High-Throughput Continuous Flow Production of Nanoscale Liposomes by Microfluidic Vertical Flow Focusing

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Liposomes represent a leading class of nanoparticles for drug delivery. While a variety of techniques for liposome synthesis have been reported that take advantage of microfluidic flow elements to achieve precise control over the size and polydispersity of nanoscale liposomes, with important implications for nanomedicine applications, these methods suffer from extremely limited throughput, making them impractical for large-scale nanoparticle synthesis. High aspect ratio microfluidic vertical flow focusing is investigated here as a new approach to overcoming the throughput limits of established microfluidic nanoparticle synthesis techniques. Here the vertical flow focusing technique is utilized to generate populations of small, unilamellar, and nearly monodisperse liposomal nanoparticles with exceptionally high production rates and remarkable sample homogeneity. By leveraging this platform, liposomes with modal diameters ranging from 80 to 200 nm are prepared at production rates as high as $1.6 \text{ mg min}^{-1}$ in a simple flow-through process.

1. Introduction

Nanoscale unilamellar lipid vesicles (liposomes) represent the most clinically advanced class of nanoparticle drug carrier,[1] with over ten liposomal drugs approved for clinical use[2] and more than 30 additional liposomal therapeutics currently in the pipeline.[3] Due to their ability to encapsulate hydrophilic drugs within their aqueous cores and lipophilic compounds within their lipid bilayers while supporting tailored surface chemistries for targeted delivery and extended blood circulation time, liposomes can serve as versatile and multifunctional nanoparticles for numerous drug delivery applications.[1,4] Liposomal drugs have been shown to increase therapeutic index while minimizing toxicity compared to the use of free drugs,[5] with significant applications in the treatment of cancer[6–10] and a variety of other pathologies.[11–15] Liposomes also offer notable advantages for vaccine development,[16] biosensors,[17] and diagnostic imaging.[18,19]

Conventional liposome preparation is based on batch methods such as film hydration,[20,21] reverse-phase evaporation,[22,23] detergent depletion,[24] electroformation,[25] crossflow injection,[26] or alcohol injection,[27–29] and typically combined with postprocessing steps such as membrane extrusion[27] or sonication[29] to reduce the size and polydispersity of the final liposome populations.[30] These steps are generally performed in discrete vessels under manual operation, resulting in cumbersome, time-consuming, and labor intensive processes that require significant effort to scale from benchtop production to larger volume batches. Overall, liposome technology remains constrained by the challenges of scaling conventional preparation methods to produce large quantities of liposomes in a controlled, reproducible manner.[31] The development of new liposomal nanomedicines would greatly benefit from novel synthesis methods supporting robust and reproducible generation of liposomes across the full range of production scales.

Microfluidic technologies including pulsed jetting,[32–34] droplet emulsion transfer,[35–38] and electroformation[39] have
been explored as alternatives to bulk-scale liposome production,[40] but these methods are low throughput and generally produce liposomes that are too large and polydisperse for drug delivery applications.[41] In contrast, microfluidic hydrodynamic focusing (MHF) is a simple technique for nanoscale liposome formation that provides several advantages compared with both conventional nanomanufacturing and other microfluidic methods.[42–45] In MHF-directed liposome production, a center fluid stream of alcohol and dissolved lipid is sheathed by two oblique streams of aqueous buffer enabling controlled diffusive mixing over length scales on the order of several hundred nanometers. The controlled codiffusion of lipid, alcohol, and aqueous buffer results in the self-assembly of unilamellar lipid vesicles of remarkably consistent size that may be adjusted by changing the ratio of buffer to alcohol volumetric flow rates.[42–45] Whereas batch liposome preparation involves turbulent flows in a sequence of large fixed-volume reactors, microfluidic flow-focusing takes advantage of controllable laminar flow streams and short diffusion length scales in a small-volume process enabling continuous-flow synthesis of liposomes, with small and uniform liposomes achieved without the need for postprocessing steps to decrease the size or polydispersity of the resulting sample.

