm filter. %CVs are measured for quadruplicates run on 5 instruments.

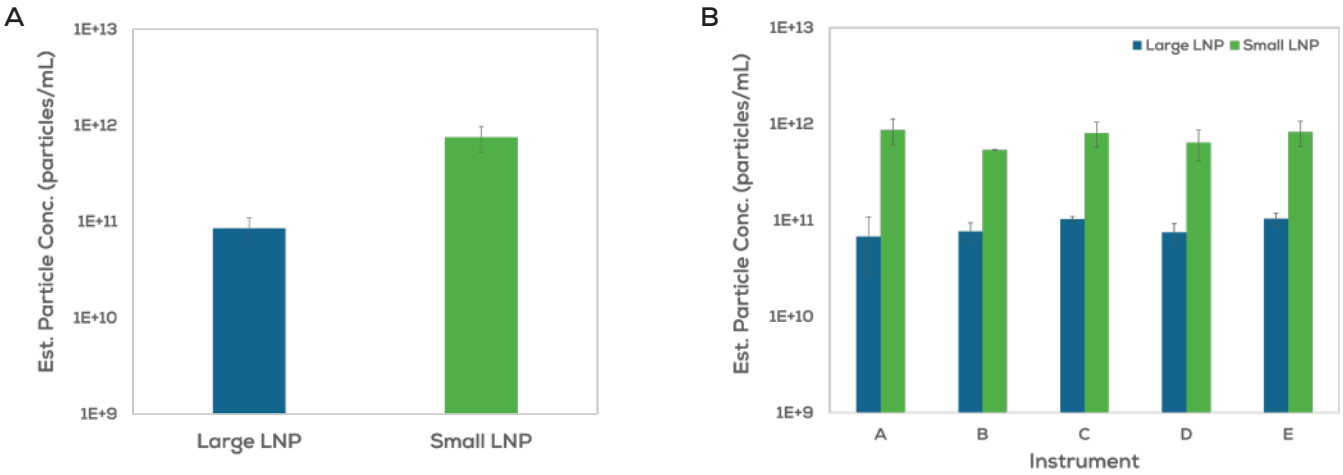

Figure 5: The particle concentration for large and small LNP samples. Error bars are the standard deviation across 4 replicates on 5 instruments each (**A**). Instrument-by-instrument particle concentrations for the same two samples. Error bars are the standard deviation across 4 replicates each. The means of all five instruments are within 20% of the average concentration value for each particle (**B**).

Diluting the small LNP samples in a 5-fold series down to 625-fold dilution shows the linearity and the wide dynamic range for particle concentration (Figure 6). The starting concentration was 1.0e12 particles/mL and final dilution was measured at 1.2e9 particles/mL. Across the entire dilution range, an unweighted linear regression fitting has a R^2 value of 0.9983. For the lowest concentration the CV was only 32%, suggesting that the lower limit was not reached. It is important to note that the lower limit of LNP particle concentration will vary from particle-to-particle and sample-to-sample as it depends on average size, composition, and buffer. For example, for 81 nm polystyrene beads, a six log dynamic range (10^7-10^{13} particles/mL) were observed (data not shown).

Quant

For LNP samples, Stunner's built-in applications for RNA-LNP, DNA-LNP, or protein-LNP payloads makes quant simple and easy. Stunner cuts through the turbidity of an LNP with Unmix analysis to separate out the absorbance of each pre-set payload from any particle or buffer absorbance. For LNPs loaded with any kind of RNA, the RNA-LNP application will help quantify the total amount of RNA present in a sample (Figure 7) without any kind of sample preparation and

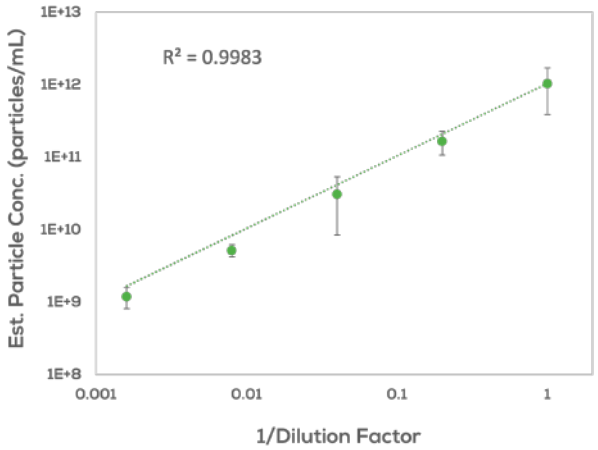

Figure 6: Estimated particle concentration across a five-fold dilution series of the small LNP sample down to 625x dilution. Error bars are the standard deviation across 4 replicates on 5 instruments each.

