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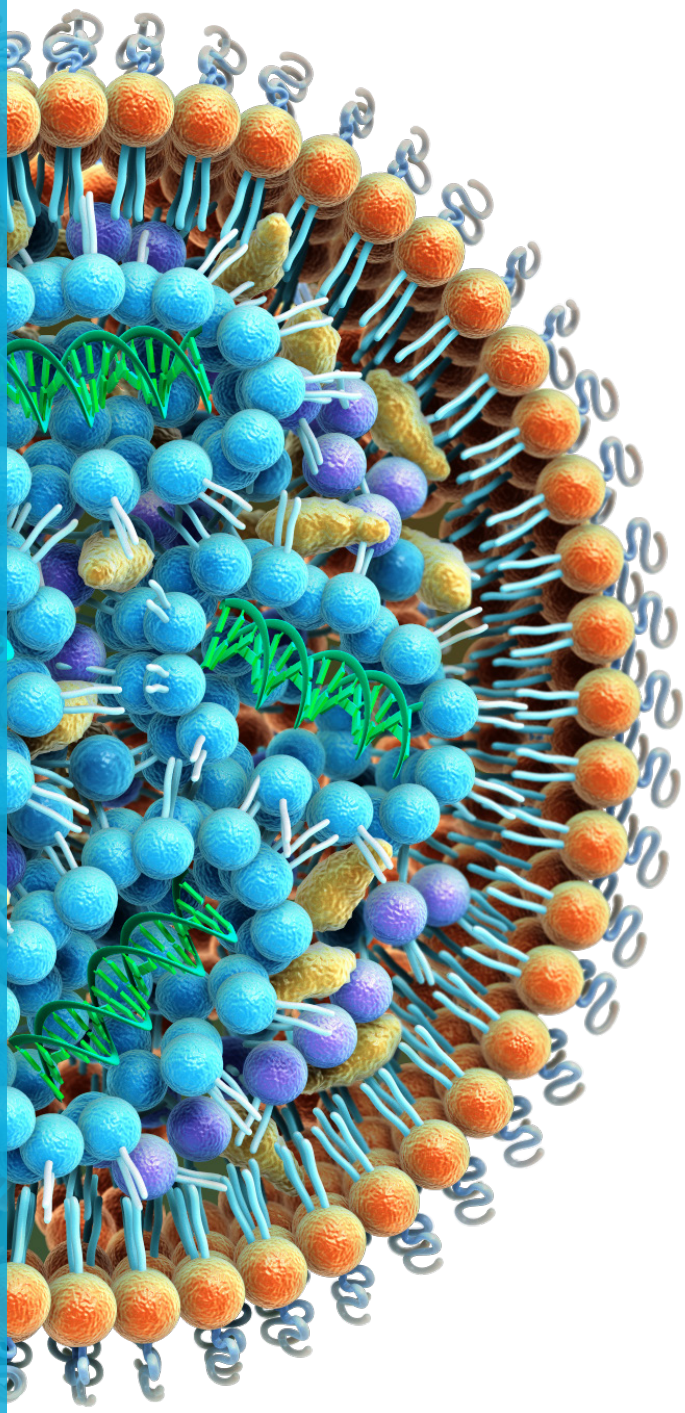
Nucleic Acid Lipid Nano-particles

Seamless scale up of siRNA-LNP formulations using the NanoAssemblr® platform



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Abstract

Lipid nanoparticles (LNPs) are the leading technology for delivering therapeutic nucleic acids. Their quality and efficacy are influenced by their physico-chemical properties, which are sensitive to the method of production. Hence, a robust and reproducible process is crucial to making high quality LNPs for preclinical and clinical development.

Laminar flow microfluidic mixing, as employed across the NanoAssemblr platform, affords exceptional process control and consistency that is also easily scalable. Consistency of the process across the NanoAssemblr suite is demonstrated by producing LNPs encapsulating siRNA against Factor VII in batch sizes that span three orders of magnitude produced using the NanoAssemblr Benchtop, Blaze and Scale-Up systems, the latter employing 8 microfluidic mixers in parallel. Formulations were found to be indistinguishable in size, size dispersity, composition, and *in vivo* biological activity across the three systems. Seamless scale up was demonstrated by transferring parameters optimized on the Benchtop, to the Blaze and 8x Scale-Up systems without modification. Finally, the process produced robust results throughout a 5 L formulation using a single mixer with the Scale-Up system, even at 8-fold higher concentrations, as determined by comparing physico-chemical properties of the formulation collected in fractions at different time points.

Taken together, these findings suggest that a 25 L formulation encapsulating > 34 g of siRNA in 4.5 h is well within achievable limits while maintaining high product quality with minimal process development. The NanoAssemblr platform is therefore apt to accelerate the development and translation of genetic medicines.

