Cytosolic delivery via escape from the endosome using emulsion droplets and ultrasound

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Abstract
Vaporizing emulsion droplets may aid in endosomal rupture as a drug delivery route to the cytosol. Upon insonation, emulsion droplets formed from perfluorocarbon liquids may vaporize with sufficient expansion to disrupt liposomal or endosomal membranes. Emulsion droplets of perfluorohexane (PFC6) or perfluoropentane (PFC5) were prepared as free droplets in calcein or as droplets encapsulated within liposomes containing calcein. Folate-stimulated endocytosis created an experimental model, wherein calcein was self-quenched until released from the vesicles. Upon release, calcein was diluted below its self-quenching concentration and its release quantified by fluorescence. In this experimental model, folated emulsions or folated eLiposomes were incubated with folate-starved HeLa cells. Samples were exposed to two seconds of 20-kHz ultrasound (US) at 1 W/cm². Fluorescence microscopy identified released intracellular calcein. Upon insonation, both free emulsion samples and eLiposome samples produced calcein release to the cytosol. Calcein fluorescence was more intense in samples containing PFC5 compared to PFC6. Insonation of samples without emulsion droplets produced no cytosolic delivery. Likewise, cells that took up emulsion droplets but were not exposed to US did not exhibit fluorescence throughout the cell. These results suggest that vaporizing emulsion droplets are internalized into the cells and can produce endosomal escape of a therapeutic payload.

Keywords
Cytosol, drug delivery, eLiposome, liquid emulsion nanodroplet, perfluorocarbon emulsion, ultrasound

Introduction
Targeted drug delivery is currently an area of intense research attention. The ability to direct therapeutics to a specific site offers intriguing opportunities to localize a drug’s therapeutic effects while eliminating unwanted side effects. Other benefits of targeted drug delivery may include increased drug concentration at the target site and/or a reduction in the total amount of drug administered in order to achieve a therapeutic concentration at the desired site. Strategies have been investigated to deliver drugs not only to targeted tissues, but also within specifically targeted cells [1–3].

To be effective, therapeutics that require intracellular delivery – including genetic treatments and many drug therapies – may enter the cell directly through the cell’s plasma membrane or by endocytosis; in the latter case, the therapeutics must escape from the endosome before they are degraded [4–6]. Controlled escape from the endosome is one of the challenges to effective intracellular delivery. Several techniques have been investigated to accomplish controlled and efficient endosomal escape. So-called “passive” techniques include pH buffering, pore formation in the endosomal membrane, fusion with the endosomal membrane and photochemical disruption of the endosomal membrane [7–12]. Although these endosomal escape techniques have shown some promise, they present a number of disadvantages. For example, many of these techniques involve polymeric or cationic materials that are toxic, and most of these methods lack the ability to specifically control the location and rate of release [13,14].

Another potential strategy for drug targeting that may have applications for endosomal escape as well as targeting to a specific volume is “actuated targeting”. This strategy refers to techniques that employ an external trigger to actuate the delivery or the efficiency of treatments. For example, ultrasound (US) has been investigated over the past decade for its ability to activate drug carriers to release a sequestered drug load at a specific target site [15,16]. More recently, US has been used in combination with nano-sized emulsion droplets. Liquid emulsion droplets formed from a high-vapor-pressure liquid may be induced to vaporize when exposed to US [17–24], a phenomenon called acoustic droplet vaporization. In contrast to other ultrasonically responsive carriers (such as microbubbles), stable emulsion droplets may be formed at sizes that are small enough to pass into tissues and even into cells, where targeted drug delivery is most desired; it is possible that a nanodroplet may pass from capillaries into tissues and/or into cells and then be converted
into bubbles [20], whereas bubbles would be excluded from capillary escape and endocytosis due to their larger sizes. The ability of smaller particles to pass into tissues has been termed the enhanced permeability and retention (EPR) effect, and is a major motivation for designing drug-carrying particles between 50 and 300 nm in diameter [25,26]. Perfluorocarbon (PFC) emulsion droplets are an excellent candidate for ultrasonic targeting because they are easily formed within this size range.

For drug delivery, emulsion droplets may act as drug-carrying particles themselves or as part of another drug delivery system. For example, our research group has formed eLiposomes to take advantage of nanoemulsion droplet vaporization in a liposomal drug carrier [27–31]. eLiposomes are defined as liposomes with encapsulated emulsion droplets. These vesicles release more of their encapsulated contents at low (medically relevant) US intensities than do conventional liposomes. As the emulsion droplets vaporize and expand, the liposomal membrane is disrupted and the encapsulated contents are released [30].

