Screen and prep LNPs with Nunchuck

Introduction

Lipid nanoparticles (LNPs) are a valuable tool in the repertoire of gene and vaccine delivery vectors, but LNP development is a complex, multi-step process. Screening formulations, controlling the size and quality of the LNPs, and improving encapsulation efficiency all require significant and tedious optimization. Then, when you have the perfect method, you must transfer it to another platform for scale-up and repeat all that work. Nunchuck is a simple, robust, and scalable microfluidic mixing platform capable of quickly producing 0.5–200 mL of LNPs to speed you through finding the perfect LNP (Figure 1).

Particle size impacts everything in LNPs, from tissue targeting to payload release. Nunchuck is the first and only platform that combines two critical steps needed to make the perfectly-sized LNP – total flow rate screening and lipid nanoparticle formulation. Screening 8 total flow rates (TFRs) from 0.5–30 mL/min in just 10 minutes makes it easy to get the particle size needed for a given application. Low volume runs mean formulation screening wastes less of your expensive payload and takes the guesswork out of LNP manufacturing. Then, with all the parameters dialed in, Nunchuck can make up to 200 mL of consistent, high-quality LNPs.

Nunchuck uses microfluidics to control the nano-precipitation of LNPs from aqueous and organic solutions. This leads to exceptional reproducibility and fine control over particle size simply by varying the TFR and flow rate ratio (FRR). The Nun cartridge contains mixing chambers compatible with a wide array of lipid formulations, organic components, and payloads. LNPs made with cationic and ionizable lipids produced by Nunchuck show high encapsulation and successfully maintain the chemistry-dependent behavior for the efficient delivery of mRNA.

A

B

Figure 1: Nunchuck is the ultimate one-and-done LNP maker (**A**). The Nun consumable contains novel microfluidic mixing wells and the entire fluidic pathway (**B**).

This app note shows how Nunchuck can screen a range of TFRs to make ionizable and cationic RNA-LNPs of uniform size, high encapsulation efficiency, and expected morphology, sized from 50–200 nm and in a volume range from 0.5–200 mL.

Methods

LNP Formulations

RNA-LNPs were prepared by microfluidic mixing in Nunchuck of various aqueous and organic phases. Aqueous phases consisted of mRNA (CleanCap® EGFP mRNA, L-7601, TriLink Biotechnologies) or Poly(A) RNA (Poly(A), polyadenylic acid, cat. no. 10108626001, Sigma-Aldrich) in 50 mM sodium acetate buffer, pH 4 or 5. The RNA concen-

tration was determined by UV/Vis on Stunner and adjusted with buffer to maintain a nitrogen-to-phosphate ratio (N:P) of 8 or 9, depending upon the lipid formulation and desired FRR. Empty LNPs were made with the same buffer and under the same conditions without RNA. Organic phases consisted of ionizable lipids mixed with 1,2-Distearoyl-sn-glycerol-3-phosphocholine (DSPC), Cholesterol (Chol), and DMG-PEG(2000) in ethanol at a 50:10:38.5:1.5 molar ratio, respectively. Ionizable lipids included 8-[(2-hydroxyethyl) [6-oxo-6-(undecyloxy)hexyl]amino]-octanoic acid, 1-octylnonyl ester (SM-102) and 4-(dimethylamino)-butanoic acid, (10Z,13Z)-1-(9Z,12Z)-9,12-octadecadienoic-1-yl-10,13-nonadecadien-1-yl ester (MC3). Cationic RNA-LNPs were made with 1,2-dioleoyl-3- trimethylammonium-propane (DOTAP) dissolved in ethanol in a 40:10:48:2 molar ratio of DOTAP:DSPC:Chol:DMG-PEG(2000). The total lipid concentration was 8 mg/ml for the ionizable lipid mix and 12 mg/ml for the DOTAP lipid mix. All lipids were purchased from Cayman Chemical or Avanti Polar Lipids.

The LNPs were produced with Nunchuck using either the Screening Run or Prep Run applications. A subset of the standard TFR screen was used for all LNPs: 30, 25, 20, 15, 10, 5, and 2 mL/min with the fastest TFR screened first and the rest in order of decreasing speed. We also tested two FRRs: 3:1 and 10:1. For a detailed description of how to run TFR screens on Nunchuck, please read the *RNA-LNP Screening Run on Nunchuck* technical note.