Introduction

The recent phase three clinical validation of Patisiran – a lipid nanoparticle (LNP) formulation and the first small interfering RNA (siRNA) therapeutic to reach this stage of development[1] – establishes LNPs as the premier technology for small RNA delivery. There are numerous LNP formulations being developed not only for siRNA[2,3] but also mRNA[4] and plasmid DNA[5], some of which are being tested in the clinic. Initially, thin film hydration followed by homogenization was the only method for producing cationic liposomes that are then complexed with nucleic acids, but this is a multi-step process that is challenging to scale up, and the product produced was cationic and consequently toxic. Along with ionizable lipids, second-generation methods based on in-line precipitation of LNPs from ethanol by bulk mixing with an aqueous buffer were developed to partially mitigate these challenges. Bulk mixing by ethanol injection or in a T-tube is a turbulent mixing process that is inherently heterogeneous. Nanoparticle precipitation

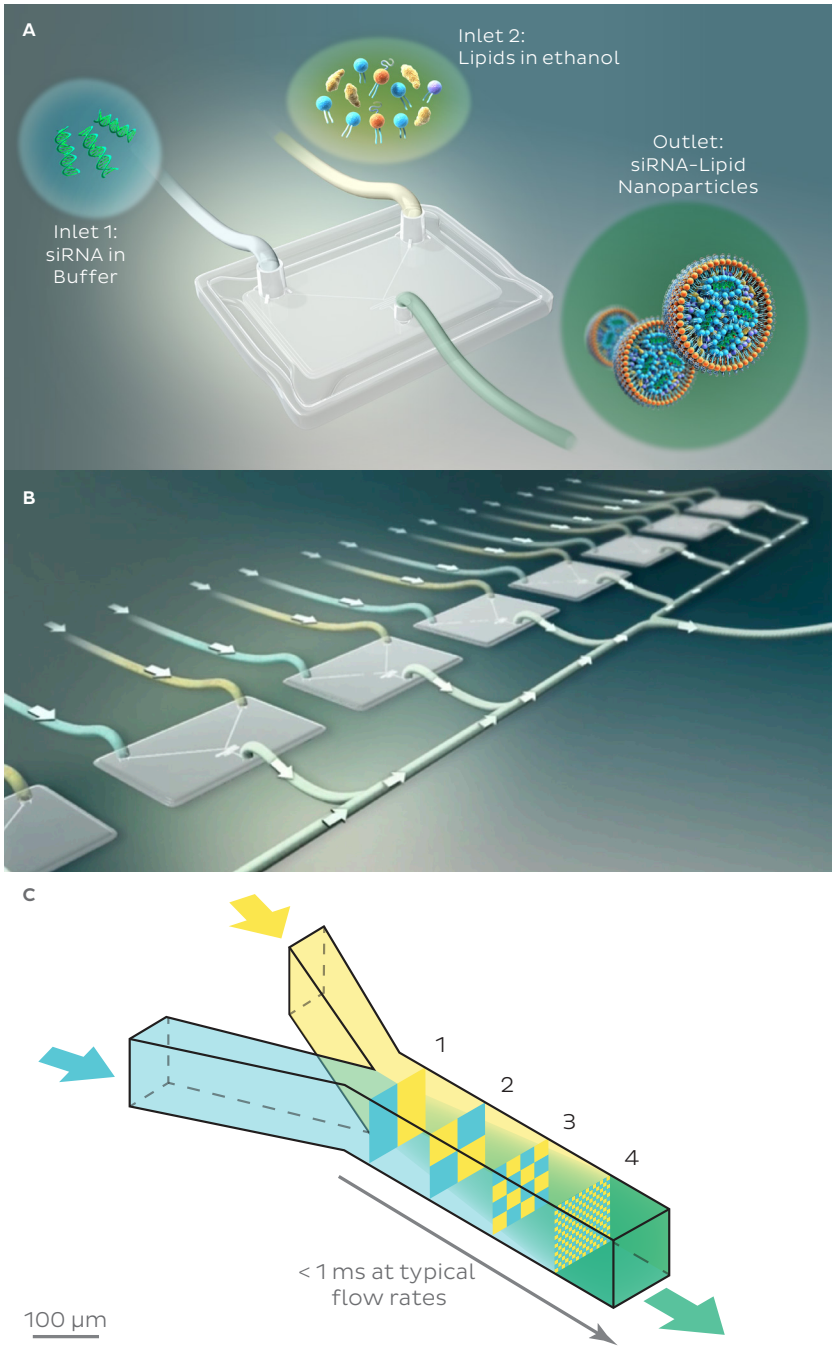


Figure 1. A) *siRNA in acidic buffer is injected into the left inlet while lipids including an ionizable cationic lipid dissolved in ethanol are injected into the right inlet of the NanoAssemblr microfluidic mixer. Following controlled mixing in microfluidic channels, lipid nanoparticles (LNPs) are spontaneously formed.* ***B)*** *The NanoAssemblr Scale-Up system uses multiple microfluidic mixers in parallel to increase throughput while preserving the physics of mixing.* ***C)*** *Diagram depicting two fluids mixing under laminar flow in a microfluidic mixer.*

1. Two fluids (buffer and ethanol) entering the channel under laminar flow do not mix.

2. Microscopic features in the channels are engineered to cause the fluid streams to mingle in a controlled, reproducible, and non-turbulent way.

