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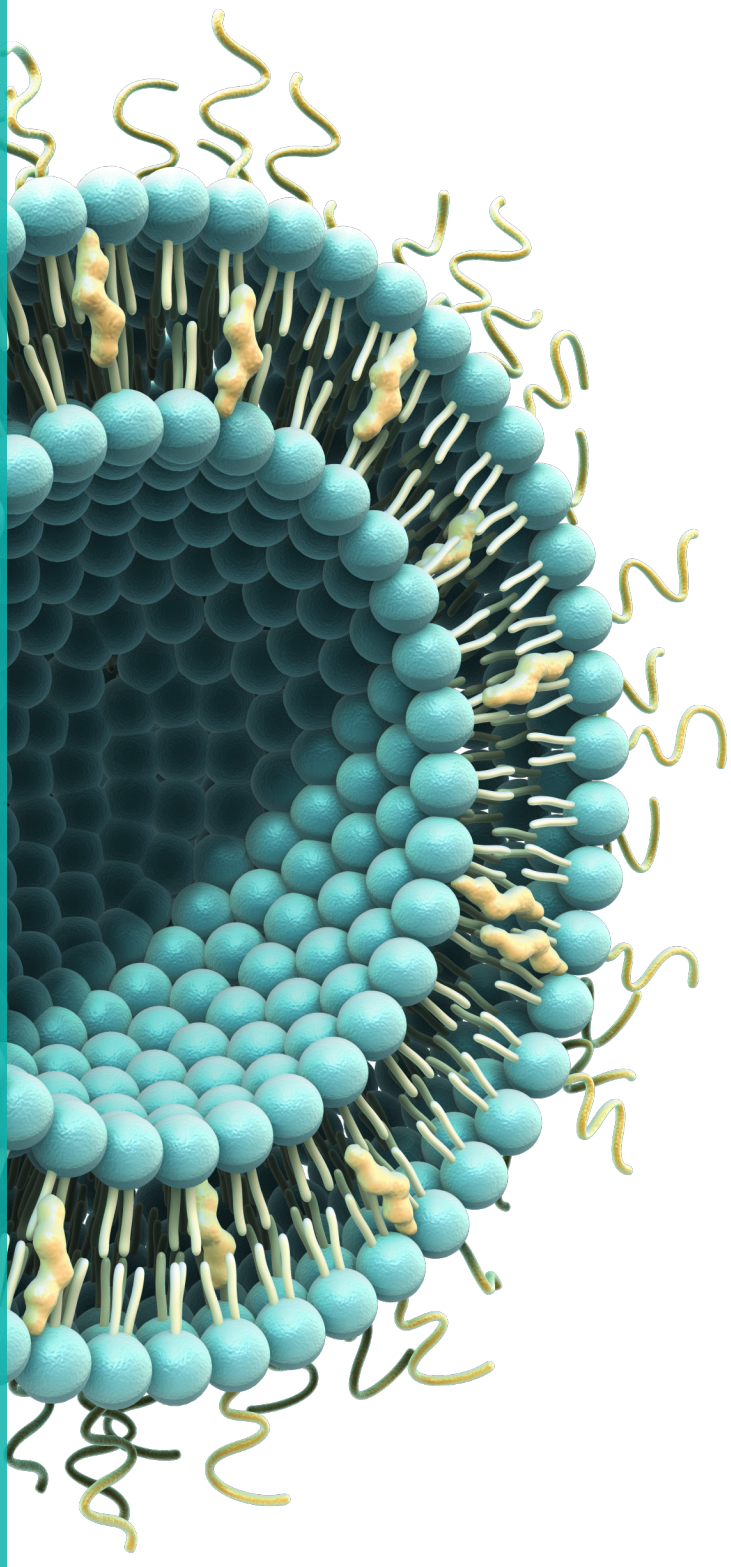
Liposomes

Using formulation parameters to tune size on the NanoAssemblr® Benchtop



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Abstract

Liposome encapsulation is a technique that has been used to enhance drug circulation time and reduce toxicity of hydrophilic molecules, or improve solubility of hydrophobic molecules. Depending on the type of drug molecule or target tissue, different liposome compositions are often needed. Additionally, size and homogeneity of the liposome population can impact liposome behavior *in vivo.* In this application note, we examine the effect of lipid composition and concentration on the size and size distribution of liposomes generated using the NanoAssemblr® Benchtop instrument. The process reproducibility afforded by the NanoAssemblr Benchtop allows observed changes in liposome size to be attributed to formulation changes. Increasing cholesterol content in liposome formulations was found to increase the size of resulting liposomes.

