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Ultrasound-Mediated Release of Hydrophilic and Lipophilic Agents From Echogenic Liposomes

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Abstract

Objective—To achieve ultrasound-controlled drug delivery using echogenic liposomes (ELIPs), we assessed ultrasound-triggered release of hydrophilic and lipophilic agents in vitro using color Doppler ultrasound delivered with a clinical 6-MHz compact linear array transducer.

Methods—Calcein, a hydrophilic agent, and papaverine, a lipophilic agent, were each separately loaded into ELIPs. Calcein-loaded ELIP (C-ELIP) and papaverine-loaded ELIP (P-ELIP) solutions were circulated in a flow model and treated with 6-MHz color Doppler ultrasound or Triton X-100. Treatment with Triton X-100 was used to release the encapsulated calcein or papaverine content completely. The free calcein concentration in the solution was measured directly by spectrofluorimetry. The free papaverine in the solution was separated from liposome-bound papaverine by spin column filtration, and the resulting papaverine concentration was measured directly by absorbance spectrophotometry. Dynamic changes in echogenicity were assessed with low-output B-mode ultrasound (mechanical index, 0.04) as mean digital intensity.

Results—Color Doppler ultrasound caused calcein release from C-ELIPs compared with flow alone ($P < .05$) but did not induce papaverine release from P-ELIPs compared with flow alone ($P > .05$). Triton X-100 completely released liposome-associated calcein and papaverine. Initial echogenicity was higher for C-ELIPs than P-ELIPs. Color Doppler ultrasound and Triton X-100 treatments reduced echogenicity for both C-ELIPs and P-ELIPs ($P < .05$).

Conclusions—The differential efficiency of ultrasound-mediated pharmaceutical release from ELIPs for water- and lipid-soluble compounds suggests that water-soluble drugs are better candidates for the design and development of ELIP-based ultrasound-controlled drug delivery systems.

Keywords

calcein; Doppler ultrasound; drug release; echogenic liposomes; papaverine; ultrasound

The clinical need for organ- or tissue-specific drug delivery, also known as targeted drug delivery, arises when systemic delivery of a drug in sufficient doses to achieve a therapeutic

effect at the target site results in deleterious systemic effects. Relevant clinical problems include delivery of chemotherapeutic drugs to tumors, delivery of thrombolytic drugs to the cerebral or coronary circulation during ischemic stroke or myocardial infarction, and delivery of vasoactive drugs to the cerebral or coronary circulation for treatment of vasospasm. Current solutions to these problems involve invasive catheterization procedures directed at selective intra-arterial infusion of a drug into the target vascular bed. Such procedures often carry major risks. In many cases, therapeutic efficacy is lost shortly after removal of the indwelling catheter and cessation of drug infusion. Additional treatments require repetition of the invasive catheterization procedures with incremental accumulation of procedure-related risks. In most cases, prolonged maintenance of indwelling catheters for sustained or repetitive treatment is associated with prohibitive risk.

Over the last 4 decades, liposomes have been explored as targeted drug delivery vehicles.^{1,2} A number of clinical and experimental therapeutic agents have been successfully loaded into liposomes.³ Liposomes, which are formed by enclosure of an aqueous core by 1 or more phospholipid bilayers, range in size from about 100 nm to several micrometers. Liposomes offer several advantages as drug delivery vehicles. Water-soluble compounds may be entrapped in the aqueous core, whereas water-insoluble compounds may bind to the lipid membrane. A liposome-associated drug (either bound to the lipid or entrapped in the aqueous core) is hypothesized to remain physiologically inactive until the physical integrity of the lipid membrane is disturbed. Drug-loaded liposomes propagated in the circulation can function as mobile drug reservoirs with the ability to deliver large pay-loads of a drug to specific target organs or tissues when appropriately triggered. Several chemical and physical methods for triggered drug release from liposomes have been explored, including acoustic pressure-, temperature-, and pH-dependent mechanisms.^{4–6}

One promising approach that is being explored for drug delivery is the use of ultrasound-activated echogenic liposomes (ELIPs). These agents are synthesized by incorporating air and a drug of interest into liposomes that are submicrometer in size (mean diameter, 780 nm).⁷ These encapsulated air pockets are probably on the order of tens or hundreds of nanometers in diameter and thus can be considered nanobubbles. Note that the perfluorocarbon gas pockets in many contrast agents used clinically today are on the order of several micrometers and thus encapsulate microbubbles, not nanobubbles.^{8,9} Exposure of ELIPs to suitable pulses of ultrasound disturbs the physical structure of the liposome and results in drug release. Because ELIP activation by ultrasound can be controlled both spatially and temporally, ELIPs are potentially powerful tools for selective tissue- or organ-specific drug delivery. Furthermore, the encapsulated nanobubbles can serve as ultrasonic contrast agents, enabling the process of drug release and activation to be imaged in real time.

The pulse repetition frequency and duty cycle dependence of the acoustic pressure destruction threshold for ELIPs has been characterized with 6-MHz pulsed Doppler ultrasound.¹⁰ Continuous wave ultrasound (2–7 W/cm²) has been investigated as a means of triggering calcein or recombinant tissue plasminogen activator (rt-PA) release from ELIP in nonflowing solutions.^{4,11,12} Recently, pulsed color Doppler ultrasound was used to trigger release of rt-PA from rt-PA-loaded ELIPs in flowing solutions.¹³ The use of color Doppler ultrasound (a scanned mode) enables a larger number of ELIPs to be exposed per unit of time than spectral Doppler ultrasound (an unscanned mode). This is important in clinical drug delivery paradigms because ELIPs circulating in the bloodstream will move rapidly through the ultrasound field. Because in vitro evaluation of ultrasound-triggered drug release must consider this aspect of clinical drug delivery, our experimental design used a flow model with a peristaltic pump to circulate ELIP solutions through an ultrasound exposure volume.