Particle Works

Controlled synthesis of lipid nanoparticles using the Automated Nanoparticle System

Application Note

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As part of Blacktrace, and under Dolomite Microfluidics brand, we have been working in the particles market for 20 years. Now with a brand dedicated to particles, Particle Works combines the expertise and trust our customers desire, with a focussed passion for what we do.

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1. Abstract

Lipid nanoparticles (LNPs) are powerful carriers for the delivery of biotherapeutics and active pharmaceutical ingredients (APIs). They are attractive due to their size and biocompatibility, which are important factors when determining the efficacy of an encapsulated API. Particle Works' leading microfluidic technology offers a unique automated nanoparticle generation platform for the production of homogeneous, controllable, and reproducible LNPs. The ability to control LNP size from as small as 20 nm allows for a wide range of end user applications. Key features of the Automated Nanoparticle (ANP) System include running protocols with importable experiment tables to generate formulation libraries, and the ability to run in continuous mode to generate large samples once the synthesis is optimised. The ANP System's robustness was demonstrated by comparing the data collected in 'protocol' library generation mode to continuous mode, where the LNP size remained consistent with a polydispersity index (PDI) of < 0.2, and values as low as 0.06 achievable after optimising the synthesis with the ANP System. By controlling the flow rate ratio (FRR), and total flow rate (TFR), facile control over LNP size is achieved. These key findings can be tailored to any formulation to expedite the development of LNP formulations for applications from gene therapy to vaccine development and drug delivery.

2. Introduction

Conventional methods for making LNPs such thin film hydration, sonication, and extrusion are laborious, and can involve large batch volumes. They lack the ability to easily control size, encapsulation efficiency (EE%) and PDI, all of which are important factors in the efficacy of liposomal APIs. The ANP System combines these features (low sample volumes, reproducibility, control over size and PDI) with microfluidic technology, employing hydrodynamic flow focussing and rapid mixing techniques to nucleate and assemble LNPs. In principle, lipids dissolved in a solvent (or a mixture of solvents) are mixed with an anti-solvent (aqueous phase), and the subsequent shift in polarity causes self-assembly of lipids into unilamellar vesicles. The ANP System combines this technology with experiment automation to allow the generation of sample libraries. Protocols can be defined off-line in a spreadsheet and imported directly into the Flow Control Centre (FCC) Software. Once set up, only a single manual step (loading nanoparticle (NP) precursor) is required at the start of the protocol, allowing walk-away operation and multiple sample generation from a single precursor loading.

Here, we demonstrate by using the FCC software accompanying the system we can control FRR, TFR, NP precursor volume, sample collection volume and allow optional in-line dilution with head and tail cuts to ensure the collected sample is representative of material produced in continuous mode. Automating fluid handling and collection ensures reproducibility from batch to batch, consistency between formulations, and prevents the variability associated with manual operation. The glass microfluidic chips are robust and reusable, reducing consumable costs by efficiently utilising expensive precursor reagents. The chips can withstand rigorous cleaning with various chemicals and can also be autoclaved. Between each experiment, the system automatically runs a wash cycle to clean the fluidic pathway and avoid cross-contamination between samples. The user also has the option to perform additional wash cycles manually in between protocol runs.

In addition to this, we demonstrate that once a formulation has been optimised in protocol mode, the system can be controlled in manual mode to continuously produce a larger batch of the formulation. The quantity collected is user defined and can be run continuously for long periods of time without compromising size and PDI.

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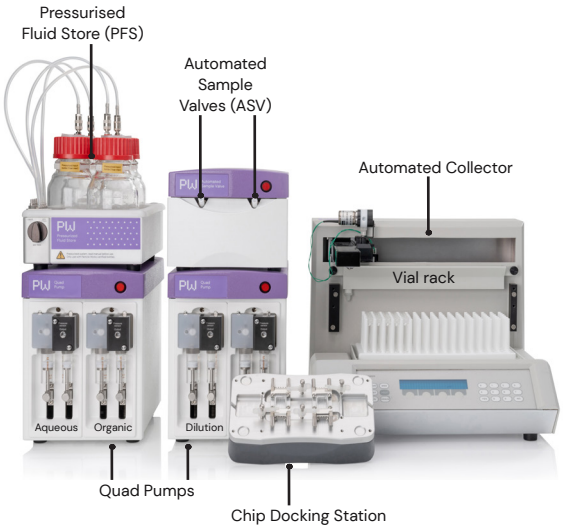

