



NEOFLUIDICS

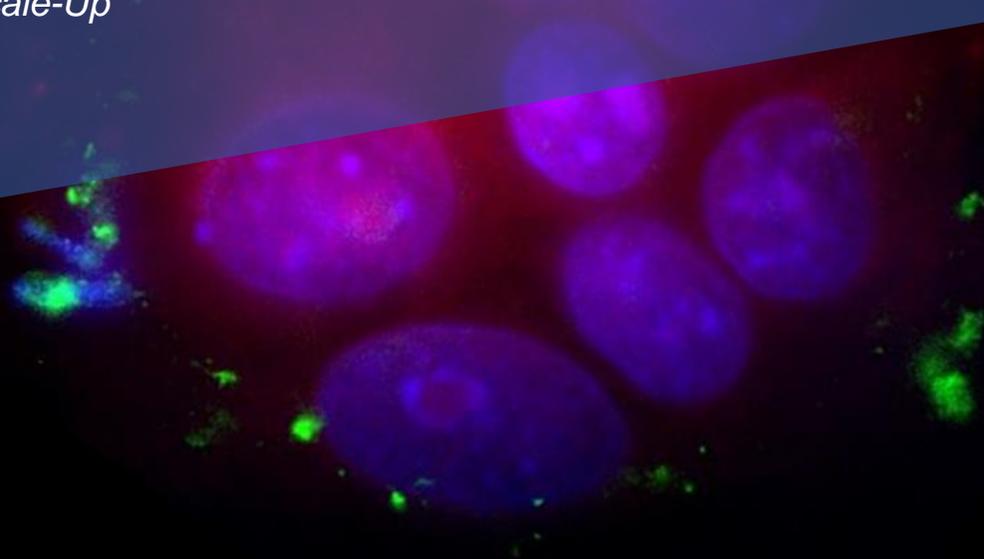
NEXT GENERATION MICROFLUIDICS FOR LIFE SCIENCES

NeoNANO™

For Microfluidic Nanoparticle Assembly

RAPID LIPOSOMAL FORMULATION DEVELOPMENT AND SCALE UP USING A SINGLE INTEGRATED SYSTEM

- *Size-Tailoring Through Flow Rate Control*
- *Batch-To-Batch Reproducibility*
- *Seamless Scale-Up*



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Liposomes are the most common and well-investigated nanocarriers for drug delivery. Size and uniformity of liposomes are vital to their efficacy, and the ability to control these factors can markedly accelerate liposomal drug research. Traditional liposome formulation methods are tedious and offer little control over size and uniformity. We have developed the NeoNANO™, a user-friendly microfluidic platform capable of producing large batches of uniform, size-controlled liposomes in a high throughput fashion. Our platform utilizes microfluidic Dean vortices, which allow for rapid mixing of aqueous and organic phase solutions, generating tunable and uniform nanoparticles. Additionally, low-shear mixing allows for encapsulation of sensitive biologics with the ability to scale up. Here, we produce liposomes ranging from sub-100 to 250 nm with high consistency (PDI < 0.2 before any post-processing) by manipulating system and formulation parameters. These results show that the NeoNANO™ platform can be readily used to develop both small-scale liposome formulations for process optimization and large-scale production.

INTRODUCTION

Liposomes are vesicles composed of a lipid bilayer that form in the shape of a hollow sphere encompassing an aqueous phase. Since their original discovery in the 1960s, liposomes have become the primary delivery agent for drugs, biologics, and nucleic acids¹. The chemical properties of liposomes allow for a wide range of therapeutic agents to be encapsulated in either the aqueous compartment (proteins, DNA, RNA)²⁻⁴ or within the lipid bilayer (hydrophobic small molecules). Further functionality can be obtained through the incorporation of functionalized lipids to increase circulation time, target disease, and provide on-demand release of active agents.

