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Microfluidic manufacture of rt-PA-loaded echogenic liposomes

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Abstract

Echogenic liposomes (ELIP), loaded with recombinant tissue-type plasminogen activator (rt-PA) and microbubbles that act as cavitation nuclei, are under development for ultrasound-mediated thrombolysis. Conventional manufacturing techniques produce a polydisperse rt-PA-loaded ELIP population with only a small percentage of particles containing microbubbles. Further, a polydisperse population of rt-PA-loaded ELIP has a broadband frequency response with complex bubble dynamics when exposed to pulsed ultrasound. In this work, a microfluidic flow-focusing device was used to generate monodisperse rt-PA-loaded ELIP (μ ELIP) loaded with a perfluorocarbon gas. The rt-PA associated with the μ ELIP was encapsulated within the lipid shell as well as intercalated within the lipid shell. The μ ELIP had a mean diameter of 5 μ m, a resonance frequency of 2.2 MHz, and were found to be stable for at least 30 min in 0.5% bovine serum albumin. Additionally, 35 % of μ ELIP particles were estimated to contain microbubbles, an order of magnitude higher than that reported previously for batch-produced rt-PA-loaded ELIP. These findings emphasize the advantages offered by microfluidic techniques for improving the encapsulation efficiency of both rt-PA and perfluorocarbon microbubbles within echogenic liposomes.

Keywords

Echogenic liposomes; Recombinant tissue-type plasminogen activator; Ultrasound-mediated thrombolysis; Microfluidic flow-focusing; Stroke treatment

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1 Introduction

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 (Hacke et al. 2004; Alexandrov and Grotta 2002; Burgin et al. 2000). *In vitro*, animal, and human studies have shown that the thrombolytic efficacy of rt-PA is significantly enhanced with concomitant exposure to ultrasound (Alexandrov et al. 2004; Braaten et al. 1997; Everbach and Francis 2000; Holland et al. 2002; Laing et al. 2012; Lauer et al. 1992; Shaw et al. 2008; Suchkova et al. 1998). Ultrasound-mediated thrombolysis (UMT) is a promising strategy to help lower the dose of rt-PA and enhance therapeutic efficacy. One of the main mechanisms responsible for UMT is acoustic cavitation, a process of sustained volumetric gentle oscillation (stable cavitation) or expansion and rapid collapse (inertial cavitation) of gaseous and vapor bubbles in a liquid due to an acoustic pressure field (Flynn 1982). Cavitation generates local velocity and pressure gradients, which can enhance the uptake of therapeutics into the surrounding tissue (Sutton et al. 2013). Previous *in vitro* studies have shown that sustained stable cavitation is necessary for promoting clot lysis (Datta et al. 2006).

Echogenic liposomes (ELIP) are phospholipid bilayer vesicles that encapsulate microbubbles. The microbubbles act as cavitation nuclei that promote sustained cavitation activity in the presence of an acoustic pressure field, and facilitate the delivery of therapeutic agents (Datta et al. 2008, 2006; Klegerman et al. 2008; Ramachandran et al. 2006; Shaw et al. 2009a, b). At an ultrasound frequency of 120 kHz, rt-PA-loaded ELIP demonstrated a significantly higher clot lysis compared to unencapsulated rt-PA with or without pulsed ultrasound exposure, attributed to the synergistic effects of stable cavitation, drug release, and fibrin targeting of rt-PA-ELIP (Datta et al. 2008; 2006; Holland et al. 2013; Laing et al. 2012; Shaw et al. 2009a, b; Smith et al. 2010; Sutton et al. 2013).

