Published in final edited form as:

Transl Stroke Res. 2015 February ; 6(1): 78–87. doi:10.1007/s12975-014-0376-4.

Plasmin-loaded echogenic liposomes for ultrasound-mediated thrombolysis

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Abstract

Plasmin, a direct fibrinolytic, shows a significantly superior hemostatic safety profile compared to recombinant tissue plasminogen activator (rtPA), the only FDA approved thrombolytic for the treatment of acute ischemic stroke. The improved safety of plasmin is attributed to the rapid inhibition of free plasmin by endogenous plasmin inhibitors present in very high concentrations (1 μ M). However, this rapid inhibition prevents the intra-venous (IV) administration of plasmin. In emergency situations catheter-based local administration is not practical. There is a need for an alternative technique for IV administration of plasmin. A possible solution is the encapsulation of plasmin in echogenic liposomes (ELIP) for protection from inhibitors until ultrasound (US)-triggered release at the clot-site. ELIP are bilayer phospholipid vesicles with encapsulated gas microbubbles. US induces oscillation and collapse of the gas bubbles, which facilitates ELIP rupture and delivery of the encapsulated contents. Plasmin-loaded ELIP (PELIP) were manufactured, and characterized for size, gas- and drug-encapsulation, and in-vitro thrombolytic efficacy using a human whole blood clot model. Clots were exposed to PELIP with and without exposure to US (center frequency 120 kHz, pulse repetition frequency 1667 Hz, peak-to-peak pressure of 0.35 MPa, 50% duty cycle). Thrombolytic efficacy was calculated by measuring the change in clot width over a 30-minute treatment period using an edge-detection MATLAB program. The mean clot lysis obtained with PELIP in the presence of US exposure was 31% higher than that obtained without US exposure, and 15% higher than that obtained with rtPA treatment ($p < 0.05$). The enhanced clot lysis is attributed to the US-mediated release of plasmin from the liposomes.

Keywords

Plasmin; Acute Ischemic Stroke; Thrombolysis; Echogenic Liposomes; Ultrasound

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CONFLICTS OF INTEREST: Authors Madhuvanthi A. Kandadai, Jason M. Meunier, Kimberly Hart, Christy K. Holland, and George J. Shaw declare that they have no conflict of interest.

Stroke is a leading cause of death and disability worldwide. [1] The landmark NINDS (National Institute of Neurological Disorders and Stroke) trial published in 1995 established the use of recombinant tissue plasminogen activator (rtPA) as a standard thrombolytic therapy for the treatment of acute ischemic stroke (AIS). [2] However, the use of rtPA is associated with an increased risk of symptomatic intracranial hemorrhage, poor recanalization efficiency, and high rates of reocclusion. [3–5] Plasminogen-independent neurotoxic effects of rtPA in the ischemic brain, which may contribute to neurologic deterioration after stroke, have been demonstrated *in-vitro* and in animals. [6, 7] Hence, there is a need for a safer and more effective alternative thrombolytic therapy.

Plasmin, a plasminogen independent direct fibrinolytic, has demonstrated potential for effective and safe thrombolysis in preclinical studies and human clinical trials. [8–11] Compared to rtPA, a greater thrombolytic efficacy of plasmin was demonstrated in an *in-vitro* human whole blood clot model, and in a rabbit distal abdominal thrombosis model under conditions of restricted blood flow to the thrombosed abdominal aorta. [12, 13] Under conditions of unimpeded blood flow to the thrombus, lysis by plasmin and rtPA were found to be similar. [12] In a rabbit ear-puncture re-bleeding model, rtPA induced bleeding of the hemostatically stable puncture sites at a dosage 25% lower than the therapeutic dosage. Whereas, plasmin treated animals showed bleeding at dosages eight-times the therapeutic dose, above which complete depletion of fibrinogen and Factor VIII occurred. [14] In an *ex-vivo* study of human cerebral thromboemboli retrieved from AIS patients, the extent and rate of lysis achieved with plasmin was similar to that achieved with rtPA. [15] A phase I human clinical trial to evaluate safety and dosage of human plasmin for hemodialysis graft occlusion, demonstrated that doses up to 24 mg injected locally were safe, and effectively lysed more than 75% of the thrombosed graft. [16] A phase 1/2a, dose escalation, safety study of catheter-based local delivery of human plasmin in acute, middle cerebral artery, ischemic stroke is currently underway. [9]

