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***In vitro* thrombolytic efficacy of echogenic liposomes loaded with tissue plasminogen activator and octafluoropropane gas**

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Abstract

Echogenic liposomes loaded with the thrombolytic recombinant tissue-type plasminogen activator (rt-PA) are under development for the treatment of ischemic stroke. These agents are designed to co-encapsulate cavitation nuclei to promote bubble activity in response to ultrasound exposure, and to enable localized delivery of thrombolytic. Stable cavitation improves the efficacy of the thrombolytic through enhanced fluid mixing. Echogenic liposomes that encapsulate air-filled microbubbles nucleate scant stable cavitation activity in response to 120 kHz intermittent ultrasound exposure, and have demonstrated thrombolytic efficacy equivalent to rt-PA alone. It was hypothesized that encapsulating octafluoropropane (OFP) gas within rt-PA-loaded liposomes instead of air will enhance ultrasound-mediated stable cavitation activity and increase thrombolytic efficacy compared to previous studies. The thrombolytic efficacy and cavitation activity nucleated from liposomes that encapsulate OFP microbubbles and rt-PA (OFP t-ELIP) was evaluated *in vitro*. Human whole blood clots were exposed to human fresh-frozen plasma alone, rt-PA (0, 0.32, 1.58, and 3.15 $\mu\text{g ml}^{-1}$), or OFP t-ELIP at equivalent enzymatic activity, with and without exposure to intermittent ultrasound. Further, numerical simulations were performed to gain insight into the mechanisms of cavitation nucleation. Sustained ultraharmonic activity was nucleated from OFP t-ELIP when exposed to ultrasound. Furthermore, the thrombolytic efficacy was enhanced compared to rt-PA alone at concentrations of 1.58 $\mu\text{g ml}^{-1}$ and 3.15 $\mu\text{g ml}^{-1}$ ($p < 0.05$). These results indicate that OFP t-ELIP can nucleate sustained stable cavitation activity and enhance the efficacy of thrombolysis.

Keywords

sonothrombolysis; ultrasound-enhanced drug delivery; echogenic liposomes; ischemic stroke; cavitation

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1. Introduction

Ischemic stroke is a major cause of death and disability world wide (Feigin *et al* 2009, Mozaffarian *et al* 2014). Recombinant tissue-type plasminogen activator (rt-PA) is the only FDA approved thrombolytic therapy (Troke and Roup 1995, Hacke *et al* 2008). However, rt-PA is administered to less than 5% of stroke patients, partly because it can trigger off-target effects such as intracerebral hemorrhage (Gore *et al* 1991, The NINDS t-PA Stroke Study Group 1997) and gastrointestinal bleeding (Schmitz *et al* 2014). Recently, mechanical thrombectomy has emerged as an effective strategy to treat ischemic stroke (Ciccone *et al* 2013, Berkhemer *et al* 2014, Jovin *et al* 2015, Saver *et al* 2015). In this minimally invasive intervention, the clot is removed physically from the blocked artery. However, a large number of hospitals lack the facilities and expertise to perform this specialized procedure (English *et al* 2016). In addition, mechanical thrombectomy is effective only in recanalizing large arteries, and is typically combined with an infusion of rt-PA to prevent clot embolization (Saver *et al* 2015). Therefore, the development of adjuvant techniques that can improve therapeutic efficacy and avoid harmful sequelae from rt-PA administration remains an active area of research.

Cumulative evidence from *in vitro*, preclinical, and clinical studies has established that ultrasound can enhance thrombolysis when used in combination with intravenous administration of rt-PA and microbubbles (Alexandrov *et al* 2004, Barreto *et al* 2013, Nederhoed *et al* 2014, Bader *et al* 2015b). Stable cavitation, or gentle microbubble oscillation observed at moderate acoustic pressure amplitudes (Flynn 1964), promotes thrombolysis by increasing the penetration of thrombolytic drugs into the clot matrix and removing fibrin degradation products (Francis *et al* 1995, Datta *et al* 2006, Prokop *et al* 2007, Sutton *et al* 2013). Echogenic liposomes (ELIP) are vesicles that can be functionalized by antibody conjugation, and serve both as targeted drug-delivery vehicles and ultrasound contrast agents (Alkan-Onyuksel *et al* 1996). Our group has developed rt-PA loaded ELIP (t-ELIP) in which rt-PA is both intercalated in the lipid bilayer and encapsulated within the aqueous core (Tiukinhoy-Laing *et al* 2007b). Liposomes coated with rt-PA are targeted to fibrin (Tiukinhoy-Laing *et al* 2007a, Klegerman *et al* 2008), a key component of clot structure. Further, encapsulation of rt-PA within the protective lipid bilayer may prevent its premature deactivation by protease inhibitors (Eccleston *et al* 1996, Shaw *et al* 2007) and reduce off-target effects through localized release upon ultrasound exposure. *In vitro* studies conducted previously using a static fluid model have shown that air-filled t-ELIP enhance thrombolysis compared to free rt-PA (Tiukinhoy-Laing *et al* 2007b, Shaw *et al* 2009a). However, in the presence of flow, the air-filled t-ELIP showed weak stable cavitation activity in response to intermittent 120 kHz insonation, and the thrombolytic efficacy of air-filled t-ELIP was equivalent to treatment with rt-PA alone (Bader *et al* 2015a). Previous work has demonstrated a significant correlation between ultraharmonic (UH) emissions from stable cavitation and thrombolytic efficacy (Datta *et al* 2006, Bader *et al* 2015b). The detected UH emissions originated in part from large, resonant sized bubbles formed via coalescence of bubbles liberated from the ultrasound contrast agent Definity® (Lantheus Medical Imaging, North Billerica, MA) which encapsulates octafluoropropane (OFP) gas. OFP and other perfluorocarbon gases have limited solubility in aqueous media, which improves the *in vivo*

persistence of echo contrast agents (Kabalnov *et al* 1998, Sarkar *et al* 2009) and improves stable cavitation activity (Haworth *et al* 2016).

In this paper, we evaluated the thrombolytic efficacy of a new formulation of t-ELIP that encapsulates OFP gas (OFP t-ELIP). It was hypothesized that encapsulating OFP within t-ELIP will enhance ultrasound-mediated stable cavitation activity and increase thrombolytic efficacy. We tested this hypothesis by performing sonothrombolysis experiments using an *in vitro* flow model equipped with a time-lapse microscopy system, and human whole blood clots. In addition, an *in silico* model was employed to determine the propensity of OFP micro-bubble shell rupture and coalescence due to secondary Bjerkness forces.