Vaporizing emulsion droplets could potentially be used to aid in endosomal escape by disturbing the endosome as they expand; the expanding vapor phase may disrupt the endosomal membrane similarly to how it disrupts the liposomal membrane in eLiposomes. By comparison, microbubbles have been shown to increase the permeability of cell membranes when exposed to US but are too large to be internalized into most cells via endocytosis [32,33]. Although nanobubbles can be formed, their lifetime is only on the order of minutes [34]. Emulsion droplets could be used alone or administered in combination with other drug delivery vectors and techniques.

Figure 1 presents a schematic hypothesis of the use of emulsion droplets surrounded by a drug solution to deliver the drug solution to the cell cytosol. A key feature is that ligands that attach the droplet to the cell also induce endocytosis. Folate is a ligand that produces endocytosis when bound to the folate receptor of many cancer cells. Some drug solution will also be endocytosed. Then ultrasonication to produce acoustic droplet vaporization would disrupt the endosome, releasing the drug to the cytosol. The purpose of this study was to test this hypothesis and examine the ability of emulsion droplets and eLiposomes to be incorporated into cells via endocytosis and then to enhance delivery to the cytosol by ultrasonic exposure. The fluorescent molecule calcein was used as a model drug.

**Materials and methods**

**Materials**

Perfluoropentane (PFC5) was purchased from SynQuest Labs, Inc. (Alachua, FL), and perfluorohexane (PFC6) was purchased from Sigma-Aldrich (St. Louis, MO). Dipalmitoylphosphatidylcholine (DPPC) in chloroform and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG2000-amine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Folic acid and N,N’-dicyclohexylcarbodiimide (DCC) were purchased from Sigma-Aldrich. Phosphate buffered saline (PBS) solution was purchased from Fisher Scientific (Fair Lawn, NJ). Sucrose was purchased from Avantor Performance Materials (Phillipsburg, NJ) and glucose form United Biochemical Corp. (Cleveland, OH). Sodium chloride and dimethyl sulfoxide (DMSO) were purchased from Mallinckrodt (Paris, Kentucky).

Gibco DMEM media, RPMI-1640 folate-free media and fetal bovine serum (FBS) were purchased from Life Technologies (Grand Island, NY). Trypsin for cell passaging was purchased from In Vitrogen (San Diego, CA). LysoTracker Red was purchased from Molecular Probes (Eugene, OR). Calcein was purchased from MP Biomedicals (Solon, OH).

Calcein was dissolved in water by slowly adding NaOH to raise the pH until the color of the solution changed from light milky orange to transparent deep red, indicating dissolution. Additional water was added to the solution to dilute the calcein concentration to 30 mM.

**Synthesis of DSPE-PEG2000-folate**

Folate was attached to DSPE-PEG2000-amine using a previously reported technique [35]. Briefly, a suspension of DSPE-PEG2000-amine, DCC and pyridine was added to a

![Figure 1](image-url)  
**Figure 1.** Schematic hypothesis of endosomal escape produced by acoustic droplet vaporization of an endocytosed perfluorocarbon (PFC) droplet. (A) PFC droplet (blue) with attached folate (F) binds to folate receptors (red) on a cell, inducing endocytosis. External drug solution (green) is also endocytosed. (B) The drug solution and folated PFC droplet is fully endocytosed. External drug solution may remain or be washed or diffused away. (C) Application of ultrasound causes an expansion of the liquid PFC to gas (light blue), which disrupts the endosome. (D) After ultrasonication, the PFC may return to liquid, but the endosome remains disrupted, releasing drug solution to the cell cytosol. Relative sizes are not to scale.
solution of folic acid in DMSO. After four hours in the dark under nitrogen, the pyridine was removed by evaporation and water was added. Unreacted folic acid, byproducts, DCC and DMSO were removed using a 3500 molecular weight cut-off dialysis bag. The DSPE-PEG2000-folate was then extracted by adding an equal volume of chloroform. A drop of hydrochloric acid was added to the aqueous phase to protonate the derivatized folic acid, making it more soluble in chloroform. During this extraction, the folate moved from the aqueous phase to the chloroform phase, as observed by the chloroform becoming yellow in color. Chloroform was evaporated to isolate the folate-conjugated lipid. Attachment of the folic acid to DSPE-PEG2000-amine was confirmed by NMR [36] (see supplementary material, Figure S1).

