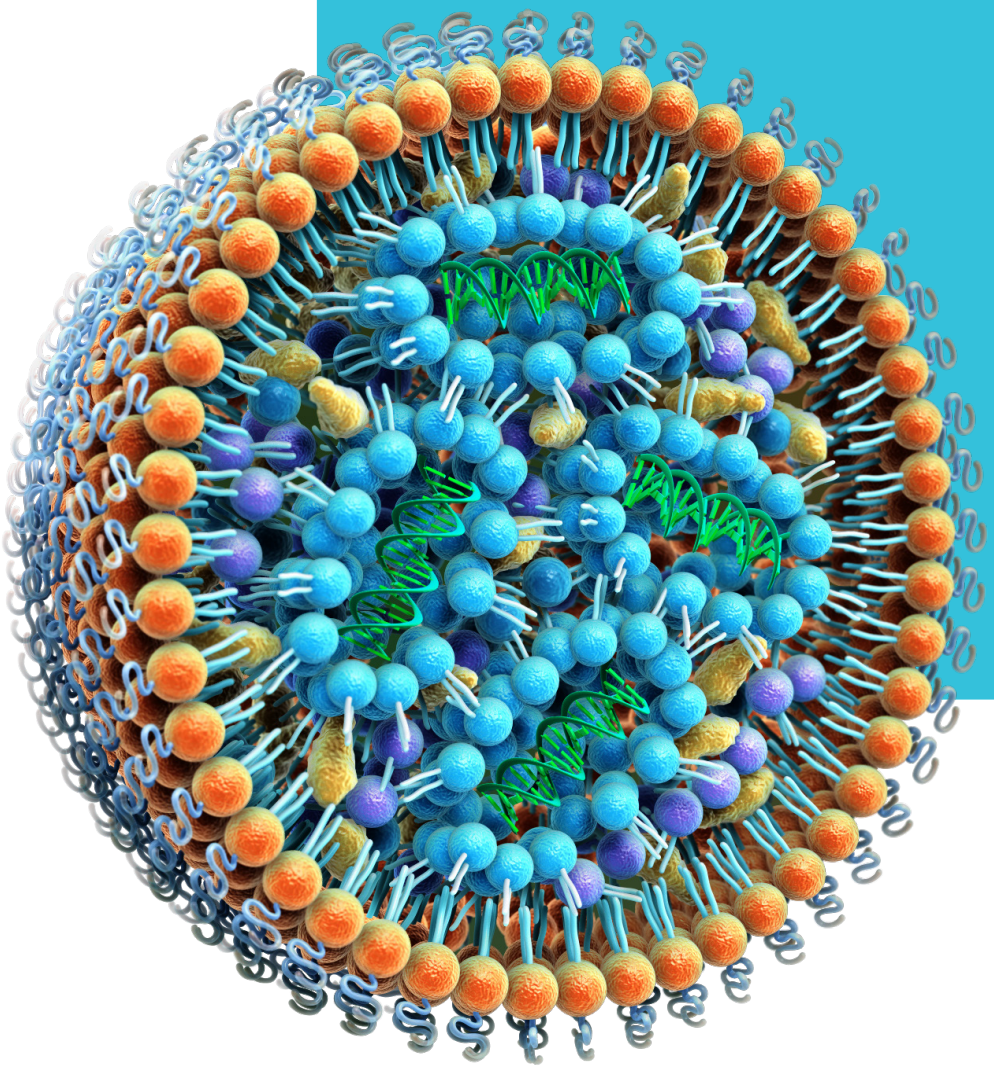
PRECISION NANOSYSTEMS

Create Transformative Medicines™

Formulating RNA Lipid Nanoparticles



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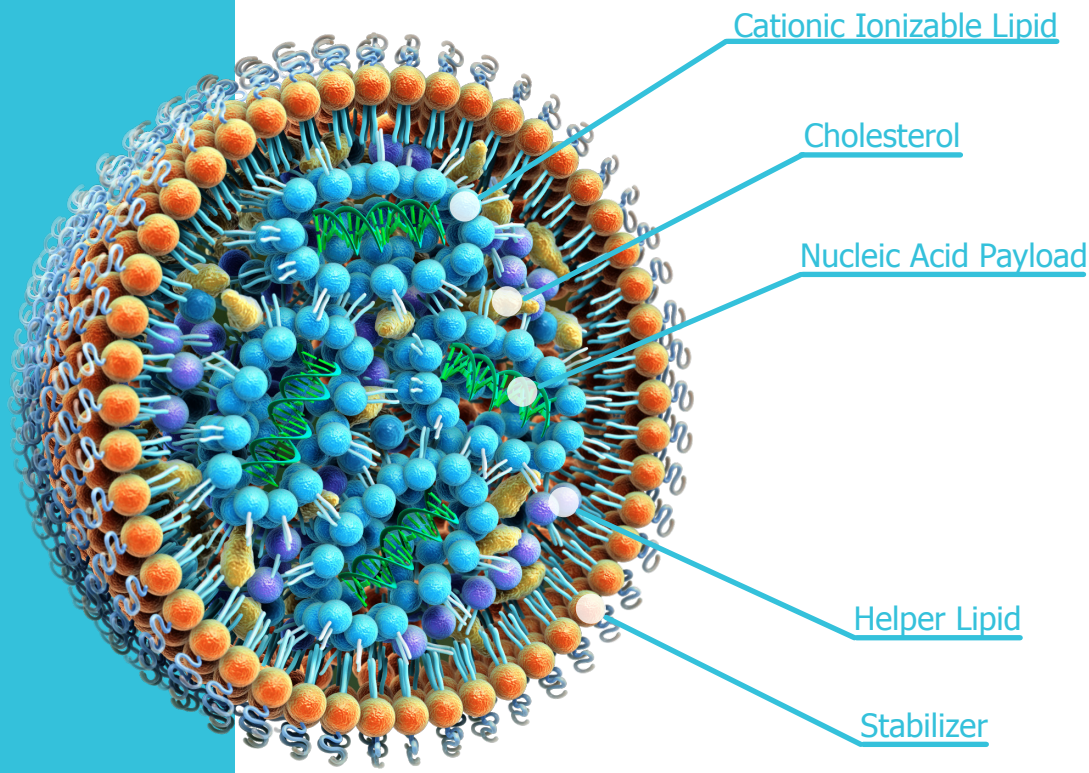
Introduction

Lipid nanoparticles (LNPs) are the most clinically advanced non-viral gene delivery system with the LNP-based small interfering RNA (siRNA) therapeutic Onpattro® having been approved by the FDA in 2019. LNPs safely and effectively deliver nucleic acids, overcoming a major barrier to the development and use of genetic medicines. In addition to siRNA delivery, LNPs are being developed to deliver a variety of therapeutic nucleic acids that can replace defective genes, edit genes using CRISPR/Cas9 or encode vaccine antigens.

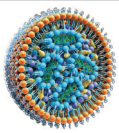
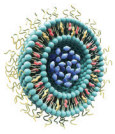
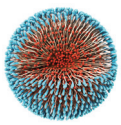
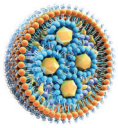
LNPs represent an evolution over cationic liposomes for nucleic acid delivery. LNPs differ from their predecessors by employing pH-sensitive ionizable cationic lipids that function to improve encapsulation of nucleic acids, lower toxicity and mediate efficient release of the nucleic acid payload into the cytoplasm. Structurally, LNPs produced using NanoAssemblr® technology differ from liposomes in that they do not have an aqueous core when observed under cryogenic electron microscopy. This structure along with high cholesterol content resembles low-density lipoproteins and are taken up by cells through a ubiquitous receptor-mediated endocytic pathway. Once in the endosome, the ionizable lipids respond to the pH change to become cationic wherein they cause the disruption of the endosome and release of the nucleic acid into the cytoplasm.



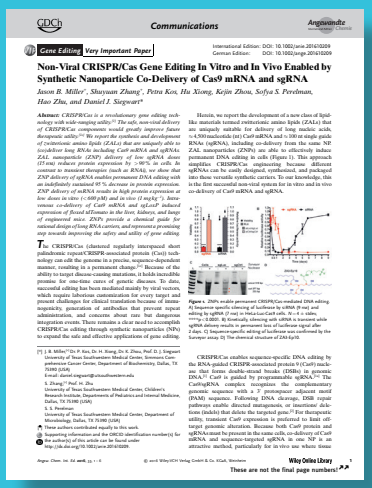
Highlighted below are several examples from peer-reviewed literature as well as application notes with original results to illustrate the multitude of applications of NanoAssemblr technology for developing and optimizing LNPs for gene delivery.

Precision NanoSystems' technology is solutions are world-leading biopharma and academic researchers to drive development of diverse nanomedicines

Versatile Applications

PARTICLE TYPE	ACTIVE INGREDIENT	EXAMPLE APPLICATION	CARRIER MATERIALS
Nucleic acid Lipid Nanoparticles (LNP) 	Nucleic Acids, Peptides and Proteins, Imaging Contrast Agents	• Rare genetic diseases • mRNA protein replacement • mRNA vaccines • Gene and cell therapy	• Ionizable lipids • Phospholipids • Cholesterol • PEG-Lipids
Liposomes 	Small Molecules, Imaging Contrast Agents	• Vaccine adjuvants • Antimicrobials • Cancer chemotherapy • Diabetes combination therapy	• Phospholipids • Cholesterol • PEG-Lipids
Polymer NPs 	Small Molecules, Imaging Contrast Agents	• Cancer chemotherapy • Targeted protein delivery • Controlled release/ biodistribution • Immuno-oncology	• Poly-lactides (ex: PLGA) • Block copolymers (ex: PEG-b-PLGA) • Polysaccharides (ex: chitosan, cellulose)
Emulsions 	Small Molecules, Imaging Contrast Agents	• Cancer chemotherapy • Drug formulation • Controlled release/ biodistribution	• Triolein/POPC • Oil/Surfactant
Organic/ Inorganic NPs 	Small Molecules, Imaging Contrast Agents	• Theranostics • Imaging	• Lipids • Noble metal NPs • Rare Earth Metals • III-V semiconductors

Featured Lipid Nanoparticle Publications


Communications

Angewandte

Gene Editing Very Important Paper

International Edition: DOI: 10.1002/anie.201610209
German Edition: DOI: 10.1002/ange.201610209

Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA

Jason B. Miller+, Shuyuan Zhang+, Petra Kos, Hu Xiong, Kejin Zhou, Sofya S. Perelman, Hao Zhu, and Daniel J. Siegwart*

Abstract: CRISPR/Cas is a revolutionary gene editing technology with wide-ranging utility.[1] The safe, non-viral delivery of CRISPR/Cas components would greatly improve future therapeutic utility.[2a] We report the synthesis and development of zwitterionic amino lipids (ZALs) that are uniquely able to (co)deliver long RNAs including Cas9 mRNA and sgRNAs. ZAL nanoparticle (ZNP) delivery of low sgRNA doses (15 nM) induces protein expression by >90% in cells. In contrast to transient therapies (such as RNAi), we show that ZNP delivery of sgRNA enables permanent DNA editing with an indefinitely sustained 97% decrease in protein expression. ZNP delivery of mRNA results in high protein expression at low doses in vitro (< 0.01 μg/mL) and in vivo (1 mg kg^{-1}). Intravenous co-delivery of Cas9 mRNA and sgLexA-induced expression of floxed tdTomato in the liver, kidneys, and lungs of engineered mice. ZNPs provide a chemical guide for rational design of long RNA carriers, and represent a promising step towards improving the safety and utility of gene editing.

