eLiposomes as a Targeted Thrombolytic Drug Delivery Vehicle During Sonothrombolysis

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Abstract

Cardiovascular diseases are a major burden on the healthcare system and are responsible for 32% of all world wide deaths. Thrombolytics are a class of powerful medications used to treat thrombotic occlusions that are a major cause of cardiovascular-related deaths, but suffer from a significant elevated risk of bleeding when used. Sonothrombolysis is a field of research investigating how to treat thrombotic occlusions using ultrasound with a reduced dosage or without of thrombolytics. A novel drug delivery vehicle, eLiposomes, is able to concurrently deliver drugs and cavitate in response to a strong ultrasonic field and have been shown to effectively deliver chemotherapy drugs selectively when uptaken by tumor cells. This study aimed to investigate the promise of eLiposomes as a targeted thrombolytic drug delivery vehicle during sonothrombolysis. By evaluating the encapsulation efficiency, ultrasound release rate, thermal stability, and monodispersity, we intended to test eLiposomes as a sonothrombolytic agent against microbubbles, nanodroplets, and free thrombolytics. We were not able to test the effectiveness of sonothrombolysis, but were able to demonstrate a statistically significant increase ($P = 0.000675$) in calcein release in eLiposomes compared to control liposomes (78% vs 32%) after 90 seconds of ultrasound treatment with a 1MHz transducer. Additionally, we were able to demonstrate a monodisperse size distribution (PDI = 0.24), statistical indistinguishability in thermal release relative to control in 4 °C, and an encapsulation efficiency of 1.5%. Further research should go into the effectiveness of eLiposomes as a sonothrombolytic agent, as well as the ultrasonic conditions behind eLiposome release.
Keywords: Liposome, nanodroplet, ultrasonic drug release, eLiposome, sonothrombolyis

Introduction

Thrombotic occlusions (thromboembolisms) are dangerous blockages of blood flow by blood clots, preventing the flow of oxygen and nutrients to parts of the body. This leads to ischemia, a state of disrupted cellular metabolism that leads to local tissue suffering impaired ion regulation, malfunctioning cellular regulatory mechanisms, and eventual cell death[1]. Depending on the size and location of the clot the ischemic injury can be mild to severe, with thrombotic occlusions to critical organs like the lungs, brain, or heart being potentially fatal. Ischemic heart disease and ischemic stroke are respectively the 1st and 6th most common causes of death in US adults in 2020, with heart disease killing over 690,000 people and stroke killing over 159,000[2]. Pulmonary embolism, while less common and less deadly, still contributes to over 47,000 deaths annually[3]. Overall, there is a high incidence and a high risk of serious complications associated with thromboembolisms, and so there is a great need for effective treatments.

Within the first three hours of presenting symptoms for acute ischemic stroke, or within the first 12 hours for acute myocardial infarction, a favored treatment for thromboembolism is intravenous administration of thrombolytics for eligible patients[4,5]. Thrombolytics are a type of medication used to dissolve blood clots by catalyzing the breakdown of fibrin, a protein that forms the mesh-like structure that holds the blood clot together. They do so by converting another protein within the clot, plasminogen, into plasmin, an enzyme that cleaves fibrin. Due to their faster clot dissolution rate compared to anticoagulants, thrombolytics are an essential tool to rapidly restore blood flow and prevent tissue damage in cases of potentially deadly blood clot occlusions[6]. Alteplase, reteplase, and tenecteplase are all genetically recombinant versions of the tissue plasminogen activator (rtPA) protein found in humans, and are all FDA approved to treat ischemic heart attacks. Alteplase alone is also approved to treat acute ischemic stroke as well as massive pulmonary embolism[7].

However, the use of thrombolytics is associated with internal hemorrhaging. The risk of intracranial hemorrhage is greatly increased after the administration of thrombolytics to treat stroke, with a 6.4% rate of bleeding within 36 hours in stroke patients administered alteplase compared to a 0.6% rate of bleeding in patients administered placebo[8]. This is thought to be associated with off-target blood clot dissolution within tissue as well as interactions with matrix metalloproteinase-9[9]. Other, but less common side effects that can occur while using thrombolytics to treat stroke, myocardial infarctions, or pulmonary embolisms are allergic reactions, hypotension, and myocardial rupture[10]. The dire threat of some of these side effects and the requirement of determining patient eligibility before treatment has led to a low rate of thrombolytic usage for patients suffering from stroke. In the United States, only an estimated 2% of ischemic stroke patients are administered thrombolytics. The reasons for the underutilization of thrombolytics in stroke treatment range from doctor unfamiliarity, time to treatment exceeding 4.5 hours, patient ineligibility for thrombolytics, as well as physician uncertainty over whether a patient is eligible or not[11].

To sidestep the risks of thrombolytics, sonothrombolysis has emerged as an experimental alternative technique that uses therapeutic ultrasound to mechanically destroy the structure of blood clots. This can be paired with cavitation agents and reduced doses of thrombolytics to heighten its effectiveness[12]. Cavitation agents mechanically disrupt the surface of the clot through cycles of expansion and compression when exposed to oscillating pressure waves while thrombolytics penetrate into the exposed portions. Various combinations of sonothrombolysis paired with lipid microspheres, microbubbles, and rtPA have already been shown to be

more successful in complete clot recanalization in patients compared to sole thrombolytic therapy. However, no significant differences were observed between intervention and control groups for mortality and intracranial hemorrhage rates[13,14].

There are some safety concerns associated with sonothrombolysis, namely excessive tissue heating, endothelial tissue damage, as well as the formation of large clot debris that can recanalize further downstream. Efforts are taken to minimize these effects by optimizing the duty cycle, peak negative pressure, and mechanical index of the ultrasound transducer, as well as the combination of therapeutic agents[15]. However, in cases where rtPA is administered during sonothrombolysis, it is almost always administered freely into circulation. This can lead it to be quickly inhibited by plasminogen activator inhibitor-1 (PAI-1) or uptaken by the liver in in vivo conditions, and in physiological conditions in humans it has a half-life of just 6 minutes[16]. Almost all rtPA released freely into the bloodstream never significantly catalyzes fibrinolysis of blood clots as a result of this, and if there was some way of increasing the amount of rtPA released locally at the clot site it would allow for more effective systemic dosage reduction whilst enhancing sonothrombolysis rates.