Formation of emulsions

PFC5 and PFC6 emulsion samples were prepared by adding 0.2 g of PFC to an optical cuvette along with 1.5 mL of water and 167 μL of hydrated DPPC suspension (30 mg/mL). The sample was chilled on ice and the 20-kHz US probe with a 3-mm tip was inserted directly into the cuvette. The sample was chilled on ice and the 20-kHz US probe with 167 μL of hydrated DPPC suspension (30 mg/mL). The sample was sonicated at 1 W/cm² while still on ice for five minutes.

If necessary, the solution was allowed to cool and was sonicated again. The resulting liposomes ranged in size from 30 to 80 nm in diameter as measured by DLS.

Interdigitated DPPC sheets were formed by adding ethanol dropwise to DPPC SUVs while stirring to a total concentration of 3 M [37]. As the ethanol was added, the solution changed from translucent pale blue to opaque white and showed an increase in viscosity as the SUVs opened into lipid sheets [37,38]. The sheets were suspended and diluted in 50 mL of water at room temperature and centrifuged at 1800 × g for three minutes, resulting in a large pellet of DPPC sheets. The alcohol-rich supernatant was removed, and the pellet was resuspended in water and centrifuged again to reduce the ethanol concentration to less than 10 mM. The pellet was again suspended in a small volume of water in order to transfer the sheets to microcentrifuge tubes, with 10 mg of DPPC allotted to each tube. The extra water was removed through one more cycle of centrifugation. If necessary, the interdigitated sheet phase was stored at 4 °C. Lipid sheets remained stable for several weeks if the temperature was maintained below the melting transition temperature (T_m) for DPPC (41 °C).

eLiposomes were formed by adding 0.2 mL of PFC emulsion to interdigitated sheets that were formed in a 30-mM calcein solution. This solution was re-pipetted and briefly vortexed to ensure complete mixing of the sheets and the emulsion droplets. The solution was then heated to 50 °C and stirred with a magnetic stir bar for 30 min, allowing the sheets to fold back into vesicles, trapping some nanoemulsion droplets inside [29]. The trapped internal concentration of calcein was assumed to be the same as the external concentration. The resulting eLiposomes were reduced in size by extrusion through a 200-nm polycarbonate filter at 50 °C using the Avanti Mini Extruder.

Empty eLiposomes, defined as liposomes refolded from interdigitated sheets without emulsion present, were prepared as a negative control. In order to avoid any unexpected difference in the liposomal membranes, the same sheet-refolding method used to form the eLiposomes was employed, but the emulsion was replaced with calcein or NaCl solution, creating liposomes without emulsions, which are hypothesized to not be ultrasonically sensitive. The size distribution was about the same as the eLiposomes.

Folate was added to some of these vesicles as follows. First micelles were prepared by dissolving 2–6 mg of DSPE-PEG2000-folate in 0.6 mL of DMSO, followed by the addition of 5.4 mL of water. The resulting solution was dialyzed against pure water using a 3500 molecular weight cut-off dialysis bag to remove the DMSO. The water was replaced after four hours; then dialysis was continued overnight. These micelles were added to a previously prepared sample of eLiposomes or control liposomes (empty eLiposomes) containing approximately 10 mg of lipid in a 1.5 mL Eppendorf (Hauppauge, NY) tube to produce a concentration of approximately 1.2 mol% DSPE-PEG2000-folate based on total lipids. Concentrations and sizes of liposome and eLiposome samples were described above and were prepared immediately prior to incubation with DSPE-PEG2000-folate. The micelles were allowed to incubate with the eLiposomes for at least one hour to allow lipid transfer from the micelles to the bilayer [36,39,40].
Purification by density separation

eLiposome and control liposome samples were purified by centrifugation on a “density cushion” to remove unencapsulated emulsions and additional external calcein from the eLiposomes [28]. Sucrose, glucose and NaCl solutions were prepared to match the internal osmolarity of the eLiposomes. The raw eLiposome formulation (containing some unencapsulated emulsion droplets) was placed at the bottom of a 1.5 mL microcentrifuge tube. Approximately 0.4 mL of the NaCl solution was added to the microcentrifuge tube. Then, using a glass Pasteur pipette, approximately 0.4 mL of the glucose solution was gently pipetted to the bottom of the microcentrifuge tube, underneath the NaCl layer. As the glucose layer was added, the salt layer was forced up, creating two distinct phases of varying densities. Finally, the sucrose solution was pipetted to the bottom of the tube, pushing up the first two layers. The resulting tube had three distinct phases of varying densities: the sucrose had a density of 1.12 g/cm³, the glucose layer had a density of 1.07 g/cm³ and the NaCl solution had a density of 1.02 g/cm³. The sample was centrifuged for 10 min at 3000 rpm (504 x g) using a fixed rotor centrifuge (Eppendorf 5415 C). Upon centrifugation, free emulsion droplets collected at the bottom of the sucrose layer due to their relatively high density (1.67 g/cm² for PFC6 and 1.63 g/cm² for PFC5). The eLiposomes, with an estimated average density of approximately 1.08 g/cm³, gathered at the interphase between the sucrose and glucose layers. Average density for the eLiposomes was calculated by averaging the volume/mass of emulsion droplets and the water within the eLiposome membrane. The densities of the layers were chosen to catch eLiposomes with one or several emulsion droplets at the desired interphase. The interphase layer was then carefully removed from the other layers with a pipette. Similarly, control liposomes (without emulsion droplets) were collected at the glucose–NaCl interphase.