The CRISPR/Cas (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein (Cas)) technology can edit the genome in a precise, sequence-dependent manner, resulting in a permanent change.[2a] Because of the ability to target disease-causing mutations, it holds incredible promise for one-time cures of genetic diseases. To date, successful editing has been mediated mainly by viral vectors, which require laborious customization for every target and present challenges for clinical translation because of immunogenicity, generation of antibodies that prevent repeat administration, and concerns about rare but dangerous integration events. There remains a clear need to accomplish CRISPR/Cas editing through synthetic nanoparticles (NPs) to expand the safe and effective applications of gene editing.

Herein, we report the development of a new class of lipid-like materials termed zwitterionic amino lipids (ZALs) that are uniquely suitable for delivery of long nucleic acids. A 4,500 nucleotide (nt) Cas9 mRNA and ~100 nt single guide RNAs (sgRNA), including co-delivery from the same NP. ZAL nanoparticles (ZNPs) are able to effectively induce permanent DNA editing in cells (Figure 1). This approach simplifies CRISPR/Cas engineering because different sgRNAs can be easily designed, synthesized, and packaged into these versatile synthetic carriers. To our knowledge, this is the first successful non-viral system for in vitro and in vivo co-delivery of Cas9 mRNA and sgRNA.

Figure 1. ZNPs enable permanent CRISPR/Cas-mediated DNA editing. A) Sequence-specific silencing of luciferase by siRNA (9 nM) and editing by sgRNA (7 nM) in HeLa-Luc-Cas9 cells. N = 4, ± stdev, ****p < 0.0001. B) Kinetically, silencing with siRNA is transient while sgRNA delivery results in permanent loss of luciferase signal after 2 days. C) Sequence-specific editing of luciferase was confirmed by the Surveyor assay. D) The chemical structure of ZA3-Ep10.

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under http://dx.doi.org/10.1002/anie.201610209.

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These are not the final page numbers! ↗ 1

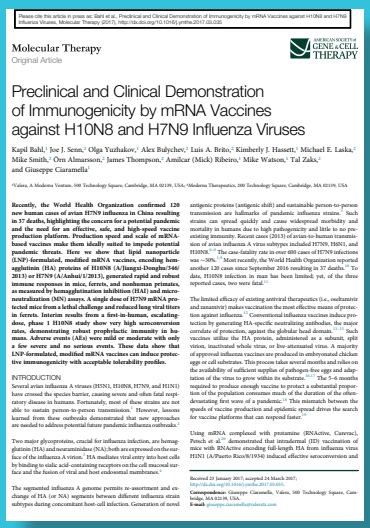
Angewandte Chemie International Edition, 2016

Non-Viral CRISPR/Cas Gene Editing In Vitro and In Vivo Enabled by Synthetic Nanoparticle Co-Delivery of Cas9 mRNA and sgRNA

Miller J, Zhang S, Kos P, Xiong H, Zhou K, Perelman S et al. Angewandte Chemie International Edition 2016; 56: 1059-1063.

Summary

- CRISPR/Cas is a gene editing technique that enables targeting disease-causing mutations with lasting and potentially curative effects, but implementation is challenging as it requires two components – a Cas nuclease and single guide RNA (sgRNA) – to be delivered in the target cell
- This can be achieved by viral vectors, but, they suffer from laborious customizations and immunogenicity that hamper clinical translation of CRISPR/Cas approach
- Dr. Daniel Siegwart's team at the University of Texas Southwestern have developed a non-viral carrier platform from synthetic lipids that effectively co-delivers mRNA encoding Cas9 and sgRNA *in vitro* and *in vivo*
- To co-encapsulate long mRNA (~4 kb) along with sgRNA, they have developed a zwitterionic lipid design that combines the roles of phospholipids and cationic lipids and used NanoAssemblr technology to formulate these into nanoparticles
- Efficacy of their formulation was first confirmed by separate delivery of sgRNA and mRNA *in vitro* – in HeLa-Luc-Cas9 and human ovarian cancer cell lines, respectively – and *in vivo*, demonstrated efficacy of the lead Luc mRNA-loaded lipid nanoparticles by injecting different mice strains and monitoring luciferase expression
- To test gene editing, mice containing a homozygous Rosa26 promoter Lox-Stop-Lox tdTomato gene in all cells were injected with LNPs co-encapsulating Cas9 mRNA and sgRNA targeting the Lox sites, which deleted the Stop cassette resulted in detectable expression of the tdTO reporter in the liver, lung, and kidneys
- In conclusion, Prof. Siegwart's study shows a proof-of-concept for using non-viral lipid nanocarriers to co-deliver CRISPR/Cas 9 components at the same spatio-temporal fashion both *in vitro* and *in vivo*, overcoming the challenge of delivering both required components in the same cell
- Further, the non-immunogenic nature and customizability make lipid nanocarriers scalable and translatable alternatives to viral carriers, bringing the field closer to realizing the promise of effective sequence-specific gene editing therapies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Molecular Therapy
Original Article

Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses

Kapil Bahl,[1] Joe J. Senn,[2] Olga Yuzhakov,[2] Alex Bulychev,[2] Luis A. Brito,[1] Kimberly J. Hassett,[1] Michael E. Laska,[2] Mike Smith,[2] Orn Almarsson,[1] James Thompson,[1] Amilcar (Mick) Ribeiro,[1] Mike Watson,[1] Tal Zaks,[1] and Giuseppe Ciaramella[1]

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Recently, the World Health Organization confirmed 120 new human cases of avian H7N9 influenza in China resulting in 37 deaths, highlighting the concern for a potential pandemic and the need for an effective, safe, and high-speed vaccine production platform. Production speed and scale of mRNA-based vaccines make them ideally suited to impede potential pandemic threats. Here we show that lipid nanoparticle (LNP)-formulated, modified mRNA vaccines, encoding hemagglutinin (HA) proteins of H10N8 (A/Jiangxi-Donghu/346/2013) or H7N9 (A/Anhui/1/2013), generated rapid and robust immune responses in mice, ferrets, and nonhuman primates, as measured by hemagglutination inhibition (HAI) and microneutralization (MN) assays. A single dose of H7N9 mRNA protected mice from a lethal challenge and reduced lung viral titers in ferrets. Interim results from a first-in-human, escalating-dose, phase 1 H10N8 study show very high seroconversion rates, demonstrating robust prophylactic immunity in humans. Adverse events (AEs) were mild or moderate with only a few severe and no serious events. These data show that LNP-formulated, modified mRNA vaccines can induce protective immunogenicity with acceptable tolerability profiles.

INTRODUCTION

Several avian influenza A viruses (H5N1, H10N8, H7N9, and H1N1) have crossed the species barrier, causing severe and often fatal respiratory diseases in humans. Fortunately, most of these strains are not able to sustain person-to-person transmission.[1] However, lessons learned from these outbreaks demonstrated that new approaches are needed to address potential future pandemic influenza outbreaks.[2]

Two major glycoproteins, crucial for influenza infection, are hemagglutinin (HA) and neuraminidase (NA), both are expressed on the surface of the influenza A virion.[3] HA mediates viral entry into host cells by binding to sialic acid–containing receptors on the cell mucosal surface and the fusion of viral and host endosomal membranes.[4]

The segmented influenza A genome permits re-assortment and exchange of HA (or NA) segments between different influenza strain subtypes during concomitant host-cell infection. Generation of novel antigenic proteins (antigenic shift) and sustainable person-to-person transmission are hallmarks of pandemic influenza strains.[5] Such strains can spread quickly and cause widespread morbidity and mortality in humans due to high pathogenicity and little to no pre-existing immunity. Recent cases (2013) of avian-to-human transmission of avian influenza A virus subtypes included H7N9, H6N1, and H10N8.[6–8] The case-fatality rate in over 600 cases of H7N9 infections was ~30%.[9] Most recently, the World Health Organization reported another 120 cases since September 2016 resulting in 37 deaths.[10] To date, H10N8 infection in man has been limited; yet, of the three reported cases, two were fatal.[11]

The limited efficacy of existing antiviral therapeutics (i.e., oseltamivir and zanamivir) makes vaccination the most effective means of protection against influenza.[12] Conventional influenza vaccines induce protection by generating HA-specific neutralizing antibodies, the major correlate of protection, against the globular head domain.[13–15] Such vaccines utilize the HA protein, administered as a subunit, split virion, inactivated whole virus, or live-attenuated virus. A majority of approved influenza vaccines are produced in embryonated chicken eggs or cell substrates. This process takes several months and relies on the availability of sufficient supplies of pathogen-free eggs and adaptation of the virus to grow within its substrate.[16,17] The 5–6 months required to produce enough vaccine to protect a substantial proportion of the population consumes much of the duration of the often-devastating first wave of a pandemic.[17] This mismatch between the speeds of vaccine production and epidemic spread drives the search for vaccine platforms that can respond faster.[18]

Using mRNA complexed with protamine (RNActive, Curevac), Petsch et al.[19] demonstrated that intradermal (ID) vaccination of mice with RNActive encoding full-length HA from influenza virus H1N1 (A/Puerto Rico/8/1934) induced effective seroconversion and

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http://dx.doi.org/10.1016/j.ymthe.2017.03.035.
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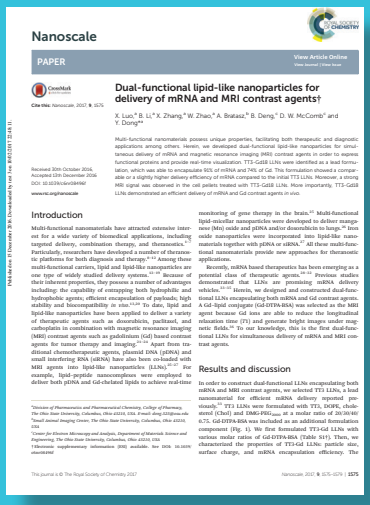
Molecular Therapy, 2017

Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses

Bahl K, Senn J, Yuzhakov O, Bulychev A, Brito L, Hassett K et al. Molecular Therapy 2017; 25: 1316-1327.