3. Intermingling of the fluids increases as they continue through the mixer.

4. Fluids emerge from the mixer completely mixed. The whole process takes < 1 ms, which is faster than particles can assemble.

is a complex process involving numerous interactions between molecules of the nucleic acid, lipids, solvent, water, and ions whose local concentrations affect the kinetics of particle formation. Hence, heterogeneous conditions produced by bulk mixing may result in a heterogeneous product. Third-generation microfluidic mixing was developed to harness laminar flow mixing to permit greater control and homogeneity of the precipitation conditions while maintaining a continuous flow process amenable to scale up manufacturing (Figure 1).

The NanoAssemblr platform (see back cover) has made microfluidic nanomedicine production accessible to researchers regardless of prior experience with

the technique, and has since been established as a robust process that can be scaled over several orders of magnitude from µL volumes to tens of liters. The platform has been used extensively in preclinical development of not only siRNA LNPs[2,3] but also antibody-targeted LNPs[6], mRNA LNPs for protein replacement therapy[4], mRNA vaccines[7,8], and delivery of CRISPR gene editing components[9,10]. The high degree of scalability is ideal for screening and optimization of nucleic acids and lipid excipients at microliter and milliliter volumes to reduce cost. The same microfluidic technology is employed in systems designed for advanced pre-clinical and clinical scale production, which allow previously optimized conditions to be replicated at larger scales.

To date, a microRNA formulation for immuno-oncology[11] as well as mRNA-LNP vaccines against Zika[7] and influenza[8] have been developed with the help of Nano-Assemblr technology and scaled up for testing in larger animal models such as canines, and non-human primates. Here, we examine the physical properties and *in vivo* efficacy of siRNA-LNP formulations at different scales of production that span three orders of magnitude. Formulations were first developed at 10 mL batch size using the NanoAssemblr Benchtop. These were then scaled up 10-fold using the NanoAssemblr Blaze designed for larger animal studies, and 100-fold using the NanoAssemblr 8x Scale-Up system designed for the cGMP environment and clinical development. Due to different volumes of product, buffer exchange to isolate LNPs from solvent and unincorporated reagents, if any, were optimized for each stage. The formulations produced on all three systems were found to perform identically in terms of their physical and chemical characteristics and *in vivo* knockdown efficacy, thereby validating the seamless transfer of formulation conditions between instruments. It also indicates that conditions remained consistent among the 8 parallel microfluidic mixers in the Scale-Up system. Additionally, process robustness was assessed by producing a 5 L formulation using a single mixer on the scale-up system at 8 times the concentration. The high concentration formulation exhibited excellent physico-chemical properties and the collected fractions of the batch were found to be indistinguishable from one another by dynamic light scattering and liquid chromatography, indicating exceptional process uniformity throughout a long formulation run.

Result

First, LNPs encapsulating siRNA against Factor VII were produced at three scales: 10 mL batches on the NanoAssemblr Benchtop, 100 mL on the NanoAssemblr Blaze, and 1000 mL on the NanoAssemblr 8x Scale-Up system using the same Total Flow Rate (TFR), Flow Rate Ratio (FRR), lipid composition and

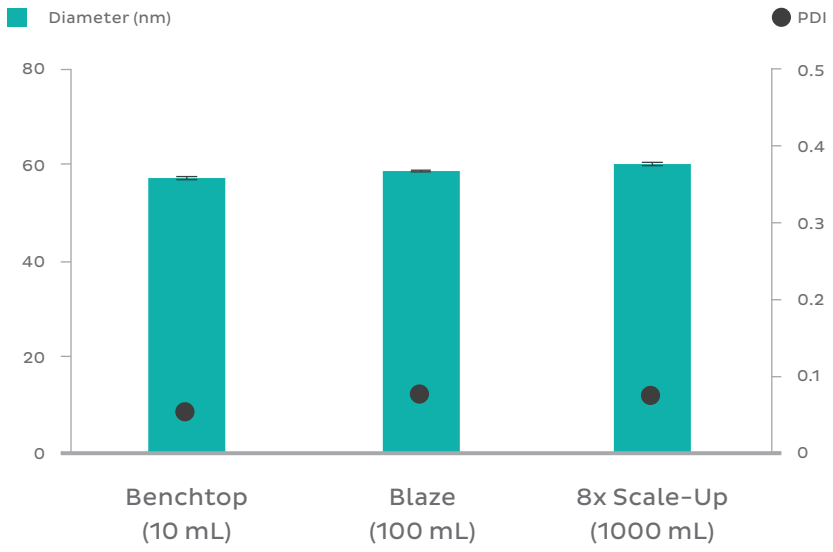


Figure 2. Factor VII siRNA LNPs maintain size and PDI when scaled by 10x and 100x on the NanoAssemblr platform.
10 mL formulations were produced on the Benchtop, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. Hydrodynamic size and particle size distribution (expressed as polydispersity index, PDI) were both determined by dynamic light scattering. Samples were measured in triplicate and values represent mean and error bars represent standard deviation of the mean.

reagent concentrations. Size and polydispersity index (PDI) – a measure of size heterogeneity - were determined by dynamic light scattering. A Size of 58 ± 2 nm and PDI of 0.05 were observed for all samples regardless of batch size and instrumentation (Figure 2). This indicates that the conditions of LNP precipitation were replicated identically between the three systems. PDI was exceptionally low, indicating highly uniform particles across the population.

