Preparation and Characterization of Lipid Nanoparticle Formulations for siRNA Delivery by a Microfluidic Approach

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Formulation of LNPs encapsulating siRNA

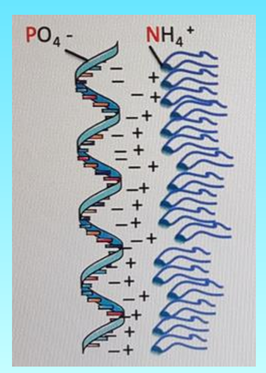
The development of mRNA-based vaccines against SARS-Cov-2 have speed up the application of Lipid Nano-particles (LNPs) to nucleic acid delivery for the treatment of rare diseases, diabetes, and cancer.

LNPs are becoming the ideal carrier: nontoxic, biomimetic, able to protect nucleic acid integrity, especially RNA molecules, safe and effective with no immunogenic responses. LNPs are able to encapsulate different genetic materials as small interfering RNA (siRNA), messenger RNA (mRNA) or plasmids and are extremely tuneable to changes in the composition and in the chemical modification.

LNP-nucleic acids assembly principle:
Adequate Ratio of Amines (NH_4+) to Phosphates (PO_4-)
to compensate charges and to protect nucleic acid cargo

After testing different N/P charge ratios, the 3:1 proportion was selected as the best balance for formulating siRNA-loaded LNPs

Optimal N/P 3 : 1



Preparations of LNPs encapsulating siRNA

LNPs encapsulating siRNA were manufactured through the Ignite NanoAssemblr platform by Precision Nanosystems-Cytiva (Fig. 1A) based on the microfluidic NxGen™ technology (Fig 1B). The organic phase was a mixture of lipids dissolved in ethanol including the ionizable amino lipid DLin-MC3-DMA, DSPC, cholesterol, PEG-DMG in a molar ratio of 50:10:38.5:1.5 to obtain a total lipid concentration of 12.5 mM [1, 2]. The aqueous phase composed of siRNA (235 µg/mL) in acetate buffer (pH 4) and the organic phase were combined in the microfluidic system in a volume ratio 1 : 3 at a flow rate of 12 mL/min (Fig. 1B). The resultant siRNA-LNPs were dialyzed against phosphate-buffered saline (PBS, pH 7.4) using GeBaFlex dialysis membranes (14 kDa MWCO) for 3 h at 4 °C to remove ethanol and then filtered through 0.2 µm filters.

The encapsulation efficiency (EE%) was calculated upon measurement by Quant-iT Ribogreen RNA assay of the RNA outside the LNPs (unencapsulated RNA) and the total RNA obtained after lysis with 2% Triton (encapsulated RNA). EE% was calculated by taking the ratio of encapsulated RNA to the total RNA (Fig. 1C). To set up the protocol, two preparations of negative control siRNAs, siRNA-NC1-LNPs and siRNA-NC2-LNPs, were synthetized providing an EE% of about 94%.

Lipid concentration was determined by the measurement of cholesterol content using an enzymatic colorimetric method.

Figure 1

A

The NanoAssemblr® Ignite™ with NxGen™ Technology

by Precision Nanosystems

Controlled Assembly: Particle size with precise control over fluid flow rates and ratios. LNPs are homogeneous in terms of lipid composition, lipid:nucleic acid ratio and size (50-100 nm)

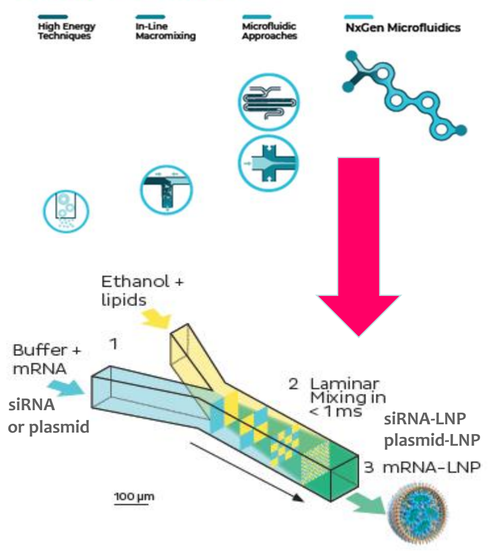
Reproducible: Reduce batch-to-batch and user variability

Scalable: Scale-up of optimized formulations for industrial production and GMP System

Accelerated timelines of drug development

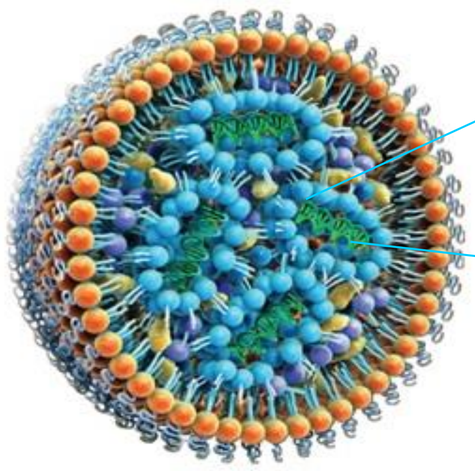
B

NxGen™ Technology



Lipids in Ethanol : siRNAs in acetate buffer Volume ratio 1 : 3

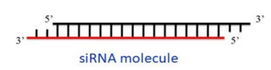
C



Diameter~ 50-60 nm

LNP manufactured using a mixture of ionizable amino lipid DLin-MC3-DMA, DSPC, cholesterol, PEG-DMG in a molar ratio of 50:10:38.5:1.5, respectively, with a final total lipid concentration of 12.5 mM [1, 2].

small interfering RNAs (siRNAs)