Summary

- mRNA therapy pioneers Moderna Therapeutics report preliminary results from first-in-human trial of mRNA vaccine against influenza
- mRNA vaccines can be substantially faster to develop than traditional vaccines and more easily engineered, promising faster and more effective response to pandemic outbreaks
- mRNA encoding HA proteins of H10N8 and H7N9 were encapsulated in LNPs and tested in multiple animal species that all exhibited significant antibody titers over baseline within 21 days
- Early results from human trials showed all patients had hemagglutinin inhibition titers ≥ 40 – a standard measure indicating 50% reduced risk of infection – with similar safety to conventional flu vaccines
- mRNA vaccines are rapidly advancing through clinical trials, and the leaders in this field use NanoAssemblr technology to formulate their product


Nanoscale

PAPER

View Article Online

Dual-functional lipid-like nanoparticles for delivery of mRNA and MRI contrast agents†

X. Luo,[a] B. Li,[a] X. Zhang,[a] W. Zhao,[a] A. Bratasz,[b] B. Deng,[a] D. W. McComb[a] and Y. Dong*[a]

Cite this: Nanoscale, 2017, 9, 1575

Multi-functional nanomaterials possess unique properties, facilitating both therapeutic and diagnostic applications among others. Herein, we developed dual-functional lipid-like nanoparticles for simultaneous delivery of mRNA and magnetic resonance imaging (MRI) contrast agents in order to express functional proteins and provide real-time visualization. TT3-GdSB LLNs were identified as a lead formulation, which was able to encapsulate 95% of mRNA and 74% of Gd. This formulation showed a comparable or a slightly higher delivery efficiency of mRNA compared to the initial TT3 LLNs. Moreover, a strong MRI signal was observed in the cell pellets treated with TT3-GdSB LLNs. More importantly, TT3-GdSB LLNs demonstrated an efficient delivery of mRNA and Gd contrast agents *in vivo*.

Received 30th October 2016,
Accepted 15th December 2016
DOI: 10.1039/c6nr09496d
www.rsc.org/nanoscale

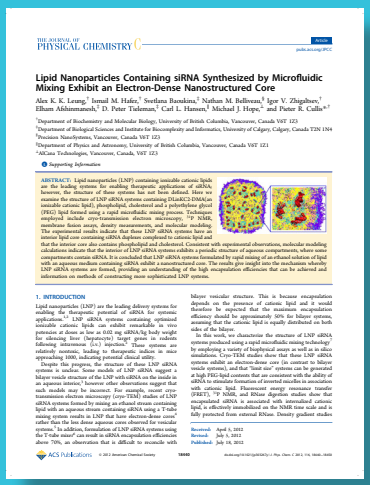
Nanoscale, 2017

Dual-functional lipid-like Nanoparticles for Delivery of mRNA and MRI Contrast Agents

Luo X, Li B, Zhang X, Zhao W, Bratasz A, Deng B et al. Nanoscale 2017; 9: 1575-1579.

Summary

- Nanoparticles are desirable for gene and drug delivery as well as medical imaging and combining these functions in the same particle can open the door to image-guided treatments with greater specificity to disease sites
- Lipid nanoparticles have made mRNA-based therapeutics a clinical reality but significant obstacles remain that limit the broad applications of mRNA-based therapeutics in clinical practice: mRNA instability and lack of efficient delivery vehicles
- Dr. Yizhou Dong's group at the Ohio State University aims to improve mRNA translation by developing lipid-like nanoparticles (LLNs) for the safe and efficient delivery of mRNA while also providing contrast function for medical imaging
- In this work, they developed dual-functional lipid-like nanoparticles for simultaneous delivery of mRNA and magnetic resonance imaging (MRI) contrast agents (Gd) in order to express functional proteins and provide real-time visualization
- mRNA-loaded LLNs were formulated using NanoAssemblr technology for *in vivo* applications and demonstrated high encapsulation and efficient delivery of mRNA and Gd contrast agents *in vivo*
- Microfluidic production of LLNs incorporating therapeutic mRNA and non-invasive MRI probes merit further development for theranostic applications



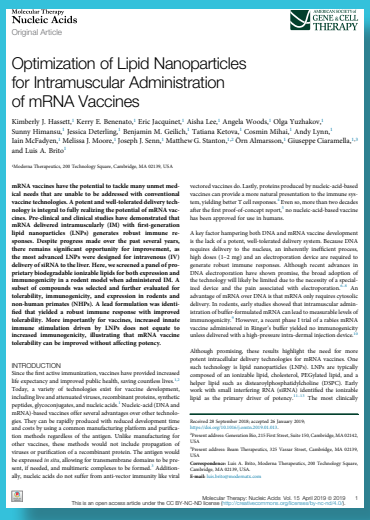
The Journal of Physical Chemistry C, 2012

Lipid Nanoparticles Containing siRNA Synthesized by Microfluidic Mixing Exhibit an Electron-Dense Nanostructured Core

A Leung A, Hafez I, Baoukina S, Belliveau N, Zhigaltsev I, Afshinmanesh E et al. The Journal of Physical Chemistry C 2012; 116: 18440-18450.

Summary

- At the time of publication, little was know about the structure of RNA-lipid nanoparticles (RNA-LNPs) and the influence of emerging microfluidic formulation techniques such as NanoAssemblr technology
- Lipid nanoparticle pioneer Dr. Pieter Cullis' lab used a combination of electron microscopy, NMR, and molecular modeling to study the structure of LNPs
- They discovered that siRNA-LNPs had a morphology that was distinct from liposomes by exhibiting an electron dense core surrounded by stabilizing lipids, rather than a hollow core observed with liposomes
- They also reported that rapid microfluidic mixing improved encapsulation efficiency of siRNA from 75% to nearly 100% and protected the RNA from RNase activity
- This new understanding of the structure provides insight into how high encapsulation efficiency and retention of RNA is achieved as well as the mechanisms of LNP formation and function



Molecular Therapy - Nucleic Acids, 2019

Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines

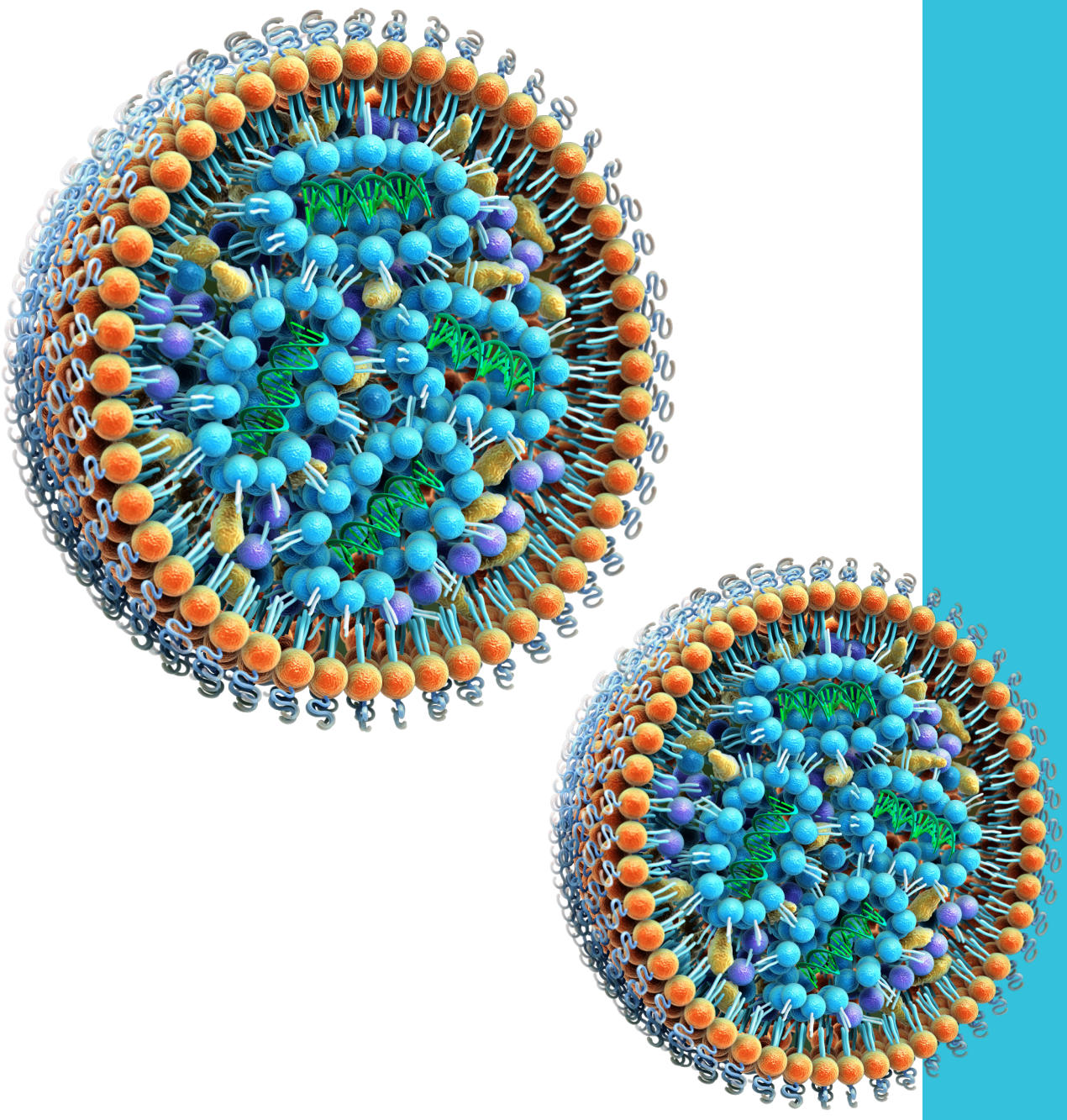
Hassett K, Benenato K, Jacquinet E, Lee A, Woods A, Yuzhakov O et al. Molecular Therapy - Nucleic Acids 2019; 15: 1-11.

