Analysis of a laminar-flow diffusional mixer for directed self-assembly of liposomes

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The present work describes the operation and simulation of a microfluidic laminar-flow mixer. Diffusive mixing takes place between a core solution containing lipids in ethanol and a sheath solution containing aqueous buffer, leading to self assembly of liposomes. Present device architecture hydrodynamically focuses the lipid solution into a cylindrical core positioned at the center of a microfluidic channel of $125 \times 125\text{-}\mu\text{m}^2$ cross-section. Use of the device produces liposomes in the size range of 100–300 nm, with larger liposomes forming at greater ionic strength in the sheath solution and at lower lipid concentration in the core solution. Finite element simulations compute the concentration distributions of solutes at axial distances of greater than 100 channel widths. These simulations reduce computation time and enable computation at long axial distances by utilizing long hexahedral elements in the axial flow region and fine tetrahedral elements in the hydrodynamic focusing region. Present meshing technique is generally useful for simulation of long microfluidic channels and is fully implementable using COMSOL Multiphysics. Confocal microscopy provides experimental validation of the simulations using fluorescent solutions containing fluorescein or enhanced green fluorescent protein.
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I. INTRODUCTION

Self-assembly forms an essential thrust of nanotechnology as it provides a general strategy for generating nanostructures.¹ Microfluidics provides a platform for directing self-assembly of nanoscopic objects.^{2–4} Laminar-flow diffusional mixers are useful for fabricating spherically shaped structures which are minimally affected by shear, such as liposomes⁵ and nanoparticles,^{6,7} and also for fabricating microfibers.^{8,9} Production of liposomes by injection of lipid-alcohol solution dates back several decades using syringes. This method provides control of liposome size based on needle diameter, flow-rate, lipid concentration, and salt concentration.¹⁰ Microfluidics provides a more integrated platform for producing liposomes.^{10,11} It additionally provides control of the shape of the interface between converging solutions,¹² of the concentration distributions and gradients, and of the residence times of the solutes.¹³ Size and geometry of microfluidic channel influence the size distribution of the resulting liposomes.¹⁴ New

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microfluidic techniques continue to emerge for generating nano-scale structures including abutting diffusion microchambers,¹⁵ tipstreaming,¹⁶ and solvent extraction.^{17,18} However, studies to date which produce liposomes utilize laminar-flow diffusional mixing.^{5,10,11,14} The laminar flow technique shows promise for fabricating liposomes of controlled size. Control of the liposome size distribution is important for drug delivery applications since liposome size is an important parameter in determining residence time in the body.¹⁹

Hydrodynamic focusing forms an integral part of a typical laminar-flow diffusion-mixer.^{5,6,13,20–23} The technique uses co-flow of miscible focusing solution to confine a solution to a sub-cross-section of a channel. Groups develop various hydrodynamic focusing architectures for mitigating sticking to channel walls,⁶ ensuring rapid mixing,^{13,24,25} and ensuring uniform residence time among the focused solutes.^{13,21,22} Three-dimensional (3D) hydrodynamic focusing sheaths a solution into a cylindrical core at the center of the channel, whereas two-dimensional (2D) hydrodynamic focusing sheaths a solution into a columnar shape spanning the height of the channel. 3D focusing reduces the variation of the velocity distribution of the focused solution from 50% variation for a 2D focused columnar core to as small as 3% variation for a cylindrical core using the present design. To date, studies which fabricate liposomes rely on 2D focusing.^{5,11,14} The present study uses 3D focusing ensuring uniform residence time in the microchannel among the newly assembled liposomes.¹³

Laminar-flow diffusion-mixers generate concentration distributions which are controllable, reproducible, and explicitly calculable. For this reason, researchers use continuous-flow diffusional mixers to study structural rearrangements in nano-scale objects such as those which occur during protein-folding.²¹ Here, knowledge of the concentration distributions throughout the device allows for interpretation of continuous flow data in terms of computed solution conditions. Previous experiments characterize the core-shaping capabilities of 3D hydrodynamic focusing manifolds⁸ and the transit times of the focused solutes.¹³ The present work is the first to examine the effect of cross-stream diffusion on the shape of the 3D focused core at long axial distances.

