Accelerate your LNP formulation screening with Sunscreen's automated microfluidic technology

Introduction

Lipid Nanoparticles (LNPs) are the leading methodology for the clinical delivery of nucleic acids (NA) applications. The potential for hugely efficient translation from concept to clinic – the first doses of SpikeVax for clinical testing were rolled out 41 days after the COVID-19 sequence was published – means that LNPs stand to revolutionise the drug development process. Sunscreen is here to boost your formulation screening and accelerate future discoveries with 96 automated experiments in under 6 hours.

The proved efficacy and outstanding safety profile of LNP technology have led to an explosion of products in development, with over 18 products currently in clinical trials. However, LNPs are anything but a simple platform technology. Each formulation must carefully select an ionisable/cationic lipid for RNA binding and delivery, a structural lipid, cholesterol derivative and a pegylated lipid, and the relative amounts of each. Newer developments are increasing the ranges of materials used and increasing the number and complexity of components, with both passive and active targeting giving the option of targeting specific organs and cell types.

The mixing of the lipid components, commonly in ethanol, and nucleic acids, in aqueous buffer, is also a critical step – the total flow rate (TFR) of materials, the ratio of materials (flow rate ratio, FRR) and the type of flow (laminar/chaotic/turbulent) affects the loading efficiency, particle structure, and particle size.

All these factors change the pKa, membrane fluidity and stability of the LNPs, adjusting how they interact with the complex biomolecular environment found in-vivo, which can affect biodistribution, expression and efficacy of the NA.

The resulting design space for LNPs is huge – testing all these factors can rapidly lead to 100s or 1000s of experiments needing to be run. When many of the microfluidic systems currently on the market are used for LNP screening, the process requires the user to individually load reagents, a time consuming and potentially wasteful process, and rely on disposable, high-cost, microfluidic mixing chips, or rely on pipette mixing, which is uncontrolled, hard to reproduce and not scalable.

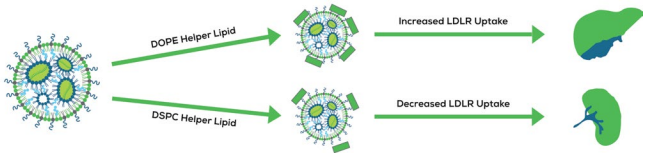

Figure 1: LNP formulation effect on biodistribution in-vivo.

Sunscreen is the first product on the market to combine automated, high-throughput testing, with the essential precision, scalability and reproducibility of microfluidics, allowing for up to 96 separate experiments to be run in one experimental set, with no intervention from the operator. Additionally, the Sunnies (microfluidic chips) used are composed of ultra-smooth, inert glass; with the automated wash step built into the experimental process, these Sunnies are indefinitely reusable.

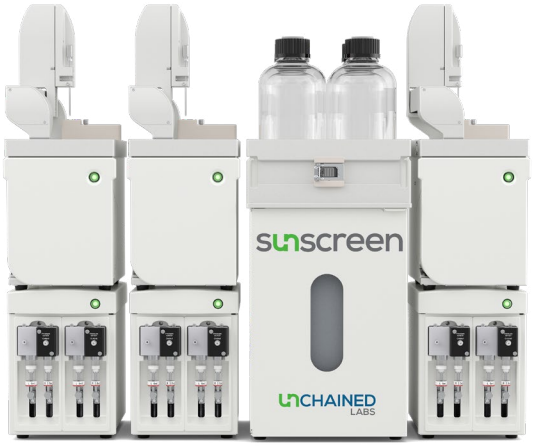

Figure 2: Sunscreen.

Sunscreen can use a range of Sunnies to affect different mixing speeds and types, giving full flexibility in how the device is run. The standard Sunny 490 Trident T utilizes hydrodynamic flow focussing (HFF), compressing a central, ethanol/lipid flow with aqueous buffer to reduce the time taken for diffusive mixing. The flows are very controlled and laminar, allowing for exquisite control of particle properties. Alternatively, the Sunny 50 Micromixer intentionally induces chaotic flow, leading to reduced mixing times at lower flow rates (and therefore smaller particle sizes).

All Sunnies are compatible with both Sunscreen and Sunshine (our process development and scale up system) - which are designed to provide a seamless transition between the stages of your LNP development pipeline.