The idea of local release has been pursued by of targeted thrombolytic drug vehicles pursues this, with successful attempts at producing microbubbles surface-loaded with rtPA and RGD peptide demonstrating success with tenfold dosage reduction compared to free rtPA and maintaining equivalent thrombolytic efficiency[17,18]. Additionally, echogenic liposomes have been produced with rtPA loaded on both the exterior and interior of the liposome that were also equivalent to free rtPA in sonothrombolysis rates[18]. Both methods rely on the cavitation of a gaseous perfluorocarbon core to release their payload.

Emulsion liposomes (eLiposomes) are a drug delivery vehicle consisting of a liposome containing a drug payload and lipid-shelled liquid perfluorocarbon emulsion within the interior. By sizing extruding the emulsions to be nanometer-scaled, the laplace pressure at their interface exerts considerable pressure and their boiling point is elevated according to the Clausius-Clapeyron relationship. This way, the emulsions can have their phase-change reactivity to thermal and pressure stresses modulated. Perfluoropentane, with a boiling point temperature of 29 °C, can be made stable at 37 °C by being sized to nanodroplets 480 nm in diameter[19]. When an ultrasonic pulse of sufficient peak negative pressure (PNP) is applied to the droplets, the local pressure inside the droplet may dip below the vapor pressure, leading to the vaporization of the liquid. The phase shift of the nanodroplet leads it to increase to be several times its liquid volume, exceeding the size of the encapsulating liposome and irreversibly lysing it, additionally releasing whatever payload the liposome carries [20]. Other studies have used eLiposomes as a targeted cancer drug delivery vehicle in cell cultures as well as evaluating its encapsulation efficiency and release rate in response to ultrasound using calcein dye, horseradish peroxidase, and mistletoe lectin[21,22]. While new research continues to further its promise as a novel drug delivery vehicle, no study so far has evaluated its potential as a sonothrombolytic agent.

Hypothesis

The hypothesis motivating our project was that the encapsulation of nanodroplets in targeted eLiposomes carrying rtPA would allow for a superior sonothrombolytic agent with increased rates of sonothrombolysis over free rtPA combined with microbubbles or nanodroplets. This would be due to the site-specific release of rtPA as well as the possibility for the liposome to penetrate into the blood clot and then release its payload.

Since we were unable to test this rigorously, we instead focused on the end state of our first stage and tested the hypothesis that the encapsulation of nanodroplets in eLiposomes carrying calcein would lead to greater releases of calcein in response to ultrasound treatment compared to control liposomes.

Project Aims

The goal of this project was to develop a targeted dipalmitoylphosphatidylcholine (DPPC) eLiposome carrying a 400 nm perfluoropentane nanodroplet and rtPA to serve as a sonothrombolytic agent.

The first aim consisted of the synthesis of 800 nm sized eLiposomes carrying a calcein payload and a 400 nm perfluoropentane nanodroplet. We would then characterize it by determining the release rate in response to ultrasound (0.6 W/cm^2, 1MHz) relative to control liposomes, thermal stability relative to control liposomes, DLS measurements, and encapsulation efficiency.

The second phase would have consisted of loading the eLiposome with rtPA instead of calcein and targeting it to activated platelets using fucoidan. We would then run a comparison of the sonothrombolytic efficacy of the eLiposomes in comparison to different treatment combinations of microbubbles, nanodroplets, and free rtPA. The scope had to be limited to accomplishing the first aim due to time constraints.

Design Constraints

To focus on and work towards achieving our goal, we also initially highlighted some essential constraints that we would strive to address and adhere to. Characterizations of our success in achieving them were summarized in **Supplementary Fig. 1**.

1. Statistically significant differences in calcein release rates between control liposomes and eLiposomes when sonicated with ultrasound.
2. Thermal stability of eLiposomes evaluated by looking at release rate of eLiposomes after 2 hours at 37 ℃. Ideally < 10%, but willing to accept up until 40%.
3. Blood clot mass reduction using eLiposome > 90% after 30 min of treatment, willing to accept >60%.
4. 1 MHz center frequency transducer.
5. 1 MPa PNP during ultrasound treatment.
6. 7.5% duty cycle during ultrasound treatment.
7. Reasonable encapsulation efficiency, ideally > 5%, but willing to accept 0.1%.
8. Good ultrasound release, ideally > 90% after 30 min, but willing to accept 50%.
9. Monodisperse, evaluated by having a polydispersity index of < 0.3.
10. Small diameter, ideally exactly 400 nm, but willing to go up to 800 nm.