Secondly, the chemical compositions of LNPs produced on each instrument were measured by disrupting the LNPs and separating components by HPLC. Samples were tested both before and after the buffer exchange process employed to isolate LNPs from un-incorporated materials, if any. Buffer exchange was not found to measurably affect composition. Additionally, the composition of LNPs produced using the three systems were remarkably uniform (Figure 3). Lipid compositions were found to be within ± 10% of target specifications in all cases. Encapsulation efficiencies were found to be >90% for all cases as determined by Ribogreen assay (not shown).

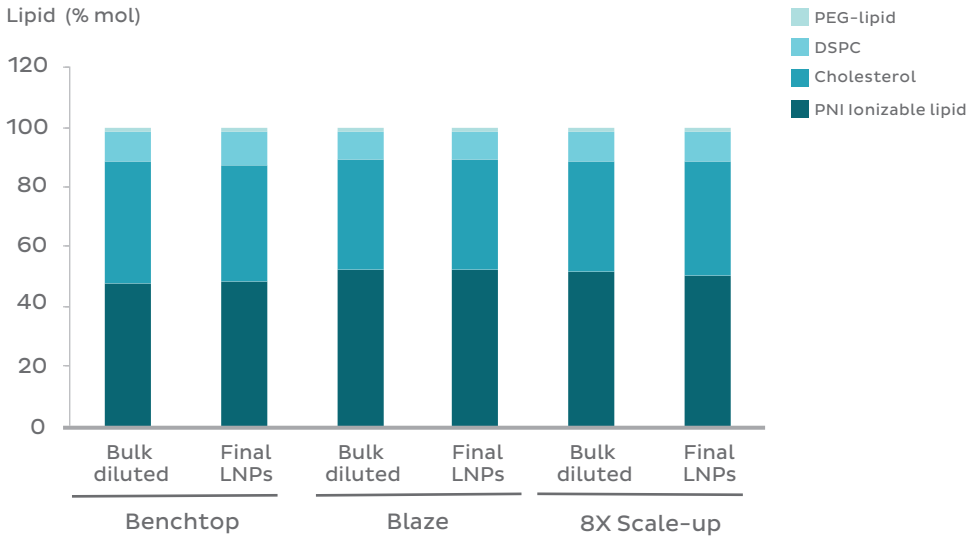


Figure 3. Factor VII siRNA LNPs maintain composition when scaled by 10x and 100x on the NanoAssemblr platform.
10 mL formulations were produced on the Benchtop, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. LNPs were disrupted, components separated by HPLC and analyzed by ELS.

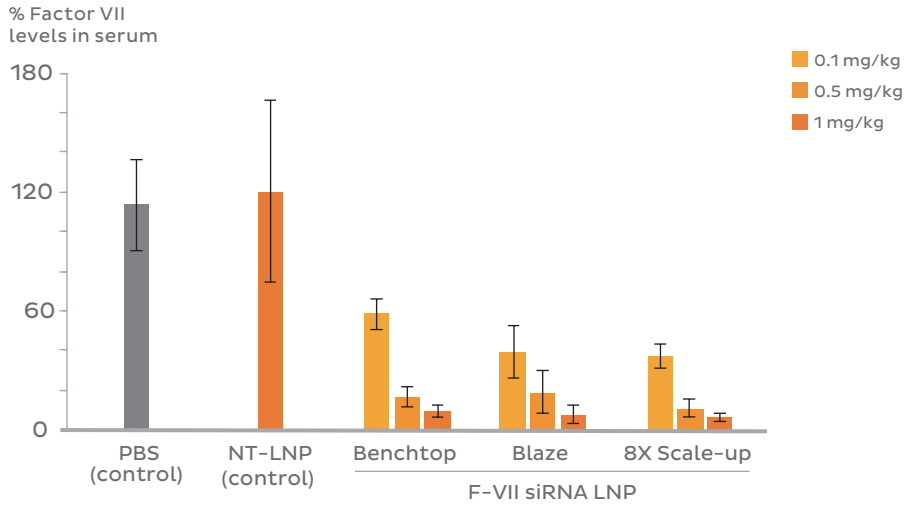


Figure 4. in vivo knockdown efficacy of Factor VII siRNA LNPs maintained when scaled by 10x and 100x on the NanoAssemblr platform.
10 mL formulations were produced on the Benchtop, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. LNPs and controls were administered to healthy mice by tail vein injection at specified concentrations. Blood samples were taken 24h later and assayed for F-VII levels. Values represent mean and error bars represent standard deviation of 3 animals.