Complex liposomal formulations allow for a high degree of tailorability and specificity for disease but in turn, significantly increase fabrication complexity. Conventional methods of liposome production are time-intensive and require rigorous cycles of sonication and extrusion to achieve a monodisperse population⁵. Predictable and reliable particle size is critical for therapeutics, as size dictates dosing, systemic clearance, and biodistribution⁶. We have developed a microfluidic platform, the NeoNANO™, to enable controlled assembly of liposomes. Microfluidic mixing of aqueous and organic solutions facilitated by Dean vortices allows ordered assembly of lipids into unilamellar liposomes. Simple inputs allow the user to control the speed of mixing and the ratio of the aqueous and organic phases to achieve a range of nanoparticle sizes. Initial iterative work can be performed on our Singlex device to conserve time and reagents, and the large-batch cartridge can be used when scaling up production. Our platform provides a simple, robust, and scalable production method for nano-formulations. Herein, we highlight the effect of several instrument parameters on final liposomal properties.

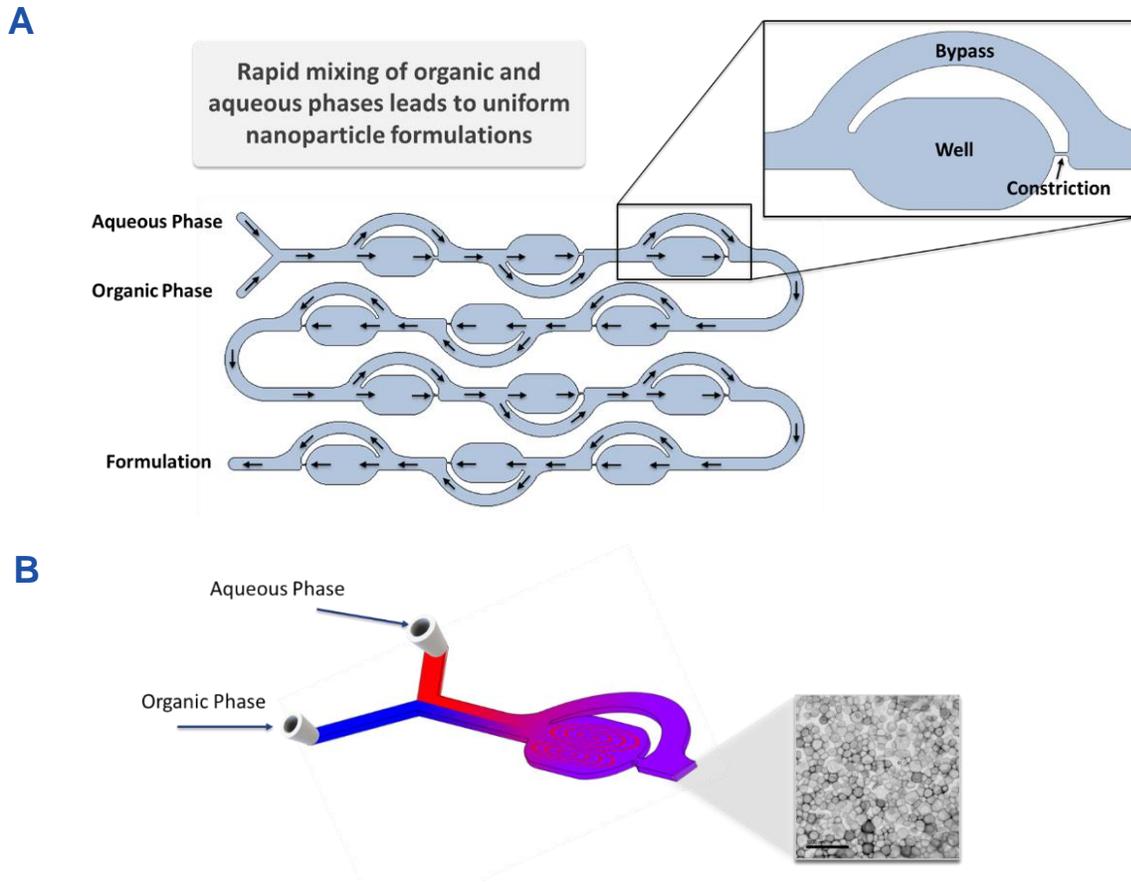


Figure 1. Large Batch Microfluidic Mixing Mechanism.