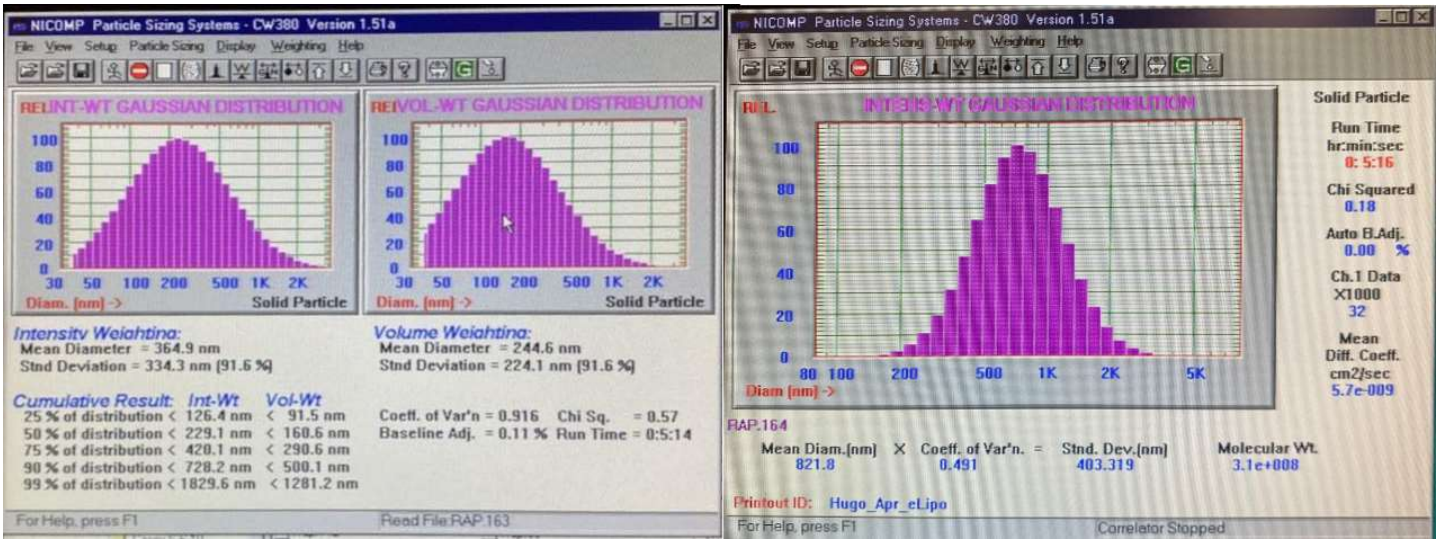

Figure 1. Characterization of particle size distributions for nanodroplets (left) and eLiposomes (right) using DLS. Nanodroplets were extruded through a 400 nm nuclepore filter while eLiposomes were extruded through an 800 nm nuclepore filter. A photo had to be taken of the machine display due to a lack of ports to retrieve screenshots.

Results

Nanodroplet and eLiposome Production and Size Characterization

Nanodroplets were produced by emulsifying perfluoropentane immersed in a mix of DSPC and DSPE-PEG-2k with 5% propylene glycol. After being extruded multiple times and spun down to get rid of large sized droplets, an aliquot was taken to measure the distribution of particle diameters via DLS. The intensity-weighted mean particle diameter was 364.9 nm ± 334.3 nm, close to the nuclepore filter size of 400 nm. The large standard deviation may have indicated the remaining presence of some larger particles in the mix.

The eLiposome mix of perfluoropentane nanodroplets, interdigitated DPPC sheets, DSPE-PEG-2k/cholesterol micelles, and calcein (diluted to a final concentration of 40 mM) was heated and extruded seven times through a 800 nm nuclepore filter. After separating out liposomes that did not encapsulate nanodroplets and nanodroplets that were not encapsulated via density purifications, the eLiposomes were extracted and washed. After resuspension, an aliquot was placed into a dynamic light scattering (DLS) machine to determine the size distribution of the nanoparticles. DLS results provided an intensity-weighted mean of 821.8 nm ± 403.319 nm, indicating a distribution of liposome diameters centered closely around the nuclepore filter size of 800 nm.

The polydispersity index (PDI), a measure of how monodisperse a particle distribution is, is given by **Eq. 1**.

$$\mathbf{PDI} = \frac{\sigma^2}{d^2} \qquad [1]$$

Where σ is the standard deviation of the distribution and d is the mean diameter.

The nanodroplet size distribution had a PDI of 0.84, indicating a broad size distribution. For the eLiposomes, the PDI had a value of 0.24, indicating a moderately monodisperse distribution[26].

The broad distribution of the nanodroplet mix is despite two consecutive spins to get rid of micron-sized droplets, which are both more reactive and unable to be internalized by 800 nm sized eLiposomes. The presence of larger particles in the nanodroplet mix may not have a strong effect on the eLiposomes, as they are more likely to vaporize during the heated extrusion step to form eLiposomes and will not be internalized. Smaller than normal nanodroplets may affect the eLiposome reactivity to ultrasound due their increased laplace pressure. It is not likely that the larger particles are microbubbles from vaporized nanodroplets, as those would be positively buoyant and leave the solvent[27].

The encapsulation of nanodroplets was verified by the comparison of depth descended by an eLiposome band in a 0.9% saline/0.15 M sucrose/0.3 M density gradient relative to control liposomes. Additionally, proof of concept tests such as 5 minutes spent in 60 °C water as well as a negative vacuum generated by a syringe both generated clear bubble nucleation within the eLiposome solution **(Fig. 2).**

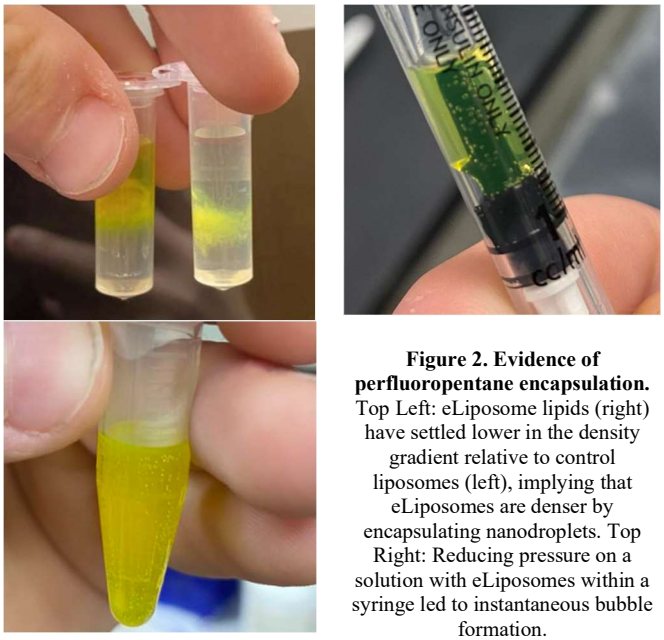

Figure 2. Evidence of perfluoropentane encapsulation. Top Left: eLiposome lipids (right) have settled lower in the density gradient relative to control liposomes (left), implying that eLiposomes are denser by encapsulating nanodroplets. Top Right: Reducing pressure on a solution with eLiposomes within a syringe led to instantaneous bubble formation. Bottom Left: Placing a solution of eLiposomes in 60 °C for 5 minutes led to the development of gas bubbles.

Thermal Release

Both control liposomes and eLiposomes were incubated in three different temperatures over the course of two hours: 4 °C, 22 °C, and 37 °C to determine the rate of thermal release of calcein across time (**Fig. 3**).

