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A Facile Route to the Synthesis of Monodisperse Nanoscale Liposomes Using 3D Microfluidic Hydrodynamic Focusing in a Concentric Capillary Array

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TOC Graphic for "A Facile Route to the Synthesis of Monodisperse Nanoscale Liposomes Using 3D Microfluidic Hydrodynamic Focusing in a Concentric Capillary Array"

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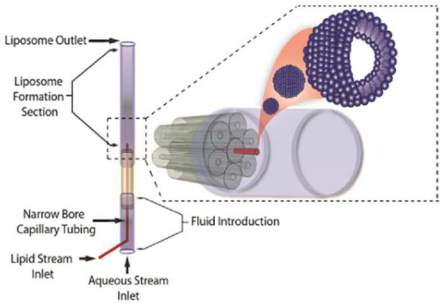

3D Microfluidic Hydrodynamic Focusing for the Synthesis of Monodisperse Nanoscale Liposomes

A Facile Route to the Synthesis of Monodisperse Nanoscale Liposomes Using 3D Microfluidic Hydrodynamic Focusing in a Concentric Capillary Array

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Abstract

A novel microscale device has been developed to enable the one-step continuous flow assembly of monodisperse nanoscale liposomes using three-dimensional microfluidic hydrodynamic focusing (3D-MHF) in a radially symmetric capillary array. The 3D-MHF flow technique displays patent advantages over conventional methods for nanoscale liposome manufacture (*i.e.*, bulk-scale alcohol injection and/or sonication) through the on-demand synthesis of consistently uniform liposomes without the need for post-processing strategies. Liposomes produced by the 3D-MHF device are of tunable size and have a factor of two improvement in polydispersity and a production rate that is four orders of magnitude higher than previous MHF methods, which can be attributed to entirely radially symmetric diffusion of alcohol-solvated lipid into an aqueous flow stream. Moreover, the 3D-MHF platform is simple to construct from low-cost, commercially-available components, which obviates the need for advanced microfabrication strategies necessitated by previous MHF nanoparticle synthesis platforms.

1. Introduction

Formed through the spontaneous assembly of amphiphilic lipid molecules dispersed in aqueous media, liposomes are biocompatible, highly versatile, micro- to nanoscale capsules that can be utilized for the storage and controlled release of a number of chemically-diverse compounds.[1-5] Potential biomedical applications for liposomes are extensive; however, current widespread pharmaceutical implementation has been limited by access to efficient and reliable manufacturing methods. Conventional techniques for liposome manufacture are known to rely on cumbersome bulk-scale post-processing strategies to yield bioavailable nanoscale products. Indeed, it is known that for successful clinical application, liposomal products should be uniformly small (20 nm-200 nm diameter) with low levels of polydispersity. These characteristics aid in promoting biological efficacy by avoiding immune cell recognition and

subsequent clearance, which is known to hinder efficient transport to the therapeutic target.[6,7] Several approaches for liposome synthesis have been investigated to improve upon conventional manufacture inadequacies; however, few have effectively demonstrated delivery of an on-demand clinically reliable product of sufficient throughput.

Incremental advancements in liposome manufacture have been demonstrated by our group and others to address synthetic throughput challenges *via* microfluidic hydrodynamic focusing (MHF), a technique that enables the one-step continuous-flow production of nanoscale vesicles using flow focusing of miscible fluids on microscale platforms.[1–5] In standard, or two-dimensional (2D) MHF for nanoliposome synthesis, a series of rectangular microchannels are engineered to impinge a center stream of alcohol-solvated lipid with one or more adjacent streams of aqueous buffer. As the aqueous streams meet with and laterally focus the miscible solvated lipid stream, the organic and aqueous phases interdiffuse producing a solvent composition in which the lipids are increasingly less soluble. This causes the lipids to self-associate into intermediate assemblies that eventually close on themselves into spherical liposomes.[5] The laminar flow profiles realized in MHF systems and their associated reproducible low Péclet mixing facilitate precise control over the magnitude and relative ratios of liposome flow inputs. As a result, MHF has been demonstrated to produce on-demand liposome preparations that are consistently more uniform than those resulting from conventional multistep manufacture strategies (*i.e.*, sonication[6,7] or bulk-scale alcohol injection[7–10]). Additionally, size control of vesicle preparations formed using MHF can be precisely tuned and easily automated using pre-programmed computer controlled flow inputs.