2. Materials and methods

2.1. Human whole blood clot preparation

Human whole blood clots adherent to silk sutures were prepared as reported previously (Bader *et al* 2015a, 2015b). Briefly, venous blood was drawn from 4 healthy volunteers, after obtaining approval from the Institutional Review Board and written informed consent. Capillaries made of borosilicate glass (1.12 mm inner diameter, World Precision Instruments, Inc., USA) were threaded with 7-0 silk sutures (Ethicon Industries, Cornelia, GA, USA) and placed in disposable borosilicate culture tubes (10 mm diameter $\times$ 75 mm height, VWR International, West Chester, PA). Blood aliquots of 500 μ l were transferred into each tube, and blood was drawn into the glass capillaries by capillary action. The culture tubes were incubated at 37 °C for 3 h, which produced cylindrical clots around the silk suture. Subsequently, the tubes were refrigerated at 4 °C for a minimum of 3 d to promote the formation of retracted clots that are resistant to thrombolysis (Shaw *et al* 2009a). Forty clots were prepared using blood drawn from each of four donors. Three clots were used per donor for each of the ten treatment and control arms reported in this study and four clots per donor were processed for histological examination.

2.2. Preparation of human fresh-frozen plasma

Human fresh-frozen plasma (hFFP) was acquired from the Hoxworth Blood Center (Cincinnati, OH, USA) and pooled to obtain consistent 50 ml aliquots of hFFP. For each experiment, 25 ml aliquots of hFFP were thawed and allowed to reach atmospheric gas equilibrium at 37 °C in a 500 ml beaker for 2 h. Recombinant tissue-type plasminogen activator (Activase®, Genentech, San Francisco, CA, USA) was procured from the manufacturer, reconstituted with sterile water to a concentration of 1 mg ml⁻¹, aliquoted into 1.0 ml centrifuge tubes, and stored at -80 °C. This protocol has been demonstrated to preserve the enzymatic activity of rt-PA for at least 7 years (Shaw *et al* 2009b).

2.3. Histological examination of clots

Clots ($n = 4$ from each donor) were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Histology slides were imaged in bright-field mode using an inverted microscope (IX-71, Olympus Inc., Waltham, MA, USA) equipped with a 12 bit charge-coupled device (CCD) camera (Retiga EXi, QImaging, Surrey, BC,

Canada). Microscopic examination was performed to assess qualitative differences in clot structure between donors.

2.4. Preparation of OFP t-ELIP

Echogenic liposomes were prepared at the University of Texas Health Sciences Center (Houston, TX, USA) using previously reported methods (Huang *et al* 2001). Briefly, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) and cholesterol (Chol) were dissolved in a 46:23:23:8 molar ratio in chloroform and the solution evaporated. The resulting film was kept under vacuum overnight to completely remove the chloroform, and then rehydrated with 200 μl of rt-PA (1 mg ml⁻¹) and 300 μl of Mannitol (0.32 M) for every 5 mg of lipid. Separation of free rt-PA from the rt-PA entrapped within the liposomes was achieved using a centrifuge (Model 5415D, Eppendorf, Hauppauge, NY, USA) at 13 200 rpm for 20 min at room temperature (25 °C). The resultant liposomal pellet was re-suspended with 400 μl of 0.32 M Mannitol, transferred to 2 ml glass vials, and kept at -70 °C for 2 h. The samples were lyophilized at -56 °C for 48 h and then kept at 4 °C overnight. To create OFP t-ELIP, the air headspace was vacuumed from vials of t-ELIP, and reloaded with 2 ml (1 atm partial pressure) of OFP.

Final vials of OFP t-ELIP containing 4 mg of lipid and 120 μg of enzymatically active rt-PA were shipped to the University of Cincinnati on ice. Prior to reconstitution with OFP, the vials were evacuated for 45 s at 10 mm Hg (1.33 kPa) and filled with 4 ml of OFP to attain a pressure of 2 atm. Three hours after pressurization with OFP, a 22-gauge needle was used to vent the pressure of OFP to 1 atm, and the OFP t-ELIP were reconstituted with 400 μl of 0.2 μm filtered, deionized, and air saturated (100% dissolved oxygen) water. Within 1 min of reconstitution, the OFP t-ELIP were diluted in hFFP to achieve enzymatic activity equivalent to rt-PA at 0.32, 1.58, and 3.15 $\mu\text{g ml}^{-1}$.

2.5. Enzymatic activity measurement

A spectrophotometric assay (Verheijen *et al* 1985) was used to evaluate the enzymatic activity of rt-PA encapsulated within OFP t-ELIP as described previously (Bader *et al* 2015b). The enzymatic activity of t-ELIP was reported with respect to a standard curve created with commercially available rt-PA (Activase®, Genetech, San Francisco, CA, USA). A chromogenic substrate (Chromogenix S-2288, DiaPharma Group, Inc., Westchester, OH, USA) was utilized that is hydrolyzed upon combination with rt-PA to produce the chromophore para-nitroaniline. Reconstituted OFP t-ELIP were diluted into a solution of 0.5% phosphate buffer solution (PBS) (Sigma-Aldrich, St. Louis, MO, USA) and 0.01% (v/v) Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) to achieve lipid concentrations of 40 $\mu\text{g ml}^{-1}$, 80 $\mu\text{g ml}^{-1}$, and 120 $\mu\text{g ml}^{-1}$ in disposable 4.5 ml spectrophotometric cuvettes (Thomas Scientific, Swedesboro, NJ, USA). Adding Triton X-100 (0.01% v/v) to the PBS prevents aggregation of rt-PA without destroying the integrity of the liposomes (Bader *et al* 2015a). The enzymatic activity of rt-PA was measured using a spectrophotometer (UV-1700, Shimadzu, Japan) equipped with a temperature controller (TCC-240A, Shimadzu, Japan) at 37 °C by measuring the change in absorbance of the solution at 405 nm.