Numerical modeling of mixing in long microfluidic channels can be computationally challenging. Multiphysics software can predict the shape of hydrodynamically focused solutions in 3D microfluidic geometries,¹² but computation of the effect of cross-stream diffusion at distances downstream requires very fine meshing in order to achieve a mesh-independent result. For long channel lengths, fine meshing leads to intractable computation times and out-of-memory errors. For this reason, multiphysics studies of advection-diffusion in long channels typically use 2D analysis. For example, Hausler *et al.* integrate out the variation of the velocity profile with channel depth,²⁶ and Lee *et al.* solve the problem entirely in two dimensions.²⁷ The present study provides the first 3D multiphysics analysis of advection-diffusion at axial distances greater than 100 channel widths. A conjoint meshing technique makes full 3D finite element computation practical at long distances by use of long thin brick-like elements in the axial flow region. Present meshing technique uses conventional tetrahedral elements for the hydrodynamic focusing region since high spatial resolution is needed at the locations of confluence between converging streams and at regions where flow is directed in multiple directions, but it uses long hexahedral elements for the extending microchannel, where flow is uniaxial. Use of long hexahedral elements reduces the number of nodes required for computation. This meshing method speeds 3D multiphysics simulation of a long microchannel, and it enables 3D simulation at longer axial distances than previously achievable.

Several previous studies develop simple house-scripted numerical models to compute cross-stream diffusion in long microfluidic channels in response to previous limitations of multiphysics software.^{20,21,28,29} These computations run faster than full multiphysics simulations thanks to their judicious use of approximations. Kauffmann *et al.* approximate a channel as infinitely wide in order to solve the advection-diffusion problem in 2D,²¹ but this approximation does not work for the present microchannel because of its square cross-section. Several laminar-flow diffusion studies use the plug flow approximation, *i.e.*, assume uniform liquid velocity over the cross-section of the channel, in order to simplify modeling to 1D or 2D,^{20,28,29} but this leads to inaccuracy in the estimation of diffusion coefficients due to neglect of hydrodynamic dispersion. CFD Research Corporation develops a 3D analytical model for advection-diffusion,³⁰ but this uses mathematics

beyond the working skill of a typical device engineer. As an alternative to numerical modeling, basic scaling laws describe cross-stream diffusion of tightly focused solutions in a straight microfluidic, notably the $t^{1/2}$ diffusive scaling law.³¹ However, the $t^{1/2}$ scaling law applies only to tightly focused distributions of initial size less than $\sim 1/100$ the size of the microchannel and consequently cannot be applied to the present device. The present work develops a custom-scripted numerical model assuming plug flow, following Kamholz *et al.*,^{20,28,29} and this effort provides numerical corroboration for the 3D multiphysics simulations.

II. METHODS

A. Microfluidic device

The microfluidic device includes a hydrodynamic focusing architecture described in detail in our previous work³² with a newly added extending microfluidic channel as illustrated in Figure 1. The microchannel length equals 120 widths, and its explicit dimensions are $125\ \mu\text{m} \times 125\ \mu\text{m} \times 16\ 000\ \mu\text{m}$. The fabricated width of the microchannel equals $125 \pm 2\ \mu\text{m}$, and the fabricated height equals $128 \pm 4\ \mu\text{m}$, as measured by inspection of the experimental images. The cross-sectional dimensions of the microchannel do not vary noticeably along its length. The present device uses a square cross-section of microfluidic channel whereas previous mixers use microchannels of high-aspect ratio cross-section.^{21,22,28}

Operation of the microfluidic device uses syringe pumps which inject the core solution at rate U_1 into the primary inlet and sheath solution at rates U_2 and U_3 into the lower and upper focusing inlets. The total volumetric flow-rate equals $U_{TOT} = U_1 + 2U_2 + 2U_3$. The volumetric rate of sheath solution to core solution, $SCR = (2U_2 + 2U_3)/U_1$, determines the initial girth of the core solution. Previous work highlights additional capabilities of this manifold including its ability to maneuver, the focused core up and down within the microchannel³² and its ability to squeeze and expand the focused core.³³

Control of mean liquid velocity requires co-adjustment to the ratio of upper and lower sheath flow-rates in order to maintain the position of the core fixed at the center-depth of the microchannel. COMSOL Multiphysics simulations provide the appropriate combinations of flow-rates U_1 , U_2 , and U_3 prior to experiment for positioning the focused core at the channel center. The present study images the device using solute residence times ranging between 0.26 s and 1.9 s as listed in Table I. Mixing between core and sheath is nearly complete after 10% of characteristic time A/D , where A is the cross-sectional area of the channel and D is the diffusion coefficient of the solute.