Knockdown efficacy of Factor VII (F-VII) was tested *in vivo* using murine models. F-VII is a blood clotting protein commonly used in proof-of-concept studies because it can be measured in plasma samples. LNPs were administered by tail vein injection at 4 different doses and compared to a PBS control and a LNP control containing a siRNA with no target in the model animal. LNP efficacy was consistent across the NanoAssemblr platform (Figure 4). All formulations exhibited a dose-dependent knockdown with ~90% knockdown at the highest doses of 1 mg/kg. Hence, formulations produced on the three systems are indistinguishable in size, composition, and biological activity, indicating consistent formulation conditions were achieved.

To test the robustness of the process, formulations were produced using the Scale-Up instrumentation employing a single microfluidic mixer at two dif-

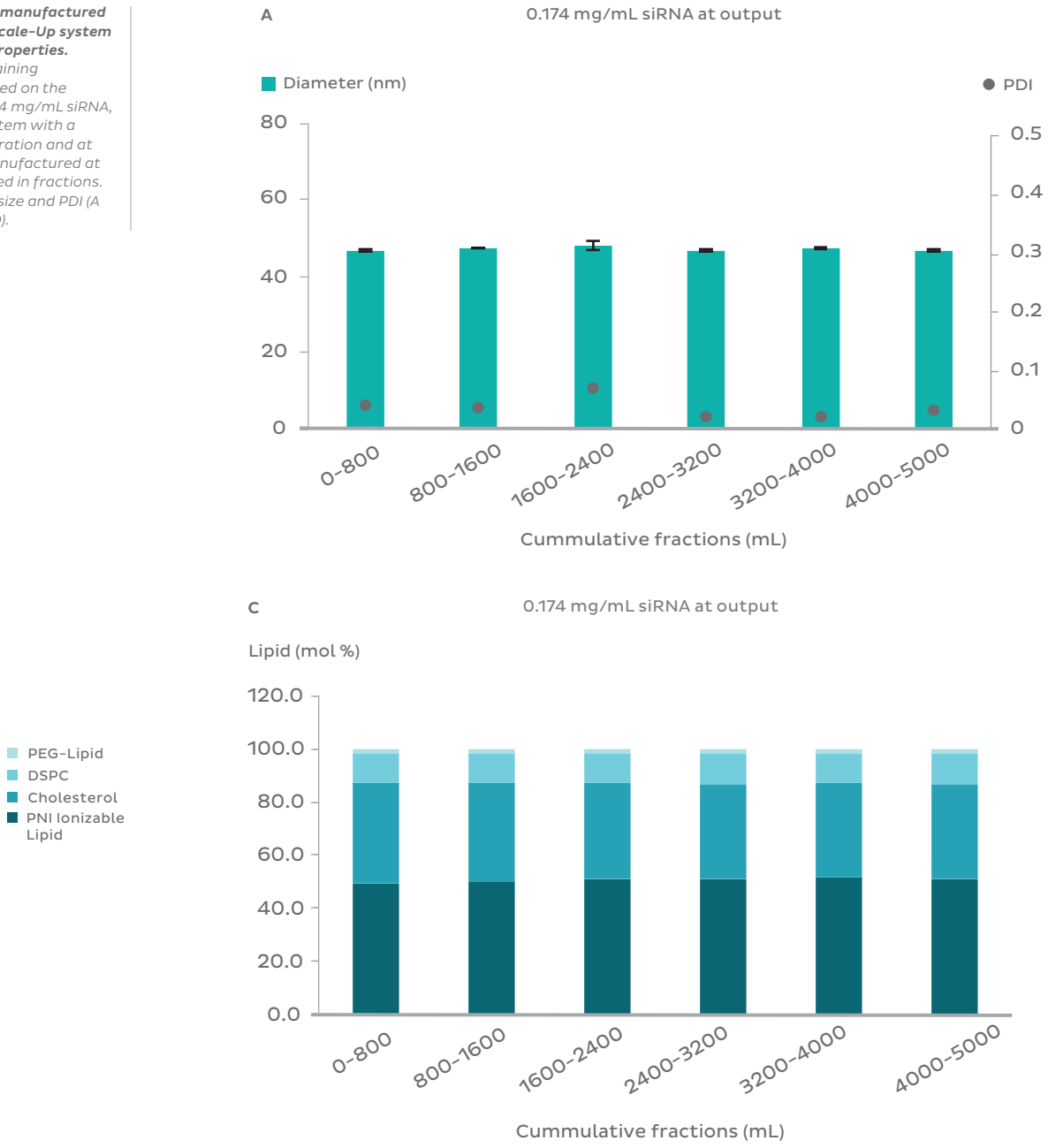
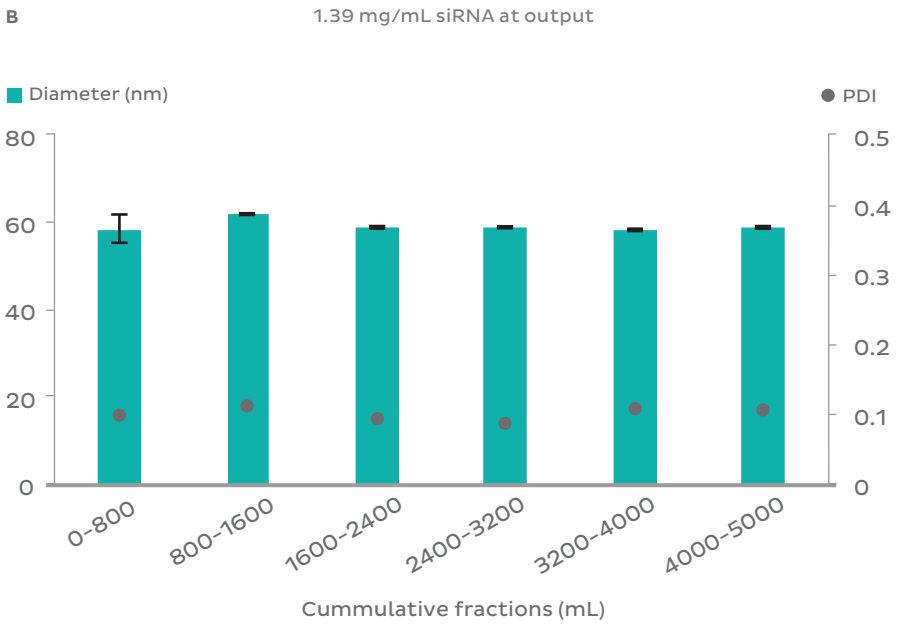
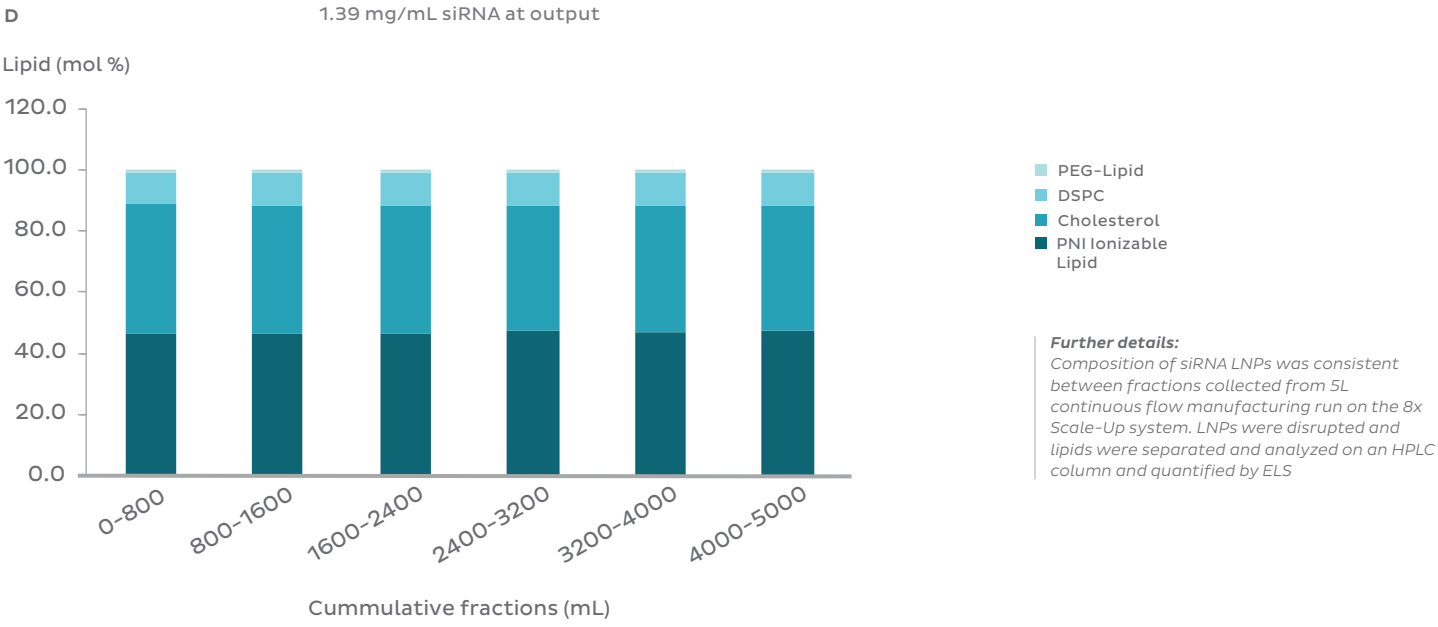