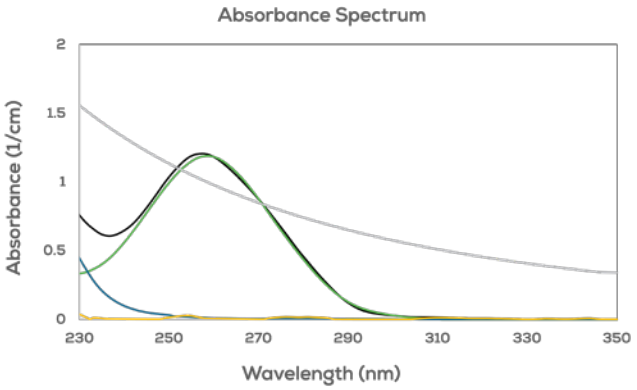

Figure 7: Stunner's RNA-LNP application is able to separate the UV/Vis signals from RNA, lipids, turbidity, and other factors. For this RNA-LNP sample, RNA absorbance (green) measures the total concentration of RNA present in an LNP sample. Turbidity is shown in gray, total absorbance without turbidity shown in black, absorbance of the particle and buffer components in blue, and residual fitting error in yellow.

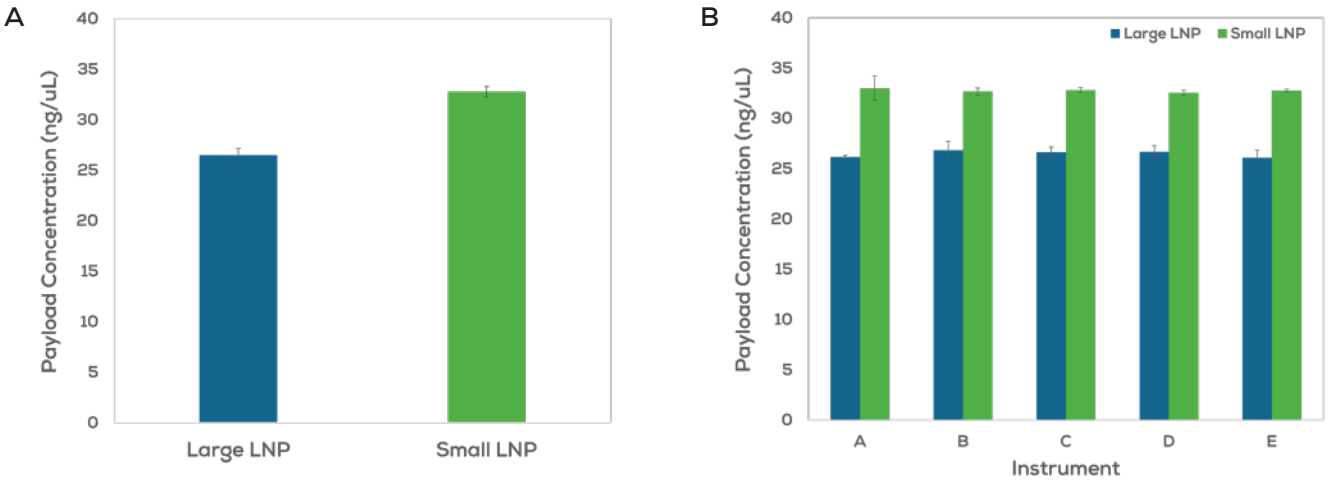

Figure 8: Total RNA concentration for the large and small LNP samples. Error bars are the standard deviation across 4 replicates on 5 instruments each (**A**). Instrument-by-instrument RNA concentrations for the same two samples. Error bars are the standard deviation across 4 replicates each (**B**).

without any standards or reagents. The total amount of RNA measured by Stunner is the sum of the RNA present in the LNP and any outside of the particle.