Cell culturing and US exposure

HeLa (CCL-2\textsuperscript{2M}) cells were grown at 37°C in 12-well plates in DMEM (Sigma-Aldrich) media supplemented with 10% FBS. Trypsin was used to passage and split the cells; the cells used in experiments were passaged less than 20 times. To encourage endocytosis, cells were grown in folate-free media for the final 48 h prior to the addition of eLiposomes or free emulsions. For free emulsion samples, the emulsion was mixed 1:1 with a 30 mM calcein solution; 200 μL of the resulting solution was added to HeLa cells that had been grown in 1.3 mL of folate-free media, resulting in an approximate calcein concentration of 4 mM around the cells. This concentration of calcein is sufficiently high to be in the self-quenching range. eLiposomes and liposomes were prepared with an internal calcein concentration of 15 mM, and most of the external calcein was removed in the centrifugation and washing steps.

The emulsion or liposome sample (200 μL) was added to each well and allowed to incubate at 37°C for two hours before application of US. Prior to insonation, the cells were washed three times with PBS to remove all suspended emulsions or eLiposomes and any external calcein. After adding 3 mL of media to the wells, US was applied to the cells by inserting the 3-mm diameter 20-kHz ultrasonic probe directly into the culture wells with the tip positioned about 2 cm above the cell monolayer. US was applied at 1 W/cm² for two seconds at room temperature. After insonation, the media was removed from all of the wells; 200 μL of fresh media or PBS was added to the wells, and cells were removed from the surface with a cell scraper. Cells were stored on ice until viewed with the confocal microscope. Fluorescence intensity in the cells was measured using ImageJ software (NIH, Bethesda, MD).

Calcein encapsulation and detection with fluorescence microscopy

Calcein was chosen to test the ability of emulsions and eLiposomes to aid in intracellular drug delivery because it does not readily diffuse through membranes and it self-quenches at high concentrations. For example, the calcein is totally self-quenched above approximately 0.5 mM. eLiposomes contained encapsulated calcein at approximately 15 mM so that they would not create a significant fluorescent signal while intact. As calcein was released from the vesicles and diluted below its self-quenching concentration, its fluorescence would be easily detected with fluorescence or confocal microscopy. Similarly, emulsion-only samples were formed in concentrated calcein so that any endosomes that formed upon endocytosis would contain calcein at a self-quenching concentration (approximately 4 mM). Therefore, the endosomes were not anticipated to significantly add to the fluorescence of cells unless the calcein was able to escape from the endosome and was diluted below its self-quenching concentration.

Intracellular delivery of calcein into HeLa cells was verified using an Olympus FluoView FV1000 confocal laser scanning microscope (Center Valley, PA). An argon laser with an excitation wavelength of 488 was used to visualize dilute calcein within the cells. The average concentration was assumed proportional to the integrated intensity within the cell boundary, which was calculated with ImageJ software. A red helium-neon laser was used to view LysoTracker Red dye within the cells.

Results

Emulsions for endosomal escape

Folated emulsion droplets suspended in concentrated calcein were incubated with folate-starved cells for two hours to allow internalization. As the folated emulsion droplets were endocytosed, it was anticipated that the newly formed endosomes would also contain calcein at a self-quenching concentration. The calcein would not create a strong fluorescent signal unless it was released from the endosomes and diluted into the cytosol. After two hours, the cells were thoroughly washed, and some samples were exposed to 20-kHz US for two seconds at 1 W/cm². The cells were then removed from the well and viewed microscopically. Control experiments were performed by repeating the procedure in the absence of emulsion droplets; a solution of calcein and DSPE-PEG2000-folate at similar concentrations to the active experiments was added to the cells, and US was applied to some wells.