On-chip microfluidic mixing using the NeoNANO™ platform. (A) The large-batch microfluidic cartridge features 12 wells in series linked by a large bypass channel and a small constriction channel. As the aqueous and organic phases enter, the fluid streams mix and split multiple times. (B) The initial laminar flow of the input streams is disrupted as the streams reach the constriction of the well, where Dean vortices are formed, driving controlled mixing of the fluids.

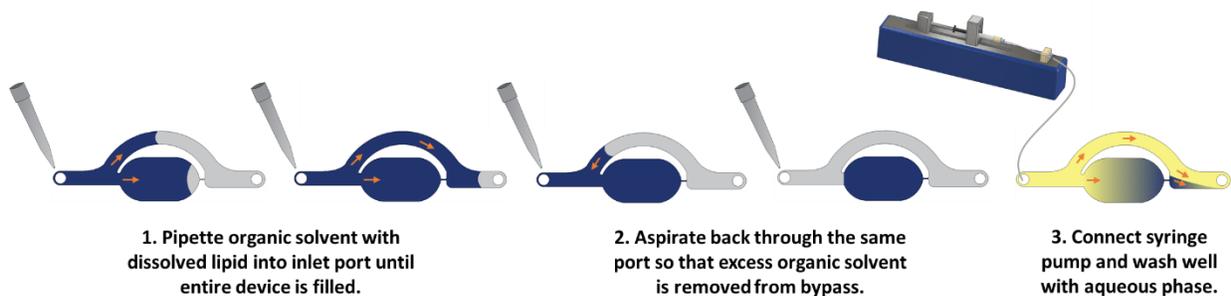


Figure 2. Small Batch Singlex Device Workflow.

Preparation of small batch formulations using the NeoNANO™ Singlex cartridge. First, the lipid in organic phase is loaded into the trap using a pipette. Then, the aqueous phase is washed through the well to produce liposomes.

SIZE-TAILORING THROUGH FLOW RATE CONTROL

Varying Total Flow Rate (TFR)

We observe that an increase in TFR significantly decreases formulation size. Using the Singlex cartridge, the size for 55:45 DPPC/Cholesterol liposomal formulations was tailored between 120 nm and 220 nm by changing the flow rate of the aqueous phase in the range of 1-20 mL/min. An identical formulation fabricated in the large-batch cartridge for large nanoparticles (LNP) resulted in diameters between 120 nm and 250 nm. In the large-batch cartridge for small nanoparticles (SNP), liposome diameter changed from 150 nm to 80nm (Figure 3).

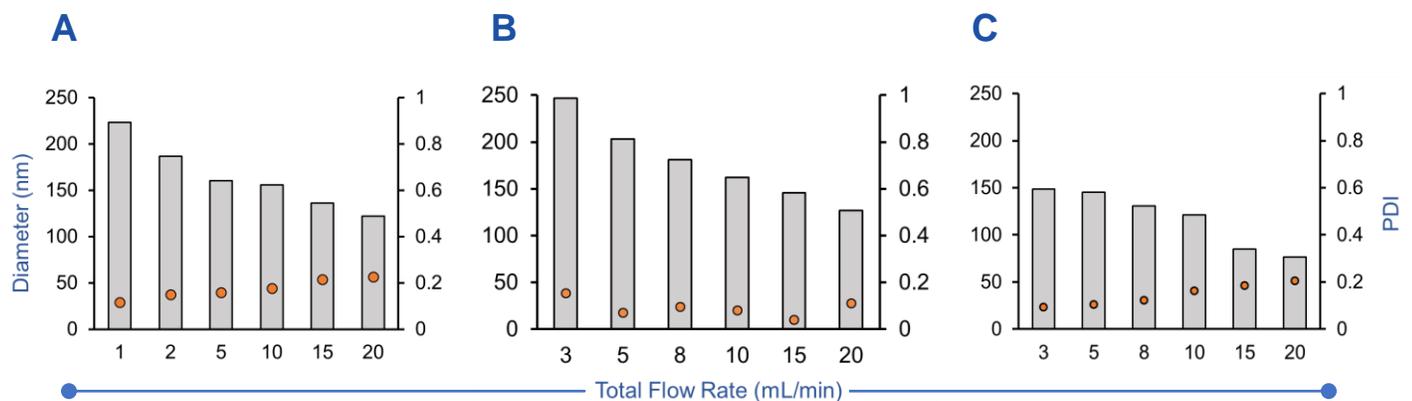


Figure 3. Effect of Increasing TFR.