LNP Characterization

LNPs were immediately diluted 10-fold with PBS, and 2 µL of each sample was loaded into a Stunner plate to measure the size and polydispersity index (PDI) by dynamic light scattering (DLS).[1] Encapsulation efficiency (EE%) was measured using a fluorescence plate-based assay employing RiboGreen reagent (Invitrogen) to measure RNA concentration with and without disruption by 10% Triton X-100 detergent. EE% was calculated as the difference between the total RNA and the unencapsulated RNA divided by the total RNA. The stability of SM-102 LNPs was evaluated using the isothermal application on Uncle at 37 °C for 4 hours to monitor the changes in the size and PDI of the LNPs at defined time points using DLS.[2]

Cryo-electron microscopy

LNP samples were dialyzed in PBS overnight before being prepared for CryoEM. Acquisitions were performed with the help of David Bulkely, Ph.D. at the UCSF EM Core Facility. 3–5 µL of each LNP was pipetted onto glow-discharged Ultrathin carbon on Quantifoil®, mounted on copper grids (Ted Pella) in a Vitrobot Mark IV (FEI) chamber with 95% relative humidity. The grids were blotted for 4 seconds and plunged into liquid ethane, cooled by liquid nitrogen. Micrographs were collected at 200 kV using a Glacios Cryo-TEM with a Gatan K3 camera. UCSF MotionCorr2 v1.3.0 was used for real-time motion correction and dose-weighting. The images were processed and analyzed manually using Fiji.[3] The particle diameters of a minimum of 40 unobstructed LNPs were determined for each sample, and a mean, standard deviation, and polydispersity index (PDI) were calculated for each sample.

Results

Control LNP size with TFR and FRR

Tuning the size is an important factor in biodistribution and cellular targeting for LNP therapeutics.[4] Increasing the TFR from 2 mL/min to 25 mL/min on Nunchuck of Poly(A)- and mRNA-loaded SM-102 LNPs decreased the hydrodynamic size of the particles from 120 and 140 nm to 70 and 90 nm, respectively (Figure 2). The PDI for all LNPs was less than 0.2, indicating monodisperse populations. EE% remained above 85% in all cases regardless of payloads or TFR. The Nunchuck microfluidic platform allows for quick, reproducible, and size-tunable assembly of nanosized particles to increase process control, limit the use of reagents, and decrease optimization time.

In addition to the TFR, we also observed that adjusting the FRR between the organic and aqueous phases can regulate particle size. Higher aqueous to organic FRR results in smaller

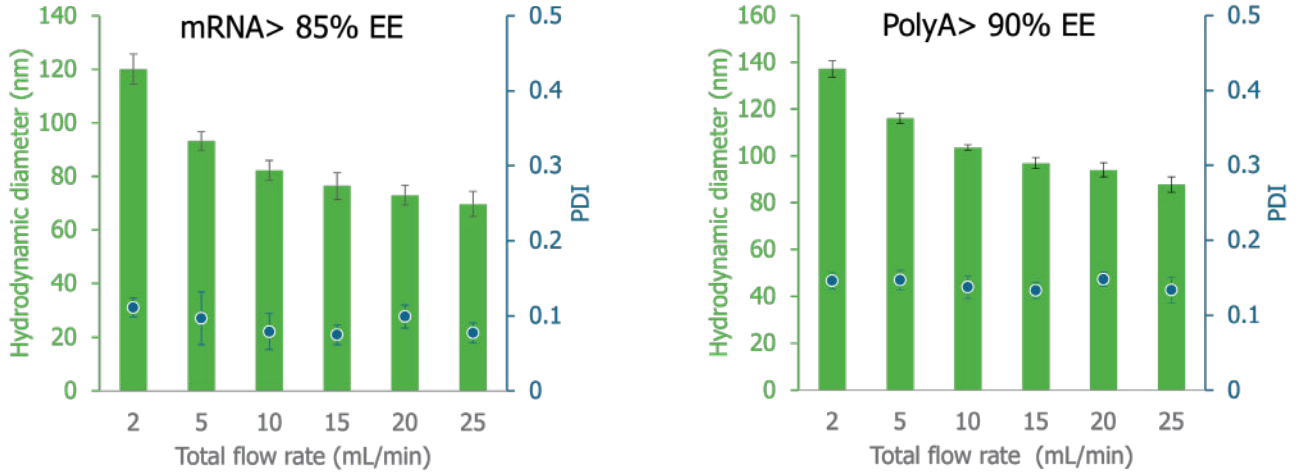

Figure 2: Effect of TFR on size and PDI for SM-102 LNPs formulated with two different payloads.

particles (Figure 3) when the TFR is constant (TFR at 25 mL/min). When the drug substance is soluble in water, the ability to tailor the flow rate ratio is essential for maximizing loading efficiency and tailoring the size of the LNP. The Nunchuck provides direct size control by controlling the input flow rates of the organic and aqueous streams and the flow rate ratio between streams.