Figure 5. Factor VII siRNA LNPs manufactured at high concentrations on the Scale-Up system retain their physico-chemical properties. *Lipid Nanoparticles (LNPs) containing siRNA against Factor VII optimized on the NanoAssemblr Benchtop at 0.174 mg/mL siRNA, were made on the Scale-Up system with a single chip at the same concentration and at 1.39 mg/mL. A 5 L batch was manufactured at each concentration and collected in fractions. Each fraction was analyzed for size and PDI (A and B) and composition (C and D).*

ferent concentrations. A final siRNA concentration of 0.174 mg/mL represents commonly reported concentrations. A formulation was also produced at 8 times that concentration (1.39 mg/mL) by increasing the concentrations of all reagents. In both cases, the batch size was 5 L. Each formulation was collected in 6 fractions without stopping the process. Excellent uniformity was maintained throughout the formulation run with no observable difference in the size or PDI between the collected fractions (Figure 5A and B). The higher concentration formulation was found to be the same diameter (60 nm, Figure 5B) as lower concentration formulations produced with the Benchtop, Blaze, and Scale-Up in its 8x configuration (Figure 2). Composition of the particles remained unaffected by the change in concentration (Figure 5C and D).



Further details:
Hydrodynamic diameter and polydispersity index (PDI) of siRNA-LNPs were consistent between fractions collected from continuous flow manufacturing on the 8x Scale-Up system. Diameter and PDI were measured by dynamic light scattering.



Further details:
Composition of siRNA LNPs was consistent between fractions collected from 5L continuous flow manufacturing run on the 8x Scale-Up system. LNPs were disrupted and lipids were separated and analyzed on an HPLC column and quantified by ELS

Discussion

Nanoparticle precipitation is an intricate process dependent on numerous intermolecular interactions. For LNPs, pH sensitive ionizable lipids must interact with components of the buffer to become cationic, following which, they interact electrostatically with anionic nucleic acids to form reverse micelles. As the polarity of the solvent increases, interactions between the exposed lipid tails and solvent molecules become unfavorable, triggering assembly of lipids around reverse micelles. PEGylated lipids, being the most soluble in polar solvent, precipitate last and passivate the surface of the particles[2,3]. Mixing of the solvent phase with the aqueous phase is critical in determining the local concentrations of reagents, as well as the local dielectric constant of the solvent, which affects electrostatic interactions critical to ionization of the novel lipid and its association with the nucleic acid. Homogeneous mixing is crucial in ensuring mixing conditions remain consistent from time-to-time, and from point-to-point within the mixing volume.

Microfluidics offers superior homogeneity in mixing which results in highly uniform batches of LNPs. Because the process is time-invariant, conditions of mixing can be replicated among different mixers bearing the same mixing geometry using the same parameters such as TFR, FRR, reagents and concentrations. Hence, it is unsurprising that formulations produced on the Benchtop, Blaze, and 8x Scale-Up, have indistinguishable physical and chemical characteristics given that these systems use the same microfluidic mixer design. These characteristics ultimately dictate biological function such as biodistribution, particle uptake, and payload release, so it follows that the biological activity is also conserved across all systems.

Consistent results across all systems allow rapid optimization of particle characteristics and performance at scales between 1 and 15 mL on the Benchtop system and production of identical particles in batches of up to 1 L on the Blaze and 25 L in 4.5 h on the 8x Scale-Up system without reoptimizing parameters.

The observation that LNPs produced with 8 mixers in parallel were indistinguishable from those produced with a single mixer, indicate excellent process control and stability with the NanoAssemblr microfluidic platform and the exceptional uniformity between individual NanoAssemblr microfluidic devices. As a consequence, the conditions of mixing induced by each device are likewise uniform. It follows that throughput can be scaled up further by increasing the number of mixers without impacting the quality of LNPs produced.

The property of time-invariance of laminar flow microfluidic mixing gives rise to consistency in LNP characteristics throughout an extended 5 L formulation run as exhibited in Figure 5 using a single mixer. These results are indicative of a robust process, wherein any arbitrary volume of reagents undergoes an identical process, yielding identical results. Such consistency is not achievable with turbulent processes whose microscopic conditions, can vary over time.

Given that process consistency was demonstrated throughout a 5 L run on a single mixer and also between 8 mixers used in parallel, it follows that a large batch totaling 25 L is well within achievable limits of the 8x Scale-Up system while maintaining high product quality with minimal process development. At final RNA concentrations of 1.39 mg/mL, this formulation would suffice to encapsulate >34 g of siRNA. At a combined flow rate of 96 mL/min such a

formulation can be accomplished in a run time of ~4.5h, which can fit into a single cleanroom shift. Employing more than 8 parallel NanoAssemblr microfluidic devices can further increase throughput of the Scale-Up System with equally robust results.

