Process optimization and pre-clinical production of lipid nanoparticles using Sunshine

Introduction

In the field of drug delivery and medicine development, nanoparticles including lipid nanoparticles (LNPs), liposomes, and polymer nanoparticles are at the forefront of a rapidly expanding class of drug delivery products termed 'nanomedicines'. They can deliver active pharmaceutical ingredients (APIs) to specific action sites while protecting the API from premature enzymatic degradation, preventing immune system overstimulation, and improving intracellular penetration and retention time. For researchers to accelerate their nanomedicine development, particle synthesis solutions are needed that are consistent, low cost, and efficient with materials. Unchained Labs provides nanoparticle synthesis solutions underpinned by microfluidics and automation, offering systems at every stage of the nanomedicine development journey; from screening and early-stage R&D, through process optimization and ultimately into manufacturing and commercialization of drug products. This application note presents example use cases for Sunshine, which offers both automated process optimization and continuous manufacturing capabilities in a single system.

After the success of COVID-19 mRNA vaccines (and other drugs before that such as the hATTR amyloidosis treatment Onpattro), LNPs have become widespread, leading to the rapid development of mRNA-LNPs for further vaccines, genetic therapies, and the treatment of many other diseases. LNPs are derived from well-established liposome technology, with the addition of ionizable lipids for encapsulation of negatively charged genetic materials, such as siRNA, mRNA, miRNA, and DNA, via electrostatic interactions. LNPs are set to transform the future of medicine, but there is a lot of research that still needs to be done for LNPs to function as optimal carriers in all their varied roles.

It is well-known that the nanoparticle assembly process is complex and challenging to control, and that optimizing the conditions for producing nanoparticles can be laborious and resource intensive. For instance, ethanol injection is commonly employed to synthesize mRNA-LNPs, in which lipids suspended in ethanol are injected drop-by-drop into aqueous mRNA solutions from a syringe. This can take a long time and can lead to inconsistent results from run to run. Other processes such as impinging jet mixing (where two fluid streams collide at high pressure in a jet mixing chamber) apply significant shear force to the particles, which can accelerate degradation and potentially cause particles to rupture. These techniques (along with traditional bulk methods such as thin film rehydration) can be challenging to scale, and struggle to generate highly homogeneous nanoparticles in terms of size, morphology, lipid composition, and mRNA payload quantity. This can result in significant variation in the in vivo behavior of mRNA-LNPs.

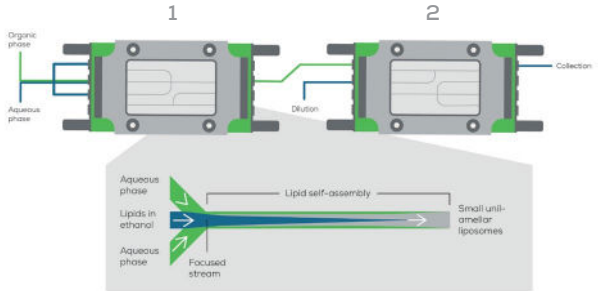

Figure 1: An example Sunny (microfluidic chip) setup. Sunny 1 shows the mixing of lipids and API (such as mRNA) by hydrodynamic flow focusing to assemble LNPs. Sunny 2 shows optional in-line dilution, which can be used to adjust pH in-line, and reduce solvent concentration in collected samples.

In contrast, microfluidic technology is based on hydrodynamic flow focusing and rapid mixing at the junction of a Sunny from separate channels to form nanoparticles using stable and predictable laminar flow (Figure 1). Consequently, microfluidic technology maintains consistent conditions for nanoparticle self-assembly and offers greater process control. This approach makes it easier to determine the formulation that optimizes the critical quality attributes of particles.

Process optimization with Sunshine

Most nanomedicine development processes start with screening a large number of formulations in order to seek "hits", or lead candidates for further development. For this process, Unchained Labs offers Sunscreen, a high-throughput automated system for screening low volumes of various formulations in a 96-well plate format. Sunscreen could be used for screening the ionizable lipid component or lipid and cargo, ratios, and so on, to find a successful formulation. After such a formulation has been selected using Sunscreen, scientists require a rapid, low-cost, and preferably automated particle production platform to optimize the size, shape, and structure of these nanoparticles ahead of final production/commercialization. With Sunshine (Figure 2), researchers can automatically optimize process parameters and generate test samples.