We have previously demonstrated that the MHF process supports the formation of nanoscale liposomes with tunable physiochemical properties[46] and online remote drug loading for highly efficient encapsulation of therapeutics for single-step preparation of liposomal nanomedicines,[47] and have leveraged the exquisite vesicle size control enabled by the MHF process to investigate size-dependent in vitro[48] and ex vivo[49] behaviors of microfluidic-enabled liposomes. Despite this progress, MHF remains a low-throughput process due to the limited volumetric flow rates supported by typical microchannel geometries, thus constraining its potential for use in full preclinical studies, clinical trials, and clinical applications where larger volumes and higher concentrations are required. Liposome throughput may be increased through the use of a multibarrel capillary flow focusing technique that can operate at higher flow rates than planar MHF devices,[50] but the large volumetric flow rates required for the aqueous sheath flow in the capillary platform result in significant liposome dilution. Alternately, the use of multiple microfluidic flow focusing elements operating in parallel has been explored for the formation of polymer nanoparticles.[51] However, it is desirable to minimize the degree of parallelization since slight variations in channel geometry and flow resistance across the flow focusing elements can degrade sample homogeneity. Furthermore, large numbers of parallel microfluidic elements can have a negative impact on the overall reliability of the continuous-flow system.

Here we report on a microfluidic vertical flow focusing (VFF) device possessing exceptionally high channel aspect ratios, and explore the ability of the high aspect ratio system to enhance the throughput of liposome synthesis. Unlike MHF devices, where the lipid stream is focused by two aqueous buffer channels located in the same plane as the lipid channel, the VFF approach takes advantage of a multilayer thermoplastic fabrication process to create a vertical stack of wide but shallow channels. The fabricated devices possess focusing channels with aspect ratios as high as 100:1, resulting in wide and thin sheets of lipids during the focusing process. The high aspect ratio system is capable of enhancing the throughput of microfluidic liposome synthesis while simultaneously reducing polydispersity of the resulting liposome populations by diminishing the impact of no-slip boundary conditions at the edges of the focusing zone. The aspect ratio of the focusing zone is limited only by the available substrate surface area, without requiring complex and costly fabrication processes typically used to achieve large out-of-plane microchannel aspect ratios.

The VFF platform is demonstrated here for the preparation of highly uniform liposomes with tunable size ranging from 80 to 200 nm at unprecedented rates of nearly 100 mg h⁻¹, using identical physics and focusing parameters as its planar counterpart. The platform offers a highly promising route toward the rapid, reliable, and automated preparation of microfluidic-enabled liposomes in sufficient quantities to support large scale in vivo experiments, preclinical and clinical studies, and pilot-scale synthesis. Using a scale-out approach, the platform may further support industrial production of liposomal drugs with minimal parallelization using small groups of VFF devices operating in parallel.

2. Results

2.1. VFF Device Fabrication

The differences in device topology between MHF and VFF device designs can be seen in Figure 1a. While rotating the focusing axis allows extremely large aspect ratios to be achieved with simple fabrication techniques, the design introduces the need for a multilayer structure comprising three stacked channels separated by thin layers of thermoplastic that serve to isolate the fluid streams before merging at the inlet to the focusing channel. The use of low-distortion, low-temperature solvent bonding combined with prepositioned alignment pins enabled the formation of a precisely aligned multilayer thermoplastic device hosting features with remarkably low aspect ratios of 1:100 (Figure 1b). Accurate alignment of the upper and lower buffer inlet routing channels was visually confirmed under a microscope. The liquid-phase solvent bonding conditions were consistent throughout the fabrication process, resulting in a uniformly sealed multilayer device that remained unaffected with no signs of delamination throughout all experiments.

2.2. Liposome Size Control

Comparisons between the populations of liposomes generated through MHF versus VFF reveal that both devices produced narrowly distributed populations of liposomes with the ability to control liposome size by altering the degree of lipid focusing through adjustment of the applied flow rate ratio (FRR), defined as the aqueous buffer volumetric flow rate divided by the lipid/solvent volumetric flow rate (Figure 2). As expected, modal diameter of the microfluidic-generated
liposomes was observed to scale inversely with FRR, and this was observed for all devices regardless of the aspect ratio, with a minimum modal diameter of \( \approx 80 \) nm achieved for the given experimental flow conditions.