The size distribution of microbubbles is a key determinant of their acoustic response (Goertz et al. 2007). The majority of microbubbles in commercial ultrasound contrast agents (UCA) range from 1 to 10 μm in diameter. Bubbles larger than 10 μm are rapidly cleared by the capillaries in the lungs (de Jong et al. 1993). Nanobubbles (<1 μm in diameter) exhibit poor ultrasound scattering efficiency from 2 to 10 MHz and have low *in vivo* clearance rates (Palma and Bertolotto 1999). Microbubbles excited at twice their resonance frequency are known to undergo stable cavitation preferentially (Bader and Holland 2013). Therefore, the size range of microbubbles can be engineered for a particular application by considering their resonant frequency and clearance rates (Feshitan et al. 2009; Shekhar et al. 2013). However, current manufacturing processes produce a polydisperse size distribution of microbubbles. Moreover, studies have shown that only <20 % of ELIP manufactured using traditional liposome manufacturing techniques possess a gas core that is responsive to ultrasound (Raymond et al. 2014; Kodama et al. 2010). Current techniques for preparation of liposomes require post-processing steps, such as freeze thawing, which may adversely affect the enzymatic activity of protein drugs encapsulated in lipids compared to native proteins (Pikal-Cleland et al. 2000). Microfluidic generation allows for fewer post-processing steps, which may preserve the enzymatic activity of a protein-drug payload. Therefore, liposomes

prepared using microfluidic techniques may improve the efficacy of ultrasound-mediated drug delivery. Microfluidic flow focusing has been previously reported to manufacture lipid-coated microbubbles with a narrow size distribution for use as UCAs (Kodama et al. 2010; Hettiarachchi et al. 2007; Talu et al. 2006; Dhanaliwala et al. 2013; Dixon et al. 2013), and to encapsulate lipophilic drugs suspended in oil (Shih et al. 2013). However, loading of protein-drugs in phospholipid-based microbubbles using microfluidic flow-focusing has not been previously demonstrated. The objective of this work was to develop rt-PA-loaded ELIP (μ tELIP) with a perfluorocarbon gas core using a microfluidic flow focusing technique, and to characterize their size distribution, acoustic attenuation, and drug loading efficiency.

2 Materials and methods

2.1 Materials

A phospholipid mixture containing the phospholipids 1, 2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000) (Avanti Polar Lipids, Alabaster, AL, USA) in chloroform in the molar ratio 9:1 was dried at 52 °C under argon gas for 1 h. The flask was further dried under vacuum overnight, rehydrated in 0.9 % saline solution, and sonicated in an ice bath until a clear solution was obtained (~1 h). Propylene glycol and glycerol (Sigma, St. Louis, MO, USA) were added to the lipid solutions for a final concentration of 10 % (v/v) concentration each. A 25 % Pluronic F-127 (Sigma, St. Louis, MO, USA) surfactant solution was prepared by adding the appropriate amount to 0.9 % saline, leaving overnight at 4 °C, and further mixing in an ice-bath using a stir-bar to achieve a homogenous distribution. The surfactant solution was diluted in the lipid mixture to a final concentration of 5 % (v/v). To prevent blockage of the microchannels with impurities, the saline solution and lipid mixture were filtered twice through a syringe filter (0.2 μ m cellulose acetate filtration media GE Lifesciences, Pittsburg, PA, USA) prior to use. A 0.5 % (w/v) bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) solution in 0.9 % (w/v) saline was prepared. The drug rt-PA (Genentech, Inc., San Francisco, CA, USA) was suspended in 0.9 % saline to obtain a 1-mg/mL solution. Perfluorocyclobutane gas (C₄F₈, Specialty Gases of America, Inc., Toledo, Ohio, USA) was used directly from a pressurized cylinder for microbubble entrainment.