Summary

- LNPs are most frequently administered intravenous injection (IVJ), though intramuscular injections (IMJ) are more practical in clinical settings, e.g. for vaccines
- Researchers at RNA-medicine leader Moderna have found differences in activity of some formulations when injected intravenously vs intramuscularly
- To optimize LNP formulations for IMJ they screened 5 different biodegradable ionizable lipids by formulating them into LNPs using NanoAssemblr technology and found the best lipids for IMJ had a higher pKa than those optimal for IVJ
- Additionally, they examined immune response to mRNA-LNP vaccines and found vaccine effectiveness did not require formulations to have a strong self-adjuvanting effect
- These findings provide clear guidelines to rationally optimizing LNP formulations for vaccines
- Rapid, reproducible LNP formulation with NanoAssemblr technology was ideal for efficiently screening numerous LNP formulations

mRNA Lipid Nanoparticles

Robust low-volume production for screening high-value nanoparticle materials



Chelsea Cayabyab, Andrew Brown, Grace Tharmarajah, Anitha Thomas

Abstract

In 2018, the FDA approval of Patisiran, a lipid nanoparticle (LNP) formulation and the first small interfering RNA therapeutic to receive FDA approval, established LNPs as the premier technology for non-viral RNA delivery. Concurrently, the NanoAssemblr® platform, which harnesses microfluidic mixing, has been demonstrated as a simple, robust and scalable production method for LNPs encapsulating various types of nucleic acids with near 100% encapsulation efficiencies. LNPs provide a versatile option for gene knockdown or gene expression studies *in vitro* and *in vivo*. The NanoAssemblr Spark™ exploits an additional advantage of microfluidic mixing: microliter-scale formulation that conserves high-value materials such as novel lipids and mRNA. This unique combination of ultra-low volume formulations with a rapid, simple and reproducible process makes Spark an ideal platform for screening and early preclinical development of mRNA-LNP formulations. Effective screening programs can significantly narrow the parameter space for developing and optimizing next-generation delivery technologies and nanoparticle therapeutics.

Introduction

Screening an array of active pharmaceutical ingredients, excipients and formulation parameters in early nanomedicine discovery and development enables more focused and efficient development in later stages by narrowing the parameter space. To this end, fast, reproducible nanoparticle production at low volume is needed to ensure observed differences in the properties or activity of the formulation can be attributed to controlled changes in composition or conditions, which informs rational design of nanomedicines. Furthermore, achieving this at low volumes minimizes the use of API and excipients, which at the discovery stage, may be limited in availability, expensive to acquire or laborious to produce.

In particular, active ingredients used in the genetic manipulation of cells such as small interfering RNA (siRNA), guide RNA (gRNA), messenger RNA (mRNA) or plasmids are either scarce or very expensive. While non-viral nucleic acid delivery systems are enabling revolutionary treatments such as mRNA vaccines,[1,2] immuno-oncology,[3] targeted oncology,[4-6] CRISPR/Cas9 gene editing,[7–9] and the treatment of rare diseases,[10,11] there remains a substantial need for improving the fundamental understanding of nucleic acid delivery systems and for further innovation to improve the quality and performance of gene-delivery nanoparticles. Specifically, further innovation of nanoparticle excipients used to encapsulate, protect, and deliver these payloads into diseased cells is necessary to advance the field. These excipients are, by their innovative nature, largely unavailable at large, low-cost commodity scales.

So, there is an unmet need in the field for robust and reproducible low volume production of nanoparticles containing genetic payloads appropriate for researchers in the discovery space. For instance, minimum volumes for T-tube mixing are on the order of 10 mL when only microliters are required for *in vitro* screening. For this reason, researchers are using crude pipette-mixing methods to perform bottom-up nanoprecipitation of complex nucleic acid-nanoparticle formulations. Mixing with pipettes offers little control, is operator-dependent, and mixing conditions that dictate the

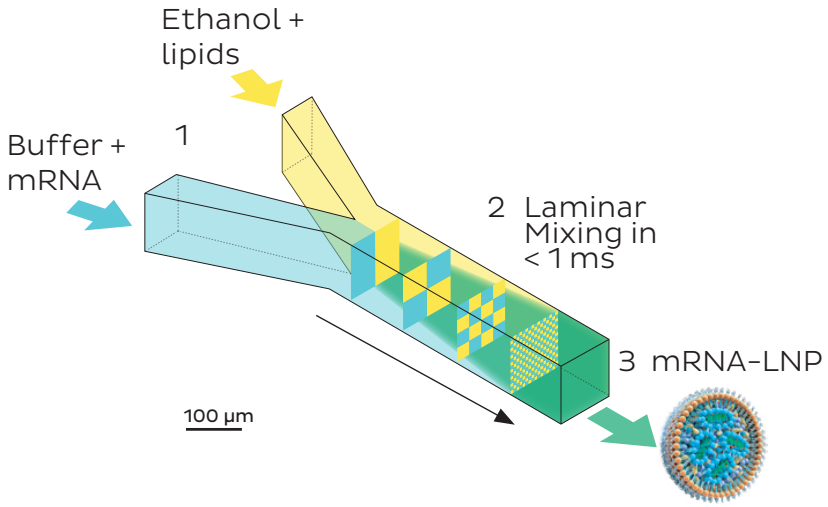


Figure 1.) Microfluidic mixing technology for manufacturing nanoparticles: *An organic solvent containing dissolved lipids and an aqueous solution containing nucleic acids are injected into the two inlet channels of the NanoAssemblr cartridge. Under laminar flow, the two solutions do not immediately mix, but microscopic features engineered into the channel cause the two fluids to intermingle in a controlled and reproducible way, where molecules interact with each other by diffusion. Within 1 millisecond, the two fluids are completely mixed, causing a change in solvent polarity that triggers the homogenous self-assembly of nanoparticles loaded with nucleic acids.*

properties of resulting nanoparticles cannot be repeated with fidelity. Hence, nanoparticle attributes and payload protection vary, resulting in high variability in functional activity studies. This hampers the innovation in this field. Furthermore, once lead formulations are identified, the production conditions have to be scalable such that formulations have the potential to advance through pre-clinical development and clinical manufacturing.

Time-invariant conditions afforded by NanoAssemblr mixers ensures the conditions of self-assembly remain consistent throughout a single formulation and between individual formulations. This allows formulations to be scaled in volume across several orders of magnitude to suit various stages of development. Figure 1 describes the process of mixing in a NanoAssemblr microfluidic mixer in more detail.

Here, we describe the NanoAssemblr Spark for the controlled and reproducible manufacturing of nucleic acid-containing lipid nanoparticles (LNPs) at volumes ranging from 100 to 250 µL. The Spark realizes the advantages of microfluidic mixing in a format that allows consistent formulation at microliter scales in seconds (Figure 2). The resulting formulation can be applied directly to cells in culture. This makes Spark ideal for rapidly producing numerous formulations for *in vitro* testing while conserving rare or costly materials. Consistent conditions allows any observable differences in the outcomes to be attributed to the differences in formulations to inform rational design of gene-delivery vehicles.

Similar lipid delivery systems have been well described in the literature for the delivery of siRNA, mRNA and plasmid payloads.[12–15] We demonstrated how mRNA-LNP formulations can be screened by systematically varying LNP composition, reagent concentrations, and the N/P ratio (the ratio between cationic amines in the lipid excipient and the anionic phosphates on mRNA). We also demonstrated how the Spark can be used for encapsulating various lengths of mRNA with consistent results. In all, we demonstrated the utility of the Spark as an efficient screening platform that allows a large number of different mRNA-LNP formulations to be rapidly produced using sub-milligram quantities of both API and nanoparticle excipients at a scale well suited for downstream physical characterization or *in vitro* functional screening.

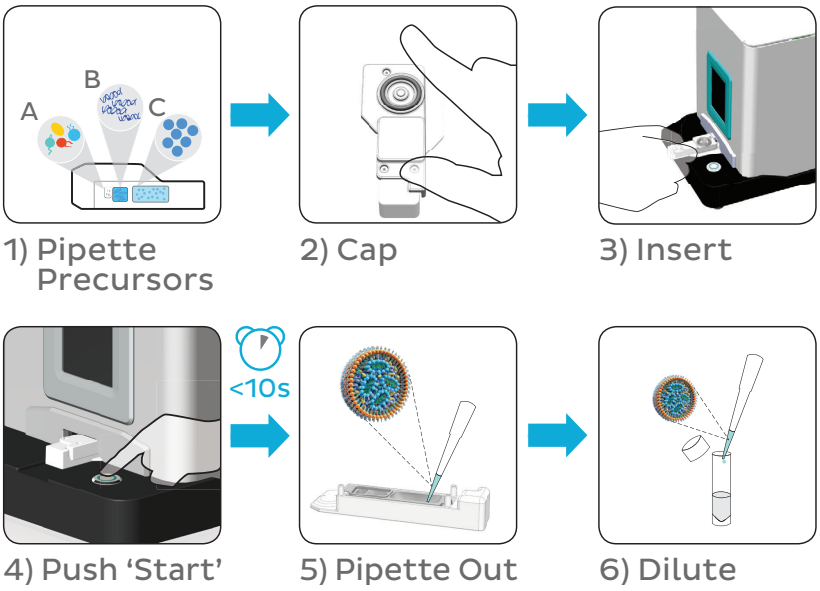


Figure 2.) The NanoAssemblr® Spark™ workflow for producing nanoparticle systems:
Step 1 – Precursor solutions were pipetted into the wells of the Spark microfluidic cartridge as follows: a) 12 µL of lipid mix in ethanol, b) 36µL of mRNA in acetate buffer, c) 48 µL of PBS.
Step 2 – The cartridge cap was fitted,
Step 3 – The cartridge is inserted into the Spark instrument. Setting 2 was selected via the touch screen interface.
Step 4 – The "Start" button was depressed to begin mixing. Mixing takes ~3s.
Step 5 – The resulting mRNA-LNP suspension is pipetted out of the collection well.
Step 6 – The LNP suspension (96 µL) is mixed with 96 µL of dilution buffer in a microcentrifuge tube.