Stunner's RNA-LNP app was put to the test for the same large and small LNP samples as above. In the same read as the particle concentration and size measurements, Stunner reported that the large LNP samples had an average payload concentration of 26.5 ng/µL with a CV of 2.5% while the small LNP samples had an average payload concentration of 32.7 ng/µL with a CV of 1.7% (Figure 8A). Comparing these values across five instruments (Figure 8B) showed differences from the mean of less than 1.5% for the large LNPs and less than 1.1% for the small LNP samples.

From one read of less than two minutes per sample, Stunner now knows the particle concentration and the total payload concentration of RNA in a sample. As part of setting up a Stunner run you can tell it a few details about the RNA present in an sample, so it knows everything needed to convert that RNA concentration into a count of total molecules. With all that knowledge in hand, Stunner automatically reports out the average number of RNA molecules per particle.

Sunscreen was used to produce a range of LNPs at different total flow rates (TFRs) from 6-12 mL/min and samples were characterized on Stunner. As flow rate increased the mean diameter for the LNPs steadily decreased (Figure 9A), which is expected as higher TFRs correspond with increased shear rates during mixing. For this screen at constant lipid concentrations and flow rate ratios (FRRs), the smaller LNP diameters formed at higher TFRs also correlate with higher concentrations of particles (Figure 9B). TFR did not correlate with RNA concentration (Figure 9C), which is expected as long as FRR and the concentration of RNA in the aqueous phase are both constant. Lastly, with constant RNA concentrations and higher particle concentrations, Stunner calculates the number of RNA molecules per particle (Figure 9D) as a way to easily visualize the impact of different LNP formulation and process conditions.

Conclusions

With RADLS and UV/Vis data on every sample Stunner delivers high-throughput answers on particle concentration, size, PDI and total RNA payload all at once. Walk away from DLS that runs samples one-by-one or only takes data from one angle on large samples. Crazy simple UV/Vis measurements give reagent-free, standard-free and hassle-free quants. A suite of LNP applications, along with the Custom Nanoparticle app, helps you analyze your nanoparticle, no matter what's loaded inside: RNA, DNA, protein or small molecules. Free yourself from complicated disruption workflows, costly dyes, wasteful standard curves and one-at-a-time DLS sizing with Stunner's dye-free, label-free, standard-free, and hassle-free nanoparticle characterization.