Applying the benefits of liposome synthesis provided by 2D-MHF towards widespread commercial manufacture is known to be limited by several practical aspects of the technique including: (i) inherent drag forces that affect symmetric flow focusing in rectangular microchannels, (ii) low throughput due to characteristically low microfluidic flow rates, and (iii) expensive, time-consuming microfabrication techniques necessary to develop the liposome manufacture devices. Here, we attempt to ameliorate these shortcomings by extending application of the technique to a facile, cost-effective annular coaxial flow system composed of an array of commercially available capillaries that enables three-dimensional (3D) fluid focusing for liposome synthesis. In 2D-MHF, the horizontal surfaces of the rectangular microfluidic channels are wetted with the soluble lipid stream (bearing a no-slip boundary condition), which causes the organic stream to migrate further from the channel center and retard lipid convection to the mixing interface. In the coaxial 3D approach introduced here, there are no surfaces present in the interfacial mixing region wetted by the organic phase and, thus, fluidic lipid elements avoid and are unaffected by a no-slip boundary condition. As a result, complete radially symmetric mixing of the fluidic inputs is realized, facilitating the production of highly uniform nanoscale liposomes manufactured at previously unfathomable rates.

2. Materials and methods[1]

2.1. Microfluidic device fabrication

Concentric capillary arrays were assembled from a stock capillary array (World Precision Instruments, Sarasota, FL) (Figure 1). The multibarrel capillary contains seven identical borosilicate glass capillaries, each possessing an inner diameter of 0.58 mm, an outer diameter of 1.0 mm, and are collectively fused in a circular pattern with an equivalent outer diameter of 3 mm. The multibarrel capillary array is 152 mm in length, but can be sectioned into smaller pieces for a device assembly. Poly(ether ether ketone) (PEEK^{TM}) tubing (510 μm outer diameter (OD), 65 μm inner diameter (ID) unless otherwise specified) (Upchurch Scientific, Inc., Oak Harbor, WA) served as the solubilized lipid feed line and was threaded through the center of the multicapillary array. The lipid feed line was connected through minitight fittings (Upchurch Scientific, Inc.) to a glass Gastight® syringe (Hamilton, Reno, NV) that delivered the lipid solution. The solubilized lipid infusion was controlled by a programmable syringe pump (Harvard Apparatus Inc., Holliston, MA). Polyvinyl chloride (PVC) tubing (3.96 mm ID) (Cole-Parmer Instrument Co., Veron Hills, IL) was used as the support line for the extra-annular aqueous sheathing flow. A continuous supply of aqueous sheath flow was delivered by a quaternary pump (Agilent Technologies, Santa Clara, CA) at programmable volumetric flow rates. The junction between the lipid feed line and multicapillary device was sealed on the downstream end using UV-curable epoxy (NOA81) (Norland Products Inc., New Brunswick, NJ).

2.2. Lipid mixture and buffer preparation

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG2000) (Avanti Polar Lipids Inc., Alabaster, AL) were dissolved in chloroform (Mallinckrodt Baker Inc., Phillipsburg, NJ) in a molar ratio of 61:30:9 DPPC:cholesterol:DSPE-PEG 2000. The lipid mixture was prepared in glass scintillation vials, evaporated, and then placed in a vacuum desiccator for at least 24 h to ensure complete chloroform removal. The dried lipid mixtures were then dissolved in anhydrous ethanol (Sigma Aldrich) with 1 wt % of a lipophilic membrane dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C_{18}) (Life Technologies, Carlsbad, CA), Carlsbad, CA)) for a total lipid concentration of 10 mmol/dm^3. A 1x Phosphate Buffered Saline (PBS) (Sigma Aldrich) solution at pH 7.4 was used as the buffer. All fluids (solvent and buffer) were passed through 0.22 μm filters (Millipore Corp., New Bedford, MA) before being introduced to the microfluidic device.

2.3. Microfluidic liposome synthesis

[1] Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

2.3.1. Synthesis of liposomes using the 3D-MHF annular device

Liposomes were prepared *via* 3D-MHF by injecting an ethanol-lipid solution into the central intra-annular PEEK tubing line and 1x PBS into the extra-annular PVC line to generate an aqueous outer sheath. For typical operation, a 65 μm ID intra-annular lipid-ethanol feed line was used and placed at a 5 mm protrusion length from the face of the multicapillary. The buffer volumetric flow rate was set to 5 mL/min and the flow rate ratio (FRR), or the ratio of the volumetric flow rate of buffer to the volumetric flow rate of solvent, was set to 5000:1. This condition yields an alcohol concentration of 0.02% and a corresponding lipid concentration of 2 umol/dm^3 at the mixing interface.