2.5.1. Loading and encapsulation ratio measurement—The loading ratio represents the total rt-PA associated with OFP t-ELIP (either on the exterior surface of the liposome, or encapsulated within the liposome) relative to the total amount of rt-PA added to the ELIP lipids during OFP t-ELIP manufacturing. To estimate the loading ratio of rt-PA on or in the OFP t-ELIP, the rt-PA enzymatic activity was measured after the addition of 1% Triton X-100. The loading ratio (LR) was defined as:

$$LR = \frac{A_1}{A_{\text{total}}} \times 100 \%, \quad (1)$$

where A_{total} represents the enzymatic activity of the total amount of rt-PA added ($400 \mu\text{g ml}^{-1}$), and A_1 represents the enzymatic activity of the rt-PA obtained after exposing OFP t-ELIP to 1% Triton X-100 solution. In this study, the loading ratio was used to determine the appropriate dilution ratio for OFP t-ELIP to achieve equivalent enzymatic activity as free rt-PA at 0.32, 1.58 and $3.15 \mu\text{g ml}^{-1}$ concentrations.

The encapsulation ratio is defined as the rt-PA within the aqueous core of OFP t-ELIP relative to the total rt-PA associated with OFP t-ELIP. To estimate the encapsulation ratio of OFP t-ELIP, the enzymatic activity was measured before and after the addition of 1% Triton X-100. This concentration of Triton X-100 exceeds the critical micelle concentration (0.015%, Triton X-100 Product Information Sheet), which disrupts the lipid shell and releases the encapsulated rt-PA (Bader *et al* 2015a). The encapsulation ratio (ER) was defined as:

$$ER = \frac{A_1 - A_{0.01}}{A_1} \times 100 \%, \quad (2)$$

where A_1 and $A_{0.01}$ represent the enzymatic activity measured in the presence of 1% and 0.01% Triton X-100.

2.6. OFP t-ELIP size and attenuation characterization

A sample of the reconstituted OFP t-ELIP suspension was diluted (1:40 000 v/v) into air-saturated PBS at room temperature, and the size distribution was measured using a Coulter counter (Multisizer 4, Beckman Coulter, Brea, CA, USA). For each measurement, $100 \mu\text{l}$ of the diluted sample was analyzed using a $30 \mu\text{m}$ aperture. A number density histogram with 200 linearly spaced bins spaced between $0.6 \mu\text{m}$ and $18 \mu\text{m}$ was rendered and corrected to account for the dilution. The results from six measurements, each using a single vial of OFP t-ELIP, were averaged and the number- and volume-weighted size distributions were reported at 1:200 dilution, consistent with the dilution used for attenuation measurements described below.

The frequency-dependent attenuation coefficient was measured using a broadband substitution technique as described previously (Raymond *et al* 2014). Briefly, OFP t-ELIP was diluted (1:200) into a reservoir containing 0.5% (w/v) bovine serum albumin. The

suspension was gravity fed into a sample chamber with acoustically transparent polycarbonate film windows (CLINicell 25, Mabio, Tourcoing, France). The reservoir, sample chamber, and transducers were mounted in a test tank filled with degassed ($20 \pm 5\%$ dissolved oxygen), deionized water maintained at 37 ± 0.5 °C using a circulating water bath (Neslab EX, Newington, NH, USA). Two broadband transducers (PI-20, Olympus NDT, Waltham, MA, USA) were used to acquire the through-transmission amplitude spectrum over the frequency range 2–25 MHz (31 kPa peak negative pulse pressure). The attenuation spectrum was computed from the received spectra in the absence (diluent alone) and the presence of the OFP t-ELIP. Acoustic attenuation measurements were performed using samples from six separate vials of OFP t-ELIP. The elasticity (S_P) and viscosity (S_F) of the OFP t-ELIP shell, and the percentage of liposomes that are echogenic were determined by minimizing the sum-squared difference in errors between the estimated and measured attenuation coefficients as reported previously (Raymond *et al* 2014). The coefficient of determination (R^2) was calculated to indicate the goodness of fit. The values of the shell parameters that doubled the error function value were selected as the limits for the confidence interval (Raymond *et al* 2014).

2.7. In vitro experiments

A schematic of the *in vitro* flow model employed in this study is shown in figure 1. An acrylic tank (16 cm $\times$ 33 cm $\times$ 9 cm) was filled with approximately 3 l of degassed ($20 \pm 5\%$ dissolved oxygen), reverse osmosis water heated to 37 ± 0.3 °C. The water was continuously filtered (0.2 μ m), and the gas content and temperature were maintained throughout the experiment with a custom-built recirculation system. The walls of the tank were lined with a 1 cm thick acoustic absorber (Aptflex F48, Precision Acoustics, Dorchester, Dorset, UK).

A flow channel consisting of low-density polyethylene tubing (inner diameter 1.6 mm, outer diameter of 3.2 mm, part 1J-109-10, Freelin-Wade Co., McMinnville, OR, USA) was utilized to direct the plasma and therapeutics from a reservoir to a clot mounted along the central axis of a glass micropipette (2.15 mm inner diameter, 0.3 mm wall thickness, part number 5-000-2200, Drummond Scientific Co., Broomall, PA, USA). The micropipette was positioned over a microscope slide (Fisherbrand, 12-550C, Fisher Scientific, Pittsburg, PA, USA) on the bottom of the tank to allow imaging of the clot with an inverted microscope (IX71, Olympus Corporation, Center Valley, PA, USA). The focal area of the objective (UPlanFLN 10 $\times$, 10 mm working distance, Olympus Corporation, Center Valley, PA, USA) was approximately 1200 μ m $\times$ 900 μ m. A CCD camera (Retiga-2000R, Q Imaging, Surrey, BC, Canada) with 7.4 μ m $\times$ 7.4 μ m resolution was used to capture images at a frame rate of 2.33 Hz. Flow was maintained at 0.65 ml min⁻¹ with a programmable syringe pump (Model 44, Harvard Apparatus Co. Inc., South Natick, MA, USA) set in continuous withdrawal mode. This flow rate was within the range of physiologic flow rates measured in occluded middle cerebral arteries (Alexandrov *et al* 2010).

2.8. Ultrasound exposure and cavitation detection

A custom-designed 120 kHz transducer (H160, Sonic Concepts, Inc. Woodburn, WA, USA) was used to insonate the clot and the perfusate within the micropipette. The unfocused transducer element (30 mm diameter aperture) was excited at its resonant frequency of 120

kHz with a function generator (33250A, Agilent Technologies, Inc., Santa Clara, CA, USA) and power amplifier (1040L, ENI, Rochester, NY, USA). A custom-built impedance matching network (Sonic Concepts, Inc. Woodburn, WA, USA) maximized power transfer to the transducer. The acoustic field was measured and the *in situ* acoustic pressure calibrated along the clot with a 0.5 mm hydrophone (TC 4038, Teledyne Reson Inc. Goleta, CA, USA) mounted on a computer-controlled three-axis positioner (NF-90, Velmex Inc., Bloomfield, NY, USA).