Figure 2: Sunshine

Sunshine may be used initially in "protocol mode", where the system can sequentially execute a table of automated and low-volume experiments from a fixed pair of injected reagents, using as little reagent as 320 µL per input per experiment. In this mode, enough reagent for several experiments is injected into sample loops within the system. As the protocol runs, aliquots of these reagents are released from sample loops controlled by careful valve timings and are combined on a Sunny of your choice (Unchained Labs reusable Sunnies). To move these aliquots of reagent around the system, driving fluids are used (typically a solvent and buffer like ethanol and PBS), which push the reagent from the loops, through the Sunny and to the Automated Collector.

Once the reagent is collected, the system completes a wash cycle, and then the next experiment starts with a new set of flow parameters. This process repeats until all the planned experiments are completed, and samples can be collected for downstream purification and analysis.

Using the Sunny Suite Software accompanying the system, users can optimize the flow rate ratio (FRR), total flow rate (TFR), NP precursor volume, sample collection volume and allow optional in-line dilution to synthesize controlled, reproducible, and monodispersed nanoparticles. For example, a series of 10 low-volume experiments with a fixed pair of reagents testing at various conditions of FRR, TFR, and dilution ratio could be executed automatically in 15 minutes in the standard protocol mode as shown by the system pressure data in Figure 3.

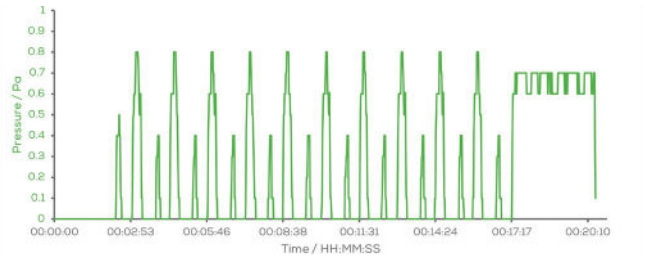

Figure 3: Pressure profile of Sunshine during a protocol running 10 repeat experiments, showing consistent pressure during the system washing and experiment steps.

Once a desired set of experimental conditions has been identified, the user can switch to continuous mode to create liters of nanoparticles for pre-clinical trials and extensive characterization. Unique in this dual capability, Sunshine bridges the gap between formulation screening and commercial scale manufacturing in a single system solution.

Herein, this application note describes how Sunshine can be used to optimize typical formulations with a view to identifying suitable process conditions ahead of continuous manufacturing. As a starting point for this study, an example LNP formulation will be used to represent a successful "hit" formulation which may have been identified using Sunscreen. In this case, we will use SM-102 as the ionizable lipid, with Luciferase mRNA with N/P ratio of 6 (see Table 1).

Parameter	Description
Aqueous Phase (A)	Luciferase mRNA in acetate buffer (50 mM, pH 4)
Organic Phase (O)	SM-102: DSPC: Cholesterol: DMG-PEG 2000 (50:10:38.5:1.5% molar) in ethanol
N/P Ratio	6
Dilution (D)	No dilution
Flow Rate Ratio (FRR)	Variable (A:O)
Total Flow Rate (TFR)	5 mL/min unless otherwise stated

Table 1: Parameters for the synthesis of mRNA-LNPs utilizing Sunshine.

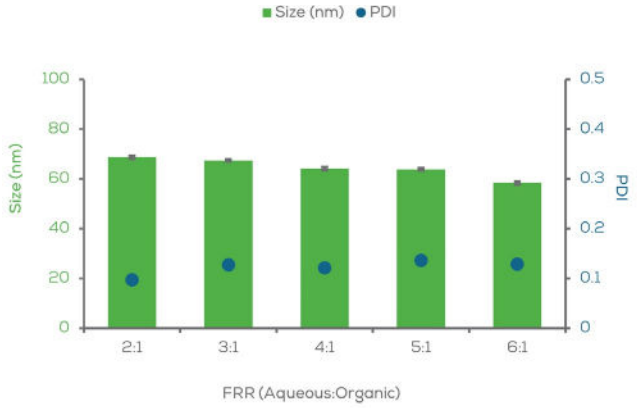

Figure 4: mRNA-LNPs produced by various FFR (aqueous to organic phase) at a fixed TFR of 5 mL/min with no dilution. LNPs ranged from 58 nm to 68 nm post-dialysis, with an average PDI of 0.12. Particle size distribution and polydispersity index (PDI) were determined by dynamic light scattering (DLS). The error bars represent standard deviation of the mean n=3.

Using Sunshine, both flow rate ratio (FRR) and total flow rate (TFR) were investigated to identify an optimal process for forming particles with this formulation. The target outcome of such work is to identify an optimized set of process conditions with which the target formulation might be efficiently manufactured in continuous flow. In this case, example criteria for an ideal particle may be a particle size around 60 nm, with a PDI <0.1, and an EE% of >90%. Using Sunshine, the nanoparticle synthesis process can be optimized towards these key criteria.

