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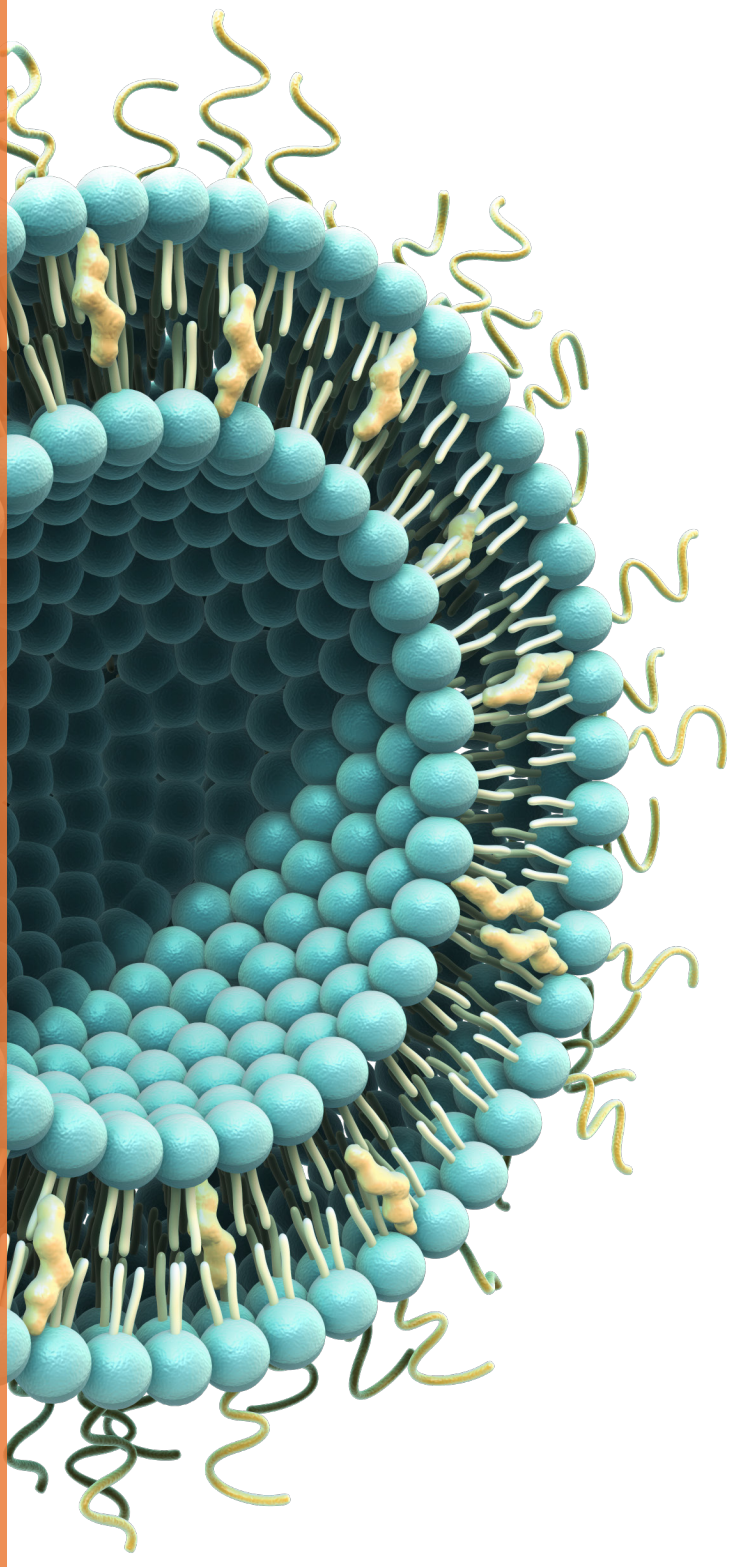
Liposomes

Using the NanoAssemblr® Benchtop instrument process parameters to reproducibly tune size



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Abstract

Liposomes are used as drug carriers to deliver a variety of therapeutic molecules such as small molecules, proteins, and nucleic acids. Liposome size and homogeneity are crucial factors that affect efficacy of liposomal drugs. Hence, a liposome production method that streamlines the optimization of these characteristics will expedite formulation development. Here we demonstrated the utility of the NanoAssemblr® microfluidic technology for rapidly and reproducibly generating homogeneous liposome populations with the ability to fine-tune liposome size through computer-controlled parameters. Bench-scale batches of liposomes were produced at sizes ranging from 40 nm to 120 nm with exceptional uniformity (polydispersity index, PDI as low as 0.05). Additionally, the robustness of the NanoAssemblr® process was demonstrated by comparing the size and PDI of 6 independent formulations conducted by different instrument operators. These data demonstrate how the technology can be used to expedite development of liposome formulations.