Cavitation emissions were monitored with a single-element passive cavitation detector (19 mm aperture, 2.25 MHz center frequency, 595516C, Picker Roentgen GmbH, Espelkamp, Germany). The signal received from the passive cavitation detector (PCD) was filtered by a 10 MHz low-pass filter (J73E, TTE Inc, Los Angeles, CA, USA) to remove noise from radiofrequency interference, and amplified with a wideband low-noise amplifier (CLC100, Cadeka Microcircuits LLC, Colorado, USA). The signal was digitized (10 ms duration, 31.25 MHz sampling frequency), and the power spectrum was computed in MATLAB® (The Mathworks, Natick, MA, USA). Stable cavitation was quantified by integrating the power in ultraharmonic (UH) bands of the power spectrum between 250 kHz and 1 MHz over a 2 kHz band centered around each UH frequency (Gruber *et al* 2014). Broadband (BB) emissions were used to quantify inertial cavitation, by summing the power in 4 kHz bands centered at each ultraharmonic band ± 10 kHz and ± 30 kHz.

An intermittent ultrasound exposure scheme was employed (Bader *et al* 2015b). The treatment was started within 5 s of the start of flow. The total treatment period was 30 min. Throughout this period, the clots were treated with 1) plasma alone (control), or 2) plasma and rt-PA, or 3) plasma and OFP t-ELIP, or 4) plasma and OFP t-ELIP with 120 kHz ultrasound. For the latter arm of the study, cavitation nuclei were insonated at a peak-to-peak pressure of 0.44 MPa for 50 s, which was followed by a 30 s quiescent period to allow a fresh influx of cavitation nuclei. The 50 s 'on' and 30 s 'off time' sequence was repeated for the entire 30 min duration of treatment. This insonation scheme has been shown to maximize UH emissions from stable cavitation (Bader *et al* 2015b). Some clots from all donors were exposed to all treatments and control arms to include potential confounding factors due to donor-to-donor variability.

2.9. Calculation of thrombolysis metrics

An image segmentation routine reported previously (Bader *et al* 2015b) was employed to quantify the instantaneous clot width from the images recorded with the CCD camera. The clot width was computed as the average width of the clot over the entire field of view in the camera. The reported clot width was corrected for suture size ($64 \pm 9 \mu\text{m}$). The fractional clot loss (FCL) and average lytic rate (ALR) were computed to evaluate the thrombolytic efficacy.

The fractional clot loss was defined as:

$$\text{FCL} = \frac{CW_0 - CW_f}{CW_0} \times 100\%, \quad (3)$$

where CW_0 represents the initial clot width, and CW_f represents the final clot width after the completion of treatment (30 min time point).

The average lytic rate was defined as:

$$ALR = \frac{FCL}{t_d}, \quad (4)$$

where t_d represents the duration of treatment. For clots that lysed completely before 30 min, t_d was set to the time to achieve 100% FCL.

2.10. Statistical analysis

Statistical analysis was performed using MATLAB[®]. The Anderson–Darling test was used to test the normality of the data obtained from experiments. Lytic efficacy of the different treatment protocols was compared using a one-way analysis of variance (ANOVA). Adjusted p -values based on post-hoc analysis by the Tukey–Kramer method were used to identify significant differences in thrombolysis between the control and treatment groups (Bader *et al* 2015b). Additionally, ultraharmonic and broadband cavitation dose was compared using the Kruskal-Wallis test. A p -value lower than 0.05 was considered statistically significant.

2.11. Numerically calculated response of OFP t-ELIP to ultrasound insonation

It has been previously reported that insonation of Definity[®] at 120 kHz resulted in resonant-sized bubbles (Bader *et al* 2015b). The formation of the resonant-sized bubbles from a population of sub-resonant nuclei occurs through a two-step process. In the first step, the expansion of the microbubble ruptures the encapsulating lipid shell (Bader and Holland 2013), liberating the gaseous core. In the second step, the liberated gas cores coalesce to resonant size through secondary Bjerkness forces (Metin *et al* 1997). To study the formation of resonant sized bubbles, calculations were performed to predict the rupture of the lipid shell and the bubble-bubble interaction forces responsible for coalescence.

The radial oscillations of encapsulated cavitation nuclei in response to 120 kHz ultrasound excitation were calculated by numerical integration of the Marmottant model (Marmottant *et al* 2005) using an adaptive fourth-order Runge–Kutta algorithm implemented in MATLAB[®] (The Mathworks, Natick, MA, USA). The Marmottant model has the form:

$$\rho \left(R \ddot{R} + \frac{3}{2} \dot{R}^2 \right) = \left(1 - \frac{3\kappa \dot{R}}{c} \right) P_0 \left(\frac{R_0}{R} \right)^{3\kappa} - \frac{2}{R} \chi \left[\left(\frac{R}{R_0} \right)^2 - 1 \right] - \frac{4\dot{R}}{R} \left(\mu + \frac{\kappa_S}{R} \right) - P_0 + p_{AC} \sin(\omega t),$$

(5)

where R represents the time dependent bubble radius, the diacritic dot denotes the temporal derivative, c represents the sound speed of the medium (1540 m s^{-1}), μ represents the dynamic viscosity ($0.005 \text{ kg m}^{-1} \cdot \text{s}^{-1}$, Holland and Apfel 1989), κ represents the polytropic

exponent of OFP (1.09, Sarkar *et al* 2009) and ρ represents the medium density (1000 kg m⁻³). The terms $\chi = S_P/2$ and $\kappa_S = S_P/12\pi$ depend upon the shell elasticity and dilation viscosity, respectively, that were derived from the measured shell parameter of OFP t-ELIP from the attenuation measurements described above. The atmospheric pressure is denoted by P_0 , the amplitude and angular frequency of acoustic excitation is indicated by p_{AC} , and ω , respectively. The rupture radius, R_{RUPT} , is defined as:

$$R_{RUPT} = R_{BUCK} \left(1 + \frac{\sigma_0}{\chi} \right)^{1/2}. \quad (6)$$

Shell rupture is thought to be critical for liberating the encapsulated gas and nucleating stable cavitation from shelled microbubbles (Bader and Holland 2013), and was investigated in reference to the size of microbubbles entrained within OFP t-ELIP. The 50 s insonation period utilized in this study would produce steady state oscillations from cavitation. The simulations employed a single ultrasound pulse of 50-cycles, which is sufficiently long for the bubbles to approach steady state oscillations (Bader and Holland 2013).