Methods

Stock solutions in ethanol of 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP), Dimethyldioctadecylammonium Bromide (DDAB), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), ,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N- methoxypolyethylene glycol-2000 (DSPE-PEG2000), 1,2-Dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) (Avanti Polar Lipids) and cholesterol (Chol) (Sigma-Aldrich), were made up. The lipids were mixed in various ratios, with total concentrations of 6 mg/mL.

PolyA (Cytiva) was dissolved in 50 mM citrate buffer, pH 6.00, to give a Nitrogen/Phosphorus (N/P) ratio of 8 for each formulation.

Unless otherwise specified, the TFR was set to 9 mL/min, with a FRR of 3:1 (Aqueuous:Organic). 50 mM citrate buffer, pH 6.00 was used as a driver fluid for the aqueous phase and ethanol was used as a driver fluid for the organic phase. Total sample size was 1000 µL, with a 100 µL head and tail cut, giving a total collected volume for each sample of 800 µL.

All particle size and PDI measurements were carried out via DLS measurements. All samples were diluted by 10x in 1x PBS. Samples were measured in triplicate (error in tables and graphs is the standard deviation of the triplicate measurements unless otherwise specified).

EE% was carried out by Invitrogen Quant-it Ribogreen assay following manufacturer's instructions. Samples were measured intact and after lysing with 1% Triton X, and Fluorescence was measured by FLUOstar Omega plate reader.

Results

Sunscreen's total of 96 experiments is drawn from the fact that it runs from a standard well-plate format. Robotic arms automate the aspiration of the re-agents from the input well plates – as little as 105 µL per input – after which the samples are held in sample loops.

The system is then primed with driving fluid to establish steady-state flow and pressure in the system, driven by precision syringe pumps. The reagents are then released from the sample loops into the main system, where the calibrated tubing and valve timings allow the two reagent packets to meet on the Sunny simultaneously, giving excellent mixing control and fidelity. The particles formed are then held in a final sample loop before being dispensed into the collection well plate.

All the flow paths, including the Sunny and fluid handling needles are automatically cleaned when not in use during each experiment, eliminating sample cross contamination.

Collected sample sizes range from 200 µL up to 2 mL, with total flow rates from 100 µL/min up to 30 mL/min and flow rate ratios of between 1:1 and 5:1, giving a huge range of flexibility to the screening process. Each sample has a completely customizable head and tail cut to ensure the centre of the sample packet, with the highest mixing quality and consistency, is collected – or the entire sample packet. Anti-dispersion technology (ADT) is used to protect the sample packets form the driving fluids and preserve high concentrations of reagents.

Sunscreen was run through a series of tests to explore its performance. The first of these looks at reproducibility across 96 experiments, essential for

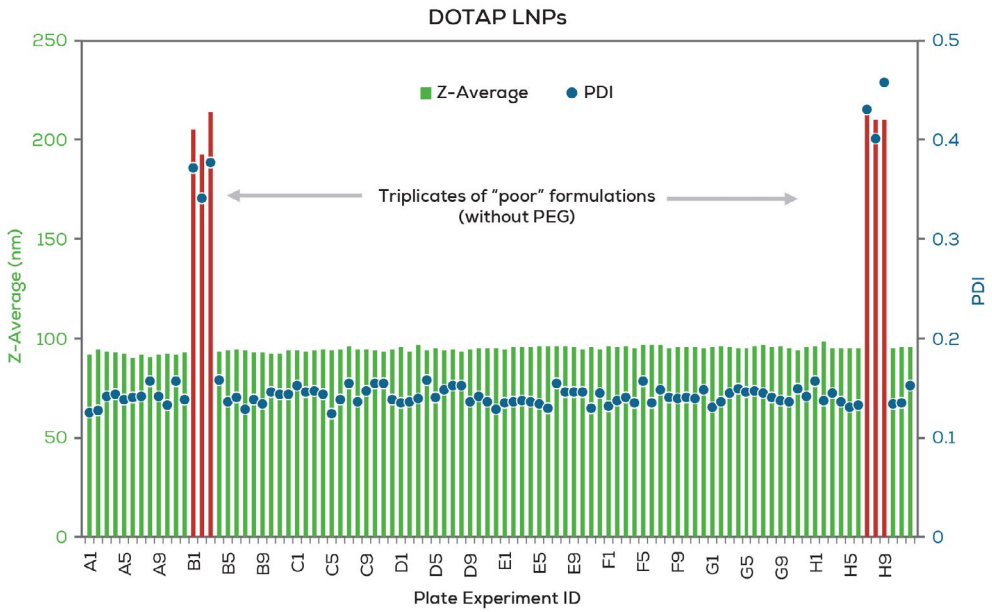

Figure 3: Reproducibility and Recoverability of Sunscreen running a DOTAP cLNP formulation over 96 experiments.