2.2 Microfluidic device fabrication

Microfluidic chips were fabricated in polydimethylsiloxane (PDMS) using the standard soft lithography process. Briefly, a 25- μ m high master was formed in SU-8 photoresist (Microchem Corp.). A mixture of PDMS base and curing agent (10:1 ratio) was poured onto the master, degassed for 120 min under vacuum, and cured for 4 h on a 60 °C hotplate. Polydimethylsiloxane (PDMS) polymer mixed with a curing agent was poured on the prepared silicon wafer. Once cured, the PDMS was separated from the master and inlet/outlet ports were punched with a 14 gauge syringe needle. PDMS was bonded to a standard glass slide using a hand-held plasma surface treater (BD-20 AC, Electro-Technic Products, Inc.). As shown in Fig. 1, the microfluidic devices contained a central gas channel, inner drug channels and outer lipid channels, which meet just upstream of the orifice. The channel

dimensions were: 50 μm and 30 μm for the outer and inner liquid channels respectively, 35 μm for the central gas channel, and 10 μm at the orifice.

2.3 Generation of μtELIP

The lipid mixture and rt-PA solutions were loaded into a syringe, placed in a syringe pump, and connected to the microchip via 1/16" o.d. tygon tubing. The lipid mixture and rt-PA were allowed to flow in the outer and inner side channels, respectively. The total combined flow rates of lipid and drug channels ranged from 35 to 115 $\mu\text{L}/\text{min}$, while a constant rt-PA flow-rate of 15 $\mu\text{L}/\text{min}$ was maintained. Octafluorocyclobutane (C_4F_8) gas was delivered to the central flow channel of the chip via a high precision pressure regulator (Swagelok, Cat. No. KLF1CFH412A20000, Cincinnati, OH, USA) using 1/16" o.d. tygon tubing.

2.4 Size and number density of μtELIP

The extruded μtELIP were observed using an inverted microscope (IX-71, Olympus Inc.) with a 12-bit high-speed CCD camera (Retiga EXi, QImaging). Particle size and number density were measured using a Coulter counter (Beckman Coulter Multisizer 4, Beckman Coulter Inc., Brea, CA, USA) fitted with a 30- μm aperture. For the size measurements the microbubbles were diluted in a 0.5 % bovine serum albumin (BSA) solution by a factor of 100 (i.e., 100 μL of liposome solution per 10 mL of BSA). The cuvette was covered with a lid provided by the manufacturer between measurements to prevent evaporation, and gently inverting several times before each measurement.

2.5 Fluorescence imaging

Nile red (Sigma, St. Louis, MO, USA), a highly lipophilic dye, was added to μtELIP to stain the phospholipid shell selectively. Nile red was purchased as a solid powder, and dissolved in acetone at a concentration of 0.5 μM . The dye calcein (Sigma, St. Louis, Missouri, USA) at a concentration of 0.1 mM was added to the rt-PA prior to μtELIP manufacturing. A 10 mM cobalt chloride solution was used to quench any fluorescence from unencapsulated rt-PA. The μtELIP were stabilized against motion in a 3D chemotaxis slide. Fluorescence emissions from calcein and Nile red were observed using 495/515 nm and 485/525 nm filters, respectively.

2.6 Attenuation spectrum measurement

Broadband attenuation of US through a μtELIP sample suspended in 0.5 % BSA was measured using a through-transmission acoustic spectroscopy system (Raymond et al. 2014; Kopechek et al. 2011; Raymond et al. 2013). Briefly, an ultrasound pulser-receiver (Panametrics 5077PR, Olympus NDT, Waltham, MA, USA) was used to generate the excitation pulse and amplify the received ultrasound signal over a frequency range of 2 to 25 MHz. The peak rarefactional pressure of the acoustic pulse was 31 kPa (Raymond et al. 2014). Test samples of μtELIP ($n = 3$) were placed 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.

For the attenuation measurements, the liposomes were diluted in a 0.5 % BSA solution by a factor of 12.5 (i.e., 400 μ L of liposome solution in 5 mL of BSA). The cassette was closed between measurements to prevent evaporation, and 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. All measurements were performed at room temperature (22.5 ± 0.5 °C).