3D focusing reduces the velocity variation of the focused solution by an order-of-magnitude compared with 2D focusing. Simulated images show the shape of the core at different focusing ratios in Figure 2, and histograms show the corresponding velocity distributions with coefficients of variation (CVs) in the range of 3%–12%. For solutes of large molecular weight, the present device maintains high uniformity of solute-velocity over the length of the microchannel.

FIG. 1. Illustration shows hydrodynamic focusing architecture of the present device as well as the initial length of the extending microchannel. The full length of the extending microchannel equals 120 channel widths and is not shown here. Syringe pumps apply core solution at rate U_1 to the core inlet and sheath solution at rates U_2 and U_3 to the upper and lower sheath inlets.

TABLE I. Operation of the device takes place at Reynolds number $Re \sim 1$. At these liquid velocities, the focused solutes reside within the 1.5-cm-long channel for $t_{\text{residence}} \sim 1$ s.

Re	u_{AVG}	$t_{\text{residence}}$
1.0	0.8 cm/s	1.9 s
2.7	2.2 cm/s	0.70 s
7.4	5.9 cm/s	0.26 s

The present device is capable of focusing to arbitrary sheath-to-core-ratio, but the core becomes less round and more slit-like at greater sheath-to-core-ratio. The present work employs experimental images acquired at sheath-to-core-ratio $SCR = 10$, for which case the initial cross-sectional area of the core equals 1/10 the area of the channel and for which the core is nearly circular in cross-section. This choice of sheath-to-core-ratio suffices for validating the numerical model.

B. Numerical modeling

Our computational method first computes the velocity field over the entire device geometry, according to the steady-state incompressible Navier-Stokes equation

FIG. 2. Contour plots show the shape of the core for sheath-to-core ratios of $SCR = 4, 10, 30$, and 100 . Histograms show the corresponding velocity distributions normalized by maximum liquid velocity. $\langle u \rangle$ indicates mean solute velocity, and CV indicates coefficient of variation.

$$\rho(\vec{u} \cdot \vec{\nabla})\vec{u} = -\nabla p + \mu \nabla^2 \vec{u}, \quad (1)$$

where ρ is the liquid density, $\vec{u}$ is the liquid velocity vector, p is the pressure, and μ is the dynamic liquid viscosity. Application of no slip, $|\vec{u}| = 0$, at the channel walls gives the steady-state solution, $\vec{u}(x, y, z)$, which feeds into the steady-state advection-diffusion equation

$$(\vec{u} \cdot \vec{\nabla})c = D \nabla^2 c. \quad (2)$$

Here D is the diffusion coefficient of the solute and c is the relative mass fraction of the solute. $c = 1$ at the core inlet, and $c = 0$ at the sheath inlets.

Our model uses a conjoint meshing technique using fine tetrahedral elements for the hydrodynamic focusing region with maximum element length $3.5 \mu\text{m}$ and coarse hexahedral elements for the axial flow region with maximum element length $100 \mu\text{m}$ and typical element width $9 \mu\text{m}$ as shown in Figure 3. Generation of the latter elements uses the swept mesh function within COMSOL to generate long thin rectangular-shaped elements across the width and down the length of the channel. The length of the extending microchannel computed equals 120 channel widths. Computation uses a personal desktop computer with 64-GB RAM running COMSOL Multiphysics 4.3 on Windows 7.

Alternative 3D computations in FLEXPDE and 2D computations in MATLAB provide numerical validation of our 3D COMSOL computations. These alternative computations begin with the COMSOL solution for the hydrodynamic focusing region and propagate this down the length of the extending microchannel outside of the COMSOL framework.

Alternative 3D computations in FLEXPDE verify the mesh-independence of the COMSOL Multiphysics solution by using fine tetrahedral elements for the entire length of the extending microchannel. Here, assumption of uni-axial flow makes this high-resolution computation possible by reducing the amount of memory required to solve the advection-diffusion equation. This computation uses a characteristic mesh length of $4 \mu\text{m}$, or $1/30$ the cross-sectional width of the microchannel, over the entire channel length thereby providing sufficient mesh density to reach a mesh-independent solution to within root-mean-square (rms) error 0.3%. Further increases in mesh density give rise to intractable computation time and out-of-memory errors, and present computation requires more than 24 h to of run time.

