I, Prithviraj Mukherjee, hereby submit this original work as part of the requirements for the degree of Master of Science in Electrical Engineering.

It is entitled:
GENERATION OF DRUG-LOADED ECHOGENIC LIPOSOMES USING MICROFLUIDIC HYDRODYNAMIC FLOW FOCUSING

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GENERATION OF DRUG-LOADED ECHOGENIC LIPOSOMES
USING MICROFLUIDIC HYDRODYNAMIC FLOW FOCUSING

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ABSTRACT

This work describes development of a microfluidic system for the synthesis of echogenic liposomes (ELIP) loaded with the thrombolytic drug rt-PA (recombinant tissue plasminogen activator). ELIP are phospholipid vesicles with encapsulated microbubbles. Drugs, genes, or bioactive gases encapsulated in ELIP can be delivered to specific tissue targets using ultrasound activation. Microfluidic devices were fabricated in Polydimethylsiloxane (PDMS), using the standard soft lithography methods and were optimized for flow rates and gas pressures to generate echogenic liposomes in the right size range and with good repeatability. The liposomes were generated in the size range of 2-20 µm, using octafluoropropane (OFP) and octfluorocyclobutane (C₄F₈) gases as core. Fluorescence microscopy was used to visualize encapsulation of the water soluble rt-PA drug. In future work, these liposomes could be used in non-invasive ultrasound-mediated drug delivery, to affect thrombolysis.
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CHAPTER 1

INTRODUCTION

In recent years, the use of gas microbubbles for ultrasound-mediated drug delivery systems has been gaining popularity [1-4]. These acoustically active carriers can be disrupted at the regions of acoustic pressure thresholds, thus providing a noninvasive, non-ionizing and real-time platform for drug-delivery [5-8]. Studies have demonstrated the use of gas-encapsulated liposomes for cancer drug delivery or clot lysis [9-12]. However, these liposomes are difficult to produce with conventional benchtop methods, which are based on mechanical agitation, or sonication. Another challenge is that the generated liposomes are highly polydisperse in size, making ultrasound imaging or activation difficult since their resonant frequency is highly dependent on the diameter [13]. Further, there is little consistency in the amount of drug encapsulated in each liposome, leading to variability in quality of drug delivery [13]. Microfluidics may offer a way to overcome these challenges and to improve performance and effectiveness of these ultrasound drug carrying vesicles. Recent research has shown that droplet microfluidics is highly effective in the generation of colloidal suspensions and microbubbles with a great degree of control [10, 14, 15].

**Droplet microfluidics**

Microfluidics has experienced intense interest in the past two decades [15-18]. It is at the intersection of fluid engineering, biotechnology and physics. It involves both development of
new microscale flow devices and applications in biology and medicine. Though most of such devices involve continuous liquid flows, a new and burgeoning area is droplet microfluidics, which involves two-phase (liquid and gas) flows. Droplet microfluidics can produce droplets in the pL to nL volume range [12]. This technique has attracted significant interest due to potential applications in petroleum industry, pharmaceuticals, medicine and food processing [2, 9, 10, 19-21].

In droplet microfluidics, immiscible streams of fluids are brought in contact and discrete droplets are formed with the help of viscous forces and surface tension. These droplets can be manipulated (split, merged, or routed) and can be analyzed individually. These droplets have been used as microreactors or carriers of drugs, genes or analytes [21, 29]. Nakajima et al. [18] demonstrated formation of droplets at a rate of 20,000/s. Nisisako and Torii [24] demonstrated that throughput can be increased even further by circularly arranging the droplet generators, introducing parallel processing and increasing the rate to 100,000 droplets/s (the size of the droplets was 100 µm, at a flow rate of 320 mL/h).

In this work, we use the flow focusing technique to generate lipid coated microbubbles, with the advantage of self-assembly. Whitesides et al. [24] have reviewed the formation of microbubbles using this technique, where two immiscible fluids flow through inlet channels of an axi-symmetric device. The downstream pressure of the fluids and the gas channel squeezes the gas channel at the orifice. The gas thread is eventually broken and a bubble is released into the outlet channel, as a result of two distinct inertial stresses exerted on the gas stream by the liquid. The gas stream pulsates at the orifice where the gas pressure \( p \) inflates the channel into the orifice, the bubble breaks off from the channel and the channel retracts back. The size of the bubbles are varied by keeping the gas pressure constant and varying the flow rate of the
surrounding fluid. The volume of the gas bubbles is dependent on the volume of the orifice, the height, width and length of the channel respectively.

This work is focused on generating drug-loaded echogenic liposomes, which will be ultimately used as ultrasound contrast agents (UCA) and drug delivery vehicles for clot lysis. The use of UCAs in screening patients with cardiovascular risk is a well-established practice. Production of phospholipid UCAs is of special interest due to use as target-specific based on surface functionalization with ligands. Maintaining monodispersity of the UCA population is important as the resonant frequency and the amount of radiation force experienced are affected by their diameter. A monodisperse population ensures a more consistent echo response of the ELIP and hence resolution of imaging.

**Echogenic liposomes (ELIP)**

Echogenic liposomes are a spherical phospholipid bilayer carrier with a gas core [24]. The gas core makes the ELIP acoustically active, which provides contrast on an ultrasound image and serves to nucleate bubble activity to trigger drug delivery. Ultrasound induces oscillations and cavitation or rupture of the liposomes at the target site, delivering the encapsulated drug to the surrounding tissue [26-28].

These echogenic liposomes serve dual purpose, as they possess both a phospholipid layer shielding the drug payload and also a gas core. With a different density and compressibility than the surrounding fluid, the gas core provides significant contrast on an ultrasound image. These ELIP can be imaged at the target site with B-mode ultrasound imaging and later ruptured using an acoustic pulse of higher energy. Huang et al. reported a 50% entrapment and retention of echogenicity, with 49.5% improved clot lysis through ultrasound triggered delivery of rtPA-loaded liposomes [5]. Meunier et al. reported an increased initial lytic rate with increasing duty
cycle of 120 kHz ultrasound enhanced thrombolysis in \textit{in vitro} human clot model [28]. More \textit{in vitro} flow model studies have shown that the thrombolysis can be enhanced by 50\% by the combined use of rt-PA-ELIP and ultrasound, than just the use of rt-PA alone [5, 10, 35-38, 40-42]. The use of ELIP also decreases the risk of hemorrhage due to rt-PA activity.