The gas core acts as a free bubble after rupture of the lipid shell (Bader and Holland 2013). The dynamics of unshelled microbubbles was investigated through the Gilmore-Akulichev formation for bubble dynamics (Gilmore 1952), which has been discussed in detail elsewhere (Bader and Holland 2013, Church *et al* 2015). To assess the propensity of bubble coalescence, the secondary Bjerkness force, F_B , produced by bubble oscillations was computed as follows (Metin *et al* 1997):

$$F_B = -\frac{\rho}{4\pi d^2} \langle \dot{V}_1 \dot{V}_2 \rangle, \quad (7)$$

where V_1 and V_2 denote the volumes of 2 microbubbles, d is the distance between the liberated microbubbles, and the angled brackets denote a time average.

3. Results

3.1. Histology of human whole blood clots

Examples of histology images of H&E-stained human whole blood clots adherent to the silk suture (obtained from 4 donors) are shown in figure 2. These images indicate the variable morphology in clots prepared from different donors. A similar variability in clot morphology has been observed in thrombi retrieved from ischemic stroke patients (Liebeskind *et al* 2011). Note that clots prepared from the same donor appeared similar (data not shown).

3.2. Characterization of OFP t-ELIP

Figure 3 shows the number and volume-weighted size-distributions of OFP t-ELIP. Nearly 99% of OFP t-ELIP particles had a diameter less than 3 μm . The mean number-weighted and volume-weighted diameters of OFP t-ELIP were $1.21 \pm 0.21 \mu\text{m}$ and $3.87 \pm 1.62 \mu\text{m}$ (mean $\pm$ SD, $n = 6$ vials).

The attenuation spectrum of OFP t-ELIP is depicted in figure 4. The solid line depicts the measured attenuation spectrum, and the dashed line represents the estimated attenuation spectrum with the measured size distribution as input (Raymond *et al* 2014). The coefficient of determination (R^2) was 0.99, indicating a good fit between the measurement and the model estimate. The 'best fit' shell parameters for OFP t-ELIP were: $S_F = 5.85 \pm 0.12 \text{ N m}^{-1}$, $S_F = 1.78 \times 10^{-6} \pm 3.02 \times 10^{-8} \text{ kg s}^{-1}$ (mean $\pm$ 95% confidence interval). The number density of OFP t-ELIP employed for the attenuation measurement was 1.9×10^8 liposomes ml^{-1} . Only $3.23 \pm 0.47\%$ of the particles in OFP t-ELIP were estimated to encapsulate bubbles (Raymond *et al* 2014). The loading efficiency and encapsulation ratio of OFP t-ELIP were $59.4 \pm 12.3\%$ (mean $\pm$ SD, $n = 6$), and $12.8 \pm 10.7\%$ (mean $\pm$ SD, $n = 6$), respectively. These results were comparable to previous studies reported with air-filled t-ELIP (Laing *et al* 2011).

3.3. Measurement of thrombolytic efficacy

Figure 5 shows the mean normalized clot width as a function of time for each experimental arm at rt-PA concentrations of 0.32, 1.58 and $3.15 \mu\text{g ml}^{-1}$. The initial width of these clots was $384 \pm 65 \mu\text{m}$, which is within the range reported for the side branches of the middle cerebral arteries (Marinkovic *et al* 1985). The clots treated with plasma alone showed less than 5% reduction in clot width over the 30 min treatment period. For all other treatment groups, a visible reduction in clot width was observed nearly 5 min after treatment onset.

The fractional clot loss (FCL) achieved with three concentrations of rt-PA are shown in figure 6. The adjusted p -values obtained using the Tukey–Kramer test comparing the FCL obtained with the control and treatment arms are shown in table 1. For all treatment arms that included a thrombolytic (rt-PA or OFP t-ELIP), a statistically significant enhancement was obtained in FCL over plasma alone. At all concentrations, there was no statistical difference in FCL between clots treated with rt-PA and OFP t-ELIP alone. At an rt-PA concentration of $1.58 \mu\text{g ml}^{-1}$, the FCL produced using OFP t-ELIP and US was greater than that achieved with rt-PA or OFP t-ELIP. At an rt-PA concentration of $3.15 \mu\text{g ml}^{-1}$, OFP t-ELIP and US exhibited a significantly higher FCL than clots treated with rt-PA alone. However, the differences in FCL between OFP t-ELIP combined with US and OFP t-ELIP alone did not reach statistical significance (table 1). Further, the FCL attained with OFP t-ELIP and US at an rt-PA concentration of 1.58 and $3.15 \mu\text{g ml}^{-1}$ were equivalent.

Figure 7 shows the average lytic rate (ALR) for the three concentrations of rt-PA used in this study. Table 2 shows the adjusted p -values obtained using the Tukey–Kramer test from comparing the ALR obtained with the control and treatment arms. For all treatment arms that included thrombolytic (rt-PA or OFP) a statistically significant enhancement in ALR was observed over plasma alone. At all rt-PA concentrations, the ALR achieved by rt-PA and OFP t-ELIP was statistically equivalent. At an rt-PA concentration of 1.58 and $3.15 \mu\text{g ml}^{-1}$, the ALR achieved with OFP t-ELIP and US was significantly higher than that achieved with rt-PA alone and OFP t-ELIP alone. Furthermore, the ALR achieved with OFP t-ELIP and US at $1.58 \mu\text{g ml}^{-1}$ and $3.15 \mu\text{g ml}^{-1}$ were comparable.

3.4. Spectral content of PCD signal

Ultraharmonic and broadband emissions detected during insonation of OFP t-ELIP are shown in figure 8(a) as a function of rt-PA concentration. The cavitation data demonstrated a non-Gaussian distribution; therefore these results were depicted using boxplots. These results show that ultraharmonic emissions were the dominant mode of cavitation activity detected by the PCD. The UH activity was significantly higher than the broadband activity for rt-PA concentrations of $0.32 \mu\text{g ml}^{-1}$ ($p = 0.0007$), $1.58 \mu\text{g ml}^{-1}$ ($p = 0.0496$) and $3.15 \mu\text{g ml}^{-1}$ ($p = 0.002$). Also, the UH activity showed an increasing trend when the concentration of rt-PA was increased from 0.32 to $1.58 \mu\text{g ml}^{-1}$. However the UH activity measured at $1.58 \mu\text{g ml}^{-1}$ and $3.15 \mu\text{g ml}^{-1}$ were comparable. Figure 8(b) compares the ultraharmonic energy detected during the insonation of OFP t-ELIP in the present study, with that observed from and air-filled t-ELIP (Bader *et al* 2015a). For all concentrations, the ultraharmonic energy received from OFP t-ELIP was higher than that received with t-ELIP ($p < 0.01$).