2.7 Chromogenic assay

A spectrophotometric assay was employed to measure the enzymatic activity of μ ELIP. This assay exploits the reaction between a chromogenic substrate (S-2288, Chromogenix, DiaPharma Group, Inc., Westchester, OH, USA) and rt-PA (Smith et al. 2010). Specifically, the chromogenic substrate is hydrolyzed by rt-PA, which results in the production of the chromophore para-nitroaniline (pNA). The enzymatic activity can be inferred by measuring the change of absorbance in solution over time at 405 nm. In this study, enzymatic activity was reported with respect to that of commercially available rt-PA (Activase[®], Genetech, San Francisco, CA, USA). Commercial rt-PA was obtained 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 °C until use. It has been demonstrated that this procedure preserves the enzymatic activity of rt-PA for at least 7 years (Shaw et al., 2009a, b). Prior to spectrophotometric measurement, the rt-PA was thawed and diluted to concentrations of 0.32, 1.58 and 3.15 μ g/mL in 1 % BSA and stored in disposable cuvettes. Measurements were performed over 5 min at 37 °C using a spectrophotometer (UV-1700, Shimadzu, Japan) equipped with a temperature controller (TCC-240 A, Shimadzu, Japan). Using linear regression, an rt-PA standard curve was generated. Next, spectrophotometric measurements were performed for a mixture of rt-PA and lipid to assess the effect of the μ ELIP manufacturing process on the activity of rt-PA. Subsequently, μ ELIP were diluted to a concentration of 3.25 μ g/mL and the rt-PA activity assessed.

2.8 Estimation of shell parameters

The shell properties of μ ELIP were estimated using a linearized acoustic model proposed by de Jong et al. in which the UCA shell is characterized by ad hoc parameters for shell elasticity (S_p in N/m) and shell friction (S_f in kg/s) (de Jong and Hoff 1993). The details of this model have been described previously (Raymond et al. 2014; Goertz et al. 2007). Briefly, this model assumes that the acoustic attenuation of a population of microbubbles is the sum of the contribution of individual microbubbles. Estimates of shell parameters were obtained by minimizing the sum-squared error difference between the estimated and measured attenuation coefficients. Suspensions of μ ELIP may also contain non-echogenic particles that do not contribute to the acoustic attenuation. The size distribution of echogenic μ ELIP was assumed to be proportional to the number of particles determined by Coulter counter measurements. The number density of μ ELIP that contributed to the attenuation (N_{fit}) was determined empirically by minimizing the difference between the cumulative deviations of model predictions from the mean of the measured response. The coefficient of determination (R^2) was calculated to indicate the goodness of fit. The values of the shell parameters that lead to the doubling of the error function value were selected as the limits for the confidence interval (Raymond et al. 2014).

3 Results

3.1 Particle size and stability

As shown in Fig. 2, the size of the extruded μ ELIP decreased with increasing lipid flow rates (40–63.5 μ L/min) for a constant drug flow rate of 15 μ L/min, and a constant gas pressure of 9–10 psi. Using lipid concentrations between 0.1 and 2 mg/mL, flow rates between 30 and 45 μ L/min, and gas pressures between 3 and 4 psi were sufficient to produce particles in a size range appropriate for use in ultrasound-mediated drug-delivery (1–10 μ m). However, these particles either aggregated to form larger particles (Fig. 3a) or rapidly contracted and disappeared within seconds. In contrast, a higher (10 mg/mL) lipid concentration using the same flow rates and gas pressures resulted in stable microbubbles (Fig. 3b). In the absence of the Pluronic F-127 surfactant, the particles demonstrated poor stability lasting on the order of 10–300 s. The size distribution of μ ELIP is shown over a 30-min time interval in Fig. 4. Both number-weighted and volume-weighted distributions peak at a particle diameter of around 5 ± 0.5 μ m. The number-weighted size distribution also shows the presence of particles smaller than 3 μ m.

3.2 Broadband attenuation

Figure 5 shows the attenuation spectrum of the μ ELIP as a function of frequency over a 30-min period. The μ ELIP spectrum peaks at 2.2 MHz, corresponding to the particles in the 5 ± 0.5 μ m size range. 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 μ ELIP. The attenuation remained stable for the first 15 min of the experiment, followed by a decrease in attenuation below 4 MHz after 30 min.