Figure 2 shows cells that had been incubated with folated PFC5 emulsion droplets suspended in concentrated calcein.
After incubation, the external calcein was washed away, and any endocytosed calcein was still at a high concentration. When the cells were not exposed to US (Figure 2A and B), almost no green could be detected throughout the cells, indicating that internalized calcein remained quenched and not detectable at the microscope parameters that were used. When the cells were exposed to US (Figure 2C and D), bright green color was observed throughout almost all of the cells (with the exception of the nucleus). These data suggest that concentrated calcein had been endocytosed along with folated emulsions into endosomes within the cells at a quenched concentration. With the application of US, the emulsion-containing endosomes were disrupted and the encapsulated calcein was released into the cytosol and diffused through most of the cell.

Confocal slices of many cells were viewed in order to verify that the green fluorescence could not be attributed to calcein on the surface of the cells. The resulting images verified that the observed calcein was on the interior of the cells.

Figure 3 shows the results of similar experiments in which cells were exposed to folated PFC6 emulsions in concentrated calcein. Once again, there was a large increase in green fluorescence after exposure to US. However, the average fluorescence intensity per cell was significantly less ($p < 0.01$, $n > 15$, t-test) than in cells that had been exposed to PFC5 emulsions. This suggests that the vaporization of PFC6 emulsions was less efficient in releasing calcein from the endosome. This is most likely because of the lower vapor pressure of PFC6, fewer droplets may have vaporized, or less gas formed and the subsequent cavitation events may have been less violent.

Figure 4 shows a typical result of control experiments done without emulsion droplets. Cells were exposed to the same concentrations of calcein and DSPE-PEG2000-folate as in active experiments, but without the presence of emulsion droplets. The cells in Figure 4(C and D) were exposed to 20-kHz US for two seconds at 1 W/cm$^2$. A barely detectable amount of fluorescence can be observed in some cells. The amount of green fluorescence is comparable to samples that had included emulsion droplets but not been exposed to US (Figures 2A and 3A) and is much less than the amount of diluted calcein observed throughout the cells when emulsion droplets were present during insonation of the cells. The same optical parameters for the confocal microscopy were used in these micrographs (Figures 2–4) to allow comparison. The baseline fluorescence could be due to some calcein passing through the plasma membrane during incubation in concentrated calcein or due to a small amount of calcein escaping through the endosomal membrane after internalization.

Negative control samples without any calcein were also viewed that had not been exposed to US, and no green was detected at the same microscope parameters that were used for samples with calcein (data not shown), indicating that the slight amount of green detected in Figures 4(A and C), 2(A) and 3(A) is indeed from calcein and not from cell auto fluorescence.
Figure 3. Confocal and light micrographs of HeLa cells exposed to a suspension of folated PFC6 emulsion droplets and concentrated calcein for 2 h. Then cells were washed to remove external calcein and droplets. When cells were not exposed to ultrasound (A and B), the quenched calcein was not released into the cytosol. Calcein could be observed through the cells that were insonated at 20 kHz for two seconds at 1 W/cm² (C and D). Panels A and C are fluorescent confocal images and panels B and D are light images.

Figure 4. Confocal and light micrographs of HeLa cells incubated with concentrated calcein and DSPE-PEG2000-folate but without emulsion droplets. Two hours later, the cells were washed to remove external calcein and folate. Cells in panels A and B were not exposed to ultrasound. Cells in panels C and D were exposed to 20-kHz ultrasound for two seconds at 1 W/cm². Without emulsion droplets present, there was not a significant difference in endosomal escape of the calcein when ultrasound was applied. Panels A and C are fluorescent confocal images and panels B and D are light images.
Vesicle uptake at different diameters

In order to test the ability of cells to endocytose vesicles the sizes of our eLiposomes, liposome vesicles (with emulsion droplets) of DPPC and DSPE-PEG200-folate were prepared containing 0.05 mM calcein at 800 nm and at 200 nm by the same method of film hydration and extrusion through membranes with 800 nm and 200 nm filter pores. At this lower calcein concentration, the calcein is not self-quenched, and the liposomes exhibit a green fluorescent signal when viewed via confocal microscopy.

Figure 5 shows that there was similar uptake of vesicles of both sizes as demonstrated by the green fluorescence with both 800-nm and 200-nm vesicles. It should also be noted that in these polydisperse samples, the 800-nm preparations also contain a small fraction of liposomes with smaller diameters, which may be internalized more readily than the larger vesicles with an 800-nm diameter. We did not measure internalization rates in this study, but other reports indicate that endocytosis of smaller particles is sometimes more rapid than larger particles [33]. While both sizes show the ability to endocytose in vitro, it is noteworthy that each size has advantages and disadvantages. The 200-nm vesicles must be used with small emulsion droplets that could lead to a lessened release of their payload when exposed to US because the smaller emulsion droplets have higher Laplace pressure (assuming the interfacial energy is equivalent) [30], but the smaller eLiposomes could penetrate into tissues beyond the capillaries via the EPR effect. In contrast, the 800-nm vesicles may contain larger emulsion droplets and thus be induced more readily to release more of their drug load, but may not be as useful in medical applications because of a reduced ability to extravasate into tissues.