In the above studies, plasmin was delivered locally to the thrombus site using catheter-based delivery, as intravenous (IV) administered plasmin undergoes rapid inhibition by endogenous plasmin inhibitors such as α 2-antiplasmin (α 2-AP), present in very high concentrations in plasma (1 μ M). [17] In one of the fastest protein-protein reactions, α 2-AP forms a 1:1 reversible stoichiometric complex with free plasmin followed by a slower reaction that results in the formation of an irreversible complex. [17] In contrast, the amount of native plasminogen activator inhibitor (PAI-1) present in healthy individuals is highly variable, ranging from 0.12–1.7 nM, much lower than the amount of plasmin inhibitors. [17] The rtPA required for thrombolysis of AIS thrombi can therefore exceed the inhibitory capacity of native PAI. Thus, a portion of rtPA is active at sites other than the culprit thrombus, possibly resulting in disintegration of hemostatic plugs at sites of vascular injury. [18] However, the rapid inhibition of plasmin, which may be responsible for the improved safety margin of plasmin compared to rtPA, is also the main hurdle in its systemic administration. A very high dose of plasmin (8 mg/kg) was required to observe depletion of α 2-AP and the simultaneous appearance of free plasmin and bleeding complications in a rabbit ear-puncture rebleeding model. [12] Plasmin has been found to be a versatile thrombolytic, safe and effective in applications other than AIS as well. Motoyama et al

found that thrombolysis with plasmin in an ex-vivo lung perfusion (EVLP) murine model of ischemia-reperfusion injury, effectively dissolved thrombi in the donor lung and reconditioned tissue for transplantation. [19] Marder et al demonstrated safety of catheter based delivery of plasmin to lower extremity arterial occlusion at dosages of 25–175 mg. [20]

In the setting of AIS treatment, catheter-based delivery directly to the thrombus may delay the initiation of thrombolytic therapy. Such treatment requires skilled personnel with the adjunct use of advanced endovascular surgical equipment. Hence, there is a need for an alternative technique for systemic administration of plasmin without inhibition of its thrombolytic activity.

In this paper, we describe the development of plasmin encapsulated in echogenic liposomes (ELIP), or bilayer phospholipid vesicles with an aqueous core containing encapsulated gas microbubbles (Figure 1). [21–25] Drugs, genes, or bioactive gases encapsulated in ELIP can be delivered to specific tissue targets using ultrasound (US). [26, 22, 27–34] Plasmin encapsulated in ELIP (PELIP) administered systemically can be protected from premature inactivation by plasmin inhibitors until US-mediated release at the clot site. In this work, we report the manufacture and characterization of PELIP, and the thrombolytic efficacy of PELIP in an *in-vitro* human whole blood clot model in the presence and absence of US exposure.

MATERIALS AND METHODS

Phospholipid mixture preparation

Phospholipids 1,2- dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero- 3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DPPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). A 10 mg/ml cholesterol solution and mannitol was purchased from Sigma Aldrich (St. Louis, Missouri). DPPC, DOPC, DPPG and cholesterol were mixed in the molar ratio 46:23:23:8 in a round bottom flask and placed in a rotary evaporator at 52 °C for 45–60 minutes (Eyela rotary evaporator, Tokyo Rikakikai Corporation Ltd., Product # N-1001, Bohemia, New York). The flask was placed in a vacuum desiccator for 3–4 hours to ensure complete removal of solvent.