Optimizing Flow Rate Ratio

Using a Sunny 190 XT, a series of experiments with various FRRs were examined to show how they affect the size and polydispersity of the LNPs. Figure 4 illustrates that the size of mRNA-LNPs was controllably altered from 68 nm to 58 nm post-dialysis by varying the flow rate ratio between the aqueous and organic phases from 2:1 to 6:1 while maintaining the constant total TFR of 5 mL/min. Alongside that, these experiments gave a consistently low PDI, with an average of 0.12 across all flow rate ratios.

Using the RiboGreen assay, the encapsulation efficiency (EE%) of mRNA was evaluated. While the TFR was maintained at 5 mL/min and the N/P ratio was kept constant at 6, the data in Figure 5 shows the encapsulation efficiency (EE%) was consistently above 90%, and reached 96% for an FFR of 6:1 (aqueous to organic phase).

Factoring in a desire to keep ethanol concentration below 30% in the collected sample, and an example target size of 50–60 nm, a flow rate ratio of 3:1 was selected for further exploration based on the data shown in Figure 4 and Figure 5.

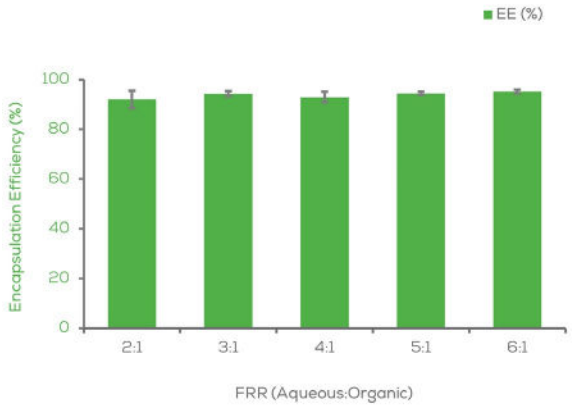

Figure 5: The encapsulation efficiency (EE%) of mRNA-LNPs was obtained by the RiboGreen assay. mRNA-LNPs were produced by Sunshine at a fixed TFR of 5 mL/min and various FRR (aqueous to organic phase) with no dilution. The N/P ratio was fixed at 6. The error bars represent standard deviation of the mean n=3.

Optimizing Total Flow Rate

The effect of TFR on mRNA-LNP particle size and EE% was examined (Figure 6). Increasing the TFR from 2.5 to 7.5 mL/m showed a reduction in the size of mRNA-LNPs, from 94 to 57 nm, with consistently decreasing PDI. There was a small increase in size when the TFR reached 10 mL/min. Overall, the best PDI and low particle size was achieved at a TFR of 7.5 mL/min, with a size of 57.7 nm and a PDI of 0.089, which also coincided with a high encapsulation efficiency of 94.7%, thus meeting all the initially desired criteria for a nanoparticle production process.

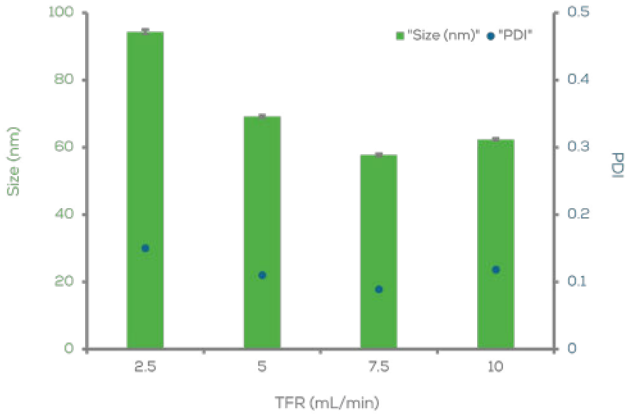

Figure 6: mRNA-LNPs produced by various TFR (aqueous to organic phase) at a fixed FFR of 3:1 (Aqueous: Organic) with no dilution. The hydrodynamic size ranged from 58 nm to 94 nm, with average PDI of 0.12. The error bars represent standard deviation of the mean (n=3).

Given the high encapsulation efficiency, low particle size and low PDI was observed at 7.5 mL/min (Figure 7). It is clear that in our example formulation, this combination of TFR, FRR, chip and formulation could be considered for larger scale continuous production for pre-clinical and ultimately clinical trials. In addition to optimizing the variables explored above, one may also consider the effect of changing the concentration of injected reagents, or the N/P ratio of the formulation selected for further optimization using Sunshine.