The size distributions of liposomes generated via VFF and MHF were also compared. Low polydispersity was maintained for all FRRs studied within the high aspect ratio VFF device, although a slight increasing trend in polydispersity index (PDI) was observed for FRR values above 40 (Figure 3). In contrast, the MHF devices generated liposome populations with consistently higher values of PDI at each FRR tested, and with significantly higher polydispersity observed when FRR was increased to its maximum value of FRR = 100.

Numerical simulations were performed to elucidate the relationships between flow conditions and the resulting velocity profile and solvent distribution during flow focusing within each microfluidic device (Figure 4). The simulations reveal that the impact of the no-slip boundary condition, which imposes zero velocity at the channel walls, diminishes as the aspect ratio increases (Figure 4a). The relationships between flow conditions and alcohol concentration within the microchannel (Figure 4b) show that for the VFF design, the alcohol concentration reaches a peak at the side walls then remains uniform across \( \approx 94\% \) of the channel width, while both aspect ratios of MHF investigated showed much larger variation in ethanol concentration.

### 2.3. Liposome Production Rate

The use of initial lipid concentrations up to 80 mmol L\(^{-1}\) was investigated to assess the maximum concentration suitable for use with the DMPC/DCP/cholesterol system, with measured modal diameters and PDI values presented in Figure 5. As revealed in this figure, increasing the initial lipid concentration, and thus the final volumetric fraction of liposomes, does not impose significant changes to the resulting liposome populations as the concentration increases from 5 mmol L\(^{-1}\) to 40 mmol L\(^{-1}\), while further increases in lipid concentration beyond 40 mmol L\(^{-1}\) result in large increases to both modal diameter and PDI.

Maximum liposome production rates for both VFF and MHF devices were also investigated and compared with each other together as well as a recently reported 3D-MHF multibarrel capillary flow focusing method. In each case, flow conditions were selected to yield liposome populations with nominal modal diameters of 100 nm. A summary of flow conditions used in each system is provided in Table 1.
A moderate initial lipid concentration of 20 mmol L$^{-1}$ was chosen for use in all experiments. As seen from the resulting throughput data in Figure 6, the VFF system enables the most rapid generation of liposomes across the three methods.

Experimental relationships between flow velocity and vesicle size distributions were analyzed (Figure 7). Overall the liposome size distributions remain constant over the full range of flow velocities, with less than 5% variation in modal diameter for flow velocities between 10 and 30 cm s$^{-1}$. Over this same velocity range, the PDI exhibited a consistent but small trend toward increasing polydispersity with higher flow rates.

**2.4. Liposome Stability**

In addition to vesicle size distribution and production rate, the stability of liposomes generated through the VFF process was also evaluated through light scattering measurements following extended incubation. Samples were characterized immediately after initial liposome formation and again at a time point 7 months post formation, with liposomes stored in glass vials.
Figure 4. Numerical simulations for each flow focusing system illustrating a) fluid velocity profiles and b) ethanol mole fraction across the channel centerline transverse to the focusing axis at a position 300 μm past the start of the focusing region for FRR = 20. As the aspect ratio increases, both flow velocity and ethanol concentration become more uniform across the width of the channel. Each profile is normalized to the corresponding channel height and flow velocity or ethanol concentration, respectively, at the channel midpoint.

Table 1. Flow focusing parameters used for comparative evaluation of liposome production throughput in VFF, MHF, and 3D-MHF systems.

<table>
<thead>
<tr>
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<th>Average flow velocity [cm s⁻¹]</th>
<th>Bulk flow rate [mL min⁻¹]</th>
<th>FRR</th>
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<tr>
<td>VFF</td>
<td>30</td>
<td>4.5</td>
<td>30</td>
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<tr>
<td>3D-MHF</td>
<td>5</td>
<td>5.0</td>
<td>1000</td>
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<tr>
<td>MHF</td>
<td>12.5</td>
<td>0.113</td>
<td>30</td>
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at 4 °C during this period. The stored samples exhibited nearly identical size distributions as the original samples, with a 2% increase in modal diameter (Figure 8) and no appearance of large aggregates in the light scattering data, as further confirmed through cryogenic transmission electron microscopy (cryo-TEM) imaging of the incubated sample (Figure 9).