3.3 Estimation of shell parameters

Figure 6 shows the measured attenuation coefficient of μ ELIP as a function of frequency and the theoretical fit based on the estimated shell parameters. The theoretical fit is in good agreement with the measured attenuation coefficients ($R^2 = 0.94$). The estimated shell parameters are shown in Table 1 with the estimated shell parameters of Definity[®] and batch-produced rt-PA-loaded ELIP reported by Raymond et al. (Raymond et al. 2014) Though the shell elasticity parameter (S_p) of μ ELIP is comparable to Definity[®], the shell friction parameter (S_f) is approximately five times higher than Definity[®]. The percentage of echogenic particles in the μ ELIP suspension ($N_{FIT}/N_{MEAS} \times 100$) was found to be approximately 35 %.

3.4 Encapsulation of rt-PA

Extruded μ ELIP liposomes with the calcein-stained rt-PA (green) and the Nile Red-stained phospholipids (red) are shown in Fig. 7. In the absence of a quenching agent, fluorescence from calcein was visible both on the μ ELIP surface and in the core (Fig. 7a–b). The addition of cobalt chloride, a quenching agent for calcein, resulted in the loss of fluorescence from calcein on the surface of the μ ELIP particles. However, fluorescence from calcein associated with the rt-PA encapsulated within the μ ELIP particles was still visible (Fig. 7c).

Figure 8 shows the results of the chromogenic assay performed on a mixture of rt-PA and phospholipids extruded through the microfluidic device and the μ ELIP particles. The rt-PA and phospholipid mixture were extruded through the microfluidic device at the same flow rates as that used for μ ELIP generation. A comparison with the rt-PA standard curve showed good agreement between the expected and measured rate of change of absorbance for the rt-PA in the extruded mixture, indicating that extrusion through the microfluidic device itself did not cause degradation of rt-PA (data not shown). The amount of rt-PA unencapsulated in suspension and associated with the shell of μ ELIP was measured to be $(64.30 \pm 3.80 \text{ } \mu\text{g/mL})$, and that associated with the rt-PA and phospholipid mixture was found to be $(148.70 \pm 30.80 \text{ } \mu\text{g/mL})$.

4 Discussion

The use of microfluidic flow focusing to manufacture monodisperse lipid-based ultrasound contrast agents has been demonstrated previously (Hettiarachchi et al. 2007, 2009; Jahn et al. 2004; Shih et al. 2013; Talu et al. 2007, 2006). However, the stability of the particles in these studies was variable and ranged from 10 min to several hours. In this work, we found that employing higher lipid concentrations improved particle stability. Specifically, a lipid concentration of 10 mg/mL DSPC was necessary to achieve particle stability of at least 30 min consistently. Although, this concentration is higher than lipid concentrations used for the microfluidic manufacturing of liposomes reported in literature (0.3–3.5 mg/mL DSPC), this higher lipid concentration is similar to the concentration used in conventional batch manufacturing of ELIP with or without drug loading (Huang et al. 2001, 2008; Swanson et al. 2010; Thomson et al. 2014). It has been demonstrated previously that increasing the acyl chain length increases the stiffness of microbubbles, resulting in higher resonance frequency and *in vivo* stability (Borden et al. 2005). However, increasing the acyl chain length may also result in a higher threshold for microbubble rupture and for cavitation emissions from microbubbles. Further, it has been shown that changing the main lipid component of microbubbles can alter ligand distribution, binding area, and bound microbubble shape (Kooiman et al. 2014). Our future studies will investigate the impact of changing acyl chain length and lipid components in μ ELIP.