Introduction

Liposomal drug delivery can greatly improve drug performance by altering pharmacokinetic and biodistribution profiles. Liposome composition can be tailored to optimize the encapsulation of drug molecules with a range of chemical properties. Additionally, certain lipids can significantly impact liposome properties that influence their efficacy as nanomedicines, such as drug retention, size, and surface charge.

Liposome size is one parameter that can influence circulation half-life and tissue penetration[1]. The NanoAssemblr® is a scalable microfluidic-based manufacturing platform that allows researchers to reproducibly fine tune liposome size. It has been shown that NanoAssemblr technology enables the formation of the smallest thermodynamically stable liposomes, so-called "limit" sized liposomes, based on the packing density of molecules participating in the nanoparticle structure[2]. The platform produces unilamellar liposomes through controlled nanopreciptitation (see ***Figure 1***). Lipids dissolved in an organic solvent, typically ethanol, are injected into one inlet of the NanoAssemblr cartridge, while water or a buffer is injected into the other inlet in laminar flow (i.e. the fluids do not mix immediately). Microscopic features within the channels cause controlled mixing at time scales more rapid than diffusion of molecules. The sudden change in solvent polarity due to mixing triggers assembly of lipids into unilamellar liposomes. Liposome size can be tuned by one or more strategies: (i) computer controlled process parameters; (ii) liposome composition; and, (iii) total lipid concentration. The instrument process parameters Total Flow Rate (TFR) and Flow Rate Ratio (FRR) are used to control the conditions of liposome self-assembly by solvent displacement (see Related Material, below, for more details). Here, we focus on the effect of liposome composition, specifically, cholesterol content, and total lipid concentration, on liposome "limit" size. We further determine the parameters required to achieve liposomes of a specific size using the NanoAssemblr Benchtop.

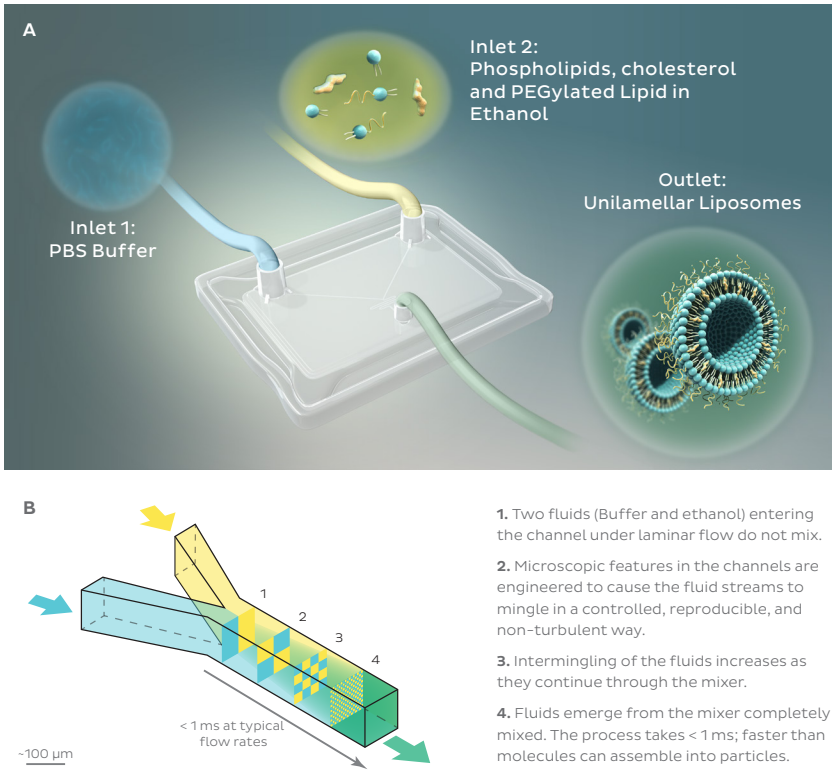

Figure 1. A) *PBS buffer is injected into the left inlet while an organic solvent with dissolved lipids is injected into the right inlet of the NanoAssemblr Benchtop microfluidic cartridge. Following controlled mixing in microfluidic channels, unilamellar liposomes are spontaneously formed. Computer 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