PELIP manufacture

PELIP were manufactured using a batch process based on the methods of Huang et al. [24, 35] Plasmin from human plasma was obtained as a lyophilized powder from Sigma Aldrich (St. Louis, MO, USA) and dissolved in filtered, deionized water (NANOPure, Barnstead 172 International, Dubuque, IA, USA) to a final concentration of 1 mg/mL. Prior to addition of plasmin, the filtered, deionised water was adjusted to a pH of 3.0 using 1N hydrochloric acid, as plasmin activity decay is curbed to a larger extent at acidic pH. [36] The dry phospholipid layer in the round-bottom flask was rehydrated with 3 mL of the plasmin solution, and sonicated in an ice bath for 5–10 minutes until a clear solution was obtained, to facilitate formation of smaller liposomes (Cole Parmer, Model # 08895-02. Vernon Hills,

Illinois). This solution was centrifuged at 14000 rpm for 20 minutes at a temperature of 4°C (Beckman centrifuge, Model#J-21C, Brea, California). The supernatant containing the unencapsulated plasmin, and the pellets containing the PELIP were separated by decantation. The pellets were rehydrated with 3 mL of filtered, deionized water (pH 3.0) and 3 mL of 0.32 M mannitol solution, stirred to form a homogenous solution, and aliquoted into glass vials. The solutions were subjected to three freeze-thaw cycles (–20°C to room temperature) to enhance gas-entrapment upon reconstitution [37] and placed in a lyophilizer (Labconco Corporation, Product # 7670520, Kansas City, MI) overnight at –48 °C and 0.02 Torr, and stored in a –20 °C freezer until use.

ELIP manufacture

ELIP devoid of plasmin were manufactured for control experiments from a phospholipid mixture with the same composition as that used for PELIP. The solvent free phospholipid mixture obtained after rotary evaporation and desiccation was reconstituted in 3 mL of filtered, deionized water adjusted to a pH of 3.0 with 1N HCl. The rest of the manufacturing process was identical to that of PELIP described above.

Size and number density measurement

The sizes and number densities of plain ELIP and PELIP were measured using a Beckman Coulter Multisizer 4 coulter counter (Beckman Coulter Inc., Brea, California) fitted with a 30 µm aperture. ELIP and PELIP were diluted by a factor of 200,000 and 10,000 respectively in 0.01 M PBS with a dissolved oxygen (DO) content of 100%, to stabilize the encapsulated gas within the ELIP or PELIP. [38]

Echogenicity measurements

Ultrasound backscatter—Ultrasound (US) B-mode images of ELIP and PELIP were obtained and image analysis was performed using the methods of Radhakrishnan et al. [38] Briefly, the liposomes were allowed to flow in a physiological flow phantom at a concentration of 0.05 mg/ml in human fresh frozen plasma (hFFP) adjusted to a DO of 100–103%. The liposomes flowed through a thin latex tube (Inner diameter 3.18 mm, Outer diameter 3.98 mm), in a water bath maintained at a temperature of 37±1°C. The liposomes were resuspended in hFFP at room temperature and gently warmed to a temperature of 37±1°C in the flow phantom for US image acquisition. The flow rate of the liposome solution was maintained at 5 ml/min using a pulsatile pump. US backscatter from the liposome solution was imaged using a diagnostic US scanner (Philips HDI 5000, Philips, Bothwell, WA, USA) equipped with a L12-5 linear array transducer with a 6.9 MHz center frequency. Images (N=3 for ELIP and N=4 for PELIP) were analyzed using a MATLAB program to obtain mean grey scale values (MGSVs) over a region of interest (ROI) in the lumen of the tubing in the flow phantom. The MGSV were converted to mean digital intensities (MDIs), which have been found to be directly proportional to the backscattered acoustic power, [39, 40] using Equation 1 shown below [38]:

$$MDI(dB) = -0.0011(MGSV)^2 + 0.48(MGSV) \quad (1)$$

Broadband attenuation—Broadband attenuation of the US signal through a suspension of ELIP and PELIP samples in hFFP, also at 0.05 mg/ml, 37°C, and a DO of 100–103% was measured using a through-transmission acoustic spectroscopy system with a usable bandwidth of 3 MHz to 25 MHz to characterize the echogenicity. [41–43] Briefly, an US pulser-receiver (Panametrics 5077PR, Olympus NDT, Waltham, MA) was used to generate the excitation pulse and amplify the received US signal over a frequency range of 3 to 25 MHz. Test samples were placed in an unmodified cell-culture cassette (CLINicell, Mabio, Tourcoing, France) with luer-lock ports used for introducing the sample suspension. The attenuation is calculated as the ratio of received signal strength with and without liposomes.