The surfactant, Pluronic F-127, combined with a 10% propylene glycol and glycerol water (PG water) added to the lipid mixture was essential to the generation of stable particles. Higher solution bulk viscosity has been shown to decrease bubble coalescence due to a decreased film drainage rate (Sanadaa et al. 2005; Talu et al. 2006). The higher bulk solution viscosity achieved with the combined use of PG water and a high concentration lipid solution likely contributed to the enhanced liposome stability. Addition of the surfactant at a 5 % (v/v) concentration effectively reduced particle aggregation post-production and significantly enhanced the number density during particle generation. Similar observations have been made in previous studies where different pluronic surfactants were used for the microfluidic generation of non-lipid or lipid shelled microbubbles (Angilè et al. 2014; Shih et al. 2013). The decreased coalescence of particles was attributed to a decrease in the surface tension caused by rapid adsorption of surfactant molecules on the liposome surface. The addition of a surfactant has also been shown to protect protein conformation and functionality (Akers and Defelippis 2000). Moreover, the surfactant pluronic F-127 is a

thermoreversible gel, and may contribute to the greater stability of the μ ELIP at physiological temperatures (Escobar-Chavez et al. 2006).

The number-weighted size distributions of μ ELIP over time show a large number of microparticles with diameters lower than 3 μ m. However, the volume-weighted distributions reveal that particles with diameters between 5 ± 0.5 μ m account for the majority of the volume fraction of μ ELIP. Therefore, liposomes with diameters between 4.5 μ m – 5.5 μ m are expected to be important for therapeutic delivery. These liposomes will also be the dominant contributors to the acoustic backscatter from μ ELIP (Gorce et al. 2000). The resonance frequency of the μ ELIP was close to 2 MHz, which may be suitable for transcranial sonothrombolysis applications (Alexandrov et al. 2004). It is noteworthy that this frequency may not be able to penetrate the skull for nearly 15 % of patients (Wijnhoud et al. 2008). For such patients, sub-megahertz frequencies may be employed (Bouchoux et al. 2012, 2014) at which microbubbles in the 20–50 μ m range are resonant. However, microbubbles larger 7 μ m are filtered by the lungs and thus for cavitation nucleation for enhancement of rt-PA thrombolysis. Resonant bubbles of 20–50 μ m could be formed by coalescence of smaller bubbles liberated from μ ELIP experiencing Bjerkness forces (Bader et al. 2015a, b). Therefore, μ ELIP could be tested for sonothrombolysis at sub-megahertz frequencies.

As shown in Fig. 7a–b, fluorescence from calcein in μ ELIP particles diluted 50 times in 0.9 % saline indicates a strong association between rt-PA and the lipid shell. The rt-PA attached to the surface of ELIP can potentially enable selective therapy through the fibrin-targeting sites on rt-PA, which could enhance localized cavitation activity and ultrasound-mediated thrombolysis (Sutton et al. 2013). Cobalt chloride was added to μ ELIP to quench fluorescence from calcein associated with the surface of the μ ELIP or that associated with excess unencapsulated rt-PA in solution. As shown in Fig. 7c, fluorescence was still observed from the μ ELIP indicating that rt-PA had been encapsulated within the μ ELIP. The amount of rt-PA encapsulated within μ ELIP was found to be about 57 % of the total free rt-PA added to the system. Previous studies have reported a total 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 (Smith et al. 2010). In this work, the chromogenic assay was performed on suspensions of μ ELIP with an intact shell and represents measurement of the rt-PA loaded onto the outside surface of the μ ELIP as well as unencapsulated rt-PA. When free rt-PA was passed through the microfluidic device, no degradation of the enzymatic activity was observed. Therefore, the amount of rt-PA encapsulated in μ ELIP was estimated from the difference between the concentration of rt-PA injected into the microfluidic device and the concentration of rt-PA either loaded on the μ ELIP or unencapsulated in the suspension.