Cells exposed to non-folated vesicles did not demonstrate detectable fluorescence (data not shown). Likewise, negative control cells (exposed to vesicles without calcein) did not show any green at the same microscope settings that were used for vesicles with calcein (data not shown).

In vitro US-induced delivery of calcein from eLiposomes to HeLa cells

In order to test the ability of eLiposomes to deliver a therapeutic to the cytosol of cells, 200-nm eLiposome samples were prepared containing concentrated (15 mM) calcein. This concentrated calcein does not fluoresce significantly while encapsulated inside of the vesicles. Conventional liposomes with encapsulated calcein at 15 mM were also prepared as a negative control. External calcein and the external emulsion droplets were removed by the “density cushion” technique; 200 μL of the various solutions were added to each well of folate-starved cells and allowed to incubate for two hours to allow the cells to endocytose the folated vesicles. After two hours of incubation, some cells in wells were exposed to 20-kHz US at 1 W/cm² for two seconds.

Figure 6 demonstrates the resulting calcein release to cells. PFC6 eLiposomes demonstrated the ability to sequester the majority of their calcein prior to insonation, followed by the release of calcein to the interior of the cells when exposed to...
US (Figure 6A–D). When US was not applied to the cells, there was no observable calcein in the cytosol. The self-quenched calcein that remained inside of the eLiposomes did not add significantly to the fluorescence of the cells. Similarly, PFC5 eLiposomes demonstrated the ability to deliver calcein to the interior of the cells when exposed to US and the ability to sequester the calcein when not exposed to US (Figure 6E–H). PFC5 eLiposomes were able to deliver significantly more calcein to the cytosol than PFC6 as evidenced by increased green fluorescence intensity in the cells as measured by ImageJ software ($p < 0.01, n > 15, t$-test). This could be due to an increased ability to vaporize and cause release from the eLiposomes or due to an increased ability to break out of the endosomes caused by more vaporization and subsequent gas bubble cavitation during the balance of the ultrasonic pulse. Control vesicles showed only a very slight ability to deliver the calcein to the interior of the cells with or without exposure to US (Figure 6I–L).

To further investigate the location of eLiposomes in cells before and after sonication, eLiposome samples were prepared with a non-quenching calcein concentration (0.05 mM). LysoTracker Red was added to cells and allowed to incubate for at least 30 min in order to allow the dye to penetrate the cells. LysoTracker dyes have no net charge at neutral pH and easily penetrate lipid membranes; however, when they diffuse into a compartment of lower pH, they become charged and cannot escape through the membrane. Various concentrations of LysoTracker Red were tested to try to improve contrast and locate the endosomes. The best concentration was determined to be 50 nM; higher and lower concentrations did reveal the location of the endosomes, but at lower concentrations, the contrast was poor, and at higher concentrations a low background of red fluorescence was observed throughout the cell. The two fluorescent molecules were imaged in series; LysoTracker dye was imaged using the helium-neon laser, and the calcein was imaged using the argon laser. Figure 7 provides an example of cells with both dyes. When US was not applied, the calcein tended to be localized in bright bunches and spots that were co-localized with the LysoTracker Red, suggesting that the eLiposomes were located in endosomes and/or other acidic vesicles (Figure 7A). After US exposure, the green calcein was spread throughout the cells. The locations of the calcein and the LysoTracker (green and red, respectively) were no longer co-localized. These results provide further evidence suggesting that eLiposomes and some external solution is internalized by the cells into endosomes prior to US exposure. The green from the calcein can be observed throughout the cell before and after sonication, but in some cases, it is also concentrated to small internal vesicles. After sonication, these vesicles are no longer observed, suggesting that the vesicles have been ruptured and their contents allowed to diffuse throughout the cell. Furthermore, there was usually less red indicator of endosomes after insonation as the low pH vesicles were disturbed and/or ruptured.