Parameter	Description
Z-Average	94.59 nm ± 0.71 nm
PDI	0.142 ± 0.01
RSD	0.76%
Total Time	05 hours 30 minutes

Table 1: Overall results of reproducibility testing. ± is given as the standard deviation of all results combined. Relative Standard Deviation (RSD) is given for Z-Average.

establishing that differences in particle properties are solely down to the formulations and conditions selected and not through random variation in the process as is often seen with, for example, pipette mixing.

The second, the formulation screen, uses a fixed set of conditions – now established to give consistent results in a stable formulation – but varies the formulation components to assess how different formulations affect particle properties, free of other variables and with low random error.

Finally, a single formulation was run with a screen of the TFR, from 5 to 12 mL/min to assess the effect on particle size and PDI afforded by adjusting flow conditions.

Reproducibility

To establish the baseline performance and reproducibility of Sunscreen, a 96 experiment series was run, with 90 repeats of a single formulation (DOTAP:DSPC:Chol:DMG-PEG2000, 40:10:37.5:2.5 molar ratio) and 6 'poor' formulations, with no PEGylated lipid, included to show system recovery after catastrophic aggregation of a sample.

Reproducibility across the 90 replicates was excellent, with an average particle size of 94.59±0.71, and an overall relative standard deviation (RSD) of less than 1% (Figure 3). PDIs were also very consistent, at 0.142±0.01 overall. Samples without PEG were highly aggregated and polydisperse but had no effect on the following samples produced, indicating no cross contamination between samples.

Formulation screen

A large screen of 32 cationic LNP (cLNP) formulations was carried out in triplicate, investigating the effects of cationic lipid identity (DOTAP vs. DDAB), structural lipid identity (DSPC vs DOPE), PEG lipid identity (DSPE-PEG2000 vs DMG-PEG2000) and PEG lipid molar % (0.5, 2.5, 5 and 10 mol%). All experiments were run from the same 96-well plate, in a single experimental run lasting 5.5 hours without any operator intervention or interruption. The particles were analyzed for Z-average, PDI, and encapsulation efficiency (EE%).