Echogenic liposomes present an attractive alternative in treatment of myocardial infarction and acute ischemic stroke, which are two of the leading causes of death in the US [29]. Recombinant tissue plasminogen activator (rt-PA) is the only FDA-approved thrombolytic drug, but it is only used in less than 1.5\% of the treatment cases [5], due to potential adverse effects such as excessive bleeding. To overcome this issue, many groups are investigating of combining the benefits of encapsulating the drug in thrombolytic liposomes (t-ELIP) and ultrasound mediated drug delivery, to improve the treatment of thrombo-occlusive diseases [1, 28, 49].

Conventionally, liposomes are produced by bulk mixing methods. Briefly, a lipid mixture of predetermined composition is mixed in an organic solvent. The solvent is evaporated under vacuum or inert gas to obtain a dry thin lipid layer. The layer is further dried in a desiccator. The contents are then rehydrated using an aqueous buffer or water, whereupon, they spontaneously self-assemble into liposomes. At this stage, the liposomes can be multilamellar with a range of sizes. Further treatment steps such as sonication, multiple freeze thaw cycles and lyophilization are carried out to cause breakdown of the multilamellar polydisperse liposomes and formation of unilamellar liposomes with a narrow size distribution. The major disadvantage of these methods is that they produce a highly polydisperse population of liposomes, and a single batch of liposomes takes 24-48 h to make. As the acoustic response is dependent on the size of the microbubble, polydispersity in the ELIP concentration is highly undesirable as it generates a non-uniform response.
The benefits of monodisperse population were demonstrated by Martz et al. who showed that different droplet sizes vaporized at unique non-overlapping acoustic thresholds [14]. The quantity of drug encapsulated in the liposomes is not consistent [38]. Thus, a monodisperse population of ELIP will result in a more uniform activation. This is schematically represented in Fig. 1. To overcome these limitations microfluidic production techniques are much more desirable. With microfluidics there is a much better control of size, density and production rate. By changing the chemistry of the continuous phase and dispersed phase in the system the characteristics of the microbubbles can be manipulated.

**Figure 1.** Proposed structure of microfluidically generated and batch formed ELIP. The batch formed ELIP may have random distribution of drug in the core. ELIP generated by microfluidics have consistent drug and gas encapsulation.

**Microfluidic generation of liposomes**

Passive droplet generation relies on proper channel geometry and dimensions, as well as flow parameters, for the successful generation. The fluids are simply driven through the channels using a syringe pump.
Droplet formation in these systems is due to the competition of viscous stresses, which deforms the fluid interface, and the capillary force resisting the deformation. The interfacial tension develops a normal stress jump across the interface. This stress jump is known as the Laplace pressure:

\[ \Delta P = P_{\text{in}} - P_{\text{out}} = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \]  

where \( \Delta P \) is the difference in pressure, \( P_{\text{in}} \) is the pressure inside the droplet, \( P_{\text{out}} \) is the pressure outside the droplet, \( \gamma \) is the interfacial tension between two co-flowing fluids and \( R_1 \) and \( R_2 \) are the inner and outer radii of curvature of the interfaces respectively. In case of spherical droplets, \( R_1 = R_2 = R \), the Laplace equation becomes

\[ \Delta P = \frac{2\gamma}{R}. \]  

In general, a fluid is dispersed in an immiscible flowing environment. When the two fluids come in contact at a junction, the interface deforms and the competition of viscous stress and capillary forces pinches into droplets [47]. The narrowest region of the dispersed fluid is the point of maximum shear-force, and the existence of this point makes it possible for a consistent generation of droplets.

There are two most common techniques by which droplet generation is done in microfluidics with either T-junction devices or the flow-focusing technique. The flow-focusing method was first introduced by Thorsen et al. [14]. This system consists of two orthogonal channels which meet at a junction. The main channel has the continuous phase flowing through it while the dispersed phase is driven into it. The shear forces deform the interfaces and eventually breaks the dispersed phase into discrete droplets. The flow regimes can be categorized into squeezing, dripping and jetting regimes based on the Ca. The T-junction devices have a simplistic design and robust operation with different fluids. But one main disadvantage is that
their generation is unstable at higher or smaller frequencies. As a result flow focusing regime [9] is a much more attractive alternative for a more stable monodisperse production of droplets.

The underlying idea of microbubble generation in flow focusing system is similar to that of a T-junction. Flow focusing technique consists of a device with two or three concentric channels which have immiscible fluids flowing through them. The middle channel(s) have the dispersed phase which are squeezed through an orifice by the continuous phase, flowing through the outer channel. When the continuous and the dispersed phase pass through the orifice there is a competition of flow, and the dispersed phase breaks up in to droplets. Two immiscible fluids flowing through an orifice, dispersed phase is squeezed by the continuous phase, and forms a jet stream. The perturbations caused by flow of the continuous phase eventually breaks the jet into smaller droplets. Using flow focusing technique with a liquid-gas two phase system, Garstecki et al. [13] developed a relation to measure the diameter of the microbubbles ($d_b$) as:

$$\frac{d_b}{D} \alpha \left(\frac{Q_g}{Q_l}\right)^{0.4}$$

(3)

where, $D$ is the orifice diameter, $Q_g$ is the gas flow rate and $Q_l$ is the liquid flow rate. In this system, the liquids had small surface tension and low viscosity values and also $Q_g / Q_l$ was less than 1. The formation of spherical drops can be explained by the tendency of the broken jet stream to attain a shape with the smallest surface area.