Plasmin encapsulation measurements

A chromogenic enzyme assay was used to determine the amount of plasmin associated with the PELIP. Reaction of plasmin with the chromogenic substrate S-2251 (DiaPharma Group Inc., West Chester, OH) results in the formation of p-Nitroaniline (pNA), which exhibits UV absorption at 405 nm. The rate of formation of pNA corresponds to the amount of plasmin activity present in the test sample, and was measured using an absorption spectrophotometer (Spectramax M5, Molecular Devices Inc., Sunnyvale, CA). Calibration curves were obtained by measuring the rate of change of absorption over 10 minutes with known amounts of plasmin (0.2–1 µg/mL). Calibration curves were also obtained with the addition of ELIP to the reaction mixture to offset contribution to absorption from the lipid bilayer and gas microbubbles in the PELIP.

The reaction mixture containing 100 µL of a phosphate lysine buffer (10 mM potassium phosphate, 70 mM sodium phosphate, 100 mM lysine buffer, pH 7.5 at 37°C), 25 µL of the S-2251 substrate (6.5 mM solution, pH 7.8 at 25°C), and 10 µL of either a blank (deionized, filtered, water) or the test solution was placed in a 96-well plate. For the calibration curve containing ELIP, 10 µL of a 1 mg/ml ELIP solution was added to the reaction mixture.

Lyophilized PELIP samples were rehydrated and diluted to a lipid concentration of 1 mg/mL, using deionized, filtered, water. A 10 µL aliquot of the PELIP solution at this dilution was added to the reaction mixture for the assay. The rate of change of absorbance shown by the PELIP test solution was used to determine the percentage of plasmin associated with the bilayer shell. The amount of unencapsulated or “free” plasmin separated during the centrifugation process was determined by assaying the supernatant. The supernatant was diluted 500x in deionized, filtered, water and 10 µL of the diluted solution was added to the reaction mixture containing 100 µL of the phosphate lysine buffer and 25 µL of the chromogenic substrate. The amount of plasmin encapsulated within the PELIP was calculated by subtracting the amounts measured in the PELIP bilayer and the supernatant from the amount of plasmin initially added to the PELIP (1 mg/mL).

Clot lysis measurements

Thrombolytic efficacy was measured using an *in-vitro* human whole blood clot model, over a time period of 30 minutes at 37°C in hFFP. [44, 45] The different treatment groups include rtPA at a 1 µg/mL concentration, and PELIP or plain ELIP, with and without US exposure. In a clinical setting 10% of the rtPA dosage is administered as a bolus, with the remaining

90% administered as a drip over a 24 hour period The concentration of rtPA used in this study was calculated based on the NINDS recommended rtPA dosage for thrombolytic therapy of AIS averaged over a time period of 24 hours for an average adult, as described by Shaw et al. [46, 47] hFFP pooled from 8 different donors was used as the medium containing the clot.

Manufacture of human whole blood clot models—*In-vitro* human whole blood clots, approximately 200–300 μm in diameter, comparable to intracerebral segments of the middle cerebral arteries were prepared. [48, 49, 35]. Briefly, whole blood was obtained from human volunteers by sterile venipuncture after Institutional Review Board (IRB) approval of the protocol and written informed consent. Aliquots were placed into 1.5 mm diameter micropipettes and allowed to clot around 7-0 silk sutures (Ethicon) at room temperature for approximately 10 minutes. The clots were incubated at 37° C for 3 hours in a temperature-controlled water bath. This method yielded sample clots that were $230 \pm 34 \mu\text{m}$ in diameter. The clots were stored at 4° C in a humidified environment to ensure platelet viability. [50]