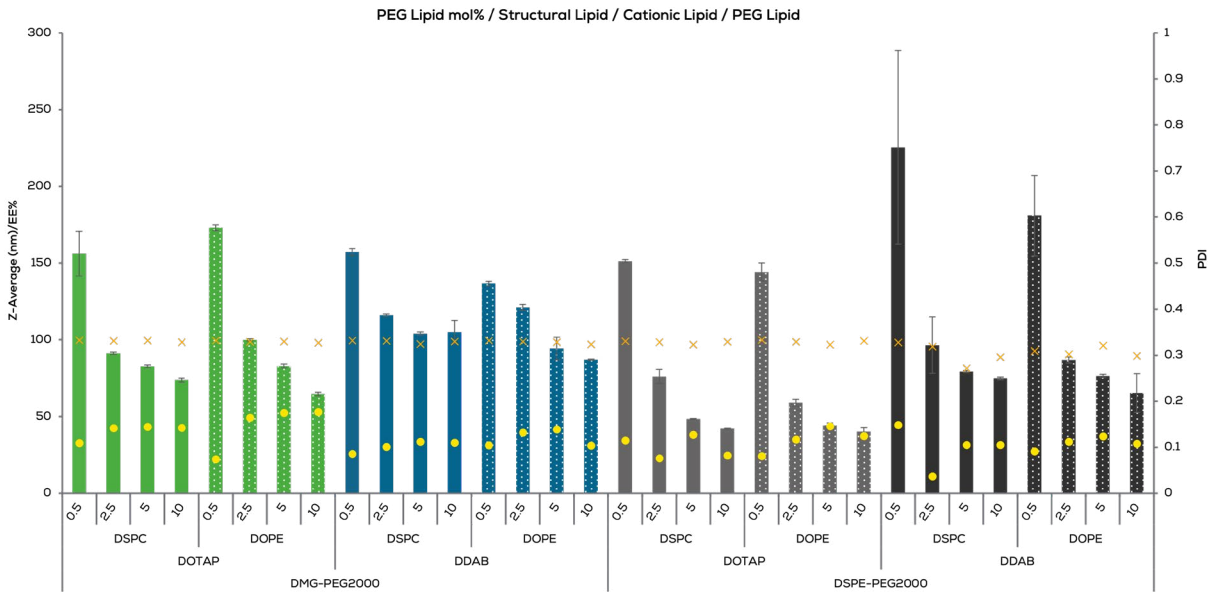

Figure 4: Results of cLNP formulation screen. Z-Average and PDIs are the average of each result (n=3), with the error bars given by the standard deviation.

The resulting wall of data is presented above, in formulation groups (Figure 4). This single experimental set, run over 5.5 hours, generated more data than a single graph can accurately convey. Some obvious trends are quickly identified – as PEG level increases, particle size drops (Figure 5). Less expected is the higher responsiveness of particle size to DSPE-PEG2000 compared to DMG-PEG2000, and the lower response to DMG-PEG2000 level on DDAB cLNP particle sizes.

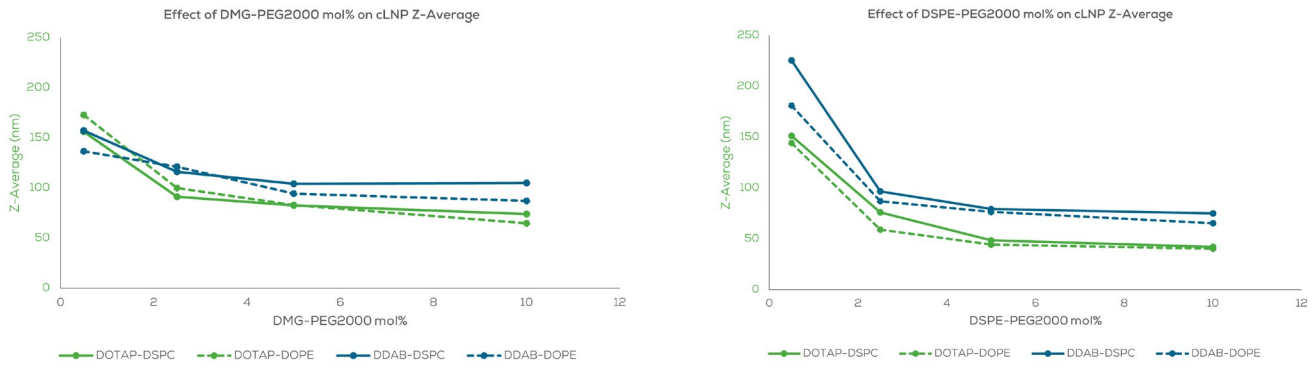

Figure 5: Effect of DMG-PEG2000 lipid mol% on particle size of cLNPs B – effect of DSPE-PEG2000 on particle size of cLNPs.

Excluding formulations with 0.5% PEG lipid, which tended to have low PDIs but high standard deviations in size measurement, indicating some level of unpredictability/instability, several other relationships were identified, including the effect of the structural lipid on PDI which was higher for DOPE in each case, and the lower encapsulation efficiency of DDAB/DSPE-PEG2000 formulations (Figure 6).