Previous studies have shown that compared to a polydisperse microbubble population, monodisperse microbubbles produce a sharper peak in the frequency dependent ultrasound attenuation (Gong et al. 2010) and can be detected with greater sensitivity when imaged close to their resonance frequency (Talu et al. 2007). Compared with the attenuation spectra of various commercial ultrasound contrast agents and rt-PA-loaded ELIP, the monodisperse μ ELIP show a sharp peak and a narrower bandwidth in the attenuation spectrum (Raymond

et al. 2014). No change in the magnitude or the peak position of the attenuation spectrum is observed for the first 15 min of the experiment, 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 passive diffusion of the gas from resonant-sized microbubbles.

One of the main limitations of batch-produced ELIP is that only a small percentage of particles encapsulate a gas-core. The percentage of μ ELIP particles contributing to attenuation ($N_{FIT}/N_{MEAS} \times 100$) estimated using the model was found to be significantly higher (approximately 35 %) than that estimated for batch produced rt-PA-loaded ELIP (1.2 %), 22.5 ± 0.5 C (Table 1). Perfluorocarbon gases are known to improve the lifetime of echo contrast agents due to low solubility in aqueous media and low diffusivity through lipid shells, and are used in commercial contrast agents such as Definity[®] (Raymond et al. 2014). Studies have shown that the cavitation activity from rt-PA loaded ELIP with an air core is lower than Definity[®], suggesting that incorporating perfluorocarbon gas within ELIP instead of air may enhance their cavitation activity and thrombolytic efficacy (Bader et al. 2015a, b). The shell friction parameter (S_F) for μ ELIP is significantly higher than Definity[®] or batch-produced rt-PA-loaded ELIP (Table 1). The enhanced rigidity of μ ELIP prepared using a longer acyl chain phospholipid (DSPC), compared to Definity[®] or batch-produced rt-PA-loaded ELIP, which contain shorter acyl chains phospholipids, (DPPC, DPPA, and DPPG) may be responsible for the higher S_F parameter of μ ELIP. Another key difference between the μ ELIP and Definity[®] formulations is the use of PEG molecules of different molecular weights. The use of lower molecular weight PEG molecules combined with a higher phospholipid concentration may cause a tighter packing of the molecules in the μ ELIP shell compared to Definity[®], contributing to the higher S_F value. The enhanced bulk viscosity due to the use of a significantly higher phospholipid concentration (10 mg/mL) in the μ ELIP formulation, compared to Definity[®] (0.75 mg/mL) may also contribute to the higher S_F parameter. A higher shell friction parameter will produce increased damping, resulting in lower back-scatter intensity and an increased threshold for nonlinear oscillations and microbubble rupture compared to Definity[®]. Although many models of microbubble oscillation have been reported (Faez et al. 2013), the model from de Jong et al. (de Jong and Hoff 1993) was chosen. When linear approximations are employed at low acoustic pressures, the results of these models are equivalent (Kumar and Sarkar 2015).

A significant limitation of microfluidic manufacturing of liposomes is the low rate of particle generation compared to batch processes. In this work, the number density of μ ELIP generated was about 2.4×10^8 liposomes/mL, significantly lower than that reported for batch produced rt-PA-loaded ELIP or Definity[®] (10^{10} bubbles/mL). A wide range of particle concentrations has been reported in literature for studies on sonothrombolysis. Our group has demonstrated thrombolysis *in vitro* for approximately 1×10^6 microbubbles/ml (Bader et al. 2015a, b), two orders of magnitude lower than the liposome concentration achieved in this work. Shih et al. have reported microbubble generation rates as high as 3×10^6 per s (Shih et al. 2013). The number density of the micbubbles post-production was not investigated over time by these investigators. Device multiplexing is a possible solution to enhance the limited μ ELIP generation rate (Fabiilli et al. 2014). Another limitation of this work is that the measurements were performed at room temperature. Additional experiments

to determine the physical and acoustic properties of the μ ELIP at physiological conditions are required. In this work, the particles were not separated from unencapsulated rt-PA post-production. The effect of separation steps such as centrifugation or dialysis on the physical properties of the particles also needs to be studied further. In addition, the effect of blood viscosity on the threshold of stable cavitation nucleation from μ ELIP should be assessed (Helfield et al. 2016).