**Discussion**

US has proven to be a potential tool for targeting drug delivery to a specific target site. The ability to focus US on a
target site, coupled with its non-invasive nature, is its key clinical advantage. US-mediated drug delivery systems have the advantage of allowing control of both the location and timing of drug delivery. Previously, US-mediated drug delivery has been connected with the cavitation of bubbles, and microbubbles have received significant attention as drug delivery particles and/or as cavitation nuclei [16,41,42]. Unfortunately, it is very difficult to form stable microbubbles sufficiently small enough and stable enough to pass from capillaries into tissues, where drug delivery would be most advantageous. Furthermore, nanobubbles have such a short lifetime [34] that only a small fraction may be extravasated before they are cleared by other means or they dissolve.

Both eLiposomes and emulsion droplets have advantages over other potential drug carriers due to their ability to extravasate into tissues, followed by US-mediated triggering of drug release. The eLiposomes can carry therapeutics in their aqueous compartment as has been carried previously [27,28,30,31]. A PFC emulsion droplet could have hydrophobic therapeutics dissolved in its volume or hydrophilic ones attached to its surface. Another strategy in using emulsions follows the experimental study of this work. There could be drugs, therapeutic proteins or stabilized nucleic acid particles surrounding the cell that cannot penetrate the cell membrane due to their charge or size. However, they could be endocytosed along with the actively targeted nanodroplets and then escape from the endosome to the cytosol as insonation expands the droplets to gas and ruptures the endosome. We have previously shown that naked PFC5 emulsion droplets are easily expanded to gas by US [30]. The gas bubble is at least five times larger than the liquid droplet, which is apparently sufficient to disrupt endosomes in this and previous research [28].

In an in vivo scenario, either construct (nanodroplet or eLiposome) could be used to treat the endothelial lining of the vascular system, but would need move beyond this barrier to interact with malignant tissues such as cancers. Fortunately, the small sizes of these constructs allow them to extravasate via the EPR effect into tumors with malformed vasculature. Many treatments, however, require the internalization into cells in order to have their effect. In fact, the perfect scenario may be one in which drug is only released inside a cell. Ideally, the drug carriers coupled with ultrasonic targeting could be used to not only penetrate tissues but also to release drugs directly to the cell cytosol. Targeting ligands can be attached to drug carriers in order to enhance their uptake into certain cells. For example, in this study, folate was attached to drug carriers because cancer cells typically overexpress the receptors for folic acid, thus increasing uptake of the carriers into cancer cells relative to other cells. Drugs and drug carriers are typically internalized into the cells via endocytosis. Because many molecules (e.g. nucleic acids) are digested in the resulting endosome, endosomal escape is an area of intense research interest; in order to be effective, many therapeutics must be internalized into cells via endocytosis and then must escape the endosome before being digested. An intriguing application of emulsions or eLiposomes (containing emulsions) is the ability to disrupt endosomal membranes by application of low-intensity US. As the emulsion is vaporized and expands, this expanding vapor phase may not only rupture the eLiposomal membrane but may also disrupt the endosomal membrane and provide a route for the escape of drugs or genes from the endosome. We had previously shown that insonation of emulsions can lyse calcine-filled eLiposomes [30], but we had no previous direct evidence that acoustic vaporization of PFC emulsions could also rupture endosomes.

In this study, folated emulsion droplets were internalized into HeLa cells via endocytosis. Subsequently, emulsions formed from both PFC5 and PFC6 were able to induce escape of calcine from the endosome when US was applied, as evidenced by fluorescence produced throughout the cytosol by the dilution of self-quenched calcine that was endocytosed along with the nanodroplets. Furthermore, folate appears to be necessary for eventual distribution of calcine into the cytosol after insonation, and folate attachment to folate receptors is known to induce endocytosis [40,43,44]. The locations of folated eLiposomes were co-localized with the locations of

![Figure 7. Confocal and light micrographs of HeLa cells incubated with PFC5 eLiposomes and with LysoTracker Red dye. Cells were imaged by confocal microscopy before (A) and after (B) ultrasound exposure. Calcein was co-localized with the LysoTracker Red in cells that had not been exposed to ultrasound, indicating that eLiposomes were located in endosomes. After ultrasound exposure, the calcein had been released into the cytosol and spread throughout the cell.](image)
acidic vesicles as revealed by Lysotracker (Figure 7), again implicating ligand-induced endocytosis as a mechanism. After insonation, the calcein had not only been released from the eLiposomes but had also escaped from the endosome (Figure 7B). As observed in other experiments, after insonation, the reduction in the red color of the Lysotracker marking the location of endosomes may also indicate that the integrity of the endosome had been compromised sufficiently by the two seconds of insonation that they could not maintain a low pH.