3. Discussion

3.1. VFF Device Fabrication

The microfluidic liposome formation process benefits from the use of high aspect ratio microchannels by reducing interactions between the focused lipid stream and the channel sidewalls which impose no-slip boundary conditions at the terminal edges of the focused stream, thus disturbing the velocity distribution, degree of focusing, and chemical species homogeneity at these regions.[52,53] In conventional planar microfabrication processes, patterning microscale features becomes increasingly difficult as the aspect ratio increases. Even when employing deep reactive ion etching or thick photore sist processing techniques, maximum aspect ratios are generally constrained to ≈20:1.[54,55] Furthermore, for the typical range of microchannel widths used in flow focusing devices, excessive substrate thickness on the order of several millimeters is required to achieve aspect ratios beyond this practical 20:1 limit. For the VFF devices described here, these limitations are overcome by orienting the focusing axis normal to the plane of the substrate, allowing the formation of high aspect ratio channels with large in-plane and small out-of-plane dimensions (Figure 1a). The use of alignment pins together with the liquid-phase solvent bonding process was found to be critical in enabling robust fabrication of the multilayer devices with no signs of delamination or device failure during operation.

3.2. Liposome Synthesis

Populations of liposomes were generated using both VFF and MHF devices to investigate the relationship between the focusing channel aspect ratio and the resulting liposome size distributions. All devices were designed with the same critical dimension of 50 μm for the flow focusing channel,

Figure 5. a) Modal diameters and b) PDI of liposomes produced through VFF under varying initial lipid concentration. Both size and polydispersity remain relatively constant for lipid concentrations up to $40 \times 10^{-3}$ M, while significant increases in both liposome size and polydispersity are observed at higher concentrations.
as measured along the focusing axis. While the planar MHF devices featured microchannel aspect ratios of 0.5:1 and 5:1, the VFF device was fabricated with an aspect ratio of 100:1.

Both MHF and VFF devices produced narrowly distributed populations of liposomes with the ability to control liposome size by altering the degree of lipid focusing through adjustment of the flow conditions. Modal diameter of the microfluidic-generated liposomes was observed to scale inversely with FRR, with vesicle size becoming insensitive to changes in flow rates for FRR values above 30. This behavior is consistent with previous studies of liposome production using planar MHF devices,[43–45] and was observed across all devices regardless of aspect ratio. Moreover, modal diameter did not significantly fluctuate between the varying platforms under the same flow conditions (<17%), which reveals repeatability within the MHF systems.

In addition to providing control over modal diameter, a key advantage of the microfluidic flow focusing process is the ability to generate liposome populations with narrow size distributions without the need for postprocessing steps, such as membrane filtration, to reduce polydispersity. The relative performance of the VFF platform toward producing vesicles with uniform size was quantified using the PDI for each liposome population. The PDI is a dimensionless parameter which in the case of a liposome population with a Gaussian size distribution is given by the square of the standard deviation of the distribution divided by the mean size. Size distributions with PDI < 0.1 are commonly referred to as monodisperse, while PDI < 0.2 indicates low polydispersity.

The high aspect ratio VHF platform generated liposomes with a lower PDI than the MHF platforms of lower aspect ratios at each FRR tested (Figure 3). The consistently lower PDI seen in VFF-generated vesicles is consistent with the hypothesis that higher aspect ratios result in more uniform fluid velocity across the width of the focusing and diffusive mixing region, thereby enabling the production of more narrowly distributed populations of liposomes.

Numerical simulations provide information on the relationships between flow conditions and the resulting velocity profile and solvent distribution during flow focusing within microchannels of varying aspect ratios (Figure 4). As the microchannel aspect ratio increases, the impact of the no-slip boundary condition which imposes zero velocity at the channel walls diminishes, and consequently the flow velocity becomes more homogenous throughout the mixing region (Figure 4a). As a direct result, nonuniformities in buffer and solvent distributions due to no-slip boundary conditions are significantly reduced in the high aspect ratio VFF system. This behavior can be seen in Figure 4b, which presents the normalized alcohol concentration across the focusing channel cross-section. The improvement in flow velocity and ethanol concentration uniformity are potentially a cause for the more uniform distributions of liposomes achieved within VFF devices.