The alternative 2D computations use finite differences to estimate the cross-stream diffusion using the plug flow approximation, essentially following Kamholz and Yager.²⁹ Computation using 2D finite differences (FD) and 3D finite elements (FE) shows a tradeoff between computation time and accuracy. The 2D plug flow computation progresses more than $1000 \times$ faster than the 3D uni-axial flow computation, as shown in Table II, but it leads to significant inaccuracies due to its reliance on the plug flow approximation.

The hexahedral meshing method in COMSOL offers the same accuracy as tetrahedral meshing and significantly reduces the computation time for advection-diffusion in a long microchannel. The computation time scales as $L^{2.0}$ for 3D uni-axial flow with tetrahedral finite elements, as

FIG. 3. 3D computations using COMSOL Multiphysics. **Top.** Conjoint mesh uses fine tetrahedral elements for the hydrodynamic focusing region and long hexahedral elements for the uni-axial flow region. Mesh shown is coarser than used in practice. **Bottom.** Contour plot shows concentration on a slice along the initial 15% length of the channel.

TABLE II. Comparison of spatial resolution and computation time for 3D hexahedral finite elements, which provides the primary simulations presented in this work, as well as 2D finite differences and 3D tetrahedral finite elements, which serve as alternative computations for numerical validation purposes. Inter-element distance for 3D hexahedral finite elements is $9\text{ }\mu\text{m}$ in the transverse direction, as listed here, and $100\text{ }\mu\text{m}$ in the axial direction.

Model	Total elements	Inter-element distance	Computation time	Softwares	Approximation
3D hexahedral FE	3×10^4	$9\text{ }\mu\text{m}$	50 min	COMSOL	None
3D tetrahedral FE	5×10^6	$4\text{ }\mu\text{m}$	1000 min	FLEXPDE	Uni-axial flow
2D FD	6×10^4	$0.5\text{ }\mu\text{m}$	0.6 min	MATLAB	Plug flow

$L^{1.7}$ for 3D flow with hexahedral finite elements, and as $L^{1.0}$ for 2D plug flow with rectangular finite differences as shown in Figure 4, where L is the length of channel computed. Note that the computation times plotted include only the axial flow region of the device.

C. Confocal microscopy

The fluorescent solutions used in the present experiments consist of 2 mM fluorescein in 50 mM potassium phosphate buffer pH 7.0, enhanced green fluorescent protein (EGFP) as purified in 20 mM Tris HCl, and 2 mM rhodamine in buffer corresponding to that of the core solution. We use EGFP in the present study because it has a significantly smaller diffusion coefficient than Fluorescein and because we already expressed large quantities of it for an unrelated effort. The supplemental material provides details on the protein-expression and purification.³⁴

Image acquisition uses a laser-scanning confocal microscope, Zeiss 710, manufactured in Germany. The present work uses the 473-nm and 561-nm laser lines to simultaneously image the core solution and focusing solution. Imaging the focusing solution provides the locations of the microchannel walls. Acquisition uses two photodetectors yielding digital 8-bit red-green images.

Digital image processing converts the two-color fluorescence micrographs into single-component concentration distributions using flat field image correction, a standard technique in quantitative fluorescence microscopy.³⁵ Figure 5 shows the various images used for image processing. These include flat-field images which use a single fluorescent solution at all inputs of the device and dark-field images which use nonfluorescent buffer at all inputs of the device. Our image processing procedure first subtracts out the spectral bleedthrough of rhodamine into the green photodetector based on the product of the red-pixel-values of the experimental image with the quotient of the green- and red- pixel-values of the flat-field rhodamine image. Next, our procedure implements flat field image correction by dividing the previous result by the green-pixel-values of the flat-field fluorescein image. Subtraction of the dark-field image

FIG. 4. Plot shows computation time versus length of channel for cross-stream diffusion during pressure-driven laminar flow. Computation time is proportional to $L^{1.7}$ for 3D hexahedral finite elements, to $L^{2.0}$ for 3D tetrahedral finite elements, and to $L^{1.0}$ for 2D Finite Differences. 3D hexahedral FE provides the primary simulations used in this work.