Clot lysis measurement—The clot attached to the suture was placed in a clean micropipette (Drummond Scientific Company, Broomall, PA), and inserted into a U-shaped sample holder composed of hollow luer lock connectors and silicone tubing (Cole Parmer, Vernon Hills, IL; outer diameter 0.125"). The sample holder was placed in an acrylic water tank with a microscope slide at the bottom allowing visualization of the clot diameter using an inverted microscope (Olympus, IX-71) with a charge-coupled device (CCD) camera (Retiga 2000R, QImaging, Surrey, BC, Canada). The CCD camera images were recorded at a rate of 6 images/minute. The average clot width (CW) was calculated, using a computer program written in Matlab 6.5 R13 (Mathworks, Inc., Natwick, MA). The positions of the two clot-plasma interfaces were determined via an edge-detection routine. The width of the clot at each coordinate along the height of the image (z) was calculated, averaged over all z values, corrected for suture width, and normalized to the average of the clot width during the first six frames. The fractional clot loss (FCL) at 30 minutes can be defined as:

$$\text{FCL} = 1 - \text{CW}_{30} / \text{CW}_0 \quad (2)$$

where,

CW_{30} = Average clot width after 30 minutes, and,

CW_0 = Initial average clot width.

US parameters—US exposure was achieved using a 120 kHz unfocused transducer (Sonic Concepts, Inc., Woodburn, WA). The transducer was mounted at one end of the tank at a 30° angle to the tank bottom allowing US exposure of the sample clot. The US parameters used were a peak-to-peak pressure of 0.35 MPa, a pulse repetition frequency (PRF) of 1667 Hz, and a duty cycle of 50%, and a time-averaged acoustic intensity of 0.5 W/cm^2 . These US parameters yielded substantial clot lysis in previous work. [48, 49, 35, 51–53, 40, 54]

Lytic rate calculation—Lytic rates were calculated using the technique published by Meunier *et al.* [48] Briefly, a non-linear least squares fit of the normalized clot width versus time, t , data to the following equation was performed:

$$\Lambda(t) = B + (1 - B)\exp(-kt) \quad (3)$$

where Λ is the normalized average clot width (dimensionless), B is the final value of the normalized average clot width (also dimensionless) and k is the exponential decay rate constant (min^{-1}). An R^2 value of at least 0.88 was obtained for the different treatment groups. For small values of time, equation (3) can be approximated as:

$$\Lambda(t) \approx 1 - (1 - B)kt \quad (4)$$

Using this relationship, the initial lytic rate (LR) is defined as:

$$\text{LR} \equiv (1 - B)k \quad (5)$$

Statistical Analysis

The differences between treatment groups was assessed using Independent Samples T-Tests. Parameter estimates and 95% confidence intervals were calculated. A p value less than 0.05 was considered significant. All statistical analyses were conducted using SPSS 21.0 (IBM Corporation, Armonk, NY).

RESULTS

Size distribution of ELIP and PELIP

The size distribution of the liposomes are shown in Figure 2, with characteristic peaks corresponding to diameters of 1.13 and 1.20 μm for ELIP and PELIP respectively. Particles ranged in size from 0.6 μm to as large as 12 μm , indicating polydispersity of the distribution. The mean particle diameter of PELIP is comparable with that of commercial US contrast agents which range in mean diameter from 1–5 μm . [55] The average number densities of ELIP and PELIP were 113×10^9 particles/mL, and 23×10^6 particles/mL respectively.

US backscatter and attenuation

US backscatter from ELIP and PELIP were observed as grayscale speckles in the flow phantom as shown in Figure 3. The average MDI for ELIP and PELIP were calculated to be 21.9 ± 2.5 dB and 21.5 ± 3.5 dB, suggesting that the echogenicities of the ELIP and PELIP samples analyzed are similar.

Figure 4a shows the mean acoustic attenuation of ELIP and PELIP. The presence of acoustically active gas bubbles increases the attenuation of the sample relative to the hFFP alone. The attenuation of the samples remained stable over time with relatively small decreases in magnitude over a 30-minute period, indicating stability of entrapped air bubbles within the ELIP or PELIP (see Figure 4b), within a clinically relevant time frame.