Conclusion

Laminar flow microfluidic mixing, as employed across the NanoAssemblr platform, affords exceptional control over the microenvironment of LNP formation that ultimately influences physico-chemical properties and consequently biological activity. Because the Benchtop, Blaze and Scale-Up systems share the same microfluidic architecture, the same parameters can be transferred between systems to produce the same results. This is substantiated in the consistent size, dispersity, composition and potent biological activity observed in Benchtop, Blaze and 8x Scale-Up batches that spanned three orders of magnitude in volume. The platform allows formulations to be produced at scales suited for any stage of development. Furthermore, this seamless transfer of optimized conditions allows developments at earlier stages to carry forward to later stages, greatly reducing time and effort spent developing and reoptimizing production processes.

The use of 8 microfluidic mixers in parallel did not measurably affect outcomes, indicating exceptional consistency in the manufacturing of individual devices. Additionally, comparisons between collected fractions of a 5 L formulation using a single mixer found no measurable difference in the physical characteristics of the particles throughout the process. Production was also scaled by increasing the concentration of reagents 8-fold with the single mixer configuration, and the resulting particles had the same size as those produced with the 8-mixer configuration while comfortably exceeding quality requirements for PDI. Taken together, these findings suggest a 25 L batch encapsulating over 34 g of siRNA in 4.5h is imminently achievable with the NanoAssemblr platform. Such robustness and scalability are essential to accelerating the development and translation of genetic medicines by enabling efficient optimization and scale-up of nucleic acid LNP formulations.

Materials & Methods

Factor VII siRNA formulation: Appropriate amounts of PNI ionizable lipid, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids), Cholesterol (CHOL, Sigma Aldrich) and PEG-lipid (NOF America Corp) were dissolved in ethanol at molar ratio of 5:1:3.85:0.15, respectively. Factor VII siRNA (Axolabs) was dissolved in acetate buffer (pH 4) at 0.232 mg/mL, 0.928 mg/mL and 1.856 mg/mL. The siRNA concentration was determined using UV spectroscopy (Nanodrop Lite, Thermo Fisher Scientific Inc).

For scale-up study, about 10 mL of formulation was prepared on NanoAssemblr Benchtop at an aqueous:organic FRR of 3:1 and TFR of 12 mL/min. The formulation was scaled-up to 100 mL on the Blaze and 1000 mL on the 8X Scale-up System using identical parameters. LNPs were manufactured at siRNA concentration of 0.7 mg/mL in all three instruments and diluted with PBS before buffer-exchange on tangential flow filtration system (KrosFlo® Research IIi Tangential Flow Filtration (TFF) System, Spectrum Labs). Finally, the particles were concentrated to 1 mg/mL, sterile filtered and stored at 4 °C before further analysis.

For single mixer experiments, two 5000 mL LNP formulations were prepared each with a single Scale-Up mixer at each of 0.174 mg/mL and 1.39 mg/mL siRNA concentration (nominal, at the output of the microfluidic process). Particle dilution and buffer exchange were performed by TFF as described above.

Particle characterization: A sample of bulk diluted and final product was used to determine physico-chemical characteristics (size, PDI, encapsulation), lipid concentration and siRNA concentration. Particle size was determined using dynamic light scattering (Malvern Zetasizer Nano ZS, UK) and encapsulation using RiboGreen assay. Lipids were separated and analyzed on a HPLC column using an ELS detector. For siRNA, LNPs were disrupted using a surfactant and analyzed by HPLC using a photodiode (UV) array detector.

Factor VII gene knockdown: CD1 mice (18-23 g; 6-8weeks) were divided into 11 groups (n=3) and siRNA LNPs were administered intravenously using a single bolus injection via tail vein at a dose of 0.1 mg/kg, 0.5 mg/Kg and 1 mg/Kg. Non-targeting (NT) siRNA containing chemical modifications and length similar to Factor VII siRNA was used for preparation of negative control. After 24 hours, blood (~ 500 µL) was collected by cardiac puncture after euthanizing mice. The blood samples were allowed to stand for 20-40 min at RT to allow the blood to clot. Serum was separated by centrifuging at 2000xg for 10 min at 15 °C and was stored at -80 °C for later analysis. Analysis of Factor VII protein levels in the serum weas performed using Biophen Factor VII assay (Aniara, OH, USA).

Related Material

About the NanoAssemblr Platform:
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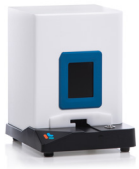
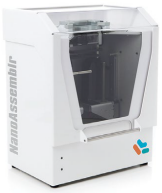
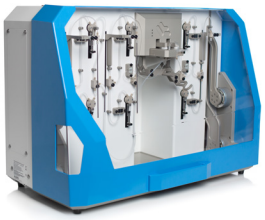
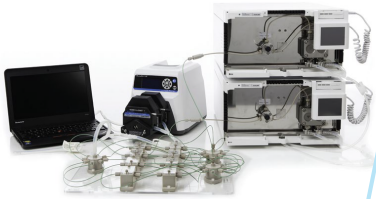
Spark™ (Lead Candidates) **Benchtop** (Optimized Formulations) **Blaze™** (Clinical Candidate) **Scale-Up** (Clinical Data)









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