Increasing TFR results in smaller liposomes when using (A) Singlex cartridge, (B) large-batch LNP cartridge, or (C) large-batch SNP cartridge.

<i>Lipid Composition</i> → DPPC/Chol 55:45 (A and B) and 70:30 (C)	<i>Organic Phase</i> → Ethanol	<i>TFR</i> → Variable
<i>Lipid Concentration</i> → 10 mg/mL	<i>Aqueous Phase</i> → Water	<i>FRR</i> → 10:1

Varying Flow Rate Ratio (FRR)

In addition to tailoring TFR, the user can drive changes in size by adjusting the FRR between the organic and aqueous phases in the large-batch cartridges. Higher aqueous to organic FRR results in smaller particles (Figure 4). At a flow rate of 15mL/min, the range achievable through FRR control was 125 to 205 nm using the LNP cartridge and 82 to 165 nm using the SNP cartridge. When using the cartridge for the fabrication of loaded liposomes, particularly when the drug is soluble in water, the ability to tailor the flow rate ratio is essential for maximizing loading efficiency.

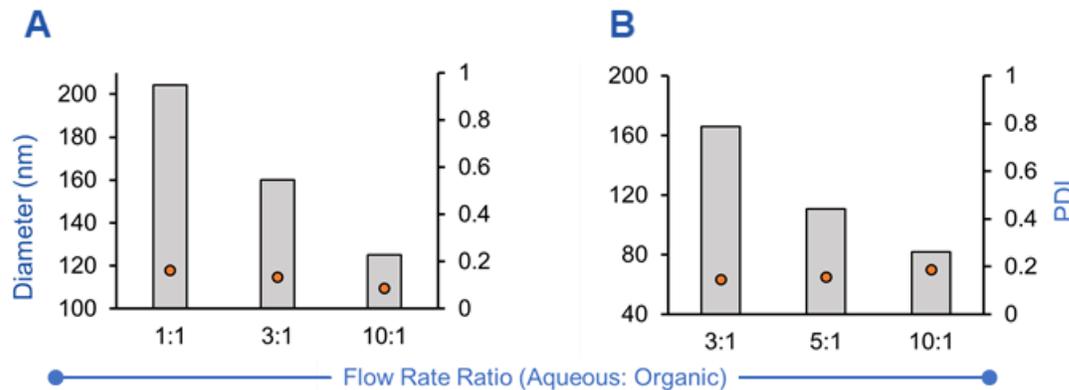


Figure 4. Effect of Increasing FRR.

A higher FRR results in smaller particles. FRR is varied from 1:1 to 10:1 with a constant TFR 15mL/min in the large-batch (A) LNP and (B) SNP cartridges.

Lipid Composition → DPPC/Chol 70:30 *Organic Phase* → Ethanol *TFR* → 15mL/min
Lipid Concentration → 10 mg/mL *Aqueous Phase* → Water *FRR* → Variable

BATCH-TO-BATCH REPRODUCIBILITY

Multiple batches of formulations with the same lipid composition were produced on different days and by multiple users (Figure 5). When using the Singlex device, less than a 5% deviation in size was observed. For the large-batch cartridges the deviation was less than 2%.