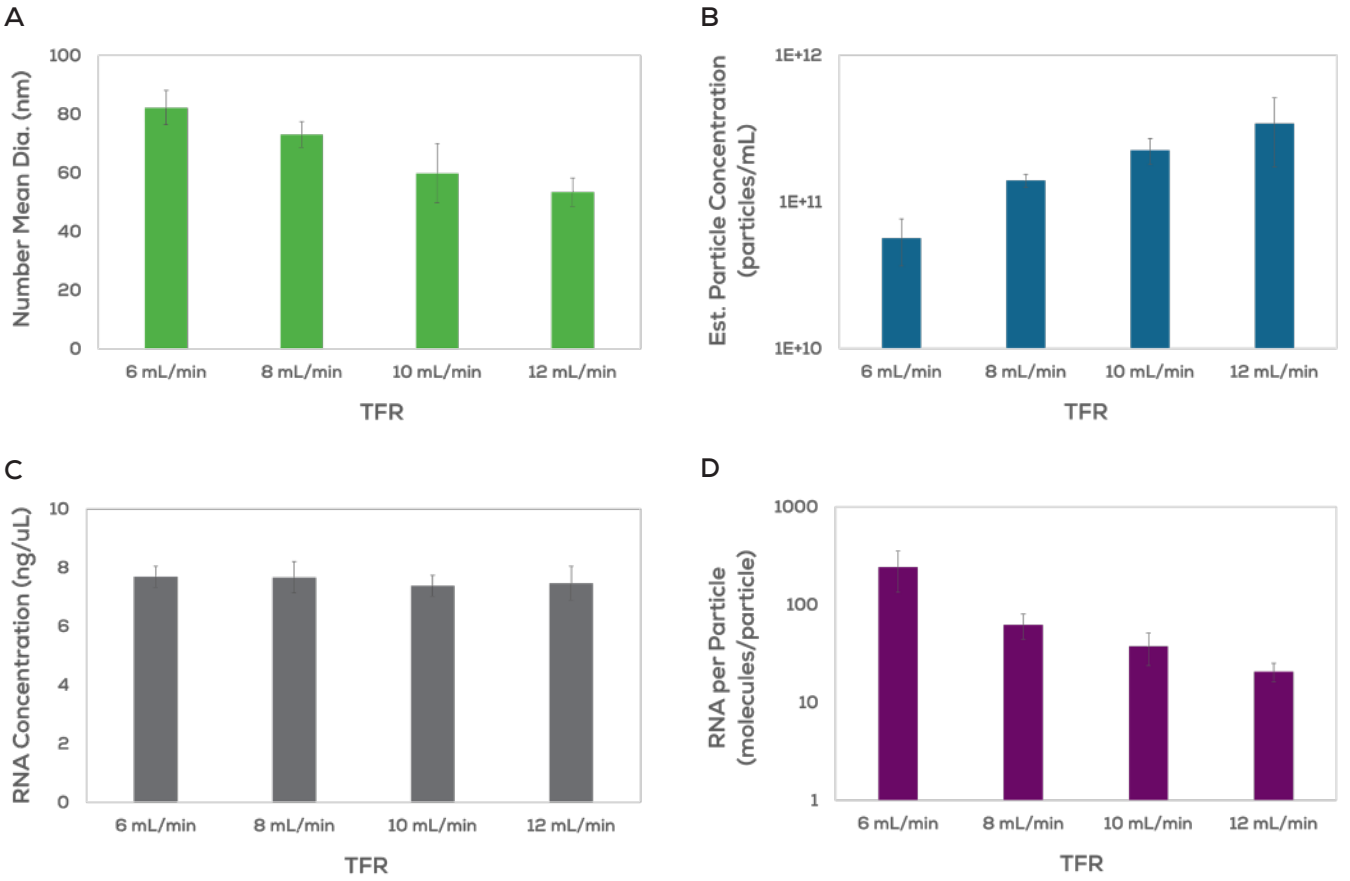

Figure 9: LNPs were generated on Sunscreen across multiple total flow rates (TFR). Stunner simultaneously gathers size (**A**), particle concentration (**B**) and a total RNA concentration (**C**) on every sample and calculates an average number of RNA molecules per particle (**D**), assuming 100% encapsulation efficiency.

Materials & methods

1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphoethanolamine (POPE), cholesterol (Chol), and 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG) lipid stocks were made in ethanol.

Calf liver RNA and calf thymus DNA were filtered with a 10 kD MWCO filter and the retentate used for LNP production.

LNPs were made using Sunscreen by Unchained Labs. LNPs were composed of DOTAP:POPE:Chol:D-MG-PEG dissolved in ethanol at 50:10:38.5:1.5 molar ratio and made with a total flow rate (TFR) of 12 mL/min and an aqueous-to-organic flow rate ratio (FRR) of 3:1. RNA-and DNA-LNPs were made with nitrogen-to-phosphate ratio (N/P) of 5, 10, and 50 by adding 100, 50, and 10 µg/mL, respectively, nucleic acid to the aqueous phase (100 mM citrate buffer, pH 4) and a final lipid concentration of 4 mM.

After production, the LNPs were buffer-exchanged into PBS, pH 7.4 to reduce residual organic solvent.

LNP particle concentration, RNA payload quantification, hydrodynamic size and polydispersity were assessed using the Stunner RNA-LNP application. PBS was used as a blank. Outliers were excluded if 2 or more angles were excluded by the software's automatic angle selection, or during analyses using Number distribution metrics, if more than 1 peak was found. A buffer viscosity and refractive index of 1.002 cP and 1.334, respectively, at 20°C and the default acquisitions of 7 angles, 5 acquisitions, and 1 second each were used for RADLS.

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