Create LNPs with expected morphology

SM-102 LNPs made using Nunchuck were predominately spherical, with empty particles adopting either a unilamellar or bilamellar structure and Poly(A)-loaded particles forming bilamellar structures (Figure 4). The loaded particles displayed morphologies typical of other RNA-LNPs, including some with a bleb compartment and others with higher electron density regions, likely dense-packed Poly(A), more fully encapsulated within the lipid particle.[5] Bleb compartments were more common in the 2 mL/min TFR Poly(A)-loaded particles, while the 20 mL/min particles were more uniform.

Poly(A) LNPs made with a TFR of 2 mL/min were larger than those made with a TFR of 20 mL/min, showing average core diameters of 60.4 nm and 40 nm, respectively. The larger particles also tended to be less uniform in size with a PDI of 0.13,

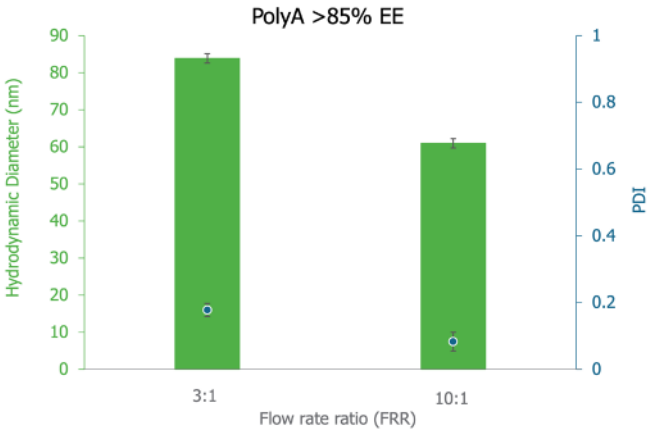

Figure 3: Effect of FRR on size and PDI.

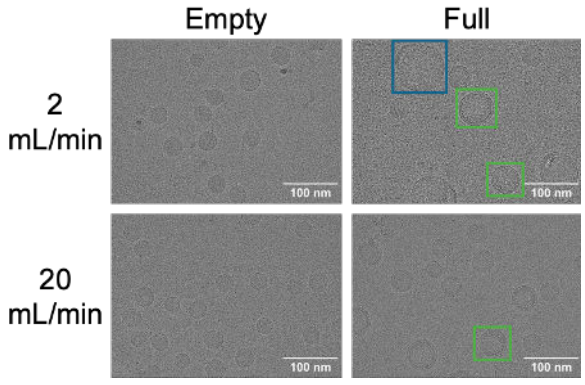

Figure 4: Poly(A)-loaded (full) SM-102 LNPs with a 3:1 FRR are larger than empty LNPs made at the same TFR and FRR without payload. Typical morphologies of full RNA-LNPs include particles containing bleb compartments (blue box) and electron-dense, fully encapsulated spherical particles (green boxes).

compared to 0.03 for particles made at 20 mL/min TFR, despite the same lipid formulation and payload. The EM core diameters of the 2 and 20 mL/min Poly(A) LNPs were significantly smaller than the Stunner measured hydrodynamic diameters of 120.6 and 76.4 nm respectively. This is most likely due to the difficulties in resolving the low contrast PEG chains on the lipids at the surface of the LNPs.[6] PEG chains and PEGylated lipids significantly impact the diffusion rate and hydrodynamic diameter of LNPs, as well as increasing circulation time and decreasing immunogenicity.[7]

Changing the TFR had a comparatively smaller impact on the empty SM-102 LNPs, with the 2 mL/min particles having an average core diameter of 34.2 nm and the 20 mL/min particles having a core diameter of 31.8 nm. Under both TFR conditions, full LNPs were significantly larger than empty LNPs. Nunchuck can be used to create consistently high-quality lipid nanoparticles time and time again.

Formulation screens

Poly(A)-loaded LNPs were made with three lipids, one cationic and two ionizable (DOTAP, MC3, SM-102), and, regardless of the chemistry, the EE% remained above 95%. However, TFR and choice of charged lipid component noticeably impacted the size of LNPs (Figure 5). Nunchuck's Screening Run application makes it easy to perform screening and rapidly select the perfect formulation.