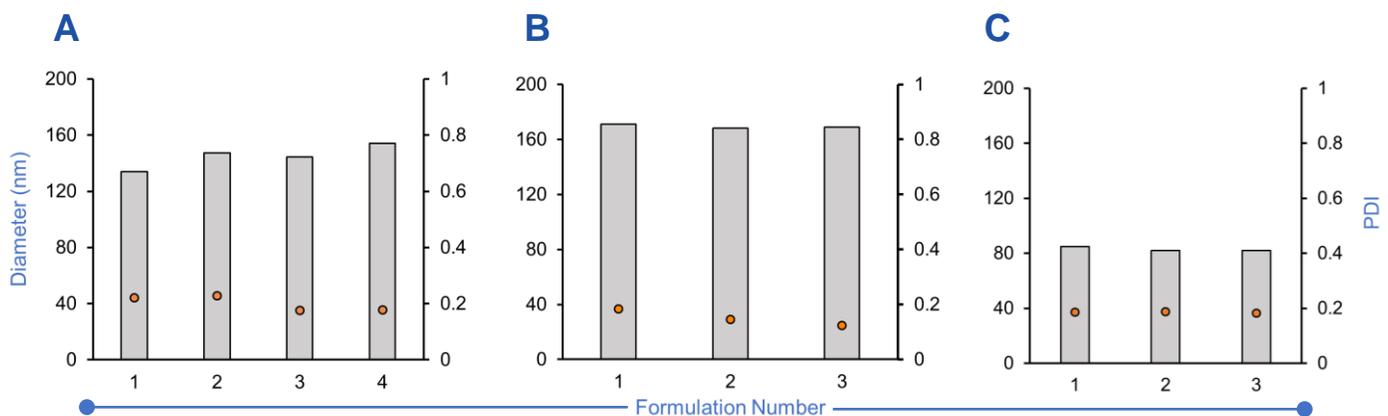


Figure 5. Batch-to-Batch Reproducibility.

DPPC/Cholesterol formulations with compositions of (A) 60:40 in Singlex device and 70:30 in large-batch (B) LNP and (C) SNP devices made over multiple days.

Lipid Composition → DPPC/Chol 60:40 (A) and 70:30 (B and C) *Organic Phase* → Ethanol *TFR* → 10mL/min (A & B)
Lipid Concentration → 10 mg/mL *Aqueous Phase* → Water *TFR* → 15mL/min (C)
FRR → 10:1

SCALE UP

Using the syringe pump-driven NeoNANO™ system with the Singlex and large-batch devices, users can iterate through many formulations to optimize size, charge, drug loading, and post-processing steps while minimizing use of materials. For *in vitro* studies, the user may not require the higher volume NeoNANO+ system. However, in a pre-clinical setting, the NeoNANO+ will be able to produce hundreds of milliliters per hour.

Formulation Uniformity over Time

To verify formulation uniformity over time, we tracked the size and PDI over a 100 mL fabrication volume (Figure 6). The average PDI was 0.12 +/- 0.02 and the corresponding size was 170.5 +/- 1.5 nm. This result is also within 1% of that shown in Figure 5b, where identical formulation parameters were used.

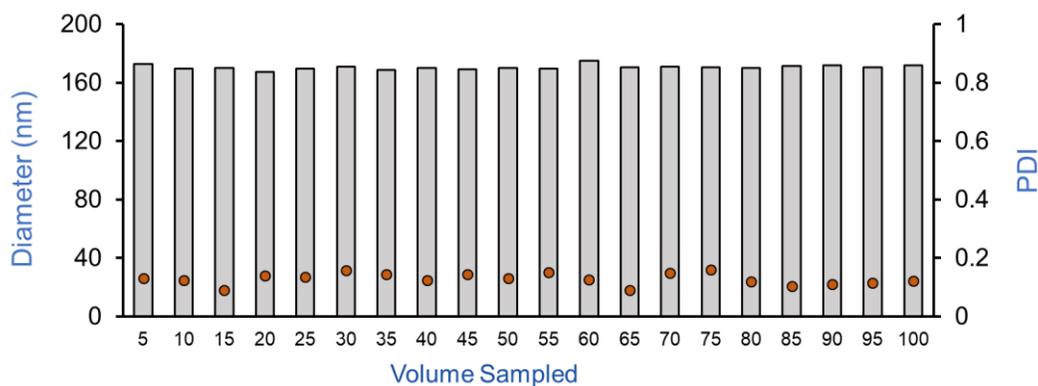


Figure 6. Uniformity Over Time.

Twenty fractions of 5mL were collected during the fabrication of 100 mL of liposomal formulation.

<i>Lipid Composition</i> → DPPC/Chol 70:30	<i>Organic Phase</i> → Ethanol	<i>TFR</i> → 10mL/min
<i>Lipid Concentration</i> → 10 mg/mL	<i>Aqueous Phase</i> → Water	<i>FRR</i> → 10:1

MATERIALS AND METHODS

Liposomes were composed of DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) and cholesterol. The organic solvent phase consisted of lipids dissolved in 95% ethanol. Reagent grade water was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NeoNANO™ or NeoNANO+ system.