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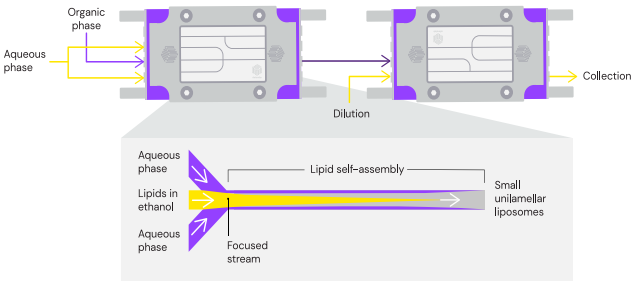


Figure 1. *a) ANP System setup. b) Microfluidic chip 1- entry of lipids in solvent and aqueous phase, hydrodynamic flow focussing, and subsequent mixing assembles LNPs, optional in-line dilution in microfluidic chip 2 further facilitates LNP growth c) depiction of LNP assembly in microfluidic chip 1.*

3. Results

The key parameters investigated to tune the LNPs' size was the FRR between the organic and aqueous phase, and the TFR. Further to this, a comparison between the protocol and continuous mode was made to demonstrate the transferability of the process parameters for bulk production of LNPs.

The FRR was investigated by changing the organic phase in relation to the aqueous phase. Data shows that by changing FRR, one can change the LNP size in a consistent, reproducible way (Figure 2). PDI of 0.06 was achieved for FRR of 3:1:1, whilst overall the PDI was < 0.2 during parameter testing and optimisation. Repeatable minimised PDI could be achieved for experiments.

TFR was also investigated to observe its influence on the LNP size, the data for the formulation investigated showed no significant change in LNP size with increasing TFR (figure 3 and 4). In the higher TFR (>10 ml/min) a decrease of 10 nm was observed (Figure 3).

To further examine the robustness of the system, the same formulation was then run in continuous mode. The results between protocol and continuous mode are comparable, demonstrating that once a formulation has been optimised it can reliably be bulk produced in continuous mode at various TFR (Figures 5 and 6).

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

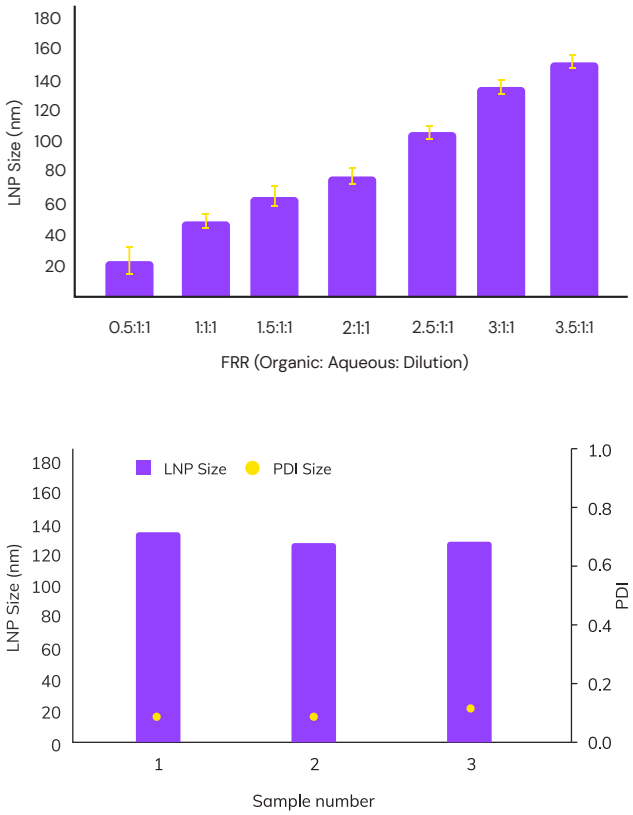


Figure 2. *i) Lipid nanoparticles produced by varying lipid to aqueous flow rate ratio (FRR) at a total flow rate (TFR) of 3 ml/min. LNP size ranged from 20 nm to 150 nm, mean (n=3). The error bars represent standard deviation of the mean. ii) LNPs produced at FRR of 3:1:1 at a TFR of 3 ml/min. Three samples collected with an average LNP size of 134 nm and PDI below 0.1. Particle size distribution and polydispersity index (PDI) were determined by Malvern dynamic light scattering (DLS) appendix figure 7. The error bars represent standard deviation of the mean.*