Drug encapsulation efficiency

Figure 5 shows the calibration curves obtained using plasmin alone ($y=17.04x+5*10^5$, $R^2=0.89$, $N=3$) and plasmin with ELIP ($y=26.46x-0.0015$, $R^2=0.96$, $N=4$). The amount of plasmin associated with the outer surface of PELIP was determined to be 0.039 ± 0.007 mg/mL ($N=5$). The amount of plasmin in the supernatant collected after centrifugation of the PELIP to separate unencapsulated plasmin from the PELIP was found to be 0.49 ± 0.23 mg/mL ($N=3$).

Clot lysis

The normalized average final clot widths were 0.83 (95% CI 0.72 to 0.95) for plain ELIP with US exposure, 0.54 (95% CI 0.36–0.72) for PELIP with US, and 0.70 (95% CI 0.63 to 0.77) for rtPA alone. The normalized average final clot width for PELIP with US exposure was smaller than the mean final clot width for rtPA (difference -0.16 , 95% CI -0.32 to -0.001 , $p=0.049$). The normalized average final clot width for PELIP with US exposure was also smaller than the normalized average final clot width for plain ELIP with US exposure (difference -0.29 , 95% CI -0.52 to -0.07 , $p=0.038$). Table 1 shows the normalized average clot width, FCL, LR, number of experiments (N), and number of donors (D) for each treatment group studied. The mean FCL obtained by exposing PELIP to US was found to be 16% higher than the thrombolytic efficacy of rtPA alone at a 1 μ g/ml concentration ($p<0.05$). In the absence of US exposure, PELIP exhibited nearly 31% lower average FCL (not significantly different from control) compared to lysis with PELIP with US exposure ($p<0.05$). The average FCL obtained with ELIP was also not significantly different from control clots in the absence or presence of US exposure. Figure 6 shows the change in clot width over time, normalized to the clot width at the start of the treatment period. The highest estimated lytic rate belonged to the group of clots exposed to PELIP and pulsed US (Table 1).

DISCUSSION

In this work, clots exposed to PELIP and US showed a significantly greater thrombolysis than clots exposed to rtPA alone. The thrombolytic efficacies of PELIP in the absence of US exposure, and ELIP with or without US exposure were not different from control, indicating that thrombolysis caused by PELIP exposed to US may have been due to the release of the encapsulated plasmin.

Several studies have demonstrated the effectiveness and safety of ultrasound in enhancing thrombolysis of middle cerebral artery occlusion (MCAo) in AIS patients. Alexandrov et al demonstrated through a phase II multicenter randomized trial that simultaneous application of 2 MHz transcranial doppler for two hours and intravenous rtPA was safe and improved the rate of complete recanalization. [56] Molina et al demonstrated increased rates of complete recanalization with the simultaneous application of 2 MHz transcranial doppler (TCD) for 2 hours, intravenous rtPA, and galactose based microbubbles, compared to intravenous rtPA and TCD, or intravenous rtPA alone. [57] The transcranial ultrasound in clinical sonothrombolysis (TUSCON) study also demonstrated safety and enhanced efficacy of simultaneous application of 2 MHz TCD, intravenous rtPA and perflutren lipid-

microbubbles for complete vessel recanalization compared to intravenous rtPA treatment alone. [58]

However, the Transcranial Low-Frequency Ultrasound-Mediated Thrombolysis in Brain Ischemia (TRUMBI) trial used combined rtPA and low frequency transcranial ultrasound (300 kHz) therapy and was prematurely abandoned due to a high incidence of symptomatic hemorrhages. [59] Studies have shown that the acoustic parameters used in the TRUMBI trial (US intensity of 0.7 W/cm² and a continuous insonication of 90 minutes) may have induced multiple internal reflections, standing waves and cavitation effects, leading to a disruption of the small vessels and BBB opening in the subarachnoid space. [60–63]