The lipid system selected to demonstrate the capabilities of the VFF platform contained dihexadecyl phosphate (DCP), an anionic lipid that has been used to enhance lipid-based drug delivery performance in a number of recent in vivo studies including siRNA delivery,[56] topical drug delivery,[57] pulmonary delivery,[58] and brain targeting.[59] To confirm that the VFF process is not limited to charged lipids, a second
lipid system with DCP replaced by a neutral lipid, namely phosphatidylethanolamine (PE) linked to polyethylene glycol (PEG), was also explored. Liposomes functionalized with hydrophilic polymers such as PEG are in widespread clinical use due to their ability to enhance steric stability and in vivo residence times while in blood circulation.[5] A comparison of vesicle populations generated by VFF and MHF flow focusing using a lipid solution with PE-PEG is presented in Figure 10. In these experiments, the modal diameter of each population was found to be nearly identical when using the same FRR value, and the VFF device exhibited the most narrow size distribution, as expected. Overall, these results confirm that the VFF technique is suitable for scalable liposome production using a wide range of lipid systems.

3.3. Liposome Production Rate

While the ability to enhance liposome size uniformity through the use of high aspect ratio focusing channels is a valuable characteristic of the VFF technology, this study was primarily motivated by the need for higher throughput and scalable synthesis of liposomes for applications in nanomedicine. As an initial step to exploring the throughput limits of the VFF platform, we first sought to enhance vesicle production rate by increasing the initial concentration of lipids introduced into the flow focusing chip. Previous studies of microfluidic-enabled liposome production using MHF devices have limited the starting lipid concentration to 5 mmol L$^{-1}$ to ensure that all lipids remain well below their solubility limits and do not form aggregates prior to vesicle synthesis.[42–45] Here we demonstrate that under the particular flow conditions, microchannel dimensions, and lipid chemistry used in this study, an initial lipid concentration as high as 40 mmol L$^{-1}$, or eight times higher than prior MHF studies, may be used to enhance the final liposome concentration without impacting the quality of resulting vesicles.

By combining the ability of the high aspect ratio platform to support higher bulk flow rates than conventional planar MHF chips with increased initial lipid concentration, liposome production rates for both VFF and MHF devices were examined. Additionally, the vertical focusing method was also compared to a recently reported multibarrel capillary flow focusing system termed 3D-MHF in which a central stream of solvated lipids is sheathed by an annular flow of aqueous buffer that provides axisymmetric focusing within a coflow tubing system[50] (Table 1). The VFF method produces ≈100 nm liposomes at a rate of 95 mg h$^{-1}$ lipid (Figure 6), far exceeding the production rate of its counterparts.

The corresponding liposome production rate was determined through NanoSight particle tracking analysis and compared with a theoretical estimate of liposome production assuming 100% yield with the known lipid content as determined from,[60]

$$R = \frac{QcN_Aa}{4\pi[(n_0)^2 + (n_e - e)^2]}$$

where $R$ is the liposome production rate (liposomes per second), $Q$ is overall flow rate (m$^3$ s$^{-1}$), $c$ is the final lipid

Figure 8. Assessment of liposome stability from populations generated through VFF. Negligible change in modal diameter and size distribution was observed after a 7 month incubation at 4 °C.

Figure 9. a) Wide-field and b) magnified and contrast-enhanced cryo-TEM images of liposomes prepared via VFF after a 7 month incubation at 4 °C, revealing intact unilamellar vesicles with negligible deterioration or aggregation despite their high concentration and long incubation time.