Materials & Methods

Solution preparation:

Messenger RNA (TriLink Biotechnologies) was diluted using sodium acetate buffer, pH 4 (Sigma Aldrich) to a final buffer strength of 100 mM and to the required mRNA concentration. Stock solutions of cationic lipid (CL), 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar lipids), Cholesterol (CHOL, Sigma Aldrich) and PEG-lipid (NOF America Corp) in ethanol were mixed at a mole fraction of 50:10:40-x:x respectively. Here, x is the mole fraction of PEG-lipid and was varied between 1.5 to 5%, substituting for cholesterol. Cationic lipids (CL) were one of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids), 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA, Avanti Polar Lipids), or a proprietary ionizable cationic lipid (PNI-ILa).

Ultra-low volume LNP preparation:

LNP samples were prepared as indicated in the Spark workflow (Figure 2) 12 µL of 35 – 65 mM lipid mix (to explore the effect of lipid concentration), and 36 µL of mRNA solution containing 10 - 25 µg of nucleic acids (as required by the N/P ratio) in 100 mM sodium acetate buffer were pipetted into the first and second wells, respectively, of the Spark microfluidic cartridge (Precision NanoSystems, Vancouver, Canada). 48 µL Ca^{2+}- and Mg^{2+}-free PBS (Corning) at pH 7.4 was pipetted into the collection well (well 3). To initiate microfluidic mixing of lipid and mRNA solutions, the loaded cartridge was capped, and inserted into the NanoAssemblr Spark (Precision NanoSystems, Vancouver, Canada) and the formulation was run with the Spark setting at "3". The resulting mRNA LNPs were immediately diluted on-chip in the collection well. The contents of the collection well were then immediately transferred to a microcentrifuge tube containing 96 µL of Ca^{2+}- and Mg^{2+}-free PBS at pH 7.4. All samples were prepared in triplicate.

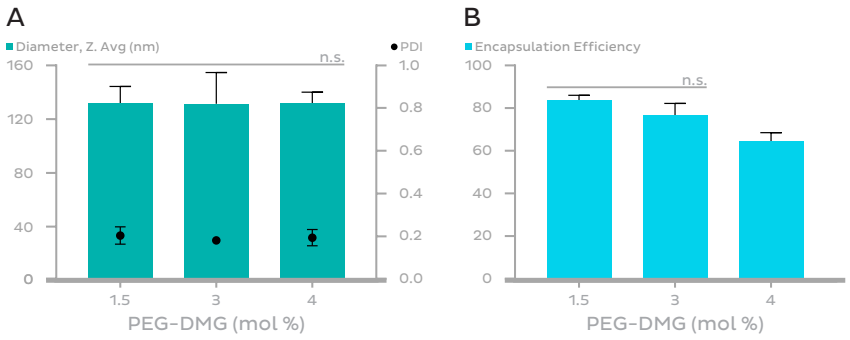


Figure 3.) Ultra low volume screening of mRNA LNP formulations for lipid-PEG amounts. *Figure shows the effect of different PEG content on the size and PDI (A), and encapsulation efficiency (B) of mRNA-LNP formulations using the NanoAssemblr Spark by changing the mole percentage of PEG (2000) -DMG. Values represent the mean from triplicate formulations. Error bars represent the standard deviation. Means grouped by horizontal bars were not significantly different (P >0.05 by ANOVA (A) and Tukey's multiple comparison test (B)). Error bars not drawn when error is less than the size of the symbol.*

Formulation Parameters

Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG 50:10:40-x:x | x = 1.5, 3, 5 mol%
Initial Lipid Concentration	50 mM
mRNA	GFP (996 nt), 0.025 mg
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS

Analysis of LNPs:

Encapsulation efficiency (EE%) was measured using a fluorescence plate-based assay employing the Ribogreen reagent (Invitrogen) as per PNI Ribogreen assay protocol[16]. This assay measures the quantity of mRNA in samples with intact LNPs to determine the quantity of unencapsulated RNA as well as in LNP samples disrupted by triton X-100 (Sigma Aldrich) to measure the total RNA. EE% is calculated as the difference between the total RNA and the unencapsulated RNA divided by the total RNA.

Size and polydispersity index (PDI) were determined by dynamic light scattering using a Zetasizer Nano ZS (Malvern Panalytical, UK). 10 µL of LNP suspension was transferred to a low-volume cuvette containing 300 µL of Ca^{2+}- and Mg^{2+}-free PBS at pH 7.4. Refractive index was set to 1.14 and temperature was set to 25°C.

Results

The effect of PEG content was examined by comparing samples containing 1.5, 3 and 4 mol% PEG lipid (Figure 3). PEG content did not have a statistically significant impact on size. The formulation PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5 mol%) had the highest encapsulation efficiency, whereas there was a statistically significant reduction in EE% at 4 mol% PEG-lipid.

The suitability of three cationic lipids for producing mRNA-LNPs was determined by comparing EE% and particle size (Figure 4). Permanently cationic lipids DOTAP and DOTMA were compared to PNI's proprietary ionizable cationic lipid (PNI-ILa). N/P ratio, concentrations of reagents and the formulation process were held constant. PNI-ILa produced significantly smaller LNPs with the lowest PDI.

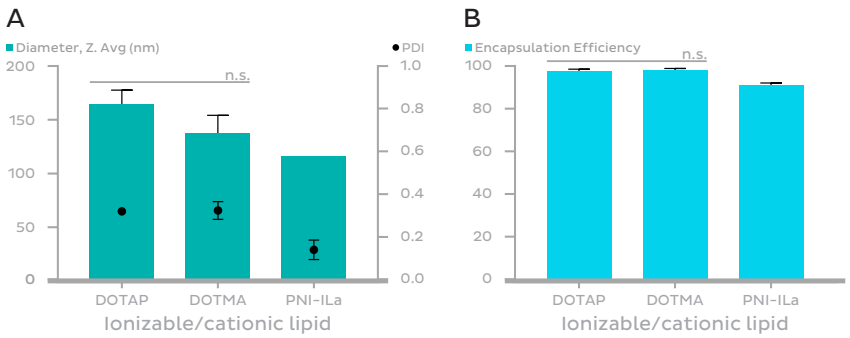


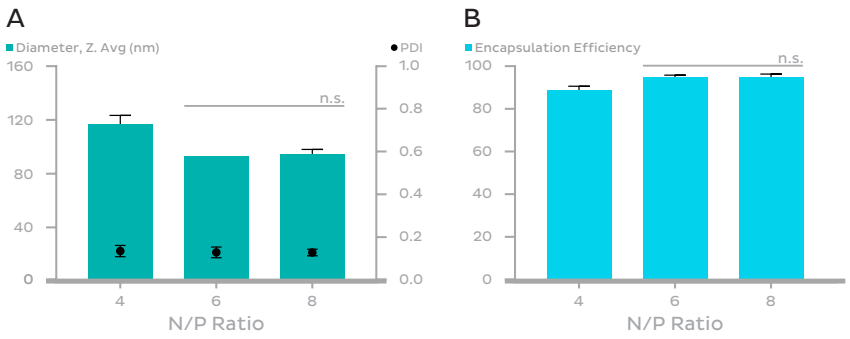
Figure 4.) Screening different cationic/ionizable lipids in low-volume mRNA-LNP formulations. *Figure shows effect of different cationic/ionizable lipids on size and PDI (A) and encapsulation efficiency (B) of mRNA LNP formulations. Encapsulation efficiencies over 90% were achieved. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by horizontal bars were not significantly different (P>0.05 by Tukey's multiple comparison test). Error bars not drawn when error is less than the size of the symbol.*

Formulation Parameters

Lipid Composition	CL:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5mol%) CL = DOTAP (18:1 TAP) or DOTMA or PNI-ILa
Initial Lipid Concentration	50 mM
mRNA	GFP (996 nt), 0.025 mg
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS

The N/P ratio has been shown to affect siRNA-LNP quality and efficacy. To determine the effect of N/P ratio on mRNA LNPs, three N/P ratios were tested: 4:1, 6:1 and 8:1 (Figure 5). At 4:1 the particle size is larger with a slight decrease in EE% compared to 6:1 and 8:1. At N/P of both 6:1 and 8:1 similar size, PDI and EE% were achieved suggesting that an asymptotic limit has been reached.

Figure 5.) Systematic screening of N/P ratios with low-volume mRNA-LNP formulations. *Increasing N/P ratios were tested. Size and PDI (A) were measured using dynamic light scattering and encapsulation efficiency (B) was measured by modified ribogreen assay. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by the horizontal bars were not significantly different (P>0.05 by Tukey's multiple comparison test). Error bars not drawn when error is less than the size of the symbol.*

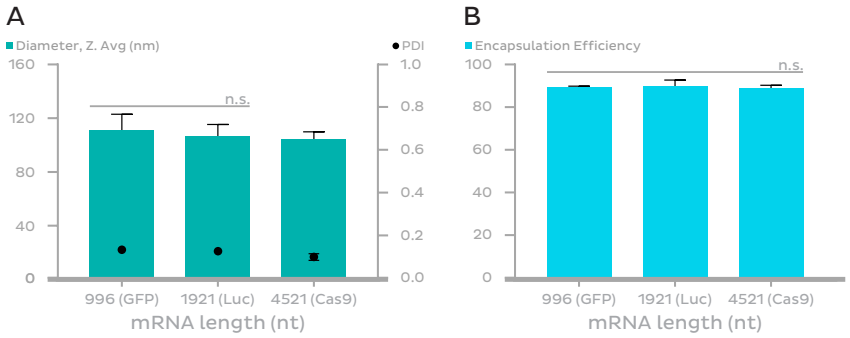


Formulation Parameters

	N/P		
	4	6	8
Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5 mol%)		
Initial Lipid Concentration	50 mM		
mRNA	GFP (996 nt), 0.025 mg	GFP (996 nt), 0.0167 mg	GFP (996 nt), 0.0125 mg
Organic Solvent	Ethanol		
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate	0.464 mg/ml mRNA in 100 mM sdoium acetate	0.347 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2		
Downstream Processing	4x dilution with nuclease-free PBS		

Three mRNAs with different lengths were encapsulated to explore the effect of RNA length. These were GFP mRNA containing 996 nt, Luc mRNA containing 1921 nt and Cas9 mRNA containing 4521 nt. Across all three mRNAs EE% of 90% were achieved with consistent size and PDI, indicating that mRNA length does not impact key physical attributes of the LNPs (Figure 6).