To use the NeoNANO™ system in combination with the Singlex device, the trap is pre-loaded with the desired organic phase (Figure 2). Tubing from a syringe containing the

aqueous phase is connected to the inlet, and flow rate is set within the range of 1-20 mL/min. Outlet tubing is connected to the microfluidic outlet port and feeds into a collection vial. A volume within the range of 100-1000 μ L was used for fabrication.

The NeoNANO™ or NeoNANO+ system was used in combination with two large-batch microfluidic cartridges, one that results in large nanoparticles (LNP) and a second version that creates smaller nanoparticles (SNP). Tubing from the organic phase and aqueous phase are connected to the inlet ports (Figure 1). Flow rate of the aqueous and organic stream are selected, with a maximum total flow rate of 22 mL/min. Outlet tubing that feeds into a collection vial is connected at the third cartridge port.

The primary factor to consider when choosing between the NeoNANO™ and NeoNANO+ is the desired batch volume. Both flow rate sources—syringe pump and peristaltic pump—can be used interchangeably to prepare formulations.

Particle size was measured using Dynamic Light Scattering (Omni, Brookhaven Instruments, NY, USA). The particle size reported was equal to the measured effective diameter.

Size-Tailoring Through Flow Rate Control

Varying Total Flow Rate (TFR)

Flow rate is set using the syringe pump interface or peristaltic pump control. For the Singlex device, the TFR is equivalent to the flow rate of the aqueous phase. In the large-batch device, the TFR is equal to the combined flow rate of the aqueous and organic streams.

Varying Flow Rate Ratio (FRR)

A true FRR sampling can only be achieved on the large-batch microfluidic device, where the cartridge accepts two coflowing streams. When using the syringe pump, the flow rate ratio can be set by loading the aqueous and organic phases into syringes of differing diameters. A 10 mL syringe filled with the aqueous phase and a 1 mL syringe filled with the organic phase will result in a 10:1 FRR (based on the diameters of the syringe barrels). When using the NeoNANO+, the flow rate ratio can be set using the integrated pump software and has more tailorability as it is not dependent on syringe diameter.

DISCUSSION

The NeoNANO™ microfluidic platform allows for quick, reproducible, and tailorable assembly of nanosized formulations. The system allows for a seamless transition from small-volume optimization to large-scale pre-clinical production. Using the Singlex cartridge, users can prepare up to a milliliter of formulation to quickly determine viable particle compositions. Promising formulations can subsequently be produced at larger volumes in the range of one to hundreds of milliliters for in-depth testing. The nature of the platform ensures particles are monodisperse, unilamellar and similarly-sized from batch-to-batch. The polydispersity of the data presented here ranges from 0.05 to 0.2 prior to any post-processing such as dialysis, filtration or centrifugation.

With the NeoNANO™ system, one can fabricate highly uniform formulations across hundreds of milliliters due to the relatively large microfluidic geometries that limit cartridge fouling over time and ensure that all incoming fluid experiences uniform vortical mixing profiles. The ability to generate particles with reliable size and homogeneity is in stark contrast to conventional methods, where average particle size is variable and polydispersity is high. Furthermore, adapting conventional protocols to test new compositions and tailor formulation properties requires substantial time and can be cost-prohibitive. Microfluidic technologies increase control at the microscale, limit the use of reagents, and decrease process time. The NeoNANO™ system provides direct size modulation through the manipulation of input flow rates of the organic and aqueous streams, as well as the flow rate ratio between streams.

CONCLUSION

We have highlighted a microfluidic platform for fabrication of liposomal formulations that provides an alternative to the inconsistency, complexity, and lack of scalability of conventional production methods. The NeoNANO™ system allows for homogenous, reproducible nanoparticle formulations within minutes. With small-batch cartridges, a suite of particle compositions can be rapidly tested without expending costly resources. Then, using the large-batch cartridge, preferred formulations can be further refined by tailoring the flow rates of the aqueous and organic streams.

Reference Materials

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