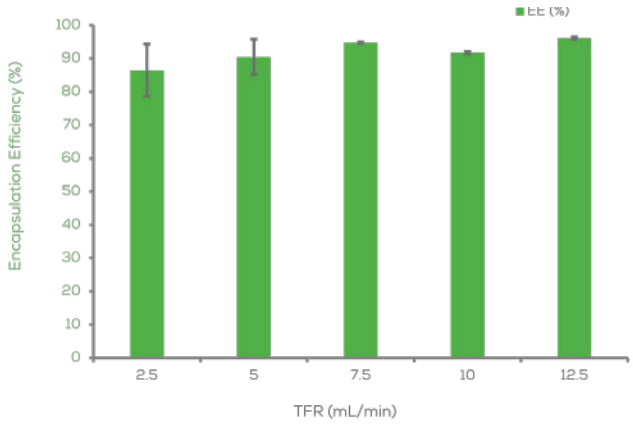

Figure 7: The encapsulation efficiency (EE%) of mRNA-LNPs was obtained by the RiboGreen assay. mRNA-LNPs were produced by Sunshine at a fixed FRR of 3:1 and various TFRs were investigated with no dilution. The N/P ratio was fixed at 6. The error bars represent standard deviation of the mean n=3.

Transitioning to continuous production

After optimization of a formulation using protocol mode in Sunshine, (producing a typical minimum of 1 mL of sample), users can then switch to a continuous mode of system operation, where Sunshine can produce up to tens of liters of sample per day (depending on the optimized flow conditions being used). To demonstrate the simple transition between protocol mode and continuous mode, a liposomal formulation was prepared using Sunshine in both operation modes at multiple flow rate ratios.

Parameter	Description
Aqueous Phase (A)	1 x PBS pH 7.4
Organic Phase (O)	Phospholipon 90G (1 mg/mL), DBAB (0.1 mg/mL) in ethanol
Dilution (D)	1 x PBS pH 7.4
Flow Rate Ratio (FRR)	2:1:2, 2:2:2, 2:3:2 (A:O:D)
Total Flow Rate (TFR)	3 mL/min

Table 2: Parameters for the synthesis of liposomes utilizing Sunshine.

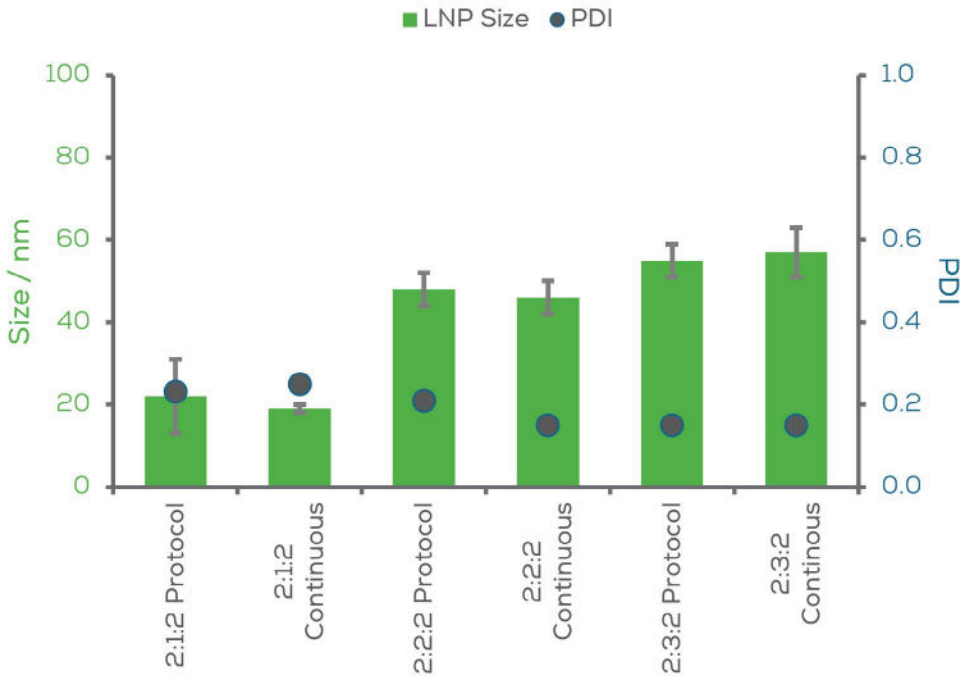

Figure 8: Liposome produced continuously at a TFR of 3 mL/min at varying FRR. Nanoparticles ranged from 19 nm to 57 nm, mean n=3. The error bars represent standard deviation of the mean. PDI remains consistent between runs-by-runs ranging from 0.15 to 0.2 with a standard deviation of 0.01.