Many groups have used hydrodynamic flow focusing to generate microbubbles. Biggs et al. [29] reported liposomes using flow focusing technique within several hundred nanometers. Microfluidic techniques were also used to achieve small sizes of 300 nm using a DPPC: cholesterol: DCP phospholipid formulation in IPA, with a polydispersity of 3%-5% [13, 24, 32]. But these did not use any gas to be encapsulated in the core. Hettiarchchi et al. [4, 12] reported a production rate of $6 \times 10^4$ per min, using a perfluorocarbon gas core. In later work, they reported
encapsulation of oil soluble drug Doxorubicin, with the smaller liposomes (7.5 ± 0.2 μm) generated at lipid/water flow rates of 0.33μL/s and gas pressure of 1 psi [3]. Hettiarachchi et al. [4,15] reported microfluidic generation of liposomes in the size range of 3.3-8.3 μm, using OFP gas. The lipid composition used was DPPC: DPPA: DPPE-PEG-5000 and the number density reported was 600 K/mL. Lee et al. [12] have demonstrated generation of nitrogen and PFB filled microbubbles with a size of ≤5 μm. Lee et al. have also reported generation of C₄F₈, Octafluorocyclobutane, of 15μm size with a polysdispersity index (σ) of 95% [3]. Recently, they reported generation of liposomes with a size of 9-16 μm [15], using C₄F₈. His work consisted of an oil soluble drug, Placlitaxel and have used oil as a continuous phase in the production of liposomes. The lipid composition used in their work was DSPC: DSPE-PEG-2000 at a concentration of 0.5 mg/ mL. The stability of these microbubbles was about 10 min. Porter et al. [58] demonstrated microfluidic generation of size-controlled monodisperse microbubbles with mean diameters ranging from 3.4-12.5 μm. Porter showed that the resonance frequency of these monodisperse microbubbles decreased as the acoustic pressure amplitude increased.

**Scope of work**

In this work, we hypothesize that microfluidics can be used to generate monodisperse ELIP in the 2-20 μm size range, by using OFP or C₄F₈ as gas core and rt-PA drug. Microfluidic devices with two different outlet types were used to generate monodisperse echogenic liposomes within the desired size range. The effect of these outlet designs in the generation and retention of size of the ELIP have been investigated. While Huang et al. [5] have previously demonstrated tPA entrapment in ELIP, this work demonstrates a successful microfluidic encapsulation of the water soluble thrombolytic drug. Different lipid compositions have been tested to yeild the size distribution and stability desired in our application. Liposome stability of 1 h, under different
induced conditions of saline, BSA and a thermo-reversible gel was observed. The echogenicity of the liposomes was measured to have a resonance frequency of 2.2 MHz. Also, 35% of the liposomes were estimated to contain a gas core, which is an order higher than previously reported rt-PA loaded ELIP.

**Chapter summaries**

Following this introduction, Chapter 2 discusses the lipid composition, gases, collection and suspension methods, as well as imaging and analysis methods used throughout the course of this project. In Chapter 3, the different aspects of device designs, results of device characterizations, flow rate gas pressure optimization are discussed. ELIP size and stability, along with drug encapsulation are also presented. Finally, in Chapter 4, a summary of the results is provided, along with recommendations for future work.
CHAPTER 2

EXPERIMENTAL METHODS

In this work, droplet generation was accomplished using a microfluidic hydrodynamic focusing technique. As discussed in the introduction, in this approach a flow stream is pinched by a transverse flowing secondary flow, which leads to a shear force that fractures the primary flow into small microbubbles. The size of the microbubbles generated in this manner is maintained by controlling the two flow rates. This chapter describes fabrication of these microfluidic devices and the experimental methods used in this work, from flow optimization to imaging and analysis of the generated microbubbles (i.e. liposomes).

Fabrication of devices

Devices in this work were fabricated using standard soft lithography methods. The process involves two key steps – master fabrication and replica casting. The master was fabricated using with a 3 in clean silicon wafer, rinsed in acetone, isopropyl alcohol and deionized water (DI) for 30 s each. The wafer was submerged in buffered oxide etch (BOE) for 30 s to remove the native oxide layer, rinsed with DI water again, and then blown dry with nitrogen. Negative photoresist SU-8 (2075, MicroChem Co.) was then spin-coated to the thickness of 25 µm (1,500 rpm for 10 s spread and 4,250 rpm for 35 s coat). The wafer was then soft baked on a hotplate at 65 °C for 10 min. Photoresist was exposed for 45 s at 5 mJ/cm² and post-exposure baked in an oven at 95 °C for 15 min. The film was developed using SU-8
developer (MicroChem Co.) and rinsed in DI water for 5 min. Any undeveloped photoresist was then cleaned using 20% oxygen plasma in a reactive ion etching system (March CS-1701 RIE) for 15 min. To complete the master fabrication process, the wafer was taped to a petri dish, treated with Sigmacoat (Sigma-Aldrich) and allowed to rest in a chemical hood overnight.

Replicas of the SU-8 master were formed in polydimethylsiloxane (PDMS). For this, the PDMS (Sylgard 184, Dow Corning) monomer was mixed with a curing agent in the 10:1 w/w ratio. The mixture was degassed, poured into the petri dish containing the SU-8 master, and degassed for the second time. PDMS was cured on a hot plate at 80 °C for 2 h. PDMS devices were then cut to size with a scalpel and rinsed with DI water for 15-20 s. Each replica was bonded to a glass slide using a corona discharger (BD-20, Electro Technic Products) using a 20 s treatment of both surfaces. The completed devices were then allowed to rest on a hot plate at 60°C for 2 h to improve bonding. In the final step, devices were primed with 100 µL of DI water. Representative images of the completed chip and resulting channel cross-section are shown in Fig. 2.