Introduction

Traditional methods of liposome formulation include sonication and extrusion. These methods are laborious and offer limited control over size and polydispersity of liposomes, both of which can affect liposome circulation time, drug retention or its ability to penetrate different tissues[1]. Prior research has demonstrated the utility of the NanoAssemblr® Benchtop instrument for formulating unilamellar liposomes[2] for remote loading of chemotherapeutics[3], in situ loading with hydrophobic and hydrophilic small molecules [4,5], nucleic acids[6], and vaccine adjuvants[7]. The NanoAssemblr platform works through controlled nanoprecipitation of liposomes ***(Figure 1)***. When lipid molecules dissolved in a low-polarity organic solvents are mixed with water or buffer, the resulting change in polarity triggers the spontaneous self-assembly of the lipids into unilamellar vesicles. The NanoAssemblr platform combines microfluidic mixing with independent computer controlled injection of both fluids to achieve control over precipitation conditions that directly influence liposome size. This method also ensures consistency among liposomes within any given batch, from batch to batch, and from operator to operator. With a reproducible process, effects of altering the formulation can be isolated (see Related Materials, below, for additional resources). Additionally, the NanoAssemblr platform offers a straightforward path towards clinical development by streamlining scale-up through continuous flow and multiple parallel mixers. Here, we demonstrated that the NanoAssemblr® Benchtop consistently formulates homogenous liposomes by comparing the size and polydispersity (PDI) of 6 independent formulations. Next, we demonstrated how precise computer control over injection speed and the ratio between aqueous and organic phases is used to finely tune liposome size and minimize heterogeneity.

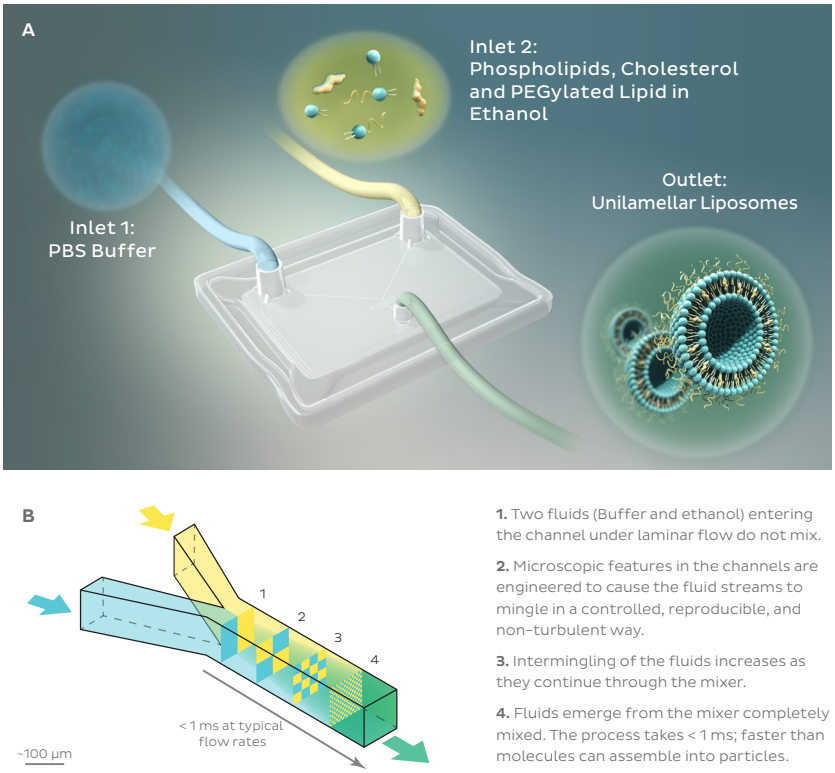

Figure 1. A) *PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr Benchtop microfluidic cartridge. Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer controlled injection allows specification of the total flow rate and the flow rate ratio of aqueous to organic solvents to control liposome size.* ***B)*** *Diagram depicting two fluids mixing under laminar flow in a microfluidic mixer. The two fluids enter the channel as adjacent streams. Specially engineered microscopic features within the channels cause intermingling of the two fluid streams, effectively increasing the surface area between them, increasing the interface between the two liquids across which diffusion can take place, and reducing the diffusion distance.*

Result

LIPOSOME BATCH REPRODUCIBILITY USING NANOASSEMBLR BENCHTOP

To assess batch-to-batch variation of liposome formulations prepared using the NanoAssemblr Benchtop Instrument, multiple batches of liposomes with the same lipid composition were formulated under identical instrument parameters by independent operators. This led to comparable liposome sizes and PDIs demonstrating consistency across batches and operators ***(Figure 2).***

TUNING LIPOSOME SIZE BY ALTERING FLOW RATE RATIO AND TOTAL FLOW RATE RATIO

The aqueous: organic Flow Rate Ratio (FRR) and Total Flow Rate (TFR) are the primary process parameters that impact nanoparticle characteristics. FRR is the volumetric ratio of the organic and aqueous phases being mixed through the microfluidic cartridge. TFR is the total speed in mL/min at which both fluid streams are being pumped through the two separate inlets of the microfluidic cartridge.