Since the TRUMBI trial, animal studies in a primate middle cerebral artery occlusion (MCAo) model have shown safety of US enhanced thrombolysis with rtPA at a frequency of 490 kHz with optimized ultrasound parameters and transducer design. [64]

The ultrasound parameters used in this work yielded the maximum ultrasound enhanced thrombolysis based on previous optimization studies using rtPA and rtPA loaded echogenic liposomes. [65, 52, 48, 49, 35]

However, given the wide range of US parameters used in US enhanced thrombolysis studies, there is undoubtedly a need for a thorough optimization of the acoustic parameters for safe thrombolysis.

The goal of this work was to develop a drug delivery system for plasmin that can effectively shield plasmin from inhibitors until site-specific US-mediated release. Given the enhanced safety profile of plasmin compared to rtPA, this therapy may significantly decrease the risk of hemorrhagic complications, increase the rate of recanalization, and prove to be more beneficial than intra-arterial delivery of plasmin directly to the clot-site in AIS patients. Studies have shown that intra-venous delivery of the lytic rtPA is more effective than intra-arterial delivery. [66] In another recent study, Bizjak et al compared the lytic efficacy of equimolar amounts of rtPA and plasmin in an in-vitro partial vessel occlusion model. [67] After initial incubation with the thrombolytics, the clot was flushed with plasma and observed for lysis over time. While rtPA treated clot continually lysed over the time period of study, plasmin treated clot did not change significantly in size after flushing. The authors attribute this to the inhibition of plasmin by inhibitors in the plasma and the insufficiency of endogenous rtPA inhibitors. The use of US mediated release allows for continuous availability of plasmin directly to the clot site without the need for a catheter.

In this study, the lytic rates were estimated to be higher for US-mediated thrombolysis with PELIP. Hence, potentially faster thrombolysis can be achieved using PELIP and US exposure, and a continuous 24 hour infusion of thrombolytics may not be necessary, further enhancing the safety profile of thrombolytic therapy using PELIP and US exposure.

From the chromogenic assay, the total amount of plasmin associated with the PELIP was measured to be about 51 % of the amount initially added to the phospholipid mixture (1 mg/mL), including about 4 ± 1 % associated with the PELIP surface.. However, the lytic activity of PELIP alone (without US) was not significantly different from control, possibly due to

rapid deactivation of the plasmin on the PELIP surface by the plasmin inhibitors. Banbula et al reported that plasmin up to a concentration of 12 mg/kg can be completely inactivated by the endogenous plasmin inhibitors in human whole blood. [68] This concentration is significantly higher than thrombolytic dosages of 1–4 mg/kg [69], or the estimated dosage of 0.5 mg administered to clots in this work.

The amount of unencapsulated plasmin in the supernatant was measured to be 49 ± 23 % of the amount initially added to the phospholipid mixture. However, due to the autodegradation of plasmin at physiological pH the plasmin detected may reflect the amount of undegraded plasmin remaining in PELIP at the time of measurement rather than the actual amount encapsulated. [36] Several measures were taken to curb the autolytic activity of plasmin in this work, including the use of a low pH solvent, low temperatures, and short sonication and centrifugation steps. However, a detailed study on the decay of plasmin during the PELIP manufacture and characterization steps are warranted.

Acknowledgments

The authors gratefully acknowledge funding from the Cardiovascular Center of Excellence (CVCoe) at the University of Cincinnati, NIH K02-NS052653, 3P50 NS044283-0651, and R01-NS047603 grants. The authors also acknowledge the valuable assistance from Kirithi Radhakrishnan and Jason L. Raymond in echogenicity measurements, and members of the Image-guided Ultrasound Therapeutics Laboratories and the Emergency-Medicine Research Interest Group at UC for useful discussions and feedback on this project.