There seems to be a clear effect of temperature on calcein release for the eLiposome group, though it isn't exactly linear. For eLiposome ANOVAs between temperature groups, significant differences were found at every time point except for 0:00. This makes some sense, as at the time 0:00 none of the samples were yet exposed to their temperature conditions, meaning they all essentially underwent identical conditions. At the time 1:30, two samples fell into their micro-centrifuge tubes while spinning, one for the 4 °C group and one for the 37 °C group. While an attempt was made to retrieve the sample and place them into their respective wells, both samples had much higher release rates compared to their own groups, with values of 103% and 79% respectively, perhaps due to agitation. These data values were excluded during data analysis as an outlier while it was still a significant result ($P = 0.00488 < 0.05$).

The relationship of temperature and calcein release seems less obvious in the case for control liposomes, with either the 37 °C group or the room temperature group releasing the most calcein at particular time points. Control liposome ANOVAs between temperature groups at different time points found no statistically different differences between groups at times 0:30 and 1:00, coinciding with the times that the room temperature group was dominant. The difference between temperature groups became statistically significant from 1:30 onwards, with the 37 °C group clearly leading release rates.

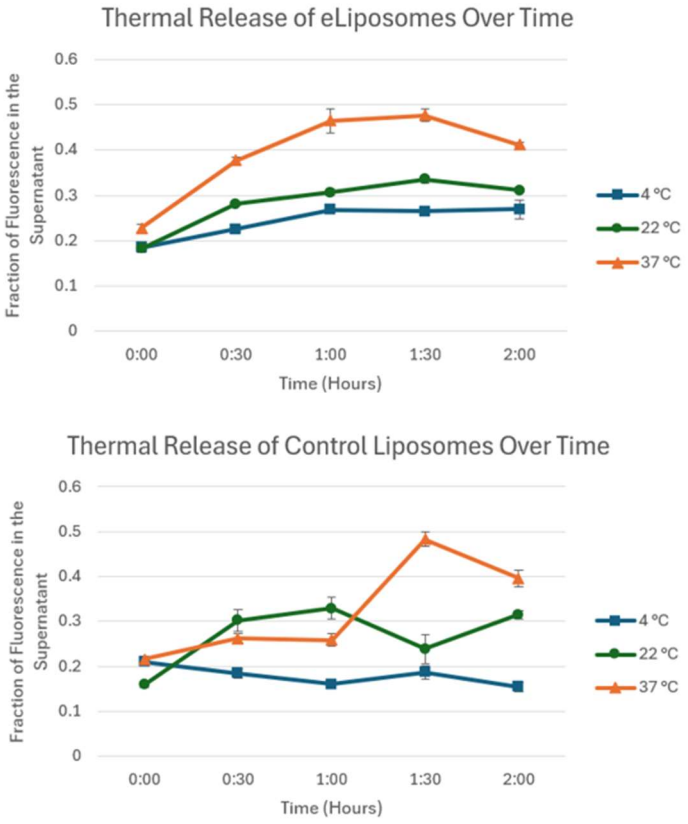

Figure 3. Thermal release over the course of two hours. ANOVA tests at each time point reveal significant differences between all groups at every time point except 0:30. Qualitatively the eLiposomes are only mildly unstable at 37 °C relative to control. Error bars are of standard error.

Two factor ANOVAs with replication for each temperature group were run and led to observations of significant differences within the 22 °C ($P=0.00676$) and 37 °C ($P = 7.3E-7$) groups, but insignificant differences between the 4 °C ($P = 0.38239$) groups. This implied that, at least for two hours, the eLiposomes are not statistically different in thermal release from control liposomes in cold conditions, but are more reactive relative to control in conditions outside of a fridge.

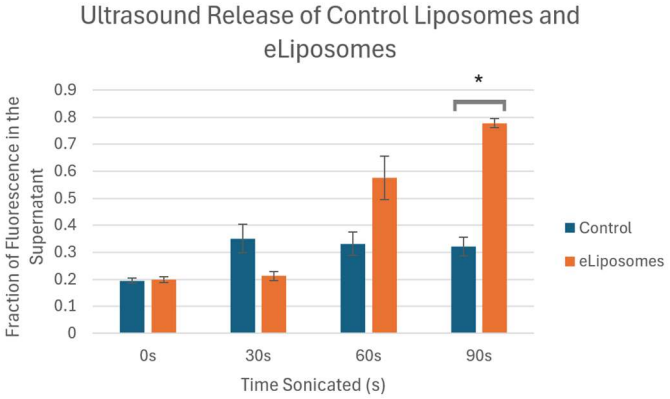

Figure 4. Ultrasound-stimulated release of calcein into the free solvent after ultrasound treatment (0.6 W/cm^2, 1MHz). * indicates statistical significance between groups as determined by a two-way t-test ($P > 0.001$). Error bars are of standard error.

Ultrasound Release

Both control liposomes and eLiposomes were exposed to continuous wave ultrasound (0.6 W/cm^2, 1MHz) for three different durations of time (30 seconds, 60 seconds, 90 seconds) in a 37 °C water bath (**Fig. 4**).

Differences in ultrasound release rates between control and eLiposomes were only significant at the end of 90 seconds as determined by an unpaired two-way t-tests. While eLiposomes have greater release rates than control liposomes at 60 seconds of sonication, the difference is not significant. At 30 seconds, one of the control liposome samples was contaminated by a pipetting error and was rejected out of an abundance of caution, despite having a relatively reasonable release rate of 0.526 relative to its other rates within the control group at that time (0.44 and 0.26). The inclusion or exclusion of this data point does not change the statistical insignificance of the difference between control and eLiposomes at 30 seconds ($P = 0.07$ including the data point, $P = 0.15$ without the data point).

At 90 seconds the effect of the presence of nanodroplets is very apparent, with a greater than twofold increase relative to control for the experimental group, with release rates of 77.8% and 32.2%, respectively. This is a very significant difference with ($P = 0.000675 < 0.05$). This evidence supports the hypothesis that internalizing perfluorocarbon nanodroplets will enhance sensitivity to ultrasound.