Organic Phase (O)	Phospholipon 90G (1 mg/ml), DDAB (0.1 mg/ml) in ethanol
Aqueous Phase (A)	1 x PBS pH 7.4
Dilution (D)	1 x PBS pH 7.4
Flow Rate Ratio (FRR)	2:1:1 (O:A:D)
Total Flow Rate (TFR)	Variable

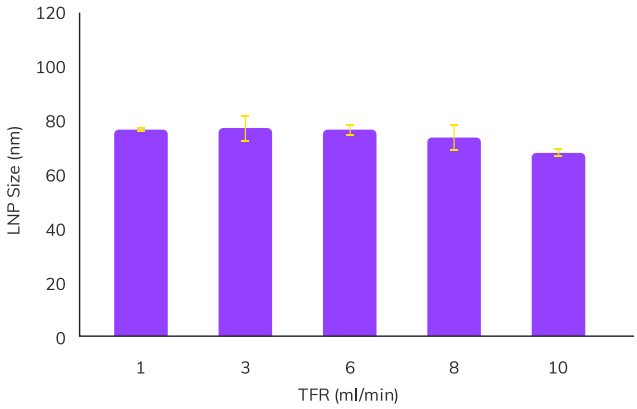


Figure 3. *Lipid nanoparticles produced by varying total flow rate (TFR) at a fixed 2:1:1 (organic: aqueous: dilution) flow rate ratio FRR. LNPs ranged from 66 nm to 78 nm, average PDI of 0.17 with 0.01 standard deviation. Mean (n=3) particle size distribution and polydispersity index (PDI) were determined by Malvern dynamic light scattering (DLS). The error bars represent standard deviation of the mean.*

Organic Phase (O)	Phospholipon 90G (1 mg/ml), DDAB (0.1 mg/ml) in ethanol
Aqueous Phase (A)	1 x PBS pH 7.4
Dilution (D)	1 x PBS pH 7.4
Flow Rate Ratio (FRR)	0.5:1:1, 1:1:1, 1.5:1:1 (O:A:D)
Total Flow Rate (TFR)	3 ml/min

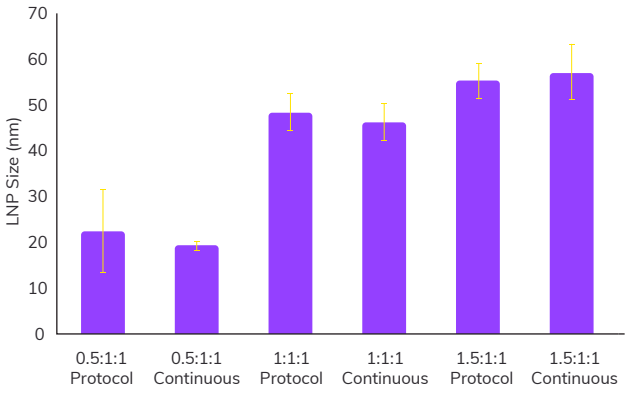


Figure 5. *Lipid nanoparticles produced continuously at a total flow rate (TFR) of 3 ml/min at varying flow rate ratios (FRR). LNPs ranged from 19 nm to 57 nm, PDI remained consistent between runs ranging from 0.15 to 0,2 with a standard deviation of 0.01. Mean (n=3) particle size distribution and polydispersity index (PDI) were determined by Malvern dynamic light scattering (DLS). The error bars represent standard deviation of the mean.*

Organic Phase (O)	Phospholipon 90G (1 mg/ml), DDAB (0.1 mg/ml) in ethanol
Aqueous Phase (A)	1 x PBS pH 7.4
Dilution (D)	1 x PBS pH 7.4
Flow Rate Ratio (FRR)	1.5:1:1 (O:A:D)
Total Flow Rate (TFR)	Variable

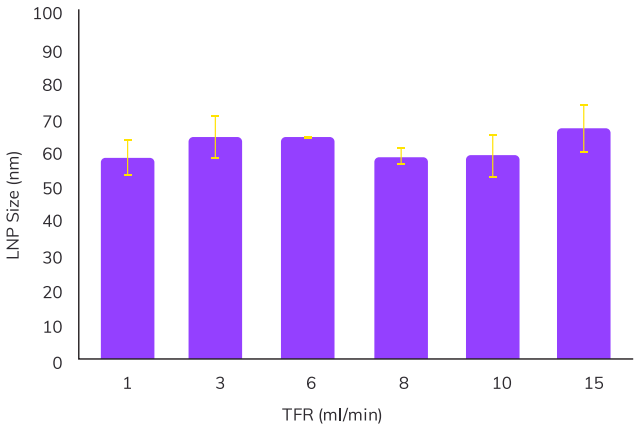


Figure 4. *Lipid nanoparticles produced by varying total flow rate (TFR) at a fixed 1.5:1:1 (organic; aqueous: dilution) flow rate ratio. LNPs ranged from 57 nm to 66 nm, average PDI of 0.19 with 0.01 standard deviation. Mean (n=3) particle size distribution and polydispersity index (PDI) were determined by Malvern dynamic light scattering.*