FIG. 5. Image processing converts a two-color fluorescence micrograph into a single-component concentration distribution using a dark-field image and two flat-field images. Contour plot shows the experimental concentration distribution generated by image processing. The color-scale varies between 0 and 1 as shown. The contour plot and gray-scale image show the same data set.

from all source images takes place prior to their use in image processing. Each image contains 8-bit pixel-values ranging between 0 and 255. Some pixels in the flat field image are saturated at 255 due to the presence of noise on top of a strong fluorescence signal. Future experiments of this nature may seek to acquire 12-bit or 16-bit images if possible in order to increase dynamic range and avoid saturation. A typical raw image uses a total depth-scan of $300\ \mu\text{m}$ using $1.0\text{-}\mu\text{m}$ steps resulting in a 2048×300 image with each pixel corresponding to

$0.12\ \mu\text{m} \times 1.0\ \mu\text{m}$. Linear interpolation remaps the image such that the final image contains pixels of $0.50\ \mu\text{m} \times 0.50\ \mu\text{m}$ area.

The raw flat field image appears slightly brighter at the channel center than near the side walls most likely due to collection of out-of-focus photons from the nearby sheath flow regions. This optical effect reduces the concentration perceived at the center of the channel from nearly 1.0 to 0.8.

D. Microfluidic production of liposomes

Microfluidic production of liposomes applies total volumetric flow-rate $20.4\ \mu\text{l}/\text{min}$ for a 100-min duration giving a sample volume of 2.0 ml. Analysis follows within 24-h of generation of multiple samples, which are collected into Eppendorf tubes. Liposome formation and storage take place at room temperature, approximately $22\ ^\circ\text{C}$.

The liposome solution consists of phospholipids 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG) dissolved with cholesterol (Chol) in ethanol in a 10:1:10 (DPPC/DSPE-PEG/Chol) molar ratio. The molecular weight of DPPC is 734; the molecular weight of DSPE-PEG is 2800; the molecular weight of cholesterol is 387.

Imaging of liposomes by transmission electron microscopy (TEM) takes place at 120 kV using a Philips Tecnai T-12 scope. TEM grid preparation uses a $5.0\ \mu\text{l}$ aliquot of sample pipetted onto a carbon-coated 200 nm mesh copper grid and stained with $5.0\ \mu\text{l}$ of sodium phosphotungstate negative stain. Measurement of the mean hydrodynamic diameter of microfluidically synthesized liposomes uses dynamic light scattering at 90° with a Melles Griot HeNe 632.8 nm laser.

III. RESULTS

Confocal microscopy images show the cross-section of the microfluidic channel with fluorescent solutes dissolved in the core solution at distances along the length of the microchannel in Figure 6. Fluorescein diffuses outwards at a noticeably faster rate than EGFP as expected based on their relative molecular weights. The core remains positioned at the center of the channel at distances downstream, and it does not rotate or twist in shape, indicating that flow remains uni-axial over the length of the channel, as expected. Mixing between core and sheath is nearly complete after 10% of characteristic time A/D , where A is the cross-sectional area of the channel and D is the diffusion coefficient of the solute, or $t_{\text{Mix}} = 0.1 A/D = 3\ \text{s}$ for Fluorescein and 20 s for EGFP.

At the beginning of the extending microchannel, both fluorescein and EGFP show similar concentration distributions since the length of the hydrodynamic focusing region is short compared with the diffusion length of the solutes. Many 3D focusing devices use longer multi-step focusing structures^{6,36} in which significant diffusion-mixing can occur in the focusing region. The short length of the present hydrodynamic focusing architecture suits it for use as a diffusion mixer.

At long distances, small molecules typified here by fluorescein diffuse across the microchannel whereas large molecules modeled here by EGFP remain confined to the central core. For production of liposomes, the salts present in the sheath solution should diffuse 2–3 $\times$ faster than fluorescein and therefore should become nearly uniformly distributed over the channel cross-section at the outlet of the channel. The newly formed liposomes, however, should migrate radially outward from center at an even slower rate than EGFP molecules. The velocity distribution broadens as the core solution diffuses outward—for the case of EGFP at mean liquid velocity $2.2\ \text{cm/s}$, the velocity variation increases from 6% at the inlet to 10% at the outlet.