3.5. Shell rupture and coalescence of bubbles

Numerical prediction of the expansion of OFP microbubbles within OFP t-ELIP exposed to 120 kHz intermittent ultrasound is shown in figure 9. The measured shell stiffness and shell friction parameter of OFP t-ELIP were converted to shell elasticity and shell viscosity (Marmottant *et al* 2005) ($\kappa_s = 2.93 \text{ N m}^{-1}$, $\chi = 4.72 \times 10^{-8} \text{ N m}^{-1}$) and used in equation (5). The peak-to-peak pressure of the 120 kHz insonation was 0.44 MPa, consistent with the experimental studies. Calculations were performed for bubbles with diameters ranging from $0.2 \mu\text{m}$ to $7 \mu\text{m}$. Bubbles larger than $7 \mu\text{m}$ were not considered because they would be trapped within the capillary beds of the lungs (De Jong and Cate 1993). Bubbles smaller than $1.05 \mu\text{m}$ demonstrated negligible expansion. Shell rupture was found to occur only for microbubbles larger than $1.05 \mu\text{m}$.

Numerical computation of the secondary Bjerkness force between the liberated gas cores is shown in figures 10(a)–(c). The distance d between bubbles in equation (6) was computed based on the concentration of OFP t-ELIP used in the study and the experimentally determined bubble encapsulation efficiency of OFP t-ELIP. Using the curve fitting tool ‘cftool’ in MATLAB®, the secondary Bjerkness was fit to microbubble diameter in the least squares sense to a two-parameter model of the form:

$$F_{B12} = a(D_1 D_2)^b, \quad (8)$$

where F_{B12} is the secondary Bjerkness force in mN exerted between bubble 1 and bubble 2, D_1 and D_2 are the initial diameters of bubble 1 and bubble 2 in μm , and a and b are fit-ting parameters. The values for the fitting parameters, along with the goodness of fit metrics (coefficient of determination and root mean square error) for the numerically generated data in figure 10 are shown in table 3. At all concentrations, the secondary Bjerkness force is directly proportional to the size of the bubble (with $b = 0.95$). As the concentration of OFP t-ELIP is increased, the distance between microbubbles decreases. Consequently, the secondary Bjerkness force increases by a factor of 4.6 when the concentration of OFP t-ELIP is increased from a target rt-PA concentration of $0.32 \mu\text{g ml}^{-1}$ to $3.15 \mu\text{g ml}^{-1}$.

4. Discussion

The results of this study shown in figures 6 and 7 revealed that OFP t-ELIP and US enhanced thrombolysis compared to rt-PA at concentrations of 1.58 and 3.15 $\mu\text{g ml}^{-1}$. The fractional clot loss (FCL) and average lytic rate (ALR) obtained with OFP t-ELIP and US at concentrations of 1.58 $\mu\text{g ml}^{-1}$ were 71.9% and 106.1% higher than that obtained with an rt-PA alone at 3.15 $\mu\text{g ml}^{-1}$. Of note, no increase in the FCL or ALR was observed with OFP t-ELIP and US when the rt-PA concentration was increased from 1.58 $\mu\text{g ml}^{-1}$ to 3.15 $\mu\text{g ml}^{-1}$. The results of the present study suggest that the encapsulation of rt-PA within OFP t-ELIP combined with ultrasound exposure would allow a reduction in the rt-PA dose by a factor of two and increase the lytic efficacy. A reduction of the rt-PA dose may avoid adverse effects such as intracerebral hemorrhage and off-target bleeding (Turi *et al* 1993, Adams *et al* 1996, Haley *et al* 2005). The depletion of plasminogen at higher concentrations of rt-PA is likely the reason for the enhanced thrombolytic effect at 1.58 $\mu\text{g ml}^{-1}$, relative to 3.15 $\mu\text{g ml}^{-1}$. This hypothesis is supported by previous studies conducted with free rt-PA (Onundarson *et al* 1992, Shaw and Meunier 2008). When rt-PA reacts with plasminogen, plasmin is formed, which is responsible for fibrinolysis. Direct delivery of plasmin to the thrombus could reduce the dependence on the availability of plasminogen (Kandadai *et al* 2015). Plasminogen inhibitors in the bloodstream deactivate plasmin rapidly, which may be overcome by encapsulating plasmin into ELIP (Kandadai *et al* 2015). The FCL obtained by these investigators using plasmin and air-loaded liposomes exposed to pulsed US ($46 \pm 18\%$) was less effective than the FCL obtained with OFP t-ELIP at an equivalent rt-PA concentration of 3.15 $\mu\text{g ml}^{-1}$ exposed to intermittent US in the present study ($65 \pm 20\%$). The difference in thrombolytic efficacy could be partly due to robust cavitation nucleated by OFP t-ELIP.

The ultrasound exposure parameters employed in this study aimed to harness stable cavitation (Datta *et al* 2006), or gentle microbubble oscillations to enhance the drug transport into the clot and to remove fibrin degradation products by inducing microstreaming. The increase in thrombolytic efficacy with OFP t-ELIP and US was likely due to the robust UH cavitation activity nucleated by OFP microbubbles encapsulated within OFP t-ELIP. Bader *et al* previously demonstrated that exposing air-filled t-ELIP to US produced scant UH activity compared to insonation of the OFP-filled contrast agent Definity® (Bader *et al* 2015a). The UH activity achieved with OFP t-ELIP in this study was over an order of magnitude higher than that reported with air-filled t-ELIP (Bader *et al* 2015a). This increase could be attributed to the higher persistence of OFP bubbles in aqueous media. However, the UH activity, FCL, and ALR attained with unencapsulated rt-PA and Definity® by Bader *et al* was higher than that reported with OFP t-ELIP in this study (Bader *et al* 2015b). Previous studies have reported the formation of resonant-sized bubbles through coalescence of sub-resonant nuclei (Postema and Bouakaz 2002), which requires rupture of the lipid shell (Bader and Holland 2013). Time-lapse microscopy has revealed dramatic thrombolytic effects in the presence of resonant-sized ($\sim 50 \mu\text{m}$) bubbles that persisted for up to 100 s (Bader *et al* 2015b). No such persistent OFP microbubbles were observed in our study. Unfortunately, the current system cannot be used to observe the coalescence of bubbles at microsecond time scales. Despite these limitations, evidence of

microbubble coalescence was observed previously with the system employed in this study (Bader *et al* 2015b). Future studies with high-speed imaging could provide further insight into bubble coalescence.