Organic Phase (O)	Phospholipon 90G (1 mg/ml), DDAB (0.1 mg/ml) in ethanol
Aqueous Phase (A)	1 x PBS pH 7.4
Dilution (D)	1 x PBS pH 7.4
Flow Rate Ratio (FRR)	0.5:1:1, 1:1:1, 1.5:1:1 (O:A:D)
Total Flow Rate (TFR)	10 ml/min

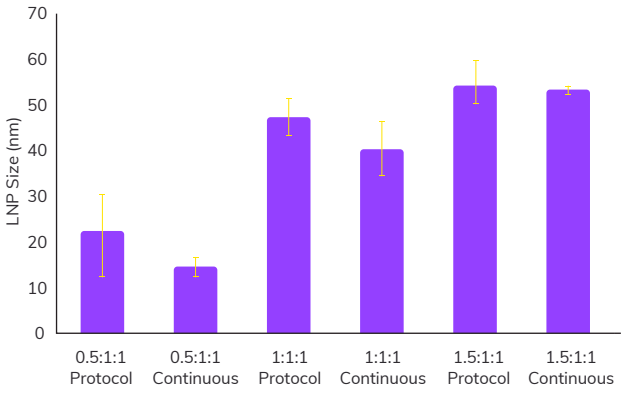


Figure 6. *Lipid nanoparticles produced continuously at a total flow rate (TFR) of 10 ml/min at varying flow rate ratio (FRR). LNPs range from 15 nm to 55 nm, PDI remained consistent between runs ranging from 0.15 to 0.2 with a standard deviation of 0.01. Mean (n=3) particle size distribution and polydispersity index (PDI) were determined by Malvern dynamic light scattering (DLS). The error bars represent standard deviation of the mean.*

4. Discussion

LNP homogeneity and size play a key role in determining the efficacy of the encapsulated API. Therefore, it is vital that the LNPs are not only controllable but also reproducible. The lipid formulation tested in this application note demonstrated that the size of LNP as well as the PDI can easily be controlled by manipulating the FRR between the organic and aqueous phase (Figure 2). Hydrodynamic flow focussing mixes the two phases entirely based on molecular diffusion in the direction of fluid flow streams, termed laminar flow mixing. The ANP System can be used with a range of chip types; mixing can also be achieved using our micromixer chips to allow mixing by chaotic advection.

As the ratio of organic to aqueous phase increases, the LNP size also increases. The increased size is caused by higher local concentrations of lipid during the liposome growth, which means more lipid is available to be integrated into the particle structure. Also, because the change in solubility of the lipid is less, there is a less aggressive particle nucleation, which potentially means that fewer particles form overall, and therefore each one grows larger given the amount of material present.

Similarly, TFR is also an important factor which can help reduce the overall LNP generation time. The TFR results (Figures 4 and 5) demonstrated that the LNP generation time can be shortened to increase throughput. In this regime, the TFR is not a limiting factor up until >10 ml/min at which point TFR starts to have more of an effect; usually FRR effects dominate at lower TFRs.

For other formulations and concentrations of lipids, TFR can influence the LNP size; this can happen because the time required for diffusion of between the organic and aqueous phase is shortened. As a result, the rate at which the lipids can self-assemble exceeds the rate of diffusion resulting in smaller LNPs.

To further examine scalability, protocol versus continuous production was investigated. The results (Figures 5 and 6) demonstrated that the LNP size was largely unaffected by the TFR and continuous production. This shows that LNP assembly remains identical to the protocol mode and the user can opt to bulk produce after library generation optimisation with no additional process development required.

The parameters investigated here demonstrate it is important to control the mixing technique and ratio to achieve a desired LNP size. Secondly, LNP homogeneity as measured by PDI (low as 0.06 for the formulation tested) is important for all end applications to improve efficacy. Reproducibility is paramount to any LNP generation process, and the results reported here show minimal variance between each repeat since the automated process conditions were identical, reliably producing LNPs with a low PDI. FCC controlled automation of process parameters and hardware ensures human error or change in operating conditions is minimised.