2.3.2. Synthesis of liposomes using the 2D-MHF planar device

Liposomes were synthesized using the microfluidic flow focusing method as described previously.[1–5] Briefly, a lipid-ethanol mixture (10 mmol/dm^3 lipid) was injected between two aqueous buffer inlets (1x PBS) into a PDMS- glass microfluidic device with channel dimensions of 300 μm tall by 50 μm wide. The FRR was set to 10:1. This condition yields an alcohol concentration of 10% and a corresponding lipid concentration of 1 mmol/dm^3 at the mixing interface. The linear flow velocity of the total flow for all FRRs was kept constant (0.11 m/s) for a volumetric flow rate of 100 μL/min.

2.4. Asymmetric flow field-flow fractionation (AF^4) with multi-angle laser light scattering (MALLS) and quasi-elastic light scattering (QELS)

Liposomes manufactured using MHF techniques were analyzed using Asymmetric Flow Field-Flow Fractionation (AF^4) paired to Multi-angle Light Scatter (MALS) and Quasi-elastic Light Scattering (QELS) detection (Wyatt Technology, Santa Barbara, CA). A vendor-supplied spacer (250 μm thickness) was used to house a 10 kDa molar mass cut-off regenerated cellulose membrane for the separation (Millipore, Bedford, MA). Flow was controlled using Eclipse 2 software (Wyatt Technology). A sample volume of 500 μL was injected at a flow rate of 0.1 μL/min while focusing at 1.5 mL/min for 5 min. The injection step was followed by a second focusing step of 1.5 mL/min for 5 min. The crossflow was ramped linearly from 0.2 mL/min to 0 mL/min over 30 min while eluting the particles at 1 mL/min. Light scattering data was collected online using a simultaneous MALS and QELS detection scheme, followed by conversion into particle size and size distributions using vendor-supplied software (ASTRA®, Wyatt Technology). Static light scattering intensity (λ=690 nm) was measured at 15 angles simultaneously. Scattering data was collected at 1 s intervals by MALS and 5 s intervals by QELS. An autocorrelation function of the QELS was fitted to a single-mode exponential decay model to yield the hydrodynamic radii of the liposomes. A coated sphere model (*i.e.,* a spherical structure with two radial regions of dissimilar refractive index) was used to convert the MALS-determined radius of gyration to a relevant geometric radius for liposome size approximation.

The differential of each fractionated liposome sample produced a monomodal Gaussian distribution, indicating monodisperse particle populations.

2.5. Computational Fluid Dynamics (CFD) simulation of ethanol-water concentration profile

A computational fluid dynamics simulation was developed to illustrate the difference in the ethanol-water concentration profiles between the two distinctive microfluidic device geometries (annular versus planar). The concentration profile of a center stream of ethanol focused by an exterior sheath of water was represented in a three-dimensional model created using COMSOL Multiphysics 4.2 software (COMSOL Inc., Burlington, MA). The non-linear relationship for ethanol-water of the mutual diffusion coefficient (D) to ethanol mole fraction (X_E) was estimated using Eq. 1, which was derived from experimental values.[11] Hydrodynamic flow focusing of a radial system and a rectangular system were analyzed using the simulation to illustrate the difference in diminishing ethanol mole fraction within the two microchannel architectures.

$$D[m^2 s^{-1} \cdot 10^{-9}] = 11.22{X_E}^5 - 24.11{X_E}^4 + 12.27{X_E}^3 + 6.45{X_E}^2 - 5.88X_E + 1.42 \quad (1)$$

For the comparison, the FRR in the simulation was set to 100:1 with total volumetric flow rate of 5.0 mL/min for the annular device and 90 μL/min for the planar device (corresponding to a linear flow velocity of 0.2 m/s for both devices). The simulations do not account for the viscosity of the mixtures, with the assumption that it does not have a critical effect on the resulting concentration profile for the experimental range investigated (*vide infra*).

3. Results and Discussion

The 3D-MHF system consisting of a concentric capillary array is depicted in Figure 1. Liposomes of tunable size are formed through continuous injection of an alcohol-soluble lipid solution into a central feed line that is radially sheathed by external aqueous buffer. As soluble lipids from the alcohol stream controllably diffuse into the aqueous stream, lipids self-assemble until they collapse into liposomes.[5] The magnitude of flow focusing and the mixing geometry at the interface between these two fluids play determinate roles in the size and size distribution of the resultant liposome products.