Figure 6.) High encapsulation efficiency achieved for mRNAs independent of mRNA length. *Size and PDI (A) was measured by dynamic light scattering, and encapsulation efficiency (B) was measured by modified ribogreen assay. Formulations were prepared in triplicate. Values represent the mean and standard deviations, respectively. Means grouped by horizontal bars were not significantly different (P>0.05 by Tukey's multiple comparison test (A) and ANOVA (B)). Error bars not drawn when error is less than the size of the symbol.*



Formulation Parameters

Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5)
Initial Lipid Concentration	50 mM
N/P	4
Organic Solvent	Ethanol
Aqueous Phase	0.695 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2
Downstream Processing	4x dilution with nuclease-free PBS

In some formulations, the initial concentration of lipid can affect the particle size. The effect on EE%, size and PDI for three initial lipid mix concentrations of 35, 50 and 65 mM were compared (Figure 7). Other parameters were held constant, particularly the N/P ratio and lipid composition. EE% remained above 80% in all cases, and no significant difference in size, or PDI were observed.

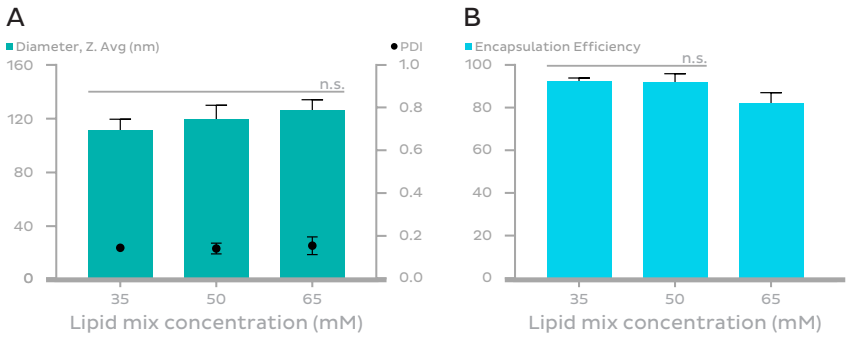


Figure 7.) Effect of lipid mix concentration on mRNA LNP. *Low volume mRNA-LNPs were formulated with different total lipid concentration between 35 – 65 mM. Size and PDI (A) were measured by dynamic light scattering. Encapsulation efficiency (B) was measured by modified Riobogreen assay. Formulations were prepared in triplicate. Values represent the mean and error bars, the standard deviation. Means grouped by horizontal bars were not significantly different (P>0.05 by ANOVA (A) and Tukey's multiple comparison test (B)). Error bars not drawn when error is less than the size of the symbol.*

Formulation Parameters

	Lipid Mix Concentration (mM)		
	35	50	65
Lipid Composition	PNI-ILa:DSPC:Cholesterol:PEG-DMG (50:10:38.5:1.5) mol%		
N/P	4		
Organic Solvent	Ethanol		
Aqueous Phase	0.487 mg/mL mRNA in 100 mM sodium acetate	0.695 mg/mL mRNA in 100 mM sodium acetate	0.904 mg/mL mRNA in 100 mM sodium acetate
Instrument Setting	2		
Downstream Processing	4x dilution with nuclease-free PBS		

Discussion

We have manufactured lipid nanoparticles encapsulating mRNA at microliter volumes for the purposes of screening materials and formulation parameters, while requiring just 10-25 µg of valuable mRNA and less than 1 mg of novel cationic lipid per formulation. Formulation parameters including PEG content, N/P ratio, choice of the cationic/ionizable lipid, mRNA length and lipid mix concentration were independently examined for their effect on nanoparticle attributes. Reproducible batch-to-batch encapsulation efficiencies, size and PDI were achieved across all variables, demonstrating how Spark is a robust platform for small-volume production of nucleic acid-loaded LNPs. It is important to note, however, that these factors may have a more dramatic effect on biological activity. Hence, further testing with biological end points is necessary. From these results, it is evident that the Spark is ideal for screening novel formulations that use scarce or expensive active pharmaceutical ingredients and formulation excipients.

An examination of PEG content in formulations containing PNI-ILa, revealed an unexpected behavior. It had been demonstrated in the past with formulations containing a different but similar ionizable cationic lipids and encapsulating siRNA, that greater PEG quantities in this range led to smaller particles.[17] Here, with mRNA as the payload, the size was unaffected by PEG content. PEG content did however reduce EE% with formulations containing 4 mol% PEG-lipid. This decrease in EE% at higher PEG-lipid was also observed in formulations produced at mL scales on the NanoAssemblr (data not shown). It was found that increasing the N/P ratios from 4:1 to 8:1 has minimal effect in encapsulation efficiency. This is expected, the mRNA will be completely encapsulated beyond a threshold N/P ratio.

We have previously shown that mRNA-LNPs produced with the Spark were effective in eliciting exogenous gene expression in rat primary neuronal cultures, and that these formulations can be scaled up for *in vivo* studies using the larger-volume NanoAssemblr instrument while maintaining consistent EE above 90%, even with different mRNA lengths.[18] Hence, once the lead nanoparticle formulations have been selected by screening using the Spark, they can be scaled up using a bench-scale NanoAssemblr systems for further optimization and early *in vivo* testing, and the the NanoAssemblr Blaze for larger *in vivo* studies or as production demands dictate.

Conclusion

Screening active ingredients, excipients and formulation conditions are important in the discovery and early development stages in order to streamline future development and rational design of nanomedicines. This work uses the NanoAssemblr Spark's unique ability to quickly and reproducibly formulate mRNA-LNPs at volumes ideal for screening (100 – 250 µL; 10-25 µg of RNA; < 1 mg of novel cationic lipid). The Spark uses proprietary microfluidic mixing technology for the rapid (< 10 seconds), controlled and reproducible manufacturing of nanoparticles. mRNA-LNPs produced using PNI-ILa were in the range of 90 – 140 nm in diameter with PDI below 0.2. The encapsulation efficiencies of mRNA were consistently over 80%. We have determined that formulation parameters such as PEG content above 3% can reduce encapsulation efficiency without detectable changes in particle size or PDI. The Spark is the only system that combines ultra-low volume formulation with reproducible conditions making Spark ideal for screening formulations that use scarce or expensive active pharmaceutical ingredients and/or excipients to inform rational design of nanoparticle drug delivery systems.

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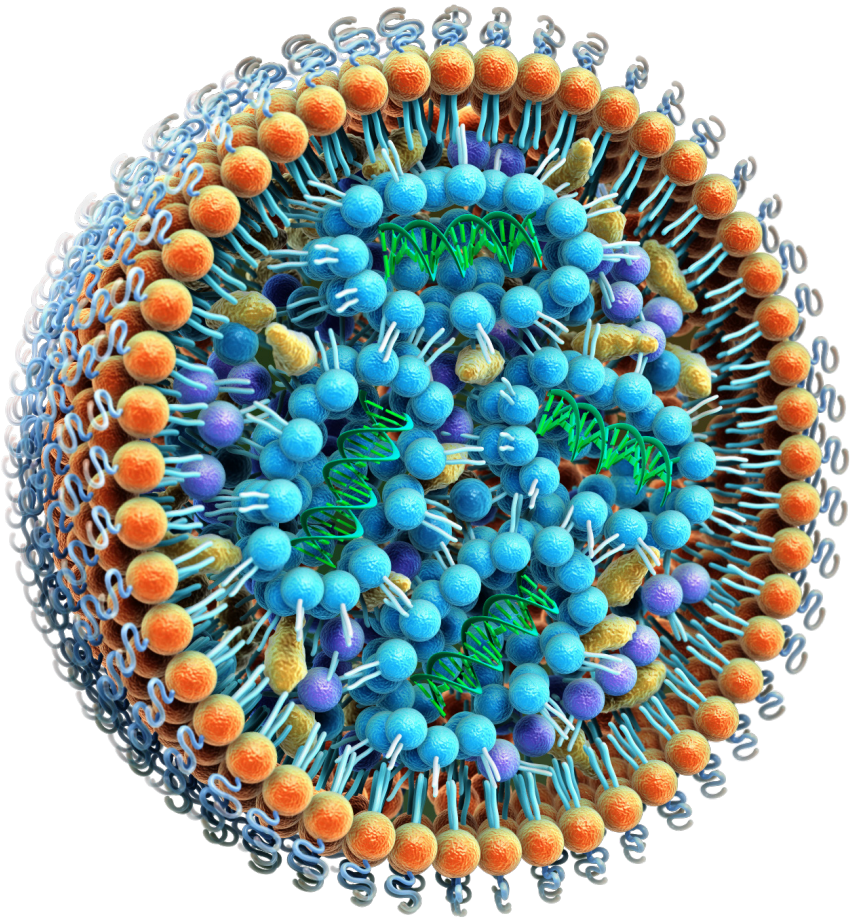
Nucleic Acid Lipid Nano-particles

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



Jagbir Singh, Kevin Ou, Anitha Thomas, Mark Ma, Ray Lockard, Shell Ip, Euan Ramsay

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 a bench-scale NanoAssemblr system, 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 at the bench scale, 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.

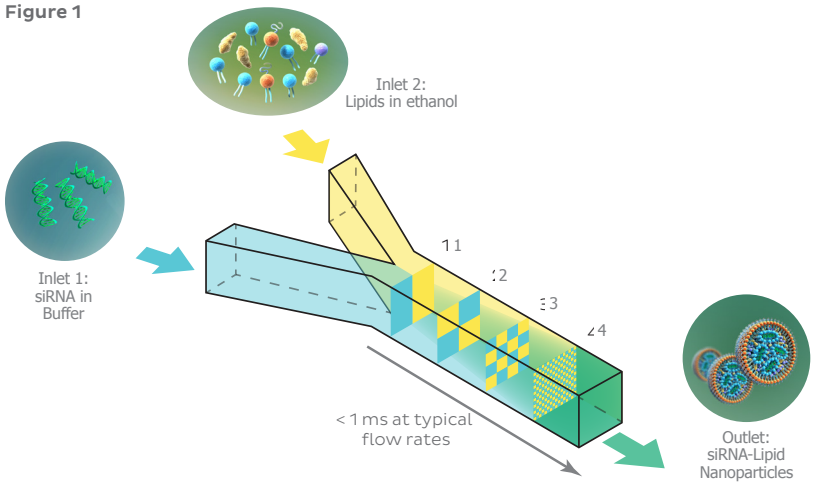
Introduction

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 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 litres. 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 preclinical and clinical scale production, which allow previously optimized conditions to be replicated at larger scales.