Figure 10. Size distributions of PEGylated neutral DMPC liposomes produced using both MHF and VFF devices at FRR = 30. The resulting liposome populations each yield a modal diameter of ~50 nm, while the VFF case exhibits the lowest measured polydispersity (PDI values given in the inset).
concentration, \( N_A \) is Avogadro’s number, \( r_b \) corresponds to the modal hydrodynamic radius of the sample as obtained through dynamic light scattering, \( e \) corresponds to the thickness of the lipid bilayer (4 nm), \( \delta \) and \( a \) is the cross-sectional area of the lipid polar head group. The cross-sectional areas of the head groups of dimyristoylphosphatidylcholine (DMPC), cholesterol, and DCP are \( \approx 0.6 \text{ nm}^2 \), 0.4 \text{ nm}^2, and 0.7 \text{ nm}^2, respectively, resulting in a molar weighted average of \( a = 0.53 \text{ nm}^2 \). An estimated production rate of \( \approx 10^{15} \) liposomes per hour was determined using both methods, confirming that nearly complete incorporation of lipids into the unilamellar vesicles occurs during microfluidic self-assembly. This demonstrated liposome production rate is nearly two orders of magnitude higher than MHF even when operating the planar device with higher initial lipid concentration than typically employed. Similarly, VFF also produced liposomes more than an order of magnitude faster than the 3D-MHF method, while simultaneously providing \( \approx 10^3 \) times higher vesicle concentration than the capillary system which results in dilution due to the high aqueous flow rates involved.

An additional parameter that impacts liposome production rate is the lipid flow velocity. While the transition from laminar to turbulent flow regimes presents an upper limit to the flow velocity that may be employed during flow focusing, in practice the maximum velocity is constrained by the pressure limitations imposed by device fabrication. At high hydrodynamic flow rates, and thus high inlet pressures, leakage can occur due to failure of the bonds between microchannel layers or failure of the world-to-chip fluidic interface. For the thermoplastic VFF chips, reliable operation was achieved for average linear flow velocities up to 30 cm s\(^{-1}\), corresponding to a total bulk flow rate within the focusing channel of 4.5 mL min\(^{-1}\) (Figure 7). This maximum maintains the flow well within the laminar regime, with an associated Reynolds number of Re = 28. Interestingly, liposome size and polydispersity were significantly increased at the lowest flow velocity of 5 cm s\(^{-1}\), suggesting that sufficiently rapid transverse diffusion of lipids and solvent occurs at this velocity to allow vesicle formation to occur prior to full development of the focused flow, thereby establishing a lower limit to the flow velocity. While the solvent bonding process used to seal the multilayer VFF chips prevents denaturation at high flow rates, the fluidic interfacing between off-chip syringe pumps and on-chip microchannels was unreliable at flow rates above 30 cm s\(^{-1}\), with a corresponding inlet pressure of \( \approx 2 \text{ MPa} \). The operational flow velocity range of the VFF chips may be further expanded by migrating to an alternate world-to-chip interface, such as threaded needle ports previously developed for high pressure liquid chromatography devices which support inlet pressures as high as 34 MPa.

4. Conclusion

The multilayer VFF concept was successfully implemented using a thermoplastic microfabrication process to form high aspect ratio channels in which the lipid stream is focused by aqueous buffer coflows oriented in a vertical stack. The VFF platform is capable of significantly increasing the throughput for continuous flow synthesis of self-assembled nanoscale liposomes while retaining the ability of conventional planar MHF systems to produce liposomes with tunable size. At the same time, the use of a high aspect ratio focusing channel was also found to result in reduced size variation for the resulting liposome populations, enabling the synthesis of nearly monodisperse vesicles. A maximum liposome production throughput of 95 mg h\(^{-1}\), corresponding to \( \approx 10^{15} \) liposomes per hour, was demonstrated using moderate values of initial lipid concentration and linear flow velocity. Using the same aspect ratio chip design, a projected throughput of 570 mg h\(^{-1}\) is possible for a single device when using the highest values of lipid concentration and velocity that ensure reliable and consistent operation of the system without degrading liposome size distributions. Further increases in throughput may be achieved by improving the world-to-chip interface which presently constrains maximum flow rates, employing higher aspect ratio focusing channels, and operating multiple devices in parallel. The exceptionally high liposome synthesis rates demonstrated in this work suggest that the automated and continuous-flow VFF platform offers significant benefits for large-scale production of liposomal nanoparticles for applications in drug delivery and beyond.