Figure 2. Photographs of the completed PDMS chip (a) and the microchannel cross-section (b). Scale bar is 25µm.
Lipid compositions

Three lipid compositions were used in this project to study the encapsulation and size dependence. The first composition consisted of DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphocholine), DOPC (1, 2-dioleoyl-sn-glycero-3-phosphocholine) and DPPG (1, 2-dioleoyl-sn -glycero-3-phospho-(1′-rac-glycerol). All three components were purchased from Avanti Polar Lipids Inc. The DPPC: DOPC: DPPG and cholesterol were mixed in a molar ratio of 46:23:28:3 in a round bottom flask and placed in a rotary evaporator connected to a vacuum pump. The evaporator and the pump were operated for 3 h to completely remove the solvent. This was reconstituted in 3mL of IPA (2-isopropylalcohol). The result was a 10 mg/mL lipid solution, which was further diluted in 0.9% saline to concentrations of 0.1, 0.5, 1 and 2 mg/mL.

A second composition contained DPPC (1, 2-dipalmitoyl-sn-glycero-3-phosphocholine), DPPA (1, 2-dihexadecanoyl-sn-glycero-3-phosphate) and DPPE-PEG5000 (1, 2-distearoyl- sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-5000]). All three components again were purchased from Avanti Polar Lipids Inc. The lipids were mixed within chloroform, in a round bottom flask, in the molar ratio of 81:8:10. The flask was placed in a rotary evaporator in a water bath at 50°C, under an inert Argon gas atmosphere for 1 h and dried in a vacuum dessicator for an additional 3-4 h. The Argon gas prevented oxygenation of the lipids. The lipids were then mixed with glycerol, propylene glycol and DI water in a 1:1:98 volume ratio. This was then placed in a sonicator for 60 min. The result was a 10 mg/mL lipid solution, which was further diluted in 0.9% saline to concentrations of 0.1, 0.5, 1 and 2 mg/mL.

The third composition contained DSPC (1, 2-distearoyl-sn-glycero-3-phosphocholine) and DSPE-PEG2000 (1, 2-distearoyl- sn -glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (ammonium salt). Both components were purchased from Avanti
Polar Lipids, Alabaster, AL. The lipids were mixed with in chloroform in the molar ratio of 9:1 in a round bottom flask. The flask was then dried at 52°C under argon gas for 1 h and further dried under vacuum overnight. The lipid mixture was rehydrated in 10 mL of 0.9% saline solution to obtain a final DSPC concentration of 2 mg/mL, and sonicated in an ice bath until a clear solution was obtained (~1 h).

The lytic recombinant tissue plasminogen activator (rt-PA) (Genentech, Inc., San Francisco, CA) was suspended in DI water to obtain a 1 mg/mL solution, and further diluted in 0.9% saline to a concentration of 0.2 mg/mL. A 40 µL of 0.1 mM Calcein was added to rt-PA per mL. Prior to experiments, the lipid and rt-PA solutions were saturated with octafluorocyclobutane (C₄F₈). The solutions were transferred to a glass vial, and the mouth was sealed using a rubber septum. The air inside was aspirated through 20 gauge needle, using a vacuum pump, for 5 s and replaced with C₄F₈ at a pressure of 5 psi for 1 min. The solutions were then allowed to rest for 5 min to allow gas to dissolve. The solutions were mixed with a small stirrer at 350 rpm. The process was repeated 3 times. The last cycle of saturation was done by feeding C₄F₈ for 5 min, followed by 5 min of rest time.

**Experimental setup**

Each bonded microfluidic chip was attached to the microscope stage with lab tape. The microscope focus was adjusted at the orifice. The lipid and rt-PA solutions were loaded into 5 mL syringes (NORMJECT, Fisher Scientific). The solutions were loaded in 5mL syringes and placed in a syringe pumps (KD Scientific KDS230, Holliston, MA). Syringe pumps were used to set flow rates to 17.5 µL/min for the lipids, and 10.5 µL/min for rt-PA.

Preliminary device characterization experiments were performed using octafluoropropane (C₃F₈), while later experiments were coinducted with octafluoro-cyclobutane (C₄F₈) (Xpress
Cryogenics, North Lakewood, WA). The gas pressure was maintained at 2 psi with a high sensitivity regulator (McMaster Carr, Aurora, OH). The orifice for liposome generation was generated was monitored using a microscope (IX-71 Olympus) at 10X magnification. The generated liposomes were then collected in a vial using a pipette. For this 100 µL pipette tip was enlarged (using a horizontal cut) to prevent liposome coalescence and destruction during collection.

Prior to imaging, a 1000 µg/mL Nile Red solution in acetone was added to the vial containing the ELIP, wrapped with aluminum foil to prevent photo bleaching and allowed to stain for 5 min. A 70 µL fraction of the stained ELIP solution was pipetted onto a microslide. The microslide was imaged by taking bright field images, as well as fluorescent images using FITC (495/519 nm) for Calcein and TRITC (557/576 nm) for Nile Red. An inverted epi-fluorescence microscope (IX71, Olympus Inc.) equipped with a 12-bit high-speed CCD camera (Retiga EXi, QImaging) was used for all imaging. The FITC and TRITC images were overlayed using ImageJ software to yield composite fluorescent images.

**Chromogenic assay**

A spectrophotometric assay was employed to measure the enzymatic activity of ELIP. This assay exploits the reaction between a chromogenic substrate (S-2288, Chromogenix, DiaPharma Group, Inc., Westchester, OH, USA) and rt-PA [54]. Specifically, the chromogenic substrate is hydrolyzed by rt-PA, which results in the production of the chromophore paranitroaniline (pNA). The change of absorbance in solution over time at 405 nm gives the measure of the enzymatic activity. In this study, enzymatic activity was reported with respect to that of commercially available rt-PA (Activase®, Genetch, San Francisco, CA, USA). Commercial rt-PA was purchased from the manufacturer in the form of a lyophilized powder. The
rt-PA was reconstituted in sterile water to a concentration of 1 mg/mL and stored at –80 ºC until use. It has been demonstrated that this procedure preserves the enzymatic activity of rt-PA for at least seven years [57]. Prior to spectrophotometric measurement, the rt-PA was thawed and diluted to concentrations of 0.32, 1.58 and 3.15 μg/mL in 1% BSA and stored in disposable cuvettes. Measurements were performed over 5 min at 37 ºC using a spectrophotometer (UV-1700, Shimadzu, Japan) equipped with a temperature controller (TCC-240A, Shimadzu, Japan). Using linear regression, an rt-PA standard curve was generated. Spectrophotometric measurements were performed for a mixture of rt-PA and lipid to assess the effect of the ELIP manufacturing process on the activity of rt-PA. Subsequently, ELIP were diluted to a concentration of 3.25 μg/mL and the rt-PA activity assessed.