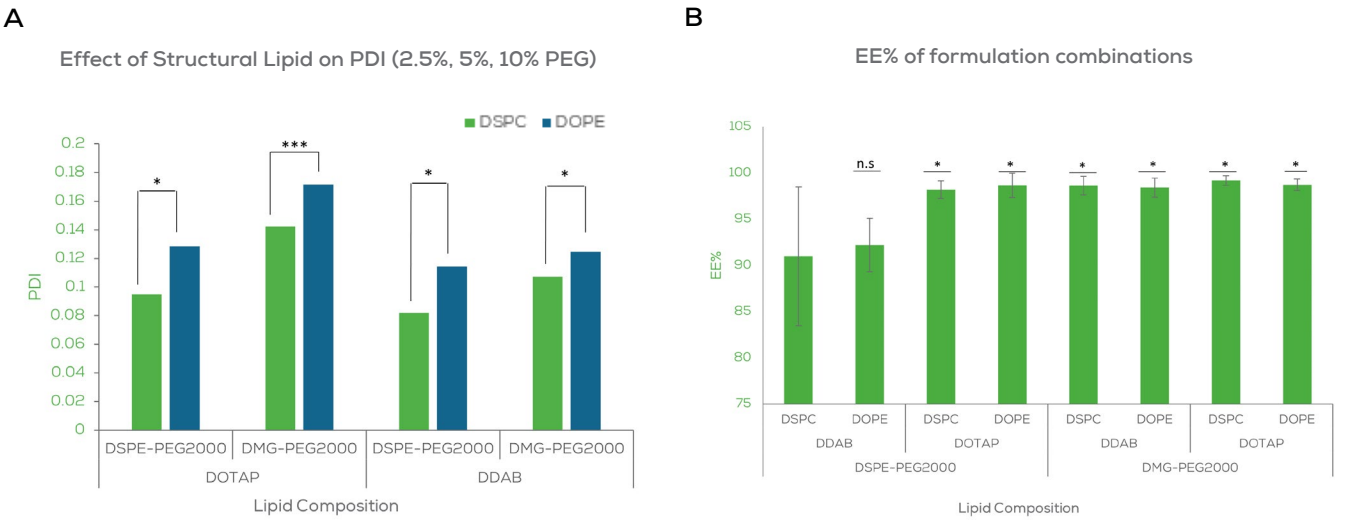

Figure 6: A- Effect of structural lipid on cLNP PDI. Significance level calculated via 2 tailed Students T-Test. B – encapsulation efficiency of cLNP formulations. Significance level calculated via Dunnet's Test against DDAB/DSPC/Chol/DSPE-PEG2000 formulations.

Flow rate screen

The last test intentionally varied flow conditions to adjust particle size with a set formulation (DOTAP:DSPC:Chol:DMG-PEG2000, 40:10:37.5:2.5 molar ratio). Particles were formed at 5, 7, 9 and 12 mL/min. As expected, the particle size drops as the flow rate increases, as the focusing of the central ethanol flow occurs faster and mixing times are decreased.

This is clearly evident in the data, with a statistically significant difference in particle size between each set of flow rates, demonstrating the excellent control and reproducibility of LNPs produced on Sunscreen (Figure 7).

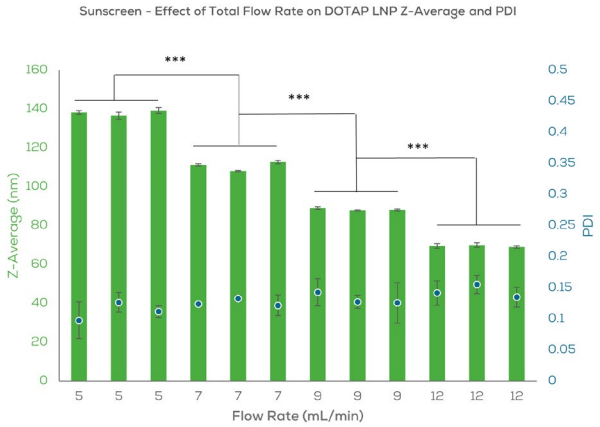

Figure 7: Effect of TFR on DOTAP cLNP size and PDI. Error bars represent the SD of 3 measurements. Significance level calculated via 2 tailed Students T-Test. Significance levels are indicated by * p = 0.05, ** p = 0.01.

TFR (mL/min)	Z-Average (nm)	PDI
5	137.9 ±1.11	0.11 ±0.01
7	110.6 ±1.96	0.13 ±0.00
9	88.24 ±0.52	0.13 ±0.00
12	69.48 ±0.38	0.14 ±0.00

Table 2: Effect of TFR on DOTAP cLNP average size and PDI. Error is given by the SD (n=3).

Conclusion

Sunscreen's winning combination of automation and microfluidics mean it is an essential tool in any LNP development laboratory. When paired up with a Stunner for 96-well plate format particle size analysis, a full plate of 96 formulations can be synthesized and characterized within a working day – all using just a single Sunny.

The consistency provided by precision microfluidic mixing guarantees high quality, reproducible particles, and the automation allows integration into complex, parallel work flows to supercharge your throughput. Whether you are screening cargos, novel lipids, formulation compostions, or production parameters, Sunscreen leaves everything else in the shade!

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