The mean liquid velocity directly determines the residence times of the solutes. One can achieve a particular extent-of-diffusion either by adjusting the total volumetric flow-rate or by translating the location of observation upstream or downstream. Images show fluorescein at three different liquid velocities in Figure 7. These appear similar to one another when viewed downwards-right diagonally because our experiment uses logarithmic steps between successive

FIG. 6. Experimental confocal micrographs show the cross-section of the microchannel at axial distances with fluorescein and EGFP in the core solution. The mean liquid velocity equals 2.2 cm/s. Plots show profiles acquired horizontally across the micrographs.

liquid velocities and also between successive imaging locations. This result corroborates efforts by other groups which fix the location of observation and simply adjust liquid velocity.¹³

The velocity-switching experiment and substance switching experiment take place on different days using replica devices of the same design. Switching of objective lenses takes place between the two experiments in order to accommodate greater-than-anticipated device thickness for the second device.

Experiment and computation show agreement regarding the shape of the concentration distribution at distances along the microchannel as shown by direct visual comparison in Figure 8. Noticeably, the multiphysics model predicts higher concentrations at the center of the core than observed experimentally. This disparity becomes evident at the entrance of the microchannel, $z = 0.1$ mm, where the model predicts concentrations of 0.95 at the channel center whereas the experimental data show concentrations of 0.80, and the disparity propagates to the end of the channel. Here, the experimental measurement yields artificially small magnitude of concentration due most likely to the use of partially saturated flat field images in image processing. Improvement of the flat field correction procedure for confocal diagnostics of microfluidic transport is possible with additional experimentation, but this falls beyond the scope of the present study. The root-mean-squared pixel-by-pixel error between the images and simulations typically falls in the range of 3%–7%.

Alternative computations using classic 3D tetrahedral elements for the entire length of the microchannel require longer computation times yet give results which are indistinguishable from the present computations.³⁴ Alternative 2D computations, however, show visually apparent inaccuracies. 2D modeling predicts reduced mean concentration over the cross-section of the channel at long distances downstream because it does not conserve mass. Specifically, the 2D method conserves $\int c u_z da$ instead of $\int c u_z da$. The 2D plug flow model gives rise to additional inaccuracies due to mismatch between the plug flow velocity and the travel speed of the core. For this reason, 2D computations predict a core whose cross-section becomes circular in shape at shorter distances than predicted by 3D FE as evident in Figure S1 of the supplementary material.³⁴ Near the entrance of the channel, where the core remains tightly focused, the 2D model marches downstream at a slower speed than the core leading to over-prediction of the core width by as much as 30%.³⁴ Downstream, after the core has broadened significantly, the 2D model marches at approximately the same velocity as the core, but the broadened core succumbs to additional mixing due to hydrodynamic dispersion, which the 2D treatment ignores.

FIG. 7. Experimental confocal micrographs show the cross-section of the microchannel at axial distances with fluorescein in the core solution. This experiment uses three different liquid velocities indicated at left. Plots show profiles acquired horizontally across the micrographs.

2D computation may be appropriate for long microchannels in situations where approximate results are needed quickly, but final data analysis requires 3D computations.

Our computations use diffusion coefficients $D_{\text{fluor}} = 6 \times 10^{-10} \text{ m}^2/\text{s}$ and $D_{\text{EGFP}} = 9 \times 10^{-11} \text{ m}^2/\text{s}$. Both of these coefficients match reasonably well with the previously reported values for $D_{\text{fluor}} = 4.0\text{--}5.5 \times 10^{-10} \text{ m}^2/\text{s}$ (Refs. 37–39) and $D_{\text{EGFP}} = 8.3\text{--}8.7 \times 10^{-11} \text{ m}^2/\text{s}$ (Refs. 40 and 41) assuming the diffusion coefficient of EGFP equals that of native GFP.

Additional forces might contribute to mixing besides diffusion and hydrodynamic dispersion which could give rise to slightly faster-than-expected diffusion for fluorescein. For example, any twisting or bending of the channel due to the flexibility of the surrounding polydimethylsiloxane would ultimately augment mixing. In addition, the walls of the channel may not be perfectly flat and straight making the effective hydrodynamic diameter of the channel slightly smaller than optically perceived. Interestingly, a previous microfluidic laminar-flow experiment gives an estimated diffusion coefficient for fluorescein which is moderately higher than other values found in the literature.⁴²

Microfluidic fabrication of liposomes uses lipids-in-ethanol solution in the core and aqueous solution in the sheath. Diffusion of the lipids from the core into the sheath leads to self-assembly of liposomes. Characterization by TEM shows the liposomes to be approximately spherical. Figure 9 shows TEM images of liposomes produced using sheath-to-core ratio $SCR = 15$. The images reflect dehydration of the sample since TEM imaging takes place under vacuum. Specifically, the images show a dehydrated lipid film with spherical pockets corresponding to the inner regions of the liposomes normally occupied by water phase.