First, we examined the effect of increasing Flow Rate Ratio (FRR), which is the ratio between the aqueous (antisolvent) phase and the organic (solvent) phase being mixed in the microfluidic cartridge on liposome size as a function of total lipid concentration. The TFR was held constant at 12 mL/min. The liposome composition tested comprised the high transition temperature lipid DSPC (T_m = 55°C), cholesterol and DSPE-PEG_{2000}. Liposomes with a high DSPC content should be formulated at temperatures above the lipid's transition temperature. Having the lipids in a fluid state facilitates the formation of liposomal structures. ***Figure 2*** shows that at total lipid concentrations of ≤ 75 mg/mL, increasing the FRR results in a decrease in liposome size until a plateau is reached at FRR > 2:1 where the liposomes reach limit size (~ 60 nm). At a 1:1 FRR liposome size was comparable and independent of total lipid concentration. However, at total lipid concentrations ≥ 100 mg/mL, increasing the FRR did not result in a decrease in liposome size.

Liposome concentration can also be increased following formulation by using centrifugal filtration (CF). CF is routinely employed in formulation development to remove unencapsulated drug, exchange buffer, and increase the concentration of liposomes irrespective of the method used to form the liposomes. POPC/Chol/DSPE-PEG_{2000} liposomes were formulated with a starting concentration of 10 mg/mL in ethanol. Following liposome formulation on the NanoAssemblr under limit size conditions, liposomes were dialyzed against PBS to remove ethanol, and thereafter concentrated more than 12-fold using centrifugal filtration. The final lipid concentration was determined to be 25 mg/mL using a cholesterol assay kit.

Hydrodynamic size and PDI measurements before and after the concentration step are compared in ***Figure 3.*** These data indicate that limit size liposomes produced on the NanoAssemblr Benchtop are robust, and retain their integrity following downstream processing.

Next, we examined how the lipid composition affects liposome size when operating at instrument process parameters shown to promote limit size. Cholesterol is an integral part of biological membranes and plays an important role in the physical properties of liposomes[3]. Additionally, cholesterol is known to influence drug release profiles in liposomal formulations[4]. To examine the role of cholesterol on the liposome limit size, the cholesterol content was systematically varied in a series of POPC/cholesterol/DSPE-PEG_{2000} formulations prepared at a FRR of 4:1 and a TFR of 12 mL/min. ***Figure 4*** shows that liposome limit size increased from 20 to 42 nm as the cholesterol content was increased from 0 to 45 mol%. Additionally, we observed that ethanol removal by dialysis did not affect liposome size.

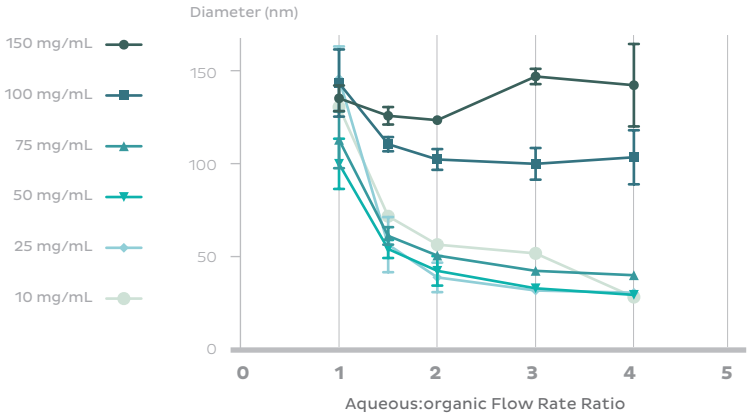

Figure 2. The effect of lipid concentration and Flow Rate Ratio (FRR) on liposome size. *Liposomes with identical lipid composition were generated at different total lipid concentrations and at different FRRs. The Heating Controller was set to 65 ˚C. Liposomes were dialyzed against PBS pH7.4 to remove residual solvent prior to measuring hydrodynamic size by DLS. Data represents the mean of 3 samples and error bars represent standard deviation.*