3.1. Interfacial Mixing Geometry Affects Liposome Particle Size Characteristics

Liposome preparation *via* MHF has been previously demonstrated in planar microfluidic systems, where microchannel depth-to-width aspect ratio was determined to significantly impact the size characteristics of the resultant liposomal products.[4] It is known that hydrodynamic (or parabolic) flow in a rectangular channel experiences a non-uniform velocity profile across the vertical plane due to no-slip boundary conditions of flow streams sandwiched between the walls

of the device. This is shown by the numerical CFD simulation in Figure 2A. In a controlled-flow particle assembly system like that required for nanoscale liposome formation, these so-called "edge effects" induce asymmetric mixing at the interface between the aqueous sheathing fluid and the solubilized lipid at the point of particle formation, which can contribute to an increase in particle size polydispersity.[12] These effects can be partially alleviated if the microchannel depth-to-width aspect ratio becomes increasingly high (*i.e.*, exceeds five); however, the feasibility of engineering rectangular microfluidic devices of increasing aspect ratio become increasingly more difficult and expensive.[13] Strategies for three-dimensional hydrodynamic focusing in planar microchannels have been proposed,[14–16] however, those demonstrated involved piece-wise focusing which, if applied to liposome synthesis, could result in delayed mixing of the lipidic input with the aqueous solvent to a point downstream from the initial interaction of the two fluids. Since liposome assembly is thought to occur at the onset of the alcohol-water interface in these systems[4], instantaneous hydrodynamic focusing at the point of particle formation is required for controlled vesicle assembly.

The 3D-MHF device presented in this investigation enables an important modification in the mixing condition at the miscible fluid interface when compared to the planar 2D-MFH device (Figure 2B). Here, the lipid input is entirely encapsulated in the annular sheathing fluid, resulting in radially symmetric mixing and subsequent assembly of liposome populations with previously unforeseen size uniformity (see Section 3.3). Comparative simulation data of interfacial ethanol concentration from the 2D- and 3D-MHF device geometries at a distance 150 μm downstream from the fluid mixing interface are displayed in Figure 2. Differences are observed regarding the presence of alcohol at the channel wall when comparing the two techniques. Numerical simulation of the planar device flow shows that a significant fraction of alcohol (≈0.4 mole fraction to 0.6 mole fraction) interacts with the device wall beyond the mixing interface. However, in the 3D device, the alcohol concentration is completely depleted in the region approaching the device boundaries. As a result, the annular flow format facilitates rapid, radially symmetric diffusion of alcohol components into the buffer phase, which ultimately results in the assembly of monodisperse populations of lipid nanoparticles. It should be noted that microfluidic coaxial flow systems have been explored previously for various particle synthesis applications, including the generation of emulsions[17–19] and the formation of photopolymerizable structures[12], but never for the formation of nanoscale liposomes. Here, we apply the benefits realized by coaxial flow towards controlled and efficient liposome assembly in a process suitable for scale-up to satisfy relevant commercial manufacturing demands.[20]

Representative liposome preparations made by MHF using the planar device (red trace) versus the annular device (blue trace) are shown in Figure 3. Flow conditions were chosen such that the average liposome radius generated by each device was similar for comparison (51 nm for 2D-MHF versus 53 nm for 3D-MHF). The annular flow device shows an improvement in optimal liposome size uniformity relative to the planar microfluidic device (Figure 3). This observation can be quantified from the particle size polydispersity index (PDI), defined as the ratio of the

square of the standard deviation of particle size to the square of the mean diameter, a normalized measure of the size distribution.[21] Interestingly, the average PDI of the resultant liposome suspensions decreases by half (0.083 to 0.044) when comparing the planar microfluidic to the annular microfluidic method. Under some flow conditions, the annular flow platform was observed to produce liposomes with PDIs as low as 0.007 (*vide infra*, Figure 5, red trace). From a process manufacturing perspective, the 3D-MHF annular flow system maintains significant advantages over its planar flow counterpart, where it has been demonstrated to reliably produce uniform nanoscale vesicles up to a rate of 10^9 liposomes/min (final lipid concentration of approximately 4 μmol/L) versus the optimized operational parameters of the planar device which yield liposomes at a lower rate 10^5 liposomes/min (final lipid concentration of approximately 400 μmol/L).

3.2. 3D-MHF Device Design Parameters Affect Liposome Size and Polydispersity

The effect of intra-annular capillary orifice size on the resultant liposome size characteristics was investigated under fixed flow conditions. The dimension of the capillary orifice is related to the effect of microchannel size and aspect ratio in the planar MHF.[1-4] It was observed that a reduction in intra-annular capillary ID from 255 μm to 125 μm to 65 μm correlated with resultant average particle radii of 72 nm, 63 nm, and 53 nm, respectively (Figure 4). Here, a fixed applied volumetric flow through a smaller orifice will generate a greater linear velocity of the soluble lipid stream and subsequent focusing condition. This, in turn, reduces the total diffusion distance of lipids from their solvated state in alcohol to the aqueous buffer where they accumulate to form vesicles. Although a decrease in average vesicle radii was observed with a reduction in intra-annular capillary ID, there was no discernible effect on the resulting size distribution. The PDIs were uniformly monodisperse at 0.02, 0.04, and 0.03 for the 65 μm, 125 μm, and 255 μm ID intra-annular capillaries, respectively.