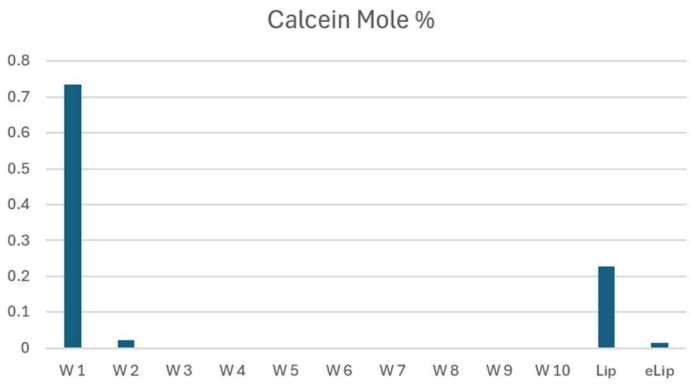

Figure 5. Distribution of moles of calcein between washes and the eLiposomes and the nonencapsulating liposomes. Note that washes after W2 do have values, they are just several orders of magnitude smaller than the visible values.

Encapsulation Efficiency

The supernatant of ten serial washes, unencapsulating liposomes filtered from eLiposomes, and an aliquot of the eLiposome solution were taken and had their 485 nm/535 nm fluorescence analyzed by a fluorescence well plate reader to determine the ratio of calcein within the eLiposomes (**Fig. 5**). After compensating for differing volumes, the encapsulation efficiency of moles of calcein within eLiposomes was found to be 1.57% **(Eq. 2)**.

$$EE\% = \frac{(c_{eLip} * V_{eLip})}{(c_{eLip} * V_{eLip}) + (c_{Lip} * V_{Lip}) + (\sum_{i=1}^{10} c_{W,i} * V_{W,i})} \quad [2]$$

Where c_j and V_j are the concentration of calcein and volume of fluid within a particular tube j, respectively.

Discussion

We have demonstrated heightened drug release compared to control liposomes for eLiposomes in response to 1 MHz of ultrasound treatment at an intensity of 0.6 W/cm^2, achieving a maximal release rate of 77.8% from the longest duration treatment on the created eLiposomes. Compared to the most immediately comparable study by de Matos et al., who also used DPPC eLiposomes with perfluoropentane emulsions to quantify ultrasound-triggered release using a 1.3 MHz transducer, we achieved similar rates of ultrasound release (57.6%) at our 1 minute condition compared to their best 1 minute release rates at 2 MPa PNP (~58%)[21]. The 2 MPa PNP is a similar driving force condition to ours, with 0.6 W/cm^2 being roughly equivalent to 1.34 MPa PNP in water from **Eq. 3**.

$$P_{pnp} = \sqrt{2 * I * Z} \quad [3]$$

Where P_{pnp} is the peak negative pressure in MPa, I is the intensity of ultrasound in MPa, and Z is the acoustic impedance of water (1.5 MRayl).

This is only a rough estimate and can depend on the frequency of the transducer and temperature of water[28].

However, there are several differences between de Matos et al.'s study and this study:

- This study sonicated samples at 37 °C as opposed to them sonicating at room temperature
- This study used a small molecule calcein as out payload instead of a macromolecular-sized payload like HRP or ML1 to determine encapsulation efficiency.
- This study defined release rate using supernatant and pellet measurements as compared to their use of measurements of free calcein fluorescence and total calcein fluorescence released by TX-100.
- This study used continuous wave ultrasound whereas they used a duty cycle of 1%.
- They observed no more than 2% background release, while this experiment consistently had background release around 20%.

Additionally, eLiposome release rates of calcein from from Lattin et al. were superior to ours, with 94% release within 10 seconds[22]. However, they use a 20kHz transducer at an intensity of 1 W/cm^2. This is not clinically translatable due to the use of high PNP combined with very low frequency, which risks damage to the body. The mechanical index **(Eq. 4)** is an approximate measure of how likely an ultrasound treatment is to cause dangerous inertial cavitations that can harm the body. Inertial cavitation likelihood is mainly determined by the peak negative pressure that the transducer exerts but also by the frequency of the transducer[29]. A mechanical index greater than 1.9 exceeds the FDA's limit for diagnostic ultrasound and risks harm to the body from bioeffects[30].

$$MI = \frac{P_{pnp}}{\sqrt{f_c} * (1(\frac{MPa}{\sqrt{MHz}}))} \quad [4]$$

Where MI is a unitless number, P_{pnp} is the peak negative pressure in MPa, f_c is the center frequency of the transducer in MHz.

Our ultrasound treatment has a MI of 1.34, whilst Lattin et al.'s has a MI of 12.24 (using I = 1 W/cm^2 to get an estimate of P_{pnp} = 1.73 MPa from **Eq. 7**). The increased risk of cavitation from higher PNP also allows for nanodroplets to vaporize more easily, leading to improved release. This has to be balanced against MI considerations when pursuing clinical translation.

Ultrasonically activatable drug release can allow for better drug localization for hazardous but life saving drugs like rtPA and chemotherapeutics. However, there were some problems with our current workup. First, the background release of calcein was excessive despite heavy washing, indicating that these liposomes are heavily le4aky despite attempts at cholesterol incorporation. The molar ratio of DPPC to cholesterol were, however, low, with an estimated 0.2 mg of cholesterol per 100 µl of the dil/DSPE-PEG-2k/cholesterol micelle solution pairing up with up to 100 mg of DPPC, a molar ratio of ~0.4%. What may also be occurring is the spontaneous vaporization of 400 nm nanodroplets within liposomes, but it should not be frequent enough to account for 20% of all fluorescence. It is a relatively constant background value, however, and does not increase over time. It may have originated from contamination of TX-100 or DPBS by calcein, but with every plate measured three wells tested the mix of DPBS with 5% TX-100 to control for this. There was never a strong fluorescence signal in any of those wells.

Second, the lack of any significant release at all at 30 seconds after sonication for the experimental group, when put in comparison with the rest of the groups, is confusing. A previous study found a lack of reactivity from eLiposomes using pulsed ultrasound that was resolved by switching from to continuous wave ultrasound and it was thought to be attributed to an interplay between mechanical cavitation and local temperature increase[27]. The success of these eLiposomes to release with longer treatments may be due to local heating that increases with the duration of continuous wave treatment, not mechanical cavitation. Although measurements of the temperature of the water bath were

conducted between swapping out samples, we did not always focus on the focal spot directly underneath the transducer so increased local heating may not have been noticed. Heat may also have been carried quickly away into the body of water anyway and hard to detect using an infrared thermometer gun.