Composition	DSPC/cholesterol/DSPE-PEG_{2000} (52:45:3 mol%)
Initial lipid concentration in organic solvent	As indicated
Organic solvent	Ethanol
Aqueous solvent	PBS pH 7.4
Total Flow Rate (TFR)	12 mL/min
Flow Rate Ratio (FRR)	n:1 as indicated on horizontal axis

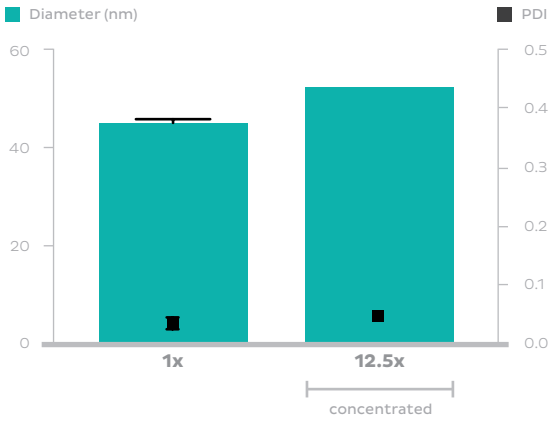

Figure 3. Liposomes were concentrated using centrifugal filtration with minimal effect on size. *Six samples were prepared with a final concentration of 2 mg/mL and mean size and PDI with SD is shown (1X). Samples were then pooled together and concentrated to a final concentration of 25 mg/mL (12.5X) in PBS pH 7.4 by centrifugal filtration.*

Composition	POPC/Chol/DSPE-PEG$_{2000}$ (52:45:3 mol%)
Initial 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)	4:1

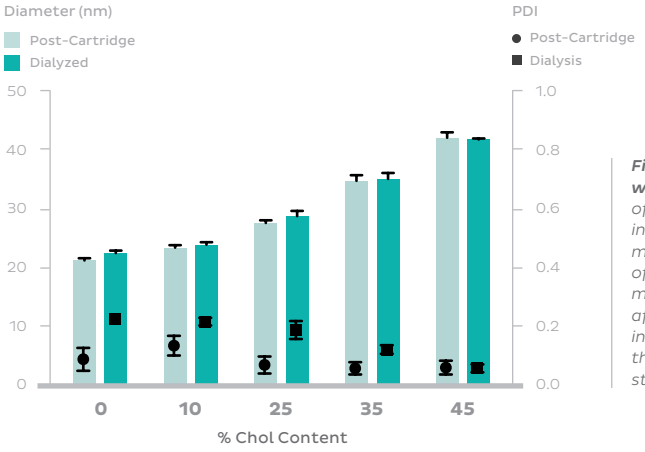

Figure 4. Liposome limit-size increases with cholesterol content. *Addition of cholesterol led to a size increase in liposomes. Liposomes contained 3 mol% DSPE-PEG and varying amounts of POPC and cholesterol. Size was measured soon after formulation and after dialysis. Samples were prepared in triplicate. Measurements represent the mean and error bars represent standard deviation.*

Composition	POPC/Chol/DSPE-PEG$_{2000}$ ((97-x):x:3 mol%) x as indicated
Initial 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)	4:1

Discussion

Self-assembly of lipids into liposomes occurs when lipids dissolved in a good solvent are mixed with a polar aqueous antisolvent. Rapid, homogenous mixing creates a sudden change in the polarity of the environment of the lipid molecules, and the magnitude of this polarity change is proportional to the driving force for self-assembly into bilayers and liposomes. The magnitude of the polarity change can be dictated by the aqueous-to-solvent flow rate ratio (FRR), which is a software-controlled parameter on the NanoAssemblr instrument. Higher FRRs lead to greater polarity changes and, typically, smaller liposomes. At sufficiently high FRR, the self-assembly process becomes kinetically limited, which leads to smaller liposomes. The lower limit of liposome size is dictated by the mechanical packing density of lipid molecules, which leads to the asymptotic behavior observed in ***Figure 2.*** Additionally, at sufficiently high lipid concentrations, the concentration gradient between free lipids and those assembling into nascent liposomes is great enough to overcome kinetic barriers. This leads to changes in the profiles observed at initial lipid concentrations of 100 mg/mL and greater.