Figure 1. *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.*

Figure 1



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 process takes < 1 ms; faster than molecules can assemble into particles.

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 NanoAssemblr 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 a bench-scae NanoAssemblr system. 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 bench-scale system, 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 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).

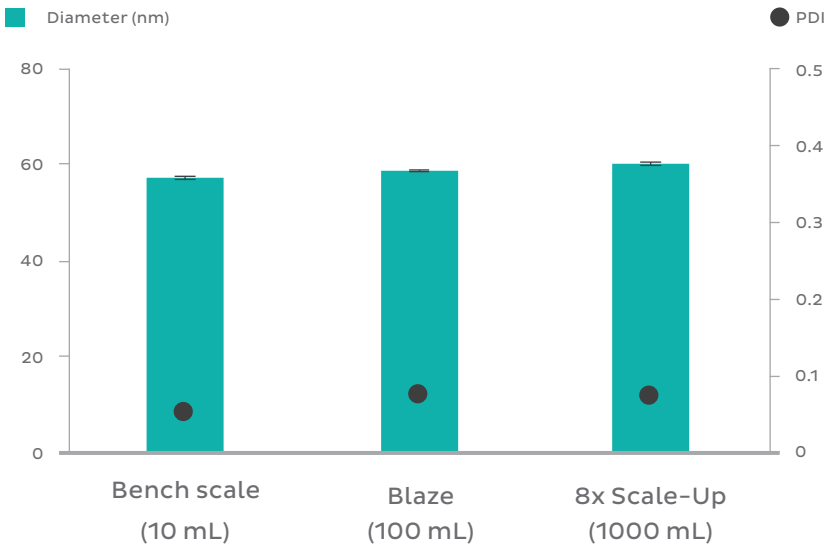


Figure 2. *Factor VII siRNA LNPs maintain size and PDI when scaled by 10x and 100x on the NanoAssemblr platform. 10 mL formulations were produced on a bench-scale system, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. Hydrodynamic size and particle size distribution (expressed as polydispersity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

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 different concentrations. A final siRNA concentration of 0.174 mg/mL represents commonly reported concentrations.

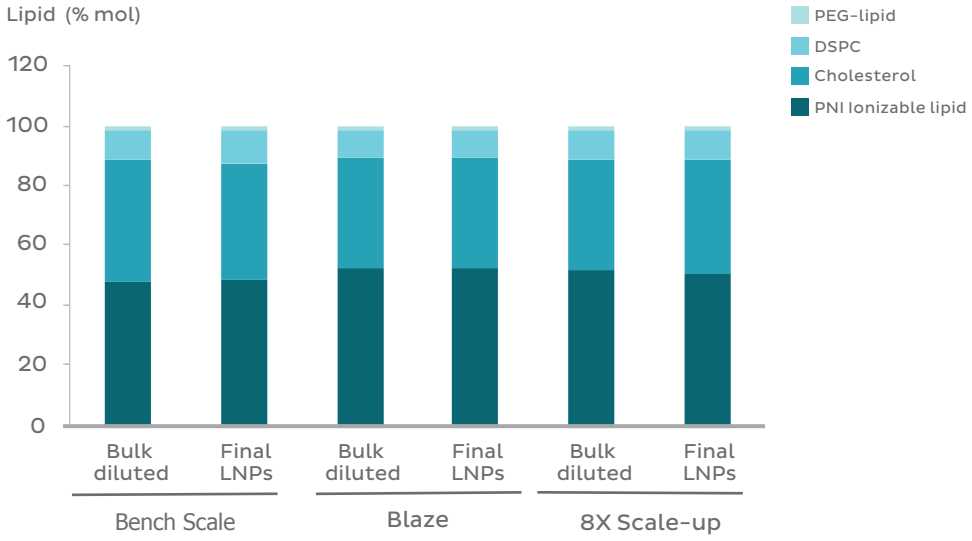


Figure 3. *Factor VII siRNA LNPs maintain composition when scaled by 10x and 100x on the NanoAssemblr platform. 10 mL formulations were produced on a bench-scale system, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. LNPs were disrupted, components separated by HPLC and analyzed by ELS.*

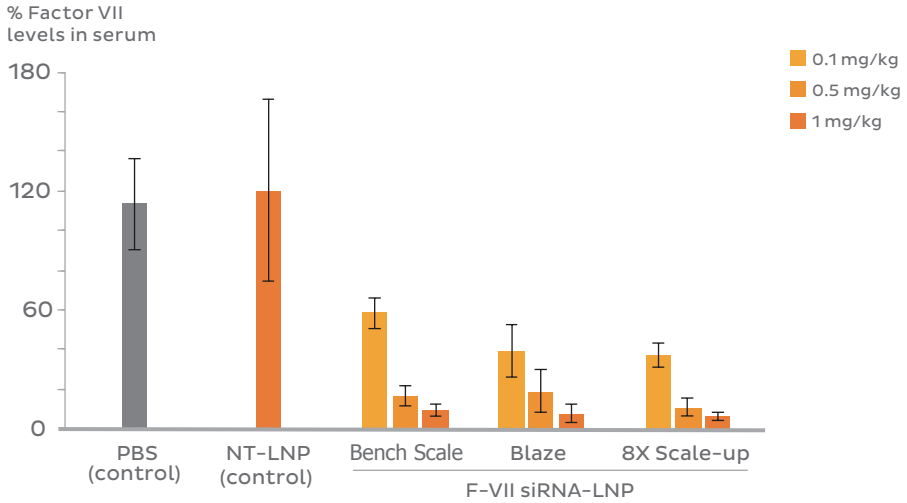
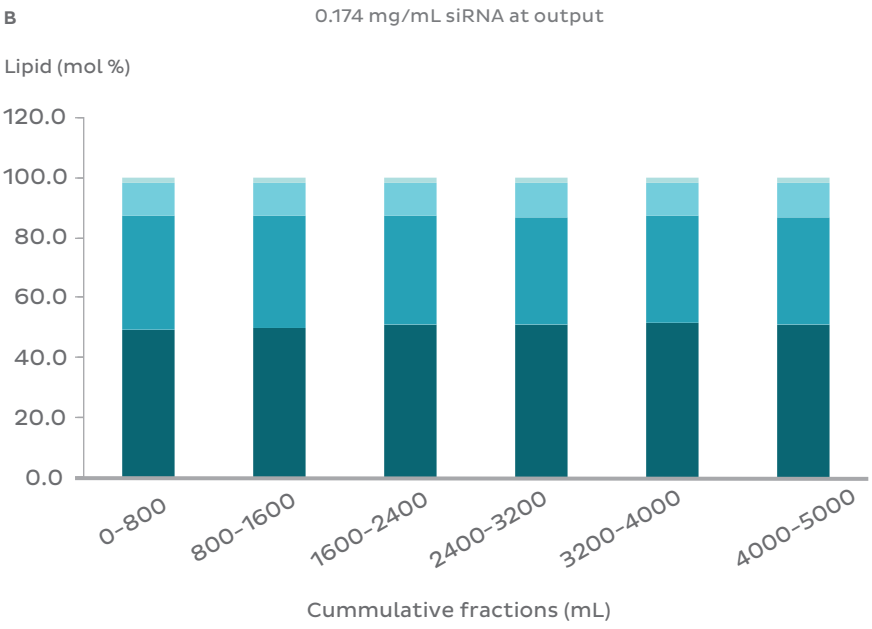


Figure 4. *in vivo knockdown efficacy of Factor VII siRNA LNPs maintained when scaled by 10x and 100x on the NanoAssemblr platform. 10 mL formulations were produced on a bench-scale system, 100 mL on the Blaze and 1 L on the 8x Scale-Up systems. LNPs and controls were administered to healthy mice by 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.*

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 C). The higher concentration formulation was found to be the same diameter (60 nm, Figure 5C) as lower concentration formulations produced on a bench-scale system, Blaze, and Scale-Up in its 8x configuration (Figure 2). Composition of the particles remained unaffected by the change in concentration (Figure 5B and D).

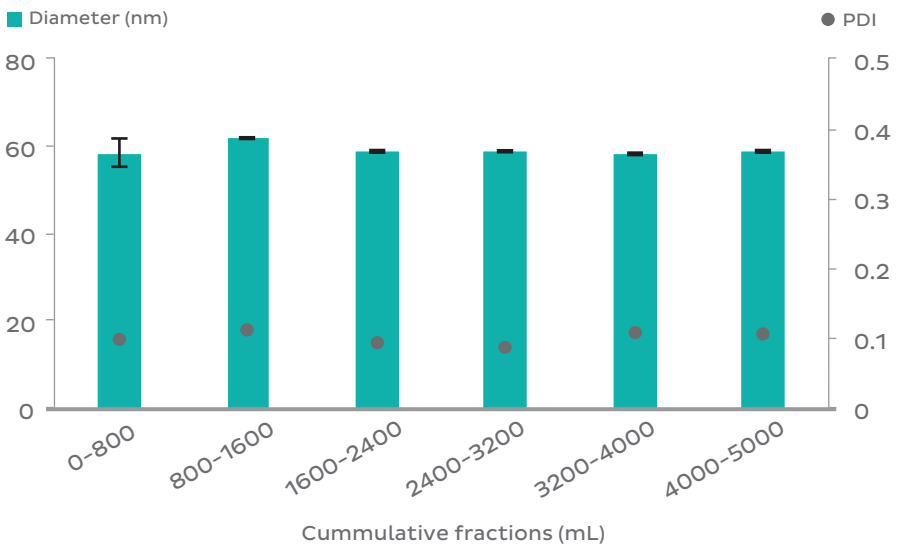
Figure 5. *Factor VII siRNA-LNPs manufactured at high concentrations on the Scale-Up system retain their physico-chemical properties. Lipid Nanoparticles (LNPs) containing siRNA against Factor VII optimized at the bench scale at 0.174 mg/mL siRNA, were made on the Scale-Up system with a single chip at 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 C) and composition (B and D).*