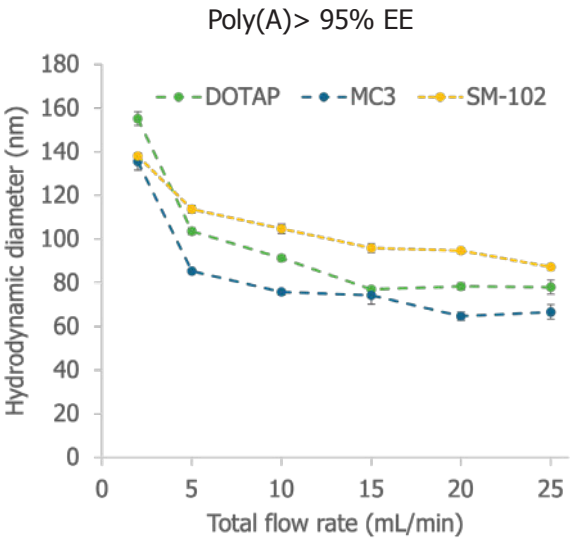

Figure 5: Effect of different chemistry on size.

Stability of LNPs

The stability of RNA-LNPs is an essential factor during storage and circulation under physiological conditions. We held Poly(A)-loaded SM-102 LNPs made with Nunchuck at 37 °C in Uncle for 4 hours and monitored them for the size or PDI. The hydrodynamic diameters of LNPs remained significantly stable and monodisperse (PDI <0.2) over the course of 4 hours of the physiological simulation (Figure 6).

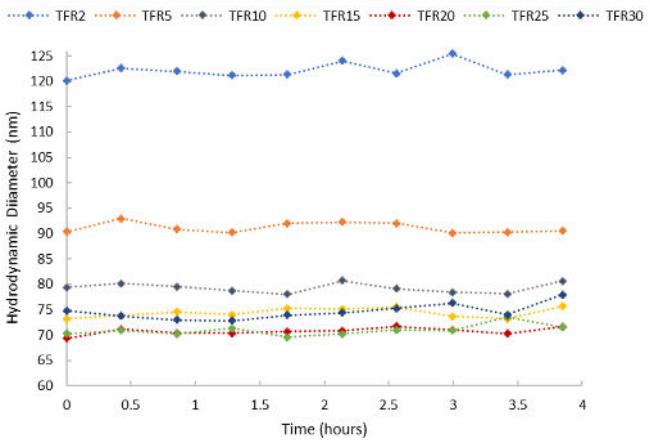

Figure 6: Poly(A)- SM-102-LNPs size change over 4 hours of incubation at 37 °C.

Differences in surface charge for cationic vs. ionic LNPs

Hepatic tissues have been shown to uptake ionizable, but not cationic, siRNA-LNPs, indicating that LNP surface charge plays a role in LNP biodistribution and organ-specific delivery.[8,9] DOTAP, MC3, and SM-102 LNPs, formulated on Nunchuck, were diluted 10-fold into a pH 5.2 or pH 7.4 buffer and the zeta potential was determined using Electrophoretic Light Scattering (ELS) on a NanoBrook Omni. Each LNP formulation we tested displayed different behavior. The ionizable LNPs showed a transition from a positively charged LNP at low pH to a neutrally charged LNP at physiological pH which facilitates endosomal escape, contrary to permanently positive lipid such as DOTAP which does not display a significant change in surface charge with change in buffer pH (Figure 7).

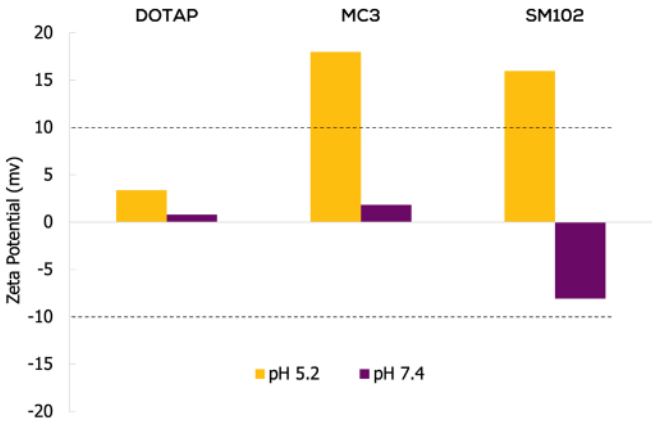

Figure 7: Zeta potential for various apparent pKa.