The trends observed in ***Figure 2*** can be used to tune particle size. First, these data can be used as a look-up-table to determine the FRR, required to achieve a specific size for this formulation. Second, increasing the starting lipid concentration is a means by which to scale up liposome production, hence the change in self-assembly behavior of the lipids at higher concentrations provides guidance on which concentration range to choose in order to achieve the highest "tunability" of liposome size. The total throughput can be calculated by considering the lipid concentration in the organic phase as well as the FRR and TFR. For instance at an initial lipid concentration of 75 mg/mL, a FRR of 2:1 and a TFR of 12 mL/min, total liposome output through the NanoAssemblr cartridge is 300 mg/min. At a TFR of 18 mL/min, total liposome output would be 450 mg/min. Additionally, these findings provide a means to achieve large liposomes by increasing solvent-phase lipid concentrations above 75 mg/mL with this formulation. Identical studies can be easily performed on the NanoAssemblr Benchtop to understand the behavior of different lipid compositions.

If desired, liposome concentration can be adjusted as desired by dilution or by centrifugal filtration following NanoAssemblr formulation. ***Figure 3*** demonstrates that liposomes can be formulated at a particular concentration and concentrated to a final concentration as required with minimal effect on liposome size and identical PDI.

From ***Figure 4,*** it is clear that liposome limit size increases with the proportion of cholesterol in the formulation. This can be understood by considering the effect of cholesterol on the properties of lipid bilayers. It has been demonstrated that the presence of cholesterol in lipid bilayers consisting of phospholipids with at least one saturated tail tends to increase its mechanical rigidity[3]. This rigidity limits the curvature of a bilayer, leading to larger equilibrium liposome size. Hence, as cholesterol content rises, this stiffens the mechanical properties of the bilayer, leading to larger liposomes with larger radii of curvature.

Having a reproducible process is crucial both to observing the differences in liposome size and attributing these differences to formulation parameters such as initial lipid concentration and lipid composition. Poor reproducibility in the preparation method makes it difficult to separate the influence of random process variations from the influence of formulation parameters. This would make the trends observed in the present study difficult to discern. Given that process parameters influence liposome size and dispersity, it is easy to see that an extremely well controlled process achieved with the NanoAssemblr Benchtop is necessary to fine-tune liposome formulations.

Conclusion

The size of liposomes generated with the NanoAssemblr Benchtop can be controlled by instrument process parameters as well as lipid concentration and composition. In this application note, we demonstrated that varying lipid concentration can affect the relationship between liposome size and FRR. Over a wide range of initial lipid concentrations, this trend remained unchanged. This offers a means of scaling quantities and throughput of liposome formulations by increasing the concentration of lipids in the organic phase. At initial lipid concentrations of 75 mg/mL, limit size liposomes were produced at a rate of 300 mg/min. Alternatively, liposomes can be concentrated after NanoAssemblr Benchtop production by centrifugal filtration without appreciably affecting size. Additionally, the amount of cholesterol in a liposome formulation was found to significantly alter the limit size of vesicles. In the case of POPC/chol/DSPE-PEG_{2000} formulations, a greater proportion of cholesterol increases the stiffness of the bilayer, favoring the formation of larger liposomes, allowing for less strain during bending of the bilayer into a sphere. Ultimately, the reproducibility of the NanoAssemblr microfluidic process was crucial to attributing changes in lipid concentration and composition to the observed differences in size. Hence, the NanoAssemblr Benchtop is the ideal tool to develop and tune liposomal drug formulations.

Materials & Methods

Liposomes were composed of cholesterol and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) or DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]). Lipids were dissolved in absolute ethanol as the organic solvent phase. 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 various Flow Rate Ratios at a Total Flow Rate of 12 mL/min. Formulations with high DSPC content were heated at 65 °C during the mixing process using the NanoAssemblr Heating Controller accessory. The Heating Controller accessory for the NanoAssemblr Benchtop can adjust the formulation process temperature with an accuracy of ±1 °C. Formulations were then dialyzed against PBS for ethanol removal. 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 Dynamic Light Scattering (Zetasizer, Malvern Instruments, UK). Where indicated, formulations were prepared in triplicate, unless otherwise noted, and size and polydispersity index (PDI) were represented as the mean of 3 samples, and error bars represent standard deviation of the mean.

Related Material

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