In a review detailing the different measurements for the vaporization threshold for perfluoropentane droplets, most of the droplets had vaporization thresholds above 1MPa, although it did not disclose if any were sonicated at body temperature or room temperature[31]. Hydrophone measurements of the RNAse tubes used to hold samples during ultrasound treatment indicated that the thin plastic wall still attenuated approximately -3.9 dB of acoustic amplitude, implying that the 1.34 MPa PNP estimate for 0.6 W/cm^2 is reduced to about 0.86 MPa on the inside of tube. This would be below virtually all the estimates of vaporization thresholds for perfluoropentane droplets, even those that were over a micron in size.

What may have occurred is a local increase in temperature working in concert to decrease the vaporization threshold as the area around the tube begins to heat up, leading to nanodroplet vaporization and liposome lysis. Continuous wave therapeutic ultrasound can quickly produce heat after running for a short time, although to gain considerable amounts of heat over the course of a minute would typically require intensities higher than 1 W/cm^2, while we only used 0.6 W/cm^2[32]. In any case, our findings support our hypothesis of increased ultrasound sensitivity from nanodroplet encapsulation. In the event of a heating dependent mechanism for vaporization it may be less acceptable for use in human treatment, however. In addition to the mechanical index, there also exists a thermal index considered in ultrasound safety to prevent ultrasound treatments from dangerously raising the internal temperature of patients[33].

For targeted eLiposomes binding to clots, it may benefit to have a diminished or delayed release to an ultrasound signal that increases over time. If the drug vehicle is too reactive to ultrasound, then unbound eLiposomes may be vaporized while moving while exposed to the ultrasound signal, leading to systemic release of the payload. By requiring the target to be stationary and exposed to the ultrasound treatment for a prolonged period, you would isolate release to just those bound to the clot. Unbound eLiposomes would be less at risk to vaporizing while merely moving above the clot site, and their payload would be destroyed when they are eventually processed by the reticuloendothelial system[34].

Conclusion

This study has successfully created monodisperse 800 nm sized liposomes encapsulating 400 nm perfluoropentane nanodroplets that greatly increased ultrasound sensitivity relative to control liposomes. The eLipsomes were able to encapsulate 1.5% of a calcein payload, and notably achieved significant release in less than 2 minutes with a 1 MHZ transducer using a much lower intensity relative to other studies. While demonstrating enhanced thermal release in room and body temperature conditions, they were shown to be stable in 4 °C conditions. The underlying mechanism of their ultrasound release remains unclear and should be further investigated.

Future improvements to this study could be evaluating the effects of local heating using a thermometer immersed under or near the focal region to get real-time heating data during sonication. Using a programmable high intensity focused ultrasound (HIFUS) transducer, an attempt to examine the effects of duty cycle, peak negative pressure, and treatment duration on the rate of calcein release after treatment may allow us to determine to what extent does heating or mechanical agitation influence calcein release at 37 °C.

Future work should also continue to work towards the creation of a sonothrombolytic agent using these techniques. The question of whether or not a eLiposome sonothrombolytic agent could be superior to current cavitation agents with free rtPA still does not have an answer. Development of this needs to decide on some considerations:

- The choice of an optimal targeting agent to blood clots on the surface of the eLiposome between RGD, fucoidan, CREKA, or other ligands[35].
- The choice of the thrombolytic inside the eLiposome.
- The exact ultrasound specifications to use when running an in-vitro sonothrombolysis trial in consideration of how to emulate in-vivo conditions.
- How to better incorporate choleserol into eLiposome membranes produced through interdigitated membranes.

However, we do now know of a verified technique to produce eLiposomes reliably, albeit potentially leaky and with inferior loading capabilities compared to other studies. Nonetheless, they are reactive with minimal intensity and show an interesting trend of delayed release to continuous wave ultrasound over time, which may be exploited in the design of a sonothrombolytic agent.

Materials and Methods

eLiposome Creation

eLiposomes were created using a mix of interdigitated sheets, perfluorocarbon nanodroplets, micelles with diD, cholesterol, and DSPE-PEG-2k, and 100 mg/ml calcein. In a cold room with an ambient temperature of 4 °C, 100 µl of each solution of the interdigitated sheets, nanodroplets, micelles, and calcein were pipetted into a 1.7 ml tube and mixed via gentle pipette agitation until the solution was uniform in color and consistency. Then, 400 µl of the solution was pipetted directly into a 600 ul syringe, which was then depressed to remove air and bubbles. The syringe was then attached to a manual mini-extruder with a 800 nm nuclepore filter. The mini-extruder was placed in a 45 °C water bath, with moderate pressure applied to both ends to both pressurize the contents whilst also preventing movement across the membrane while the solution was forming liposomes and eLiposomes. After 4 minutes, the mixture was then pushed through the 800 nm filter 7 times to size the eLiposomes.

The mixture was removed from the mini-extruder syringe and placed into a 2 ml tube, to which 1.6 ml of cold 0.9% saline was added to dilute the free calcein. This tube was counter-weghted and spun down at 10000 rcf for 5 minutes, with 1.95 ml of the supernatant taken and placed into a tube to preserve the wash. Another 2 ml of cold 0.9% saline was added to the eLiposome tube and the pellet was resuspended via gentle pipette agitation and the process was repeated a total of 10 times to determine how much calcein was not encapsulated by the formed liposomes and left in the supernatant.

To separate the eLiposomes from non-encapsulating liposomes as well as perfluorocarbon nanodroplets, the final washed pellet was resuspended in 400 µl and was deposited on top of a density gradient consisting of 500 µl layers of 0.9% saline, 0.15 M sucrose, and 0.3 M sucrose going from top to bottom. The gradient tube was counterweighted and spun at 10000 rcf for 15 minutes to separate

nanodroplets and liposomes from the central band of eLiposomes. 400 ul of the central band was extracted, placed into a 2 ml tube that was then filled with 1.6 ml of 0.9% saline that was then counter weighted and spun at 5000 rcf for 10 minutes to separate sucrose from the pellet of eLiposomes, and then resuspended the pellet in 500 µl of 0.9% saline.