**ELIP collection and size analysis**

The generated ELIP were collected in a reservoir near the chip outlet. A 100 µL volume of surfactant (1% w/v BSA) was added to the reservoir. The ELIP were analyzed in the reservoir or on a glass slide. Images were taken immediately following generation (at t = 0 min) and every 15 min over a period of 1 h. These images were analyzed using CellSens software (Olympus, Pittsburg, PA, USA) to measure the diameter of the liposomes and to generate their count. The data were imported into MS Excel and values with sphericity <0.65 were considered debris and discarded. The remaining data were used in further analysis.

Particle size and number density were also measured using a Coulter Counter (Beckman Coulter Multisizer 4, Beckman Coulter Inc., Brea,CA,USA) with a 30 µm aperture. The microfluidically generated ELIP were suspended in a cuvette, in 0.5% BSA solution (100 µL of ELIP in 10 mL BSA). The cuvette was covered with a lid in between measurements to prevent
evaporation and gently inverted several times before each measurement to redistribute the ELIP within the solution.

**Attenuation spectrum measurements**

An ultrasound pulser-receiver (Panametrics 5077PR, Olympus NDT, Waltham, MA, USA) was used to generate an excitation pulse and amplify the received ultrasound signal over a frequency range of 2 to 25 MHz [55, 56]. The liposomes, diluted in a 0.5 % BSA solution (400 μL of liposome solution in 5 mL of BSA) were suspended in an unmodified cell-culture cassette (CLINICell, Mabio, Tourcoing, France) with luer-lock ports to introduce the sample suspension. The attenuation was calculated as the ratio of received signal strength with and without liposomes in BSA. The cassette was closed between measurements to prevent evaporation, and also gently inverted several times before each measurement. Measurements with only the diluent, 0.5 % BSA, were performed, and subtracted from the attenuation spectrum of the liposome suspensions.
CHAPTER 3

RESULTS AND DISCUSSION

This chapter discusses device design, device characterization and optimization for the production of ELIP. Comprehensive results on the resulting ELIP size, stability and drug encapsulation are presented. To investigate the process of microbubble generation, the first generation microfluidic devices were designed with only two channels. After optimizing the flow parameters, and confirming the successful generation of liposomes, the three channeled second generation devices were designed. Improvements to the second generation devices based on the lessons learnt from the first generation devices are presented. We then discuss the experiments to test our hypothesis that ELIP generation and drug encapsulation can be achieved within the desired size range.

First generation devices

The first generation devices were used to perform the preliminary experiments. They consisted of two channel inlets (Fig. 3a). The channel dimensions in this design included a 50 µm wide outer channel and a 40 µm wide inner channel. The channel height was 25 µm. Devices with a range of orifice sizes of 8, 10, 12.5, 15, 17, 20 and 25 µm were fabricated. Two types of outlets were designed. The first had outlets with parallel sidewalls after the post-orifice expansion (33° angle). The second contained a gradual expansion (15° angle) into an outlet reservoir.
Preliminary experiments were performed with oil and DI water to produce droplets (Fig. 3b). It was observed that increasing the flow rate of the continuous oil phase, keeping the flow rate of the dispersed DI water phase constant, decreased the size of the droplets generated. In another set of experiments, these devices were used to determine the self-assembly of lipids to form stable bilayer liposomes. DPPC: DOPC: DPPG-chol was run through the inner channel as the dispersed phase and BSA was run through the outer channel as the continuous phase.

Microbubble generation was monitored at the orifice with a microscope. It was observed that the devices with bigger orifice sizes yielded larger liposomes (Fig. 3d). Table 1 summarizes the average size of liposomes generated by the devices with different orifice sizes. In all experiments, the outer oil channel was 50 µm wide and the inner DI water channel was 45µm wide. The width of the lipid and drug channels were 50 µm and 45 µm, respectively. The devices with orifice size of 8 µm and 10 µm generated liposomes of the desired range.

It was also observed that the outlet with parallel sidewalls was able to generate liposomes with more control. The liposomes were observed to flow in a single stream, with the continuous

<table>
<thead>
<tr>
<th>Orifice size (µm)</th>
<th>Average size of ELIP generated ( µm )</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>6.6</td>
</tr>
<tr>
<td>10</td>
<td>9.5</td>
</tr>
<tr>
<td>12.5</td>
<td>10.</td>
</tr>
<tr>
<td>15</td>
<td>12.3</td>
</tr>
<tr>
<td>17</td>
<td>21.3</td>
</tr>
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<td>20</td>
<td>25</td>
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<td>30</td>
</tr>
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</table>
Figure 3. (a) First generation microfluidic chip design. (b) Droplet generation done with oil-water interface. Smaller droplets are produced with increasing the oil flow rate ($Q_{oil} = 4-8 \mu$L/min), keeping the water flow rate constant at $Q_{water} = 1 \mu$L/min. (c) Effect of different types of outlets on the output flow. Control on the size of the bubbles was lost with expanded outlet. (d) Representative images of different size of liposomes generated using different orifice sizes of 17 µm (i), 12.5 µm (ii), 10 µm (iii) and 8 µm (iv) respectively.
phase creating a sheath flow around them. The gradual expansion of the reservoir created disturbances within the outlet which resulted in coalescence of liposomes into larger ones (Fig. 3c). One major challenge was the occurrence of channel clogging. Running the lipids in isopropyl alcohol and BSA led to protein aggregation in the PDMS channels. The debris in the reagents and the protein aggregation led to clogging in the channels and also at the orifice. Based on these observations, a second generation device was designed. To address the clogging issue, six arrays of filters were put in the lipid inlet of the second generation devices as the lipids contained more aggregated particles.