5 Conclusions

In this work, microfluidic devices were used to generate mono-disperse rt-PA loaded microbubbles in a clinically-relevant size-range. The gas-fraction of the μ ELIP was significantly higher than that reported previously for batch-produced rt-PA-loaded ELIP. The temporal stability of the particles was at least 30 min, with the use of a high lipid concentration of 10 mg/mL, 5(v/v) % surfactant pluronic F-127, and 10 % PG water solution in 0.9 % saline. Continued investigation of the physicochemical and acoustomechanical properties, efficacy, safety and stability of the μ ELIP over time, for ultrasound-mediated thrombolysis may help in the development of an ultrasound-mediated thrombolytic therapy.

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Fig. 1.
a Microfluidic device design showing the inner drug channels, outer lipid channels, orifice, and the outlet reservoir **(b)** zoomed-in view of the extrusion orifice, and **c** the microfluidic device

Fig. 2. Microbubble formation downstream of the orifice showing the effect of increasing flow rates on particle sizes. The flow rates in images (a–c) are 40, 50, and 63.5 $\mu\text{L}/\text{min}$ respectively. Scale bar: 50 μm

Fig. 3. Microscopic images of particles obtained using **(a)** a low lipid concentration (2 mg/mL), and **b** higher lipid concentration (10 mg/mL)

Fig. 4. Multisizer measurements showing number-weighted (left) and volume-weighted (right) μ tELIP size distributions at (a–b) 10 min, c–d 16 min, and e–f 32 min

Fig. 5. Average US attenuation of μ tELIP suspended in 0.5 % BSA at 22.5 ± 0.5 °C measured after 30 min ($N = 3$). Error bars represent standard deviations

Fig. 6. Measured attenuation coefficients as a function of frequency (*dashed lines*) and theoretical fits (*solid lines*) based on the estimated shell parameters of the μELIP . The gray band denotes the 95 % confidence interval for the fit

Fig. 7.
a μ tELIP diluted in 0.9 % saline without cobalt chloride quenching demonstrating the presence of adsorbed rt-PA on the μ tELIP exterior surface **(b)** Fluorescent staining of a μ tELIP showing the lipid layer in *red* and the calcein associated with encapsulated rt-PA in *green* without cobalt chloride **(c)** and after addition of cobalt chloride to quench fluorescence from rt-PA on the exterior of the liposomes

Fig. 8. The amount of rt-PA associated with the rt-PA and lipid mixture extruded through the microfluidic device ($148.70 \pm 30.80 \mu\text{g/mL}$) compared with that associated with the μtELIP shell ($64.30 \pm 3.80 \mu\text{g/mL}$)

Table 1
Estimated number density and shell parameters of μ ELIP compared to Definity[®] and batch-produced rt-PA-loaded ELIP^{*}

	Number/mL				
	$N_{meas}/10^9$	$N_{fit}/10^9$	S_p (N/m)	$S_p/10^{-6}$ (kg/s)	R^2
μ ELIP [*]	0.02 ± 0.00	0.007 ± 0.00	1.75 ± 0.68	2.71 ± 0.99	0.94
Definity ^{**}	9.64 ± 0.90	8.58 ± 1.78	1.76 ± 0.16	0.47 ± 0.05	0.93
rt-PA ELIP ^{**}	33.20 ± 8.00	0.41 ± 0.20	3.69 ± 0.76	1.88 ± 0.23	0.90

^{*} Values for μ ELIP after correcting for dilution (1:1000)

^{**} Values for Definity[®] and the batch-produced rt-PA-loaded ELIP at 25 °C computed from Raymond et al. (Raymond et al. 2014) after correcting for dilution (1: 2000 for Definity[®] and 1:200 for rt-PA-loaded ELIP)