Control Liposome Creation

Control liposomes were created the same as the eLiposomes above, with the exception that instead of 100 ul of the concentrated nanodroplet solution, 100 µl of 0.9% saline was used. Additionally, no density gradient was required for separating out components of a mixture, and while the control liposomes were washed, the supernatants were not kept. The final washed pellet was resuspended in 2 ml 0.9% saline, with 200 ul of this solution then placed into a separate 2 ml tube, filled with 1.8 ml of 0.9% saline, spun down at 5000 rcf for 10 minutes, and then resuspended in 500 ul. This was to have solutions of somewhat similar numbers of liposomes, as there was a much larger amount of control liposomes in the wash pellet compared to the eLiposome wash pellet.

Interdigitated Sheet Creation

Interdigitated DPPC sheets were formed from DPPC liposomes and ethanol. 160 mg of DPPC powder was weighed out into a 20 ml glass vial on an analytical scale and then dissolved in 8 ml of 0.9% saline to create a 20 mg/ml solution. The solution was heated to above 50 °C to create a heterogeneous mix of liposomes and ensure full dissolution of DPPC within the saline. The solution was then sonicated on the lowest setting of the Q700 Sonicator (QSonics, Newton, CT) for 5 minutes to create more uniformly sized liposomes with a mean diameter of around 150-200 nm. To create a 3 M ethanol concentration for interdigitation, 1.7 ml of ethanol was added to the solution while it was vortexing. The solution was then left to sit at room temperature for 10 minutes for the liposomes to interdigitate and the solution to thicken.

Afterwards, to clear out the ethanol from the sheets, 1 ml of the solution was placed into 8 separate 1.7 ml tubes to spin down at 10000 rcf for 15 minutes. The supernatant of ethanol was then disposed of, and 0.9% saline was added to fill the tubes to the top, with the pellet of DPPC sheets resuspended by pipette agitation. The sheets were washed three more times with 10000 rcf spins of 5 minutes each, with the supernatant disposed of each time. For the final wash, the supernatant was removed until it just barely covered the surface of the sheets. Sheets were then used to make liposomes and eLiposomes, and were good to use until a month after creation.

Perfluoropentane Nanodroplet Creation

Perfluoropentane nanodroplets were created using perfluoropentane (97%, 85% n-isomer) (Alfa Aesar, Ward Hill, MA) and a nanodroplet lipid shell mix consisting of 10mg/ml of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 10mg/ml of 1,2-Dimyristoyl-sn-glycero-3-methoxypolyethylene glycol (DSPE-PEG-2k) sonicated to be 20-50 nm liposomes in 5% v/v propylene glycol. In a cold room with temperature of 4 °C, 400 µl of the nanodroplet mix was deposited into a 600 µl syringe using extra-long 200 µl flexible pipette tips to avoid bubbles in the syringe. 50 µl of perfluoropentane was added on top of the nanodroplet mix, with the syringe lightly tapped to lower the perfluoropentane fluid below the surface of the nanodroplet mix. The syringe was then depressed to remove all air and bubbles and then attached to a manual mini-extruder with a 400 nm nuclepore filter.

The mix was passed through the filter 21 times to create nanodroplets and then placed into a 1.7 ml microcentrifuge tube. 600 µl of cold saline was added to the tube, and the nanodroplets were counterweighted and spun in the cold room at 400 rcf for 2 minutes to pellet out micron-sized perfluoropentane droplets. This was done twice, with the supernatant placed into another 1.7 ml tube and the pellet and original tube disposed of. The tube was then spun at 8000 rcf for 5 min to pellet out the nanodroplets, leaving the nanoliposomes in the supernatant, which was then disposed of. The droplets were then resuspended in 200 µl of 0.9% saline to form a concentrated solution.

DiD Dye Micelles

Micelles were created through thin-lipid hydration of a 10 mg cholesterol, 5 mg DSPE-PEG-2k, and 0.15 mg DiD dye. This was rehydrated in 5ml of 0.9% saline and sonicated with the Q700 Sonicator (QSonics, Newton, CT) for 15 minutes on the lowest setting to ensure solute dissolution, mixing and the formation of minimally sized micelles. The 5 ml were then split into three separate 2 ml tubes and spun down at 500 rcf to pellet out titanium debris from the sonicator tip.

The micelles had to be created and incorporated separately from the lipid sheets during the heating step, since lipid mixes with considerable DSPE-PEG-2k or cholesterol could not interdigitate in the presence of ethanol.

Thermal Release

For each control and the eLiposome groups, Three sets of fifteen 200 µl tubes were filled with 190 µl of 0.9% saline and then filled with 10 µl of either the control or eLiposome solution. Each set of fifteen tubes corresponded to a temperature group, with the fifteen tubes being further split up into five groups of three tubes. This was so three measurements could be made at five different time points.

The three temperature groups were cold (4 °C), room temperature (22 °C), and hot (37 °C). The cold tubes were incubated in cool water in the cold room, the room temperature tubes were incubated on a bench in the lab, and the hot tubes were incubated in an incubator set to 37 °C. Every 30 minutes, 3 tubes from each group would be collected and spun down at 10000 rcf for 10 minutes in the cold room, with the 200 µl tubes nestled in lidless 500 µl tubes nestled in lidless 1.7 ml tubes to fit in the centrifuge slots. Afterwards, 100 µl of the supernatant would be pipetted into one well filled with 100 µl DPBS with 5% Triton X-100 (TX-100), with the remaining 100 µl and the pellet being agitated and then pipetted into the adjacent well, also containing 100 µl DPBS with 5% TX-100. The decision to evenly split the volumes of supernatant and pellet was made to lower the chance of disturbing the pellet during pipetting. Calcein fluorescence was measured with 485/535 nm wavelengths using a SPECTRAmax Gemini XS (Molecular Devices, San Jose, CA) fluorescence plate reader. Calcein release was calculated according to **Eq. 5-8** as a percentage within each tube as opposed to comparing raw fluorescence values between tubes. This was to prepare against a case where some 200 µl tubes received a slightly different amount of liposomes or eLiposomes from others due to spatial changes in concentration, since eLiposomes were noted to sink in 0.9% saline.