It was reported that t-ELIP have similar efficacy to rt-PA alone for thrombus dissolution in the abdominal aortas of New Zealand white rabbits *in vivo* (Laing *et al* 2012). Laing *et al* found no difference in thrombolytic activity of air-filled t-ELIP alone versus t-ELIP exposed to pulsed US, likely due to scant stable cavitation nucleation and activity (Bader *et al* 2015a). In this study, we show that OFP t-ELIP nucleate robust stable cavitation activity when exposed to pulsed US, and enhance thrombolysis significantly over OFP t-ELIP alone and rt-PA. These results suggest that OFP t-ELIP might enhance efficacy over t-ELIP alone when used *in vivo* in future studies.

The size distribution of the encapsulated microbubbles (Bader and Holland 2013, Shekhar *et al* 2013) is an important determinant of stable cavitation activity. The majority of OFP t-ELIP used in this study had diameters less than 2 μm , as shown in figure 3. Simulations shown in figure 9 suggest that echogenic liposomes coalesce through two mechanisms. First, rupture occurs only for those shelled microbubbles larger than 1 μm in diameter. Second, the attractive force between ruptured microbubbles is directly proportional to their size (figure 10 and table 3). For instance, the Bjerkness force between two microbubbles of 2 μm diameter is an order of magnitude less than that of two microbubbles of 7 μm diameter, regardless of the concentration of OFP t-ELIP (figure 10). Therefore, bubbles with a diameter of 5–7 μm are more likely to coalesce and create resonant-sized, persistent microbubbles which contribute significantly to the lytic efficacy (Bader *et al* 2015b). Therefore, increasing the mean diameter of OFP t-ELIP 5 fold may help encapsulate suitably sized gas nuclei as well as a larger rt-PA payload facilitating enhanced thrombolysis. However, bubbles formed *in situ* by coalescence that are larger than 30 μm may be associated with embolism and inflammation (Muth and Shank 2000). The final size of microbubbles formed by coalescence is dependent on the resonant frequency (Leighton 1995). By increasing the driving frequency, the resultant bubble size will decrease, reducing the likelihood of embolization. For instance, at 220 kHz, the resonant size of a bubble would be 23 μm , a size at which reversible bioeffects in primate brains have been reported previously (McDannold and Arvanitis 2012).

A limitation of this study was that histology was performed only on clots prior to exposure to rt-PA. Clots treated with rt-PA were either lysed completely (100% FCL) or were very soft and difficult to process for histological examination. Previous studies have enumerated the limitations of the *in vitro* sonothrombolysis model employed in this study (Cheng *et al* 2005, Shaw *et al* 2007, 2009a, Bader *et al* 2015b). Briefly, this *in vitro* system does not model physiological conditions completely (Cheng *et al* 2005). The flow rate was fixed and hemodynamic pressures that may significantly alter cavitation and thrombolysis were not incorporated (Bader *et al* 2015a, 2015b). Additionally, the venous clots employed in this study were prepared from the clots of healthy donors and may differ in morphology compared to arterial clots (Shaw *et al* 2007). Nonetheless, the clot model used in this study is resistant to thrombolysis (Shaw *et al* 2006), which is consistent with the low lytic efficacy observed for clots treated with plasma alone (figures 6 and 7). Another limitation of this

study was that the OFP t-ELIP had low encapsulation ratio ($12.8 \pm 10.7\%$). The low encapsulation of rt-PA in OFP t-ELIP may partly explain why the thrombolytic efficacy of free rt-PA and OFP t-ELIP in the absence of ultrasound exposure were similar (figures 6 and 7). Further, a large vial-to-vial variability was noted within OFP t-ELIP, both with respect to the encapsulation ratio and cavitation emissions. Improving the encapsulation ratio further will prevent the inactivation of rt-PA by serine proteases and enable localized delivery of thrombolytic (Eccleston *et al* 1996, Shaw *et al* 2007). Further, only 3.23% of the OFP t-ELIP particles were echogenic, which may limit stable cavitation and thrombolytic efficacy. The OFP t-ELIP used in this study were prepared by hydrating the lipid film with an rt-PA solution followed by lyophilization. This process generated a $59.4 \pm 12.3\%$ loading ratio with rt-PA adsorbed on the interior or exterior of the lipid shell or encapsulated in the aqueous interior of the liposomes. Modifying the rt-PA to lipid ratio, the lipid formulation or the liposome preparation methods could potentially improve the encapsulation ratio. Further modification by including an extrusion method might allow us to improve the encapsulation ratio of OFP-tELIP. Recently, Koudelka *et al* (2016) used an extrusion step before lyophilization and obtained a rt-PA encapsulation ratio of 18%. Our group has also recently reported that monodisperse ($5 \mu\text{m}$ diameter) octafluorocyclobutane and rt-PA-loaded liposomes ($\mu\text{t-ELIP}$) can be manufactured using a microfluidic device (Kandadai *et al* 2016). Employing microfluidic techniques may allow the size distribution of E-LIP to be tailored according to the insonation frequency, and also produce more consistent rt-PA and perfluorocarbon gas encapsulation. Further, specialized chirp-coded pulsing schemes could be used to encourage a bubble growth to resonant size by exploiting rectified diffusion and coalescence, which is expected to enhance cavitation activity and lytic efficacy.

The liposomes used in this study have a unique advantage because they coencapsulate gas bubbles and rt-PA. The gas bubbles act as cavitation nuclei to cause volumetric pulsation and concomitant lipid shell stretching and shedding to release rt-PA at the site of ultrasound exposure. The rt-PA loaded in the liposome was encapsulated within the aqueous core, and/or associated with the bilayer, and/or adsorbed on the outer shell. The release kinetics of rt-PA were not reported in this study. We plan to perform future studies to address the release kinetics of OFP t-ELIP. In addition, the safety and efficacy of OFP t-ELIP and US should be further investigated *in vivo*.