The effect of the protrusion distance of the intra-annular lipid feed line beyond the exit of the extra-annular sheathing fluid was also investigated at fixed flow conditions. Here, the intra-annular capillary was positioned at 0 mm from the multicapillary outlet (flush to the multicapillary face) and then at a length 5 mm beyond the outlet. Resultant liposome particle size was found to be partially linked to the extension of the intra-annular lipid feed line into the aqueous sheath. At a 0 mm capillary protrusion length, the average liposome size was 96 nm with a PDI of 0.030 compared to the 5 mm protrusion length where the average liposome size was 53 nm with a PDI of 0.007. The difference in particle size and distribution for the two device constructs demonstrates that there is a critical distance from the inlet, Z_e, after which the flow is fully developed yielding conditions for systematic particle assembly.[22] This distance can be calculated using Eq. (2) for $1 < Re < 100$:[22]

$$Z_e = (0.619 + 0.0567Re)D_H \quad (2)$$

where *Re* represents the Reynolds number, which for pipe flow is :[23]

$$Re = \frac{QD_H}{\nu A} \quad (3)$$

Here, Q is the volumetric flow rate, A is the cross-sectional area of the annulus, ν is the kinematic viscosity (assumed to be that of water at room temperature) , and D_H is the hydraulic diameter of the pipe, which is defined as $D_{(outer)}$ - $D_{(inner)}$ (3.46 mm).[23] The required length that the intra-annular capillary must reside distal to the multicapillary outlet of the 3D-MHF device was calculated to be 6.7 mm at a typical operational volumetric flow rate of 5 mL/min. The 5 mm protrusion length used in these experiments approaches this distance, which within placement error, enables for a platform where the sheath flow is nearly fully developed before particle formation to result in the production of uniformly small liposomes.

3.3 3D-MHF Device Operational Flow Parameters Affect Liposome Size and Polydispersity

The magnitude of the ratio of sheathing buffer to soluble lipid flow rate (FRR) and total flow rate of MHF systems have been determined to play significant roles in liposome particle size characteristics. Previous work using planar (2D) microfluidic platforms have demonstrated a relationship between liposome size and polydispersity linked to the degree of focusing experienced by the central lipid feed line. 2D-MHF demonstrated that a greater magnitude of focusing, or higher FRR, the smaller and more uniform the resultant liposomal populations were.[1–5]

To investigate the effect of flow focusing on liposome assembly in the 3D-MHF system, the flow rate ratio (FRR) of buffer to alcohol-soluble lipid was varied from 500:1, 1000:1, and 5000:1 and the resulting liposome populations were analyzed by light scattering. As the FRR of sheathing buffer to alcohol-solubilized lipid increased from 500:1 to 1000:1 to 5000:1, the resulting average size of liposomes decreased from a radius of 66 nm to 56 nm to 53 nm, respectively (Figure 5). The trend observed here correlates with that from previous planar device syntheses and is likewise attributed to the increasing degree of focusing experienced by the center stream of solubilized lipid.

To investigate the effect of total flow rate on liposome production, 3D-MHF devices were operated with the volumetric flow rate of the sheathing buffer at 1 mL/min, 2 mL/min, and 5 mL/min (*Re* = 4.7, 9.4, and 23.6, respectively). In these studies, a device with 65 μm ID PEEK tubing extending 5 mm from the multicapillary interface was used and FRR was fixed at 5000:1. The average particle size of the resulting liposomes decreased as the input flow rate increased. Indeed, as the total flow rate of the system increased from 1 mL/min to 2 mL/min to 5 mL/min, the resulting liposome average particle sizes are reduced from 88 nm to 80 nm to 70 nm.

Although all flow parameters investigated are laminar and significantly below the transition threshold into the turbulent flow regime (an inherent characteristic of most microfluidic systems) a modest increase in *Re* appears to expedite lipid accumulation and subsequent assembly into smaller particles. Notably, an increase in buffer flow rate does not play a role in particle size polydispersity, likely due to a fixed alcohol concentration (fixed FRR) at the mixing interface which can stabilize of liposomal intermediates prior to ordering into spherical vesicular structures.[24]

The simplicity of the 3D-MHF platform assembly (handmade from common laboratory supplies) has made it such that there are likely subtle device-to-device variations. This could explain minor differences observed for liposome particle size data from two different 3D-MHF platforms operated at FRR = 5000:1 (FRR investigation and investigation of total flow) which yielded average particle sizes of 53 and 70 nm. Notably, analysis of the relative changes of liposome particle size as a function flow input parameters for experiments conducted on the same device trend reliably and in agreement with previous investigations using 2D-MHF.[1-5]