Scale up

To ensure the formulation uniformity from batch-to-batch over time and transferability from screening runs to prep runs, we tracked the size and PDI of batches made up to a 200 mL formulation volume (Figure 8). The hydrodynamic diameter was 66.5 ± 1.2 nm and the PDI was 0.13 ± 0.02. Nunchuck allows for a seamless transition from small-volume optimization to large-scale, pre-clinical production. Nunchuck can produce a highly uniform formulation across 0.5 -200 ml due to the novel microfluidic geometries that ensure all incoming fluid experiences uniform vortical mixing profiles.

Conclusion

Screening payloads, lipid formulations, and mixing conditions of LNPs takes time. Nunchuck quickly and reproducibly synthesizes RNA-LNPs at volumes as low as 0.5 ml to minimize the use of scarce and expensive payloads. The Nun uses a novel microfluidic mixing technology for the rapid (<10 minutes for total 8 TFRs), controlled, and reproducible formulation of RNA-LNPs. The size of LNPs could be controlled on Nunchuck by varying the TFR or FRR and resulted in particles 50 –200 nm in diameter with PDIs typically below 0.2. The encapsulation efficiencies of mRNA or Poly(A) were consistently over 85% to ≥99% encapsulation. The choice of charged lipid also impacted the particle size, PDI, and surface charge of the LNPs.

Nunchuck is the only system that combines low volume screening with high-volume prep runs. Optimized parameters such as reagent concentrations, TFR, and FRR were transferred directly from a formulation screen to a prep run to produce at 200 mL of LNPs, reducing the need to re-optimize the process when changing the batch size. Dump the one-flow-rate-at-a-time, back-and-forth workflows for the ultimate one-and-done LNP maker.

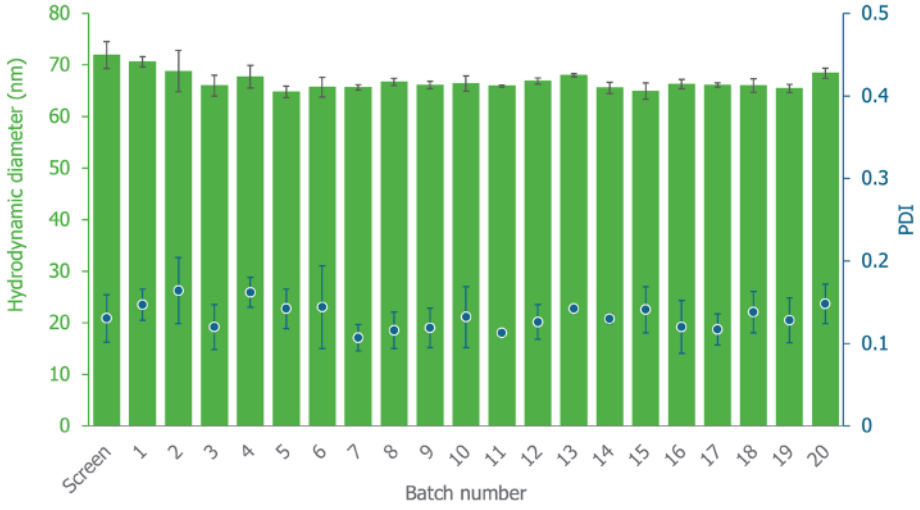

Figure 8: Twenty fractions of 10 mL were collected during the fabrication of 200 mL of DOTAP formulation.

References

1. Get the skinny on LNPs with Stunner. Unchained Labs. Application Note. 2021.
2. Uncle stays hot for proteins. Unchained Labs. Application Note. 2022.
3. Fiji: an open-source platform for biological-image analysis. J Schindelin, et al. Nature Methods. 2012; 9(7):676–682.
4. Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA. S Chen, et al. Journal of Controlled Release. 2016; 235:236–244.
5. Encapsulation state of messenger RNA inside lipid nanoparticles. ML Brader, et al. Biophysical Journal. 2021; 120(14):2766–2770.
6. Deconvoluting Lipid Nanoparticle Structure for Messenger RNA Delivery. Y Eygeris, et al. Nano Letters. 2020; 20(6):4543–4549.
7. PEGylation as a strategy for improving nanoparticle-based drug and gene delivery. JS Suk, et al. Advanced drug delivery reviews. 2016; 99(Pt A):28–51.
8. Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms. A Akinc, et al. Molecular Therapy: The Journal of the American Society of Gene Therapy. 2010; 18(7):1357–1364.
9. Selective ORgan Targeting (SORT) nanoparticles for tissue specific mRNA delivery and CRISPR/Cas gene editing. Q Cheng, et al. Nature nanotechnology. 2020; 15(4):313–320.

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