The intensity of calcein fluorescence was greater in cells that had been incubated with PFC5 emulsions compared to those incubated with PFC6 emulsions. The difference in calcein release can likely be explained by the difference in vapor pressure of the two liquids – PFC5 has a vapor pressure of 83 kPa, and PFC6 has a vapor pressure of 28 kPa at 24°C, the temperature of these experiments. Emulsion droplets that are formed from a liquid with a higher vapor pressure are vaporized at lower US intensities and likely experience a more complete phase change [45]. Due to their higher vapor pressure, it is likely that PFC5 emulsions form more persistent bubbles when exposed to US compared to PFC6. The expansion of these persistent PFC5 bubbles may be larger than that of PFC6, resulting in more cavitation and more release from the endosomes. While PFC5 may have the advantage of more completely releasing contents from the endosome, PFC6 may also be useful. The lower vapor pressure may be advantageous due to increased stability of the droplets, especially at biological temperature; at 37°C, the phase change of PFC5 may be spontaneous and irreversible, whereas the phase change of PFC6 may be more easily controlled and may also be reversible.

Published models of acoustic droplet vaporization of PFC5 show that insonation at 20 kHz creates very large bubbles, with a size of several microns, compared to insonation at 1 MHz, which produces sub-micron-sized bubbles [45]. For this study, US parameters and PFC liquids were used that were expected to cause this large bubble expansion in order to test the ability of expanding emulsions to aid in endosomal release. Both the US parameters and materials may require further tuning in order to be appropriate for a clinical setting. Namely, the ultrasonic frequency used in clinical settings is typically on the MHz scale, and materials may be identified that can apply the principles discussed herein to biological temperatures.

In this study, when PFC6 was used, a significant amount of calcein seemed to remain at a quenched concentration, perhaps remaining encapsulated in eLiposomes or in endosomes. It is unclear whether some of the calcein escaped from all of the endosomes or if only some of the endosomes released all of their sequestered calcein while others remained intact. Either or both scenarios are possible. The expanding vapor phase may only temporarily disrupt the endosomal membrane, allowing only some of the sequestered load to leak out before breaks in the membrane are re-sealed. At the other extreme, some endosomes may be completely destroyed by droplet vaporization and subsequent cavitation, while others remain essentially undisturbed, perhaps due to the lack of nucleation.

Our original motivation for this research was to support our hypothesis that cytosolic delivery by eLiposomes occurred as acoustic droplet vaporization disrupted both the liposome and the endosome. In the course of the research, we developed a new drug delivery technique using naked emulsion droplets. The therapeutic need not be encapsulated inside an eLiposome. Soluble drugs, external to the cell, that may have very little penetration of the cell membrane, could be taken into the endosome along with ligand-targeted nanodroplets of PFC. A quick application of US, before the therapeutic can be degraded in the endosome or lysosomes, could provide delivery directly to the cytosol. Previous studies with similarly sized expanding emulsion droplets inside the endosomes of cells have not killed the cells; in fact, the cells were able to transcribe the delivered plasmids and produce a marker protein [28].

This technique may have application in delivery of charged drugs or large therapeutic molecules, such as proteins or nucleic acids. The ability of vaporizing emulsion droplets to aid in endosomal escape was evident whether the droplets were administered alone or as part of an eLiposome solution.

**Conclusion**

This research demonstrates that endocytosed emulsion droplets can produce endosomal escape when exposed to US. The current hypothesis is that the low-pressure phase of the ultrasonic cycle causes the emulsion droplets to vaporize and disrupt the surrounding membranes as they expand, both liposomal and endosomal membranes. The ability to aid in endosomal escape was observed whether the droplets were administered alone or contained within DPPC liposomes. This US-induced drug delivery technique has a number of potential applications, including cancer treatments, gene delivery and perhaps protein delivery. The use of US as a drug delivery technique has the advantage of controlling the location and timing of drug delivery; therapeutics may be encapsulated and sequestered in drug carriers until they arrive at the location of ultrasonic exposure. However, for efficient cytosolic delivery, the emulsion droplets and therapeutics should be directed to the targeted cells using traditional targeting strategies prior to insonation. Both passive and active targeting strategies can be used to effect endocytosis before insonation is applied. While droplets formed from PFC5 appear to result in more intracellular delivery compared to those formed from PFC6, it is yet unclear which of these liquids is most advantageous for use in an efficient and safe drug delivery system. Due to the challenges associated with escaping the endocytic pathway, it is encouraging that acoustic droplet vaporization has the potential to be used as a triggering method of enabling endosomal escape.

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**Declaration of interest**

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Supplementary material available online

Supplementary Figure S1.

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