Figure 3 indicates the effect of FRR on liposome size. At a constant liposome composition, particle size decreased from 90 to 50 nm as the FRR increased from 1:1 to 3:1. At very high FRRs, size plateaus as liposomes form the smallest thermodynamically possible structures defined as their “limit” size[2].

The effect of TFR on liposome size can be observed in ***Figure 4.*** Liposome size decreased from 100 to about 50 nm when the TFR was increased from 1 to 12 mL/min. The FRR, lipid composition, and total lipid concentration were kept constant throughout the experiment.

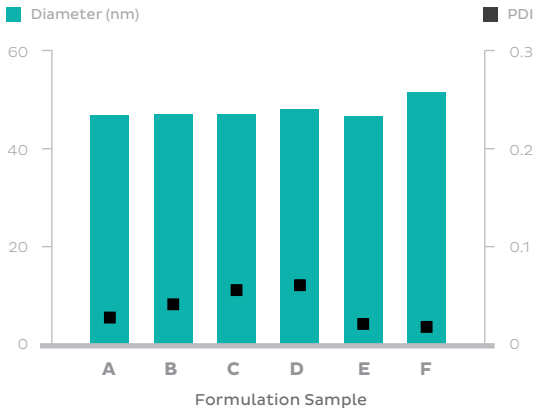

Figure 2. Liposome size and polydispersity were consistent across batches prepared by different operators. *Six independent batches of liposomes, with identical lipid composition and NanoAssemblr process parameters were generated by different operators. Liposomes generated had a size range of 46–54 nm and consistently low polydispersity indices (PDI).*

Composition	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	5:1

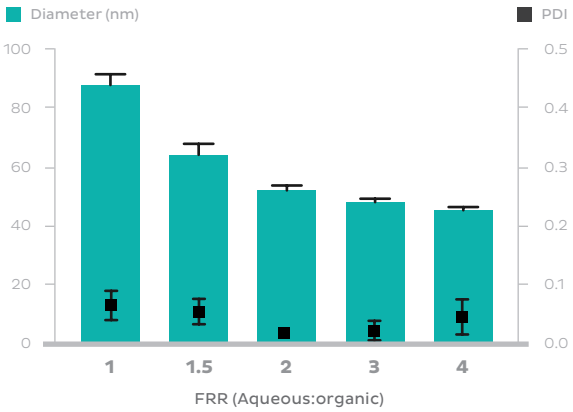

Figure 3. Liposome size tuning by Flow Rate Ratio. *Higher aqueous:organic Flow Rate Ratio (FRR) reduces liposome size asymptotically. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*

Composition	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	n:1, n as indicated

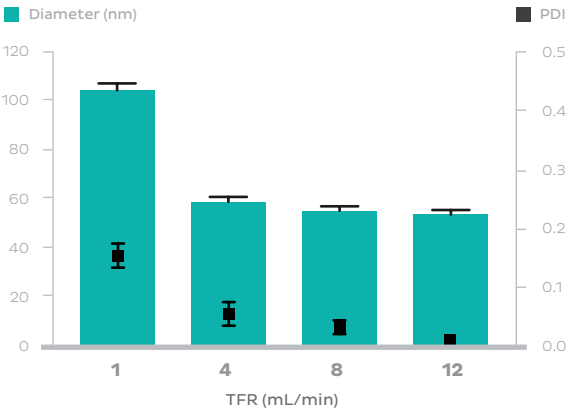

Figure 4. Liposome size tuning by Total Flow Rate. *Increasing Total Flow Rate (TFR) increases mixing speed and reduces liposome size. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*

Composition	POPC/Chol/DSPE-PEG_{2000} (52:45:3 mol%)
Lipid concentration in organic solvent	10 mg/mL
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	As indicated (mL/min)
Flow Rate Ratio (FRR)	2:1