5. Conclusions

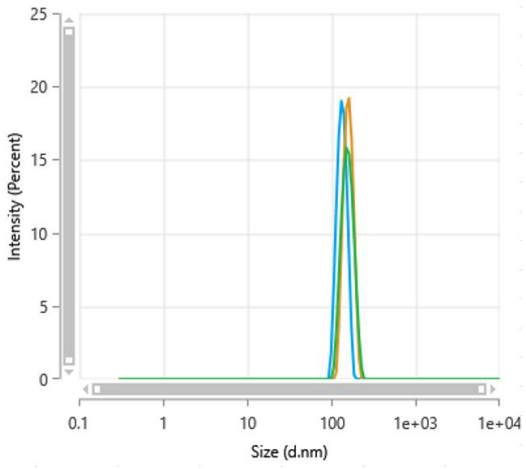
The ANP System automates the development of LNP formulations ensuring consistent results from run-to-run, offering its users savings in both time and reagent costs, due to its ability to work with small sample volumes. The experiments can be set up with ease in Excel and simply imported into the software, and the hardware offers precise control over experimental parameters such as TFR and FRR.

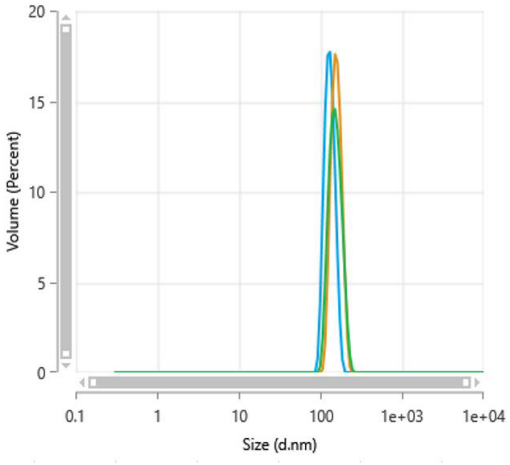
The ANP System can readily tune LNP size and uniformity, produce formulation libraries, and offer scalability to the user. The protocol automatically runs importable experiments with only a single manual step at the start of the protocol run. The ANP System demonstrates excellent reproducibility with consistent sample-to-sample generation of formulation libraries. This single platform allows the user to go from generating small volume LNP libraries to bulk production via a single step, with no need to re-validate the process. With no additional licencing or expensive consumable costs, the system components are reusable allowing user directed wash steps between experiments.

6. Materials and Methods

Lipids used in this study, Phospholipon 90G (Lipoid, Switzerland) and Dimethyldioctadecyl-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 Automated Nanoparticle (ANP) System protocol. The flow rate ratio of (organic: aqueous: dilution), 0.5:1:1, 1:1:1, 1.5:1:1, 2:1:1, 2.5:1:1, 3:1:1, 3.5:1:1 with the total flow rate of 1,3,6,8,10,15 ml/min were investigated. The collected samples were analysed immediately after collection using dynamic light scattering (Malvern Zetasizer, U.K.) accounting for the ethanol ratio in sample. All samples were generated and analysed in triplicate. The relative light intensity scattered by particles was reported as intensity number distribution and polydispersity index (PDI) with error bars representing standard deviation (SD).

7. Appendix





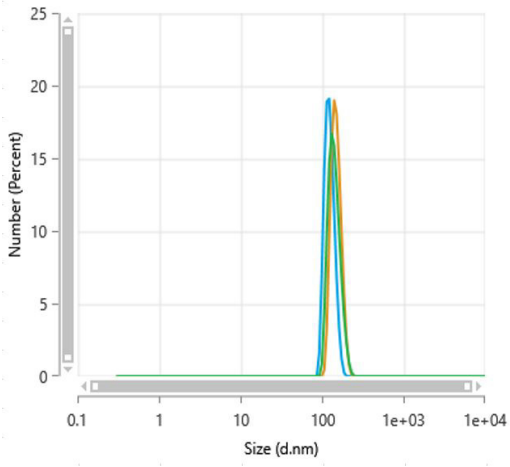


Figure 7. *Malvern mastersizer (DLS) graphs of FRR 3:1:1 at a TFR of 3 ml/min Intensity, number and volume distribution.*

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