$$\mathbf{RFU_{top\ 100\ \mu l}} = \frac{\mathbf{RFU_{supernatant}}}{\mathbf{2}} \quad \textbf{[5]}$$

$$\mathbf{RFU_{bottom\ 100\ \mu l}} = [\frac{\mathbf{RFU_{supernatant}}}{\mathbf{2}} + \mathbf{RFU_{pellet}}] \quad \textbf{[6]}$$

$$\mathbf{RFU_{total}} = \mathbf{RFU_{bottom\ 100\ \mu l}} + \mathbf{RFU_{top\ 100\ \mu l}} = \mathbf{RFU_{supernatant}} + \mathbf{RFU_{pellet}} \quad \textbf{[7]}$$

$$\mathbf{Release\ Rate} = \frac{\mathbf{RFU_{supernatant}}}{\mathbf{RFU_{total}}} = \mathbf{2} * \frac{\mathbf{2} * \mathbf{RFU_{top}}}{\mathbf{RFU_{top\ 100\ \mu l}} + \mathbf{RFU_{bottom\ 100\ \mu l}}} \quad \textbf{[8]}$$

This set of equations assumes that the volume of the pellet was virtually negligible relative to the 200 µl of solvent and that the concentration of calcein throughout the supernatant was uniform.

Ultrasound-Stimulated Release

Ultrasound treatment was performed with a Birtcher Megason VI Model 106-5 (Birtcher Corp., Los Angeles, CA), a 1 MHz therapeutic transducer that allowed for acoustic intensity to be set manually along with a timer for treatment. For each control and the eLiposome groups, three sets of three 200 µl tubes were filled with 190 µl of 0.9% saline and then filled with 10 µl of either the control or eLiposome solution. Each set corresponded to a treatment condition: 30 seconds, 60 seconds, and 90 seconds. For each treatment, a 200 µl tube was secured between a micro bar clamp and placed in degassed water in a container surrounded by a 37 °C water bath.

The transducer was positioned 0.8 cm above and turned on for the length of the treatment condition with its intensity set to 0.6 W/cm². When each group was done, they were placed in spun down at 10000 rcf in the cold room for 10 minutes and and had the top 100 µl of each tube pipetted into a with one well filled with 100 µl DPBS with 5% Triton X-100 (TX-100), with the remaining 100 µl and the pellet being agitated and then pipetted into the adjacent well, also containing 100 µl DPBS with 5% TX-100. Release rate was then calculated using (**Eq. 3-6**) after measuring fluorescence in the plate as above.

Dynamic Light Scattering

Dynamic light scattering was used to provide estimates of the size distribution of extruded eLiposomes. A 1 ml borosilicate glass tube had 500 µl of 0.9% saline added into it, and then 10 µl of either nanodroplet solution or eLiposome solution was pipetted in and thoroughly mixed. A NICOMP 370 Submicron Particle Analyzer (Entegris, Inc., Billerica, MA) was then used to measure the diameters of the nanoparticles in the borosilicate tube.

End Matter

Author Contributions and Notes

H.C.S., J.A.H., and A.L.K. designed research, H.C.S. performed research, H.C.S. analyzed data, and H.C.S. wrote the paper.

The authors declare no conflict of interest.

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Supplementary Materials

Table 1. Outline of Design Constraints, Justifications, Success, and Criteria

Design Constraint	Justification	Achieved?	How?
Significantly different calcein release rates between control liposomes and eLiposomes when sonicated with ultrasound.	If I cannot get this to work, the entire point of running other tests under the pretense that I have an ultrasound-triggerable drug delivery vehicle is impossible.	Yes	Significant (P = 0.000675) difference between eLiposome and control liposome calcein after treating with ultrasound for 90 seconds.
Thermal stability of eLiposomes evaluated by looking at release rate of eLiposomes after 2 hours at 37 °C. Ideally < 10%, but willing to accept up until 40%.	The drug delivery vehicle needs to be stable in the body for a few half-lives to avoid releasing the drug systemically.	Good Enough	Technically 41.27% of calcein was released into the supernatant at the end of two hours. However, at t = 0, 22.85% of calcein was already in the supernatant, so net loss was only 18.42% over two hours.
Blood clot mass reduction using eLiposome > 90% after 30 min of treatment, willing to accept >60%.	Desire to prove novelty and superiority over previous methods that claimed 70% clearance in 30 minutes.[1]	No	Did not test sonothrombolysis; ran out of time.
1 MHz frequency	Low enough to be clinically useful as therapeutic ultrasound, but high enough that we can also use MPa-scale PNP and not worry about MI.	Yes	Due to availability and convenience in the lab, we used a handheld 1 MHz US transducer (Birtcher Megason VI Model 106-5).
1 MPa PNP	High enough to be capable of nanodroplet vaporization, but not high enough to worry about MI.	Good Enough	0.6 W/cm^2 intensity setting on the Megason VI most closely equaled 1 MPa, ~= 1.34 MPa
7.5% duty cycle	Moderate DC, not at great risk of heating but not incapable of cavitating.	No	Due to availability and convenience in the lab, we used a continuous wave US transducer (Birtcher Megason VI Model 106-5).
Reasonable encapsulation efficiency, ideally > 5%, but willing to accept 0.1%.	Eventually we will need to encapsulate large, expensive drug particles, and so the more we're able to keep the better.	Yes	Was able to encapsulate 1.5% of the starting amount of calcein.
Good ultrasound release, ideally > 90% after 30 min, but willing to accept 50%.	Once we have a delivery vehicle bound to the clot, we want to make sure that as much of the drug gets released from its compartment.	Yes	77.8% release of calcein after 90 seconds of sonication.
Monodisperse, evaluated by having a polydispersity index of < 0.3.	We want to have a uniform product that also acts uniformly on its target. This is easiest if it is always a specific size.	Yes	PDI = 0.24.
Small diameter, ideally exactly 400 nm, but willing to go up to 800 nm.	Small (< 400 nm) diameter may have allowed for clot penetration by delivery vehicle itself, allowing for deeper release of rtPA.[3] Also a closer ratio between nanodroplet diameter and liposome diameter should increase odds of lysis after vaporization.	Good enough	Mean diameter = 821 nm. Close enough.