**Second generation devices**

The device design used for this project is shown in Fig. 4. The channel widths were set as follows: the outer lipid channel was 50 µm, the inner rt-PA channel was 35 µm, and the middle channel, which was used for C₃F₈ gas flow was 35 µm. Two orifice sizes were used, 8 µm and 10 µm. The channel height of all devices was fixed at 25 µm. The outlet was parallel based on the first generation devices. It was observed that the liposome generation depended on the downstream pressure conditions. A single downstream channel with parallel sides from the post-orifice expansion was selected out of the other outlet designs, to maintain a controlled single stream flow of the generated ELIP. This prevented coalescence that was previously observed in the angular expanded outlet channel design. The outlet channel width was 150 µm, with a length of 2.5 mm. A sheath flow of the carrier fluid was created on the periphery of the channel walls. This helped in maintaining a laminar single streamline flow of the liposomes. The lower volumetric flow rate prevented collision of the liposomes, which in turn prevented coalescence-induced destruction of ELIP.
PDMS has a high permeability to gases. Although the diffusion coefficient of perfluorocarbons (such as C₃F₈ or C₄F₈ in this work) in PDMS are several orders below the oxygen, to minimize gas diffusion through PDMS the gas inlet channel was minimized in length. This also resulted in maintaining a very low gas pressure (~2 psi) for generation of the liposomes. The size of the generated liposomes also depended on the gas pressure and the flow rates of lipid and drug.

To optimize flow rates in the system, one flow rate was kept constant and the other was varied in increments or decrements of 0.5 µL/min, until the desired liposome size was obtained. Increase in gas pressure keeping the flow rates constant, an increase in the size of the liposomes. A larger, 2mm diameter outlet port was punched in the PDMS before bonding. This was done to give enough chamber volume for the generated ELIP to be suspended during production in the reservoir. A smaller outlet port results in spatial competition which results in the ELIP coalescence, compromising in size, or even releasing of gas and drug.

Figure 4. Second generation device with three channels and outlet with parallel sides. A schematic illustrating device inputs and outputs (a) and a close-up photograph of the ELIP generation orifice (b). Width of channels was 50 µm for lipid, 35 µm for the drug and 35 µm for gas. Height of the channels was fixed at 25 µm.
Flow rate characterization

Stable generation of liposomes was observed with the appearance of a distinct interface, separating the gas, the lipid and the drug flows, upstream of the orifice. This interface was where the self-assembly of the lipid bilayer occurred. The gas thread was observed to oscillate at the orifice, where it was projected into the orifice, the surrounding lipid broke the thread and subsequently retracted. The lipid and drug pinched the central gas stream and the oscillating pressure developed at the orifice resulted in generation of liposomes. Rapid hydrodynamic oscillations at the interface were observed, which determined the break-up of the stream into liposomes. When the oscillation stopped, the ELIP generation also stopped.

For any given concentration the smallest ELIP (~5 µm) were obtained at ≤2 psi. The flow rate conditions used for lipid (DPPC: DPPA: DPPE-PEG-5000) were 17.5 µL/min, and 10.4 µL/min for rt-PA. A lower volumetric flow resulted in slow production of monodisperse ELIP. A higher production rate was observed when the lipid flow rate of lipid was increased to 42 µL/min and rt-PA flow rate was 20 µL/min. To increase the production rate, these designs can be arranged in a circular radial arrangement, with multiple lipid, drug and gas inlets.

At higher cumulative flow rates, the gas pressure had to be increased to 4 psi. This led to the liposome sizes increasing beyond the desired range (20 µm). Increasing flow rates further stopped ELIP generation altogether, as the gas flow was completely obstructed by the lipid and drug flow. High flow rates also led to a very high pressure drop inside the chip. A single stream of ELIP generation was not possible under these conditions. Although the generation rate increased, the control over the size of the ELIP was lost. Based on these observations, the cumulative flow rate was kept under 70 µL/min, at which the desired range of the liposomes could be obtained. Table 2 summarizes the cumulative flow rates of lipid and drug, and the
Table 2. Average size of ELIP generated with different flow rates used in the second generation devices.

<table>
<thead>
<tr>
<th>Total Flow Rate (µL/min)</th>
<th>Reynolds number ($Re$)</th>
<th>Average size of ELIP (µm)</th>
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<tbody>
<tr>
<td>23</td>
<td>5</td>
<td>15.5</td>
</tr>
<tr>
<td>27</td>
<td>5.9</td>
<td>13.8</td>
</tr>
<tr>
<td>37</td>
<td>8.1</td>
<td>12</td>
</tr>
<tr>
<td>52</td>
<td>11.4</td>
<td>8.9</td>
</tr>
<tr>
<td>63</td>
<td>13.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Figure 5. Representative images of different sizes of ELIP generated with increasing flow rates of lipid (a) $Q_{\text{Lipid}}$ - 45 µL/min, (b) $Q_{\text{Lipid}}$ - 50 µL/min, (c) $Q_{\text{Lipid}}$ - 60 µL/min (d) $Q_{\text{Lipid}}$ - 63.5 µL/min, keeping the flow rate of drug and gas constant. Gas ($C_3F_8$) pressure is 2 psi.
respective Reynolds number ($Re$) at which ELIP generation was observed within the desired range. Fig. 5 illustrates the change in ELIP size with increasing flow rates.

**Drug encapsulation**

Recombinant tissue plasminogen activator (rt-PA) is the only FDA approved thrombolytic for the treatment of acute ischemic stroke. However, the use of rt-PA is associated with an increased risk of symptomatic intracranial hemorrhage, poor recanalization efficiency, and high rates of reocclusion.[51, 52]. Microfluidic manufacture of rt-PA-loaded ELIP with a narrow size distribution may provide the advantage of increased thrombolytic efficacy. One of the major challenges was to prove encapsulation of the drug within the liposome.