Discussion

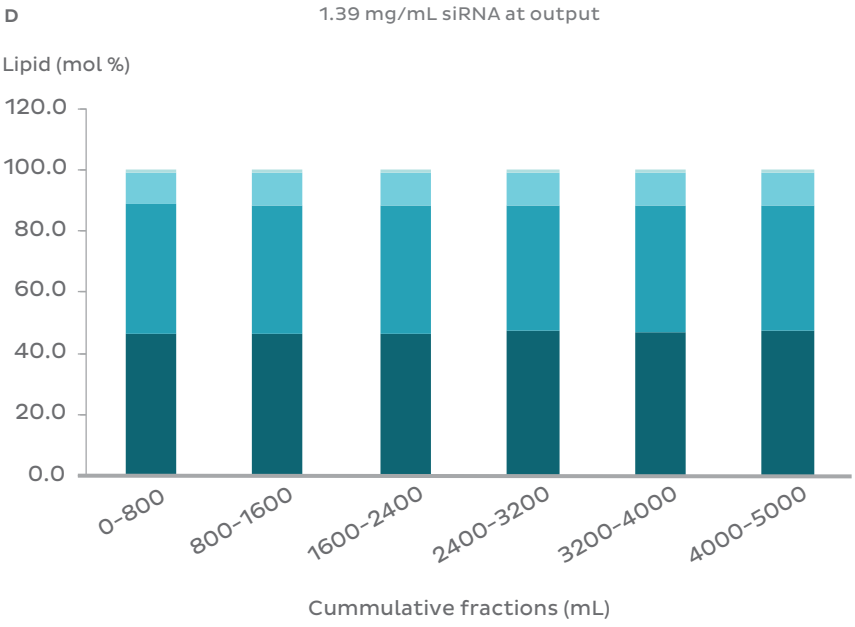
Nanoparticle precipitation is an intricate process dependent on numerous intermolecular interactions. For LNPs, pH sensitive ionizable lipids must interact with components of the buffer to become cationic, following which, they interact electrostatically with anionic nucleic acids to form reverse micelles. As the polarity of the solvent increases, interactions between the exposed lipid tails and solvent molecules become unfavourable, 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

C



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

D



PEG-Lipid
DSPC
Cholesterol
PNI Ionizable Lipid

Further details:
Composition of siRNA LNPs were 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

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 a bench-scale system, 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 bench-scale 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 bench-scale system, 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 bench-scale, 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 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 is 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 cationic 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 was 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 were performed using Biophen Factor VII assay (Aniara, OH, USA).

* The bench-scale formulations described in this work were produced using the discontinued NanoAssemblr Benchtop. The NanoAssemblr Ignite can be used in place of its predecessor, the NanoAssemblr Benchtop

Related Material

About the NanoAssemblr Platform:
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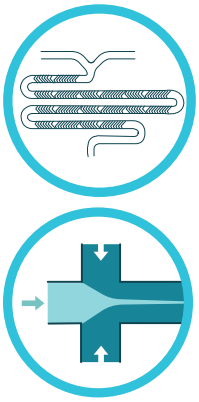
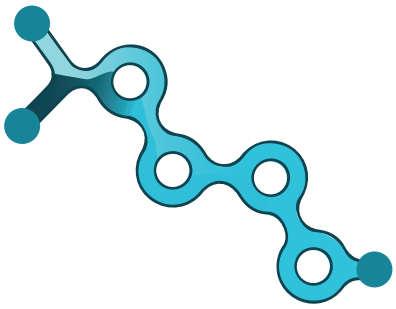
- Difficult to reproduce	- Difficult to reproduce	+ Reproducible	+ Reproducible
- Harsh process conditions	+ Gentler process conditions	+ Non-turbulent process conditions	+ Non-turbulent process conditions
- Difficult to scale	+ Improved Scalability		





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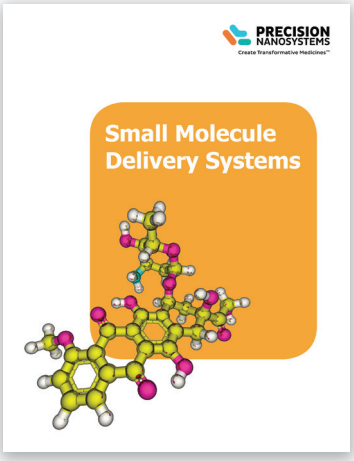

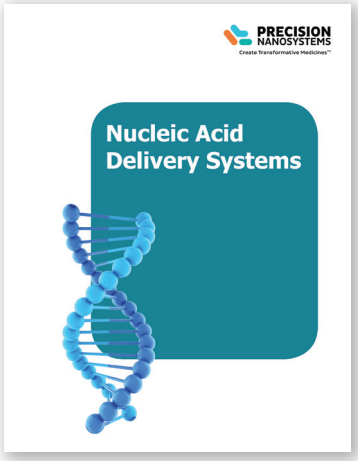

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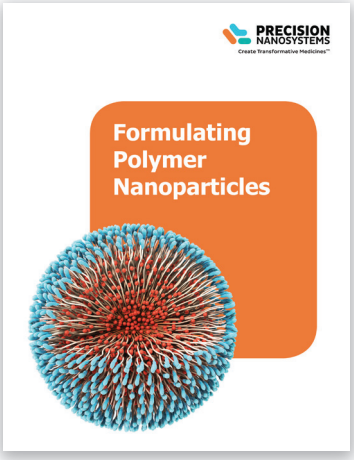

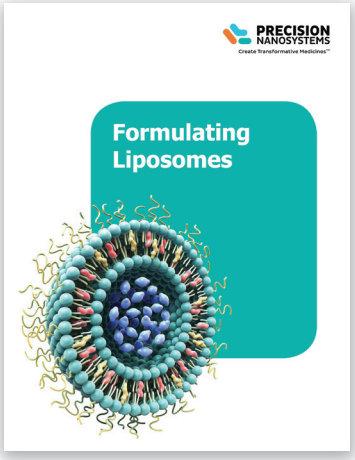

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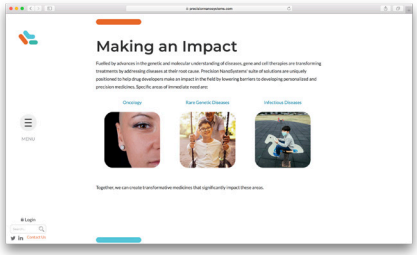

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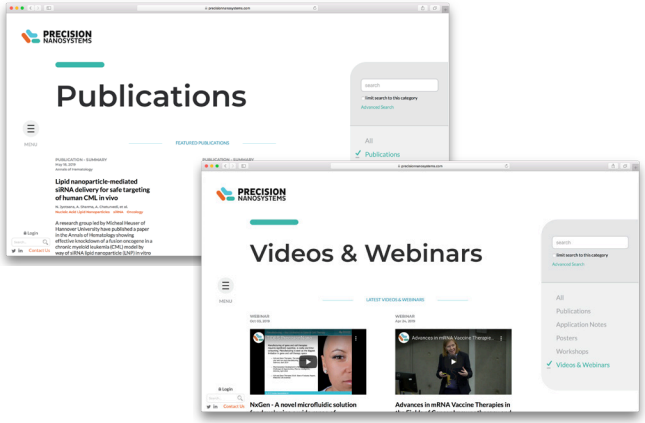

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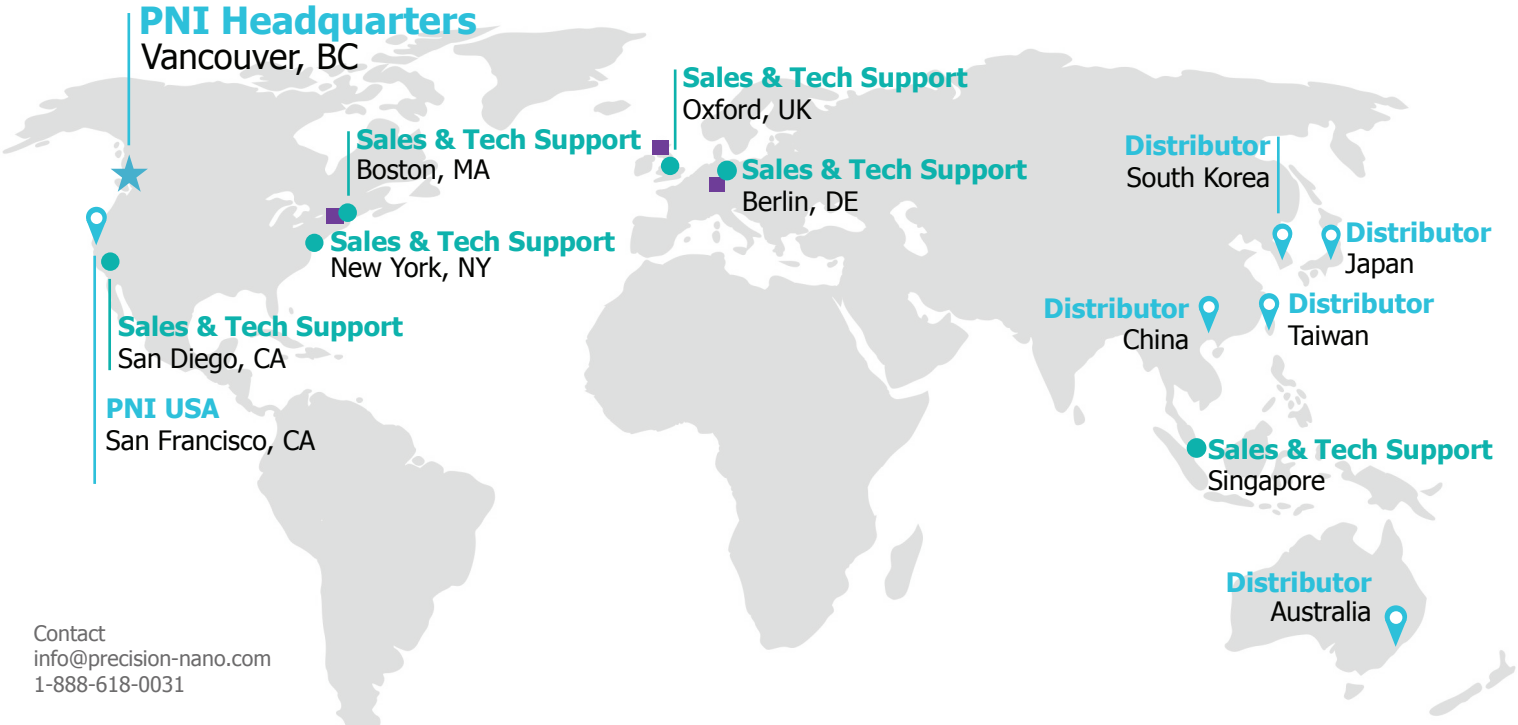

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