Figure 8 shows that liposomes based on phospholipon 90G were produced using a range of FRRs (Aqueous:Organic:Dilution) in either protocol mode or continuous production mode, using a constant TFR of 3 mL/min. The nanoparticle sizes ranged between 19 to 57 nm and differed by less than 7 nm between protocol and continuous mode, and the PDI remained consistent ranging from 0.15 to 0.2, showing an expected size increase as the proportion of organic to aqueous was increased. We can see from a process optimization perspective that lower PDIs were achieved at a flow rate ratio of 2:3:2.

Summary

In developing any nanoparticle-based drug delivery product, it is key to identify both the correct formulation and the best process conditions in order to successfully manufacture nanomedicines with the correct performance characteristics. Since particle performance is underpinned by attributes such as the size, shape and structure of the nanoparticle, the synthesis process and mixing conditions are critical to ensuring highly effective and safe nanomedicines.

Sunshine is ideally suited to optimizing the formation process of lead candidate formulations, whilst enabling a seamless transition into continuous manufacturing ahead of clinical trials. The data presented here show the principles of optimizing a selected formulation to target required key performance indicators such as size, PDI and encapsulation efficiency.

The data shown focuses on the impact of tuning total flow rate and flow rate ratio using Sunshine to rapidly complete consistent sets of triplicate data for all conditions tested, in a hands-free automated manner. In addition to these parameters, users can investigate reagent concentrations to achieve a higher yield process, tune N/P ratio to deliver more mRNA per particle, as well as test Unchained Labs' range of Sunnies to assess the ideal microfluidic mixing geometry.

Once a suitable particle formation process has been identified, the same system can be set up to execute a continuous production run with identical process conditions in a matter of minutes, allowing liters of nanoparticles to be produced for pre-clinical trials and more extensive downstream characterization.

Particles produced this way are consistent with those produced using the system's protocol mode, making the transition to continuous product manufacturing simpler than ever, with minimal process validation required.

Materials and methods

For the formation of mRNA-LNPs: 1,2-distearoyl-sn-glycero-3-phosphochloline (DSPC) (Avanti, UK) 1,2-dimyristoyl-rac-glycero-3-methoxypolyethyleneglycol-2000 (DMG PEG-2000) (Avanti, UK), cholesterol (Sigma Aldrich, UK), and SM-102 (BroadPharm, San Diego, US) were dissolved in ethanol (reagent grade 99%, Sigma Aldrich, UK). The solution of 10mM lipid formulation contained SM-102, DSPC, cholesterol and DMG-PEG-2000 in ethanol with the relative molar ratio of 50:10:38.5:1.5%. For the aqueous phase, 89.44 µg/mL of Luciferase mRNA in 50 mM of acetate buffer at pH 4 (nuclease-free) was employed.

To produce liposomal particles for continuous production comparisons, phospholipon and DDAB, phospholipon 90G (Lipoid, Switzerland) and dimethyl-dioctadecyl-ammonium bromide (DDAB) (Fischer Scientific, UK) were dissolved in ethanol (reagent grade 99%, Sigma Aldrich, UK) at 1 mg/mL and 0.1 mg/mL respectively. For the aqueous and dilution phase, phosphate buffer saline (PBS) x 1 at pH 7.4 was used. All prepared solutions were filtered with a 0.2 µm filter prior to use. The organic and aqueous were mixed automatically using the Sunshine protocol.

The organic and aqueous phase were mixed automatically using the Sunshine protocol on a Sunny 190 XT. The lipid nanoparticle samples were transferred to dialysis kits (Pur-A-lyzer™ Midi Dialysis Kit, Sigma, UK) and placed in a beaker containing 1 L of PBS 1x pH 7.4. The dialysis was run for 3 h to remove the ethanol and exchange the buffer medium. The post-dialysis nanoparticle samples were stored in the fridge for further investigation.

Particle size distribution and polydispersity index (PDI) were determined by DLS. All samples were generated in triplicate, the mean and standard deviation (SD) were reported.

The encapsulation efficiency (EE%) of mRNA-LNPs was quantified by RiboGreen assay - using Triton X-100 to break open the LNPs to analyze concentration of total mRNA in the sample and comparing this value to the concentration of free mRNA, determined in the absence of Triton. The fluorescence intensity of RiboGreen was measured on a Microplate reader (FLUROstar, BMG, Germany).

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