21/27 bp

Encapsulation Efficiency > 90%

RNA concentration was determined by Quant-iT Ribo-green RNA assay.
Encapsulated RNA (E_{RNA}) is calculated by subtracting the unencapsulated RNA (U_{RNA}) present in the whole LNP samples (unencapsulated RNA) from the total RNA (T_{RNA}) measured in the LNP samples lysed with 2% Triton (encapsulated RNA, E_{RNA}) [3]:

$$E_{RNA} = T_{RNA} - U_{RNA}$$

Encapsulation efficiency percentage (EE%) was calculated by taking the ratio of encapsulated RNA (E_{RNA}) to total RNA (T_{RNA}):

$$EE\% = E_{RNA}/T_{RNA} \times 100$$

At selected time points (0-7-15-21-30 days) we performed the physical and the chemical characterization of siRNA-loaded LNPs by particle Zetasizer (Malvern Panalytical) measurements over time. Mean hydrodynamic size of LNPs by dynamic light scattering (Z-average, nm) is reported in Fig. 2. Polydispersity index (PDI) indicating the monodispersity or aggregation of LNPs is reported in Fig. 3. Magnitude of the electrostatic repulsion/attraction between particles (Zeta-potential, mV), measured in PBS, is reported in Fig. 4.

Figure 2. Hydrodynamic size

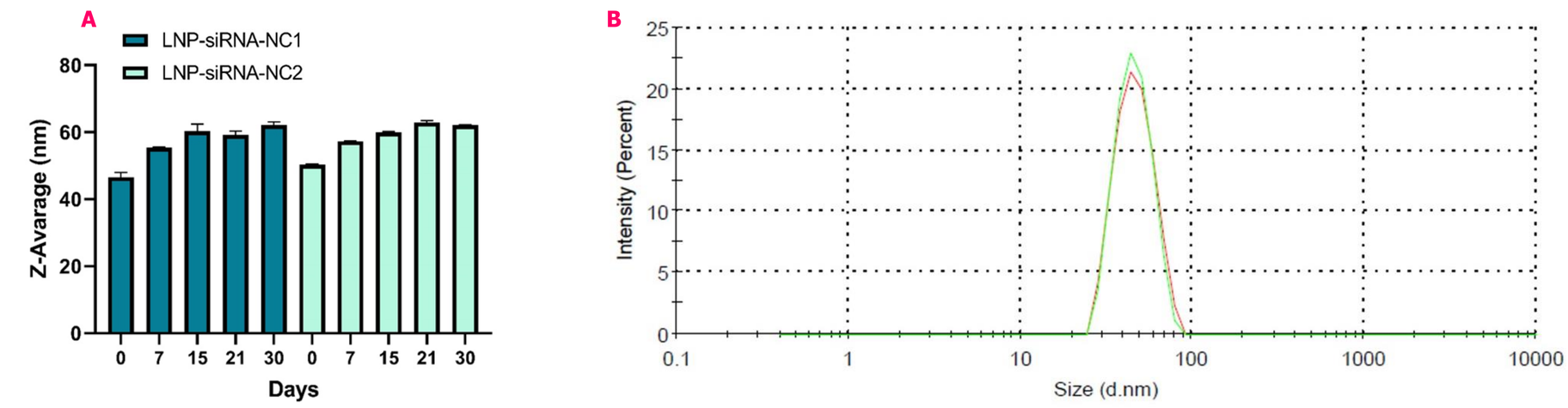


Figure 3. Polydispersity index

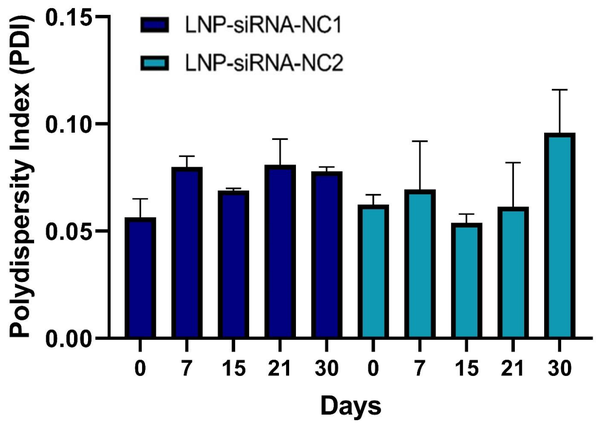
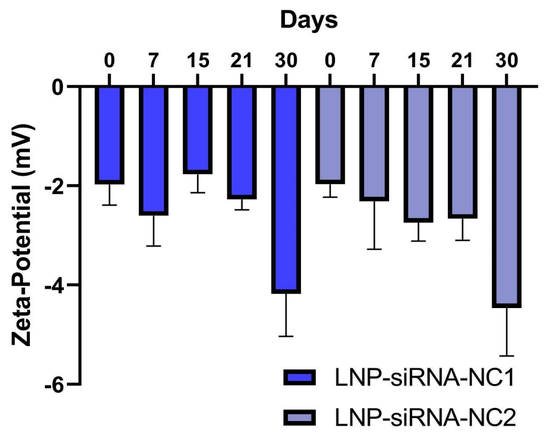


Figure 4. Zeta-potential



In conclusion, we demonstrated that to optimize the design of future RNA therapeutics, an in-depth analysis of chemical and physical characteristics provides an essential tool for the manufacturing process to reduce nucleic acid loss and the associated cost.

Acknowledgments
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[1] Leung AK et al. J Phys Chem B. 2015, PMID: 26087393
[2] Kulkarni JA et al. ACS Nano. 2018, PMID: 29614232
[3] Schober GB et al. Sci Rep. 2024, PMID: 38287070