5. Experimental Section

Device Fabrication: Multilayer microfluidic devices were fabricated using a combination of thick cyclic olefin copolymer (COC) plaques and thin COC films to produce high aspect ratio microchannels using simple fabrication techniques without the need for photolithography or clean room processing. Buffer routing channels were fabricated in 1 mm thick COC plaques (6013 grade, 5797
Topas Advanced Polymers, Florence, KY) through a precision computer numerical control milling machine (MDX-650A; Roland, Lake Forest, CA). Thin COC films (50 µm thick, 6013 grade, Topas) were patterned using an automated craft cutter (Cameo Digital Craft Cutting Tool, Silhouette America, Orem, UT) to be 5 mm in width. The COC films were used to define the multilayer channels, such that each resulting microfluidic device possessed an effective 100:1 aspect ratio in the focusing channel. The intersection between the buffer and lipid phases featured channels which were 100 and 50 µm in width, respectively (Figure 1). Alignment holes were incorporated in all film and plaque layers to assist during bonding.

The COC films and plaques were aligned and bonded together through a low-deformation, high-strength liquid-phase COC solvent bonding procedure.[64] Each 1 mm thick COC plaque was submerged in a solution of 20% decalin in ethanol for 5 min to activate the bonding surface. Following a brief wash with 100% ethanol, the COC surface was dried with nitrogen and aligned with an adjacent channel layer using the preplaced alignment pins. The COC pieces were then sealed under a pressure of 0.7 MPa for at least 24 h for complete solvent removal. The desiccated lipid mixture was redissolved in anhydrous ethanol (Sigma Aldrich) for a total lipid concentration of 20 mmol L⁻¹ unless otherwise noted. To assist in visualization during flow focusing experiments, a lipophilic membrane dye, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C18; DiI) (Life Technologies, Carlsbad, CA), was included into the lipid mixtures at 1 wt%. Phosphate buffered saline (Sigma Aldrich) solution (10 x 10⁻³ M, pH 7.4) was used as the hydration 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.

**Numerical Simulation of Ethanol Concentration Profiles and Fluid Velocity:** Computational fluid dynamics simulations were produced to illustrate the variations in physicochemical properties between the distinctive microchannel aspect ratios. The ethanol–water concentration and fluid velocity profiles of a center stream of ethanol focused by an exterior sheath of water were represented in a 3D model created using COMSOL Multiphysics 4.2 software (COMSOL, Burlington, MA). For objective comparison, the FRR was set to 20 and the total flow velocity was fixed at 0.1 m s⁻¹ in each simulation, corresponding to volumetric flow rates of 1.5 mL min⁻¹, 75 µL min⁻¹, and 7.5 µL min⁻¹ for aspect ratios of 100:1, 5:1, and 0.5:1, respectively.

**Microfluidic Liposome Synthesis:** Liposomes were synthesized as previously described for MHF devices.[42–45] A center stream of lipid solvated in ethanol was injected between two streams containing aqueous hydration buffer (Figure 1). To characterize size distributions, the FRR was varied between 10, 15, 20, 30, 40, 50, and 100 for each VFF and MHF device while holding the linear flow velocity constant at 0.1 m s⁻¹ in each test. Studies of liposome production rates were performed at FRR = 20 with linear flow velocity at 0.1 m s⁻¹, corresponding to volumetric flow rates of 1.5 mL min⁻¹, 75 µL min⁻¹, and 7.5 µL min⁻¹ for aspect ratios of 100:1, 5:1, and 0.5:1, respectively. Each microfluidic device was operated on a digital hot plate set at 35 °C throughout the entire synthesis process.[166]

**Liposome Characterization:** In all cases where liposome size is reported, the vesicles were characterized by dynamic light scattering (Nano ZS, Malvern Instruments, UK). Liposome particle count and verification of liposome diameter were obtained using a Nanosight NS300 and Nanoparticle Tracking Analysis software (Malvern Instruments). Aliquots from selected liposome samples were prepared for cryo-TEM using a CryoPlunge 3 Unit (Gatan, Inc., Pleasanton, CA) and subsequently imaged using a JEM 2100 LaB6 TEM (JEOL, Tokyo, Japan).

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