Increasing the phospholipid concentration in the core solution leads to smaller liposomes as evident in Figure 10. Phospholipid acyl chain length is known to affect liposome size.⁴³ Increasing the ionic strength in the sheath leads to larger liposomes as evident in Figure 10. At greater ionic strengths, competition for water solvation of lipids by salts likely leads to formation of larger liposomes. The larger liposomes at greater salt concentrations tend to aggregate.

FIG. 8. Contour plots show experimental and computed concentration distributions at axial distances along the length of the extending microfluidic channel. Plots show profiles acquired horizontally across the experimental concentration distributions and simulations. The model assumes diffusion coefficient $6 \times 10^{-10} \text{ m}^2/\text{s}$ for fluorescein.

Typical liposome size-distributions show standard deviations of 50%–80% with greater variation occurring at lower sheath-to-core ratio.

Notably, the present study produces liposomes which are systematically somewhat larger at sheath-to-core ratios greater than 10 than those generated by other groups. Specifically, three previous studies generate liposomes of 100-nm or smaller at sheath-to-core ratios of 10 or greater,^{14,43,44} whereas the present study generates liposomes in the range of 100–200 nm. This difference may occur as a consequence of adding DSPE-PEG, a hydrophilic and large molecular weight molecule, into the lipid solution. Previous works on the microfluidic production of liposomes use lipids of molecular weight 500–800 with cholesterol,^{11,14,43} and the present study adds DSPE-PEG of molecular weight 2800. Other parameters which can affect liposome size include the channel dimensions. Previous studies by other groups generate liposome using microchannels of smallest cross-sectional dimension equal to $65 \mu\text{m}$ (Ref. 43) and $10 \mu\text{m}$ (Ref. 14), whereas the present study uses a microchannel of smallest dimension $125 \mu\text{m}$. The size of the resulting liposomes also depends on multiple bulk parameters including temperature, bulk ratio of ethanol to water, final ionic strength, and final concentration of lipids and cholesterol.

FIG. 9. TEM micrographs show liposomes produced using the present device with mean diameter 250 nm.

FIG. 10. Plots show mean hydrodynamic diameter of liposomes produced using the present microfluidic device with different sheath-to-core ratios as measured using dynamic light scattering. Contour plot insets show the shape of the core solution at the entrance of the microchannel. **Top.** Symbols indicate total lipid concentration in the core. **Bottom.** Symbols indicating ionic strength of the sheath solution. The uncertainty in the measurement of mean hydrodynamic diameter is approximately 10% based on standard error for repeated experiments.

The present study produces lipid vesicles using a residence time about 1 s in the microfluidic channel. This residence time is sufficient for initial vesicle formation based on work by others using x-ray scattering.⁴⁵ However, subsequent size-distribution changes might take place after the freshly formed liposomes have departed the microfluidic device as they rest in vial. Stopped flow experiments show continual increase in characteristic particle size at times up to 100 s after mixing.⁴⁶ Future experiments might consider using a long serpentine channel to ensure complete formation of liposomes prior to their departure from the microfluidic channel. Microfluidics provides a potential method for achieving full control of the morphology of bottom-up fabricated vesicles. Achieving this potential may require improved understanding of the pathways of self-assembly. The present device architecture provides a potential tool for studying the dynamics of self-assembly of vesicles.

IV. CONCLUSIONS

Laminar-flow diffusional mixing provides a useful method for producing liposomes of controlled size using microfluidics. 3D hydrodynamic focusing ensures that all of the newly formed liposomes travel at a common velocity down the length of the microfluidic channel. The size of the liposomes shifts according to changes in device operation parameters, concentration of

lipids in the core, and concentration of salts in the sheath. The simulation method presented in this work uses an advanced meshing technique which allows for full 3D computation of advection-diffusion in long microfluidic channels. The method reduces computation time compared with conventional tetrahedral meshing without sacrificing accuracy.

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