Discussion

Liposome size and polydispersity are crucial factors affecting efficacy. Hence, size control and size uniformity within a batch and from batch-to-batch are crucial factors to optimize in liposomal drug formulation development. The NanoAssemblr process is highly reproducible, as shown in ***Figure 2,*** where multiple independent operators produced liposome batches with comparable sizes and PDIs. This is achieved through computer control of parameters that affect liposome size. Understanding the factors that affect liposome size provides insight into factors that affect uniformity of size and reproducibility. During nanoprecipitation, lipid molecules start off fully solubilized in a water-miscible organic solvent. When this solution is mixed with water, the polarity of the resulting solution increases, which causes the lipid molecules to self-assemble into unilamellar liposomes. Given a fixed lipid composition, two factors that affect the resulting liposome size are the magnitude of polarity change and the speed of mixing. On the NanoAssemblr platform, the former is controlled by the Flow Rate Ratio (FRR) while the latter is controlled by the Total Flow Rate (TFR). ***Figure 3*** illustrates that increasing the aqueous-to-organic FRR decreases the size of the resulting liposomes asymptotically. This is because increasing the relative amount of the aqueous phase increases the magnitude of the polarity change upon mixing, which increases the driving force for self-assembly of lipids into liposomes. Additionally faster mixing, achieved by increasing TFR through the NanoAssemblr microfluidic mixer, leads to smaller liposomes, as illustrated in ***Figure 4.*** This behavior is understood by considering the rate of polarity change compared to the rate of self-assembly. If the rate of polarity change exceeds the rate of self-assembly, lipid molecules experience an immediate super-saturation in the new solvent environment. Rates of diffusion and reorganization then limit the local supply of lipid molecules that can come together into a single liposome. This leads to smaller liposomes in greater abundance. From this understanding, the asymptotic behavior observed in both ***Figures 3 and 4*** can also be understood. As the driving force and rate of mixing are increased, they meet the limits of how small a particle can be, given the volume and packing of the constituent molecules.

With this understanding of how the solvent environment affects liposome size, it is clear that precise control over the mixing ratio and the mixing rate is necessary to achieve uniform liposome size. Within a given batch, the homogeneity of the population of liposomes is measured by the polydispersity index, where values below 0.2 are preferred for *in vivo* applications. Homogeneity of the solvent environment throughout the volume of the batch is required in order to obtain a homogeneous population of liposomes. PDIs achieved on the NanoAssemblr Benchtop were below 0.2, with many formulations below 0.1, which is difficult to achieve with conventional methods. In the microfluidic channels, the organic and aqueous phases are spatially confined, which results in laminar, as opposed to turbulent flow. Laminar mixing is gentle and highly consistent. This ensures that, under continuous flow and at steady state, each unit volume flowing through the mixer is experiencing nearly identical mixing conditions. Furthermore, the "Autoswitch" feature of the NanoAssemblr Benchtop automatically separates microliter volumes from the head and tail of the process in order to isolate the particles produced under steady state. To achieve batch-to-batch consistency the conditions of liposome precipitation must be highly reproducible. In addition to laminar flow mixing, precise computer-controlled injection of the organic and aqueous phases ensures consistent flow rates and mixing ratios between batches, and between independent instrument operators. Additionally, computer controlled injection allows more precise control over TFR and FRR and thus enables rational optimization of these two parameters.

Conclusion

We established that the NanoAssemblr Benchtop can rapidly tune the size of liposomes by adjusting instrument factors (TFR and FRR) independent of liposome composition. This is a convenient and powerful means of optimizing size and PDI of liposomal drug formulations. Additionally, it was confirmed that liposomes formulated by NanoAssemblr Benchtop maintained exceptional batch-to-batch consistency and minimal population heterogeneity. Taken together, these results demonstrate how the NanoAssemblr platform facilitates rapid optimization of liposomal drug formulations. Furthermore, the process offers a straightforward path to scaling production to clinically relevant quantities by increasing lipid concentration and implementing continuous flow and multiple parallel microfluidic mixers.

Materials & Methods

Liposomes were composed of cholesterol, POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), and DSPE-PEG_{2000} (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Lipids were dissolved in ethanol as the organic solvent and Calcium- (Ca^{2+}) and magnesium- (Mg^{2+}) free PBS buffer at neutral pH was used as the aqueous phase. The organic and aqueous phases were rapidly mixed using the NanoAssemblr Benchtop microfluidic instrument at aqueous to organic Flow Rate Ratios between 1:1 and 4:1 and Total Flow Rates between 2 mL/min and 12 mL/min. Formulations were then dialyzed against PBS to remove ethanol. At Flow Rate Ratios 1 and 1.5, formulations were diluted down to 10% ethanol immediately following the mixing process and before dialysis, since high amounts of ethanol can destabilize liposomes. Particle size and integrity were measured before and after dialysis using a Dynamic Light Scattering technique Particle size and integrity was then investigated using Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Where indicated, formulations were prepared in triplicate and size and polydispersity index (PDI) are represented as the mean of 3 samples, and error bars represent standard deviation (SD).

Related Material

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