5. Conclusions

The results of this study indicate that OFP t-ELIP without ultrasound exposure had equivalent thrombolytic activity to rt-PA alone at all concentrations investigated. Notably, OFP t-ELIP in combination with ultrasound enhanced sonothrombolysis over rt-PA alone at rt-PA concentrations of 1.58 and $3.15 \mu\text{g ml}^{-1}$. The fractional clot loss and average lytic rate attained at a concentration of $1.58 \mu\text{g ml}^{-1}$ were 71.9% and 106.1% higher than that obtained with an rt-PA alone at $3.15 \mu\text{g ml}^{-1}$. This increase in thrombolytic efficacy was likely due to robust stable cavitation from OFP t-ELIP. These results demonstrate the potential to achieve sonothrombolysis at reduced thrombolytic concentrations, which could enhance both the efficacy and safety profile of thrombolysis.

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Figure 1.

A schematic of the experimental setup used for evaluating sonothrombolysis. The direction of flow is from right to left. CCD = charged coupled device, PCD = passive cavitation detector.

Figure 2. Hematoxylin and eosin stained images of human whole blood clots on silk sutures from 4 donors.

Figure 3. The number-(left ordinate) and volume-weighted (right ordinate) size-distributions of OFP t-ELIP. The error bars represent the mean ± 1 S.D. ($n = 6$).

Figure 4.

The measured (blue solid line) attenuation coefficient as a function of frequency, and the theoretical fit (black dashed line) to the de Jong model based on the estimated shell parameters of OFP t-ELIP. The error bars represent the mean ± 1 S.D. ($n = 6$). The coefficient of determination of the fit to the data was 0.99.

Figure 5. The normalized clot width as a function of time for plasma alone and rt-PA concentrations of (a) 0.32 $\mu\text{g ml}^{-1}$, (b) 1.58 $\mu\text{g ml}^{-1}$, and (c) 3.15 $\mu\text{g ml}^{-1}$. The error bars represent ± 1 S.D. ($n = 12$ per group) at the time-point when the standard deviation was at its maximum value during the 30 min treatment period.

Figure 6.

The fractional clot loss achieved for each control and treatment arm with rt-PA concentrations of 0 (plasma only), 0.32, 1.58 and 3.15 $\mu\text{g ml}^{-1}$ (or OFP t-ELIP with equivalent rt-PA enzymatic activity). The error bars represent the mean $\pm$ 1 S.D. ($n = 12$ per group). Statistically significant differences between groups ($p < 0.05$) are indicated by asterisks.

Figure 7.

The average lytic rate achieved with each control and treatment arm at rt-PA concentrations of 0 (plasma only), 0.32, 1.58 and 3.15 $\mu\text{g ml}^{-1}$ (or OFP t-ELIP with equivalent rt-PA enzymatic activity). The error bars represent the mean $\pm$ 1 S.D. ($n = 12$ per group). Statistically significant differences between groups ($p < 0.05$) are indicated by asterisks.

Figure 8.

(a) The ultraharmonic and broadband energy detected during insonation of OFP t-ELIP with 120 kHz US ($n = 12$ per group). For all concentrations, the ultraharmonic dose was higher than the broadband dose ($p < 0.05$). (b) Comparison of the ultraharmonic energy ($n = 12$ per group) detected during the insonation of OFP t-ELIP (this study) and air-filled t-ELIP (Bader *et al* 2015a). For all concentrations, the ultraharmonic energy generated by OFP t-ELIP was significantly higher than that generated by air-filled t-ELIP ($p < 0.01$). The boxes represent the interquartile range, the horizontal red lines within the boxes represent the median, and the whiskers represent 1.5 times the interquartile range. Outliers are depicted using the '+' sign.

Figure 9.

The maximum extension (maximum microbubble diameter minus initial microbubble diameter) of microbubbles entrained within OFP t-ELIP as a function of initial microbubble diameter, calculated by numerical integration of the Marmottant equation (equation (5)). Rupture of the lipid shell occurred for microbubbles initially larger than $1.05 \mu\text{m}$ for the insonation conditions of the present study, as indicated by the red dashed line.

Figure 10.

(a) A schematic of a pair of oscillating bubbles in response to ultrasound. The calculated secondary Bjerkness force via equation (7) between bubble 1 and bubble 2 is shown in (b)–(d). The distance between the bubbles was computed based on the concentration of OFP t-ELIP at rt-PA concentrations of (a) $0.32 \mu\text{g ml}^{-1}$, (b) $1.58 \mu\text{g ml}^{-1}$, and (c) $3.15 \mu\text{g ml}^{-1}$.

Table 1

Adjusted *p*-values obtained using the Tukey–Kramer test from comparing the fractional clot loss obtained with the control and treatment arms. Statistically significant *p*-values are indicated in bold.

	Plasma only	rt-PA	OFP t-ELIP	OFP t-ELIP + US
0.32 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.001	0.001	0.001
rt-PA	—	—	0.294	0.899
OFP t-ELIP	—	—	—	0.364
OFP t-ELIP + US	—	—	—	—
1.58 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.001	0.007	0.001
rt-PA	—	—	0.511	0.001
OFP t-ELIP	—	—	—	0.001
OFP t-ELIP + US	—	—	—	—
3.15 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.001	0.001	0.001
rt-PA	—	—	0.899	0.046
OFP t-ELIP	—	—	—	0.060
OFP t-ELIP + US	—	—	—	—

Table 2

Adjusted *p*-values obtained using the Tukey–Kramer test from comparing the average lytic obtained with the control and treatment arms. Statistically significant *p*-values are indicated in bold.

	Plasma only	rt-PA	OFP t-ELIP	OFP t-ELIP + US
0.32 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.0010	0.001	0.001
rt-PA	—	—	0.287	0.899
OFP t-ELIP	—	—	—	0.371
OFP t-ELIP + US	—	—	—	—
1.58 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.020	0.014	0.001
rt-PA	—	—	0.794	0.001
OFP t-ELIP	—	—	—	0.001
OFP t-ELIP + US	—	—	—	—
3.15 $\mu\text{g ml}^{-1}$				
Plasma only	—	0.0164	0.008	0.001
rt-PA	—	—	0.899	0.016
OFP t-ELIP	—	—	—	0.031
OFP t-ELIP + US	—	—	—	—

Table 3

Best fit parameters for the secondary Bjerkness forces. The goodness-of-fit parameters coefficient of determination (R^2) and root mean square error (σ_X) are shown along with the distance between nuclei (d) used in equation (7).

(rt-PA) ($\mu\text{g ml}^{-1}$)	d (μm)	a	b	R^2	σ_X (μN)
0.32	30	2.75	0.95	0.95	5.52
1.58	17	7.99	0.95	0.95	16.04
3.15	14	12.62	0.95	0.95	25.35