Abbreviations

α2-AP	α 2-antiplasmin
AIS	Acute ischemic stroke
CCD	Charge-coupled device
CI	Confidence Intervals
CW	Clot width
D	Number of donors
DO	Dissolved oxygen
DOPC	1,2-dioleoyl-sn-glycero- 3-phosphocholine
DPPC	1,2- dipalmitoyl-sn-glycero-3-phosphocholine
DPPG	1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-rac- glycerol) (sodium salt)
ELIP	Echogenic liposomes
FCL	Fractional clot lysis
hFFP	Human fresh-frozen plasma
ICH	Intracerebral hemorrhage
IRB	Institutional review board
IV	Intravenous

LR	Lytic rate
MCA	Middle cerebral artery
MDI	Mean digital intensities
MGSV	Mean grey scale values
N	Number of trials
PAI-1	Plasminogen activator inhibitor
PELIP	Plasmin-loaded echogenic liposomes
pNA	p-Nitroaniline
rtPA	Recombinant tissue plasminogen activator
rtPA-ELIP	rtPA-loaded echogenic liposomes
ROI	Region of interest
US	Ultrasound
UV	Ultra-violet

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Figure 1.

A schematic representation of an echogenic liposome with the bilayer phospholipid shell and an aqueous core containing gas microbubbles. Protein may be encapsulated in the aqueous core, associated with the bilayer and/or adsorbed on the outer shell.

Figure 2. Size distribution of ELIP (Grey diamonds, N=4) and PELIP (Black squares, N=2), with peaks at 1.13 and 1.20 microns respectively.

Figure 3.

The average MDI calculated for PELIP (21.5 ± 3.5 dB; $N=3$) and ELIP (21.9 ± 2.5 dB; $N=4$). Images of acoustic backscatter from PELIP and ELIP flowing through the flow phantom observed as grayscale speckles are inset. Error bars represent standard deviations.

Figure 4.

(a) US attenuation averaged over measurements from different batches of ELIP (N=4) and PELIP (N=5) suspended in hFFP at 37°C. Error bars represent standard deviations. (b). US attenuation spectrum of a batch of PELIP suspended in hFFP at 37°C measured over 36 minutes (N=3). The sample was echogenic with slow decrease in US signal attenuation over the measurement period. Error bars represent standard deviations.

Figure 5. Calibration curves for plasmin alone (triangles, $y=17.04x+5 \times 10^5$, $R^2=0.89$, $N=3$), and plasmin with ELIP (diamonds, $y=26.46x-0.0015$, $R^2=0.96$, $N=4$)

Figure 6.

Change in normalized clot width over time. (The number of trials N, donors D, and 95% confidence intervals for each group can be found in Table 1) The normalized average final clot widths were lowest PELIP with US (filled squares), compared to plain ELIP with US (filled triangles) or the rtPA treated clots (open circles). In the absence of US the final clot widths of PELIP (open squares) and ELIP (open triangles) treated clots were not significantly different from control (hFFP).

Table 1

Normalized average final clot widths, fractional clot lysis and estimated lytic rates obtained for the various treatment groups.

Treatment	US ^a	N	D	Normalized average final clot widths (95% CI)	FCL	Lytic rate (Standard error)	Ref.
hFFP ^b	No	222	29	0.86 (0.85–0.88)	0.14	0.02 (0.0003)	[33, 44, 45, 54, 55]
	Yes	16	4	0.84 (0.76–0.92)	0.16	0.02 (0.001)	
ELIP	No	9	5	0.87 (0.81–0.93)	0.13	0.02 (0.001)	
	Yes	9	5	0.83 (0.72–0.95)	0.17	0.02 (0.002)	
rtPA ^b	No	42	5	0.70 (0.63–0.77)	0.30	0.02 (0.001)	[44, 45]
	Yes	11	3	0.57 (0.34–0.79)	0.43	0.033 (0.002)	
PELIP	No	5	4	0.85 (0.61–1.08)	0.15	0.02 (0.001)	
	Yes	12	5	0.54 (0.36–0.72)	0.46	0.06 (0.001)	

^a Abbreviations used: US – Ultrasound, N – Number of trials, D - Number of donors, CI – Confidence Intervals, FCL –Fractional clot lysis

^b Values from previously published work conducted in the Shaw Lab