To determine the drug loading, Calcein (0.1 mM) was added to rt-PA in a syringe prior to generation of the ELIP. After generation of ELIP, Nile Red (1000 µg/mg in acetone) was added and stained for 5 min. A 70 µL volume of the stained ELIP was then pipetted onto a chemotaxis slide. Bright field, FITC and TRITC images were taken of different areas of the outlet reservoir. Images were taken with TRITC exposure of 60 ms and FITC exposure 35 ms. Extreme care was taken so as to not cause any vibrations that might cause a shift in position of the liposomes. To quench the remaining Calcein within the surrounding fluid, 50 µL of CoCl$_2$ was added to the

![Figure 6](image)

**Figure 6.** Images showing drug encapsulation in the ELIP. Overlay images of before (a) and after (b, c) adding CoCl$_2$, to quench Calcein. Concentric rings show encapsulation of rt-PA. Red is Nile red staining the lipid bilayer and green is the Calcein in the rt-PA. Gas- C$_3$F$_8$. Scale bar is 15µm.
reservoir. Fig. 6a shows the fluorescent images of the ELIP before adding CoCl$_2$, indicating the presence of unencapsulated rt-PA in the surrounding fluid and of that may also be adsorbed on the ELIP surface. Fig. 6 shows the ELIP after adding CoCl$_2$. The inner green Calcein ring suggests the encapsulation of the drug. The fluorescence from calcein was from the inner lipid layer of the ELIP (Fig. 6c). This suggests the encapsulation of the drug. However, fluorescence signal from rt-PA that may also be attached to the outer surface of the ELIP is quenched and thus not visible in these images.

**Chromogenic assay**

The results of the chromogenic assay performed on a mixture of rt-PA and phospholipids extruded through the microfluidic device and the ELIP particles are shown in Fig. 7. The rt-PA and phospholipid mixture were extruded through the microfluidic device at the same flow rates as that used for ELIP generation. The amount of rt-PA associated with the ELIP particles was

![Graph showing the amount of rt-PA associated with different samples](image)

**Figure 7.** The amount of rt-PA associated with the rt-PA and lipid mixture extruded through the microfluidic device (148.70 ± 30.80 μg/mL) compared with that associated with the μtELIP shell (64.30 ± 3.80 μg/mL).
measured to be (64.30±3.80 μg/mL), and that associated with the rt-PA and phospholipid mixture was found to be (148.70±30.80 μg/mL), which is about 43% of the total free rt-PA added to the system (148.70±30.80 μg/mL).

Previous studies have reported a drug loading efficiency of approximately 50% for batch-produced rt-PA loaded ELIP, of which about 30% is associated with the shell surface and the remaining 20% with the rt-PA encapsulated within the ELIP [42]. The chromogenic assay performed in this work, used ELIP with an intact shell and does not account for the rt-PA that may be encapsulated within the ELIP. However, since no shear-related degradation was observed in the rt-PA after flowing through the microfluidic device, the difference between the activity of the rt-PA and lipid mixture and that of ELIP with intact shell structures is estimated to correspond to the rt-PA encapsulated within ELIP.

**ELIP size and stability**

The self-assembly of the lipid bilayer determines the stability of the liposome. Experiments were repeated with three different concentrations of lipids (0.5, 1 and 2 mg/mL) of the same composition (DSPC: DSPE-PEG2000) to confirm the repeatability of the generation of ELIP within the limited desired range and to measure the stability of the generated ELIP. Here the ELIP were observed directly on the chip with a bigger reservoir. The suspension environment was varied by pipetting 0.9% saline, 1% BSA, or a thermo-reversible surfactant, Pluronic F-127 (Sigma-Aldrich) into the chip reservoir. Images were taken at 10 zones, with periodic stirring of the reservoir ELIP solution, at intervals of 15 min for 1 h.

ELIP suspended in saline, BSA and gel at \( t = 0 \) and \( t = 60 \) min are shown in Fig. 8. These images were analyzed for size and count. Total count of ELIP was \( n = 2101 \), with a mean size \( \mu = 5.14 \mu \text{m} \pm 1.92 \), and coefficient of variation \( CV = 0.37 \). A histogram of the size distribution of
the ELIP population generated from 1 mg/mL lipid solution, is shown in Fig. 9. The total sample volume for these images was 70 µL. During image analysis, the sphericity of objects was set to 0.65. Instances with sphericity <0.65, were considered as debris in the sample and discarded. However, some liposomes were located in a different focal planes which the software was not able to count (due to lower greyscale value than threshold value), reducing precision of the counting technique.

The size distribution of the ELIP, generated from higher lipid concentration (10 mg/mL), over a time period of 30 min is shown in Fig. 10. Both number-weighted and size-weighted show a size distribution of $5 \pm 0.5$ µm. The number-weighted distribution shows the presence of particles smaller than 3 µm, and the volume-weighted distribution shows that ELIP of diameter $5 \pm 0.5$ µm constitute majority of the sample volume, which also will be the main contribution to the acoustic backscatter from the ELIP [56]. The number density was observed to gradually

![Figure 8](image_url)

**Figure 8.** ELIP at the outlet reservoir. Lipid concentration was 2 mg/mL. ELIP were suspended in 0.9% saline, 1% BSA and thermo-reversible gel. Scale bar is 200µm.
Figure 9. Histogram of ELIP population from 1mg/mL lipid solution.

Figure 10. Multisizer data showing the number-weighted and volume – weighted size distribution of ELIP at 10 (a-b), 16 (c-d) and 32 (e-f) min.
decrease over the time period (30 min) of the measurements. The total number density of the ELIP was about \(2.4 \times 10^8\) particle/mL, which is lower than the batch produced rt-PA loaded ELIP or Definity® (\(10^{10}\) particle/mL).