4. Conclusion

Microfluidic systems enable the production of monodisperse liposome suspensions whose sizes may be adjusted by controlling the device flow input parameters. Here, we demonstrate the utility of three-dimensional microfluidic hydrodynamic focusing for the rapid manufacture of nanoscale liposomes. 3D-MHF was achieved through the use of a facile and cost-effective concentric capillary array. The device enables the continuous-flow synthesis of nanoscale liposomes with unprecedentedly low levels of polydispersity. Relationships between device constructs and process parameters were investigated and observed to affect the size and polydispersity of resultant liposomes. 3D-MHF provides a platform for the on-demand, reproducible production of liposomes of tunable size without the need for any post-formation modifications to achieve nanoscale size characteristics essential to a host of commercial applications at rates relevant for industrial manufacture.

Figures

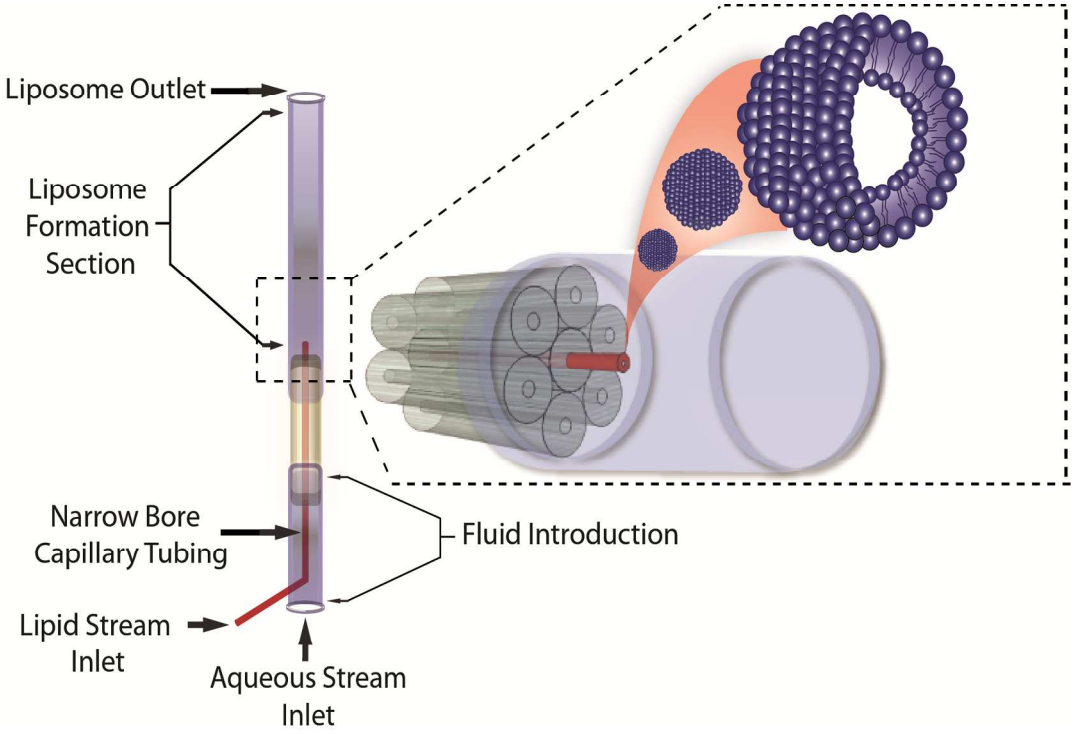

Figure 1: Schematic of 3D-MHF liposome formation device. Narrow bore capillary tubing is secured by a glass multicapillary array which serves to precisely center the intra-annular flow stream in the concentric exterior coaxial flow stream. For liposome synthesis, an alcohol-solubilized lipid solution is continuously injected into the intra-annular capillary tubing and hydrodynamically focused in three-dimensions by an exterior sheath flow of aqueous buffer. Not to scale.