It was observed that the size of the ELIP was smaller for lower concentrations of lipids. Experiments were done with a lower concentration of 0.13 mg/mL, and yielded smaller liposomes (average diameter \(\approx 4\) \(\mu\)m), as reported in Fig. 11. The diameter of the ELIP decreased by 1-2 \(\mu\)m after 15 min. This may have been due to evaporation at the reservoir which resulted in loss of ELIP or due to diffusion of the gas through the lipid membrane. The smaller (2-4 \(\mu\)m diameter) ELIP underwent quicker dissolution. This can be explained by the Laplace pressure which is inversely proportional to the diameter of the droplet (\(\Delta P = 2\gamma/R\)), hence the pressure inside the smaller ELIP was too high. The number density of the ELIP suspended in saline, generated from lower concentration lipids (0.5 and 1 mg/mL), were observed to decrease with time and were more stable in 1% BSA and the gel, as it significantly decreased the evaporation observed in saline.

The lifetime of ELIP generated from 0.5 mg/mL solution in three media and was found to be most stable in Pluronic F-127. The results are shown in Fig. 12. The lifetime of ELIP generated from all three lipid concentrations and suspended in 0.9% saline is plotted in Fig. 13. ELIP generated from 2 mg/mL are the most stable with an average lifetime of 40 min. ELIP generated from the higher concentration lipid (2 mg/mL) were stable in all three environments of saline, BSA and Pluronic F-127.

Fig. 14 shows the data for size of ELIP over a period of 60 min in three suspension environments of saline, BSA and gel for all three lipid concentrations used. ELIP generated from
Figure 11. ELIP diameter vs. concentration of lipid suspended in 0.9% Saline. Red: 0 min, Blue: 15 min.

Figure 12. Lifetime of ELIP generated from 0.5 mg/mL lipid solution in three different suspension media of 0.9% Saline, 1% BSA and Pluronic F-127.

Figure 13. Lifetime of ELIP generated using 0.5, 1 and 2 mg/mL solutions suspended in 0.9% saline.
Figure 14. ELIP sizes as a function of time for (a) 0.5 mg/mL, (b) 1 mg/mL and (c) 2 mg/mL, concentration of lipid. Data shown in individual suspension environments for 60 min. Green – 0.9% Saline, Orange – 1% BSA and Blue – Pluronic F-127.
lower concentrations of lipid did not survive more than 15-30 min in 0.9% saline or 1% BSA (due to evaporation or rapid gas diffusion in media), but survived in gel. The gel prevented the diffusion of gas and also evaporation resulting in increased stability of the suspended ELIP.

**Attenuation spectrum measurements**

Fig. 15 shows the attenuation of the ELIP as a function of frequency over a 30 min period. The ELIP attenuation spectrum peaks at 2.2 MHz, corresponding to the particles in the 5 ± 0.5 μm size range, which is similar to the size measurements done using the CellSens software. Previous studies have shown that monodisperse ELIP produce a sharper peak in the frequency dependent ultrasound attenuation [59]. The ELIP show a sharp peak and a narrower bandwidth in the attenuation spectrum, compared with the attenuation spectra of various commercial ultrasound contrast agents and rt-PA-loaded ELIP (data not shown) [55]. Negligible attenuation was observed at higher frequencies, which suggests that the smaller particles observed in the size measurements did not contribute significantly to the acoustic response of ELIP. The attenuation

![Figure 15](image.png)

**Figure 15.** Average attenuation of ELIP suspended in 0.5% BSA at 22.5 °C measured after 30 min.
remained stable for the first 15 min of the experiment, followed by a decrease in attenuation below 4 MHz after 30 min. No change in the magnitude or the peak position of the attenuation spectrum is observed for the first 15 minutes of the experiment, which is indicative of good stability of the encapsulated gas. The decrease in the magnitude of the attenuation spectrum and the shift in the peak attenuation from 2.2 MHz to 3.2 MHz after 30 min, is most likely due to diffusion of the gas from resonant-sized microbubbles.
CHAPTER 4

CONCLUSIONS

In this work, microfluidic devices were designed to test the hypothesis that drug loaded echogenic liposomes can be generated in the 2-20 µm size range. A standard soft lithography process was used in device fabrication. Microfluidic devices with two different outlet types were tested and characterized for generation of monodisperse echogenic liposomes within the desired size range. The effect of these outlet designs in the generation and retention of size of the ELIP was also investigated. Further, as part of the development process, different lipid compositions were tested in order to yeild the size distribution and stability desired in our application. The liposome stability of 1 h was achieved through changes in the media (saline, BSA, and thermos-reversible gel). The echogenicity of the liposomes was measured to have a resonance frequency of 2.2 MHz. Ultimately, these ELIP can be ultimately used as theragnostic ultrasound contrast agents.

In future work, challenges around ELIP collection and coalescence minimization still need to be addressed. Presently, collection by pipette suction is resulting in loss of about 20%-30% of ELIP generated. Increasing the reservoir volume at the outlet or integrating a collection vial at the outlet should improve ELIP collection. Collection of ELIP in an air tight vial, filled with perfluorocarbon may also result in increased stability of the ELIP. Further, redesigning the outlet channel maybe an alternative approach to minimizing coalescence of ELIP within the PDMS device. Increasing the outlet volume should also lead to a decrease in pressure drop and thus reduce flow resistance and potentially increasing survival rate of the smaller ELIP. In this work, the ELIP were not separated from unencapsulated rt-PA present in the carrier fluid. Some
approaches such as membrane dialysis or centrifugation of the ELIP need to be studied further. Redesigning the outlet system into two or three outlets for separation of excess unencapsulated drug in the carrier fluid can also be investigated.

Using microfluidics to multiplex the current design in a parallel array is another attractive prospect. A multiplexed ELIP generator would have a throughput several times higher than the current setup and would be better adapted to producing a higher number density of ELIP, which will be useful in future research.
REFERENCES