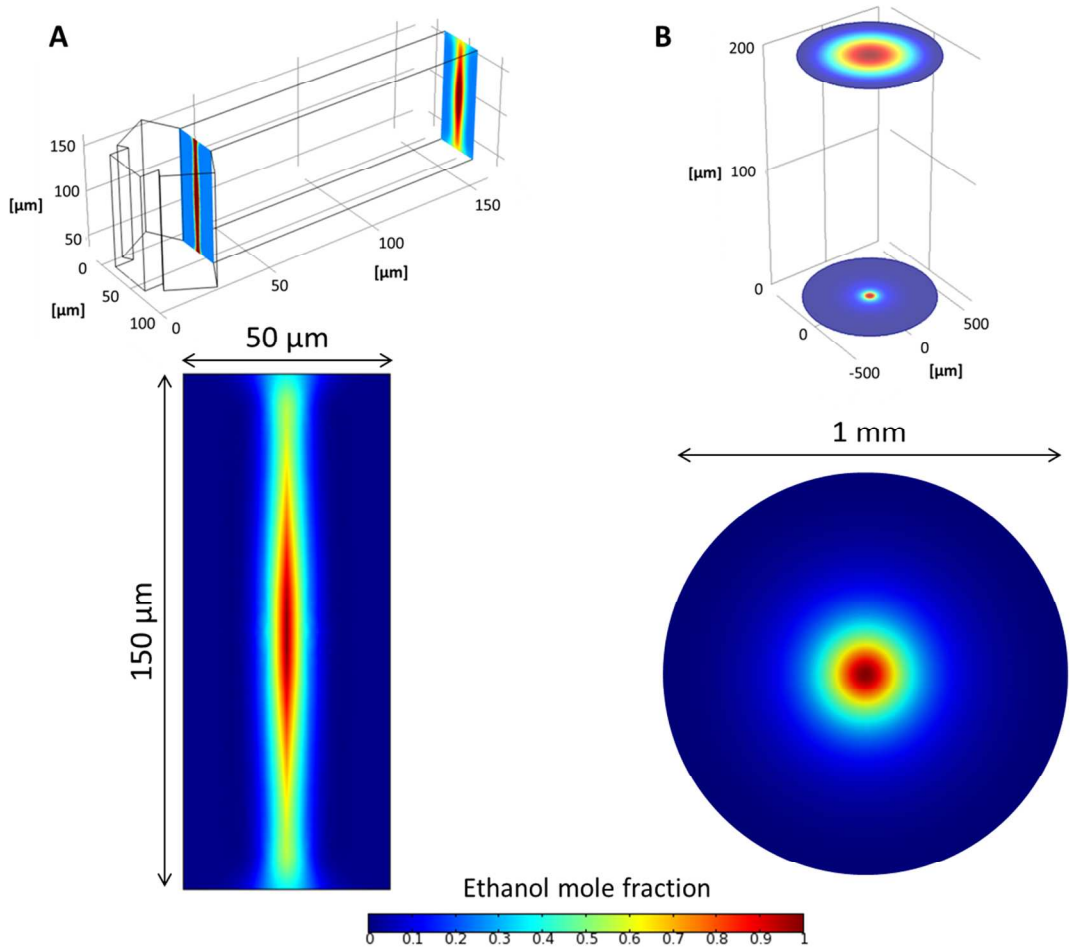

Figure 2: CFD simulation of ethanol concentration in (A) 2D-MHF vs. (B) 3D-MHF device. FRR set to 100:1 with linear flow velocity 0.2 cm/s (corresponding to a volumetric 5.0 mL/min, planar Q_{tot}=90 µL/min). Cross-sectional concentration profiles represent sections 150 µm downstream of the initial buffer-ethanol interface for both devices.

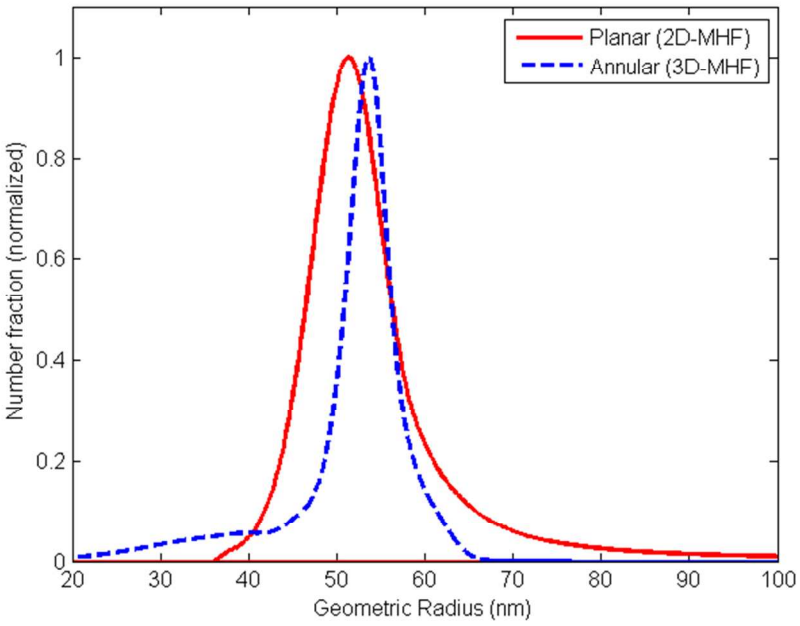

Figure 3: Comparison between 2D- and 3D-MHF liposome manufacture platforms. 2D-MHF experiments were carried out in rectangular microchannels with a 6:1 aspect ratio at an FRR = 10:1 and a total volumetric flow of 200 μL/min. 3D-MHF experiments were carried out in a device with a 65 μm intra-annular ID lipid feed line at an FRR = 5000:1 and a total volumetric flow of 5 mL/min. Average liposome radius for the 2D-MHF device was 51 nm (PDI =0.083) and 53 nm (PDI = 0.044) for the 3D-MHF device.

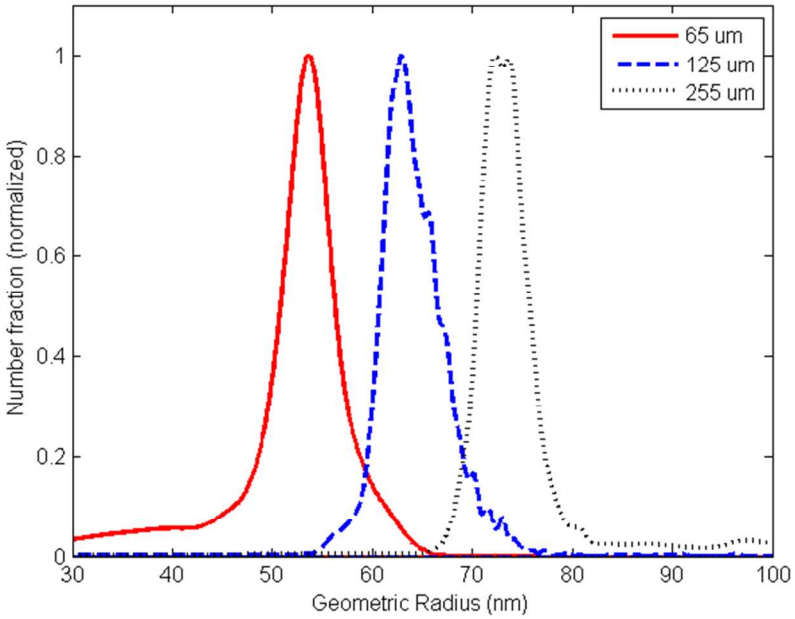

Figure 4: Effect of intra-annular orifice of lipid feed line on resultant liposome size characteristics using the 3D-MHF device. Volumetric flow rate and flow rate ratio of fluidic inputs are fixed at FRR= 5000:1 with a total flow rate of 5 mL/min. A reduction in lipid feed line orifice results in a decreased-size average liposome size, due to the increased focusing condition. Average liposome radius was 53 nm (0.044 PDI) for 65 μm ID, 63 nm (0.040 PDI) for 125 μm ID, and 72 nm (0.030 PDI) for 255 μm ID.

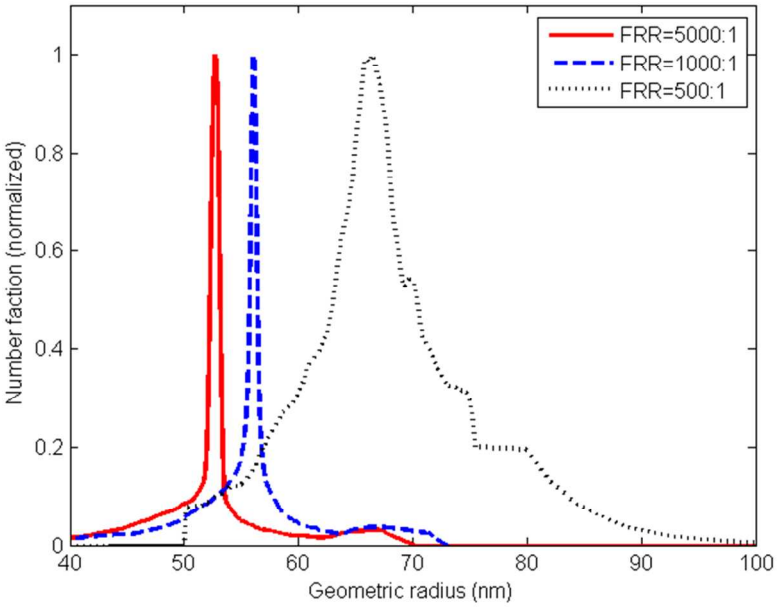

Figure 5: Effect of flow focusing on liposome size using 3D-MHF. Total volumetric flow rate was fixed at 5 mL/min. Device had a 65 µm intra-annular capillary ID. Average liposome radius was 53 nm (0.007 PDI) at FRR = 5000:1, 56 nm (0.005 PDI) at FRR= 1000:1, and 66 nm (0.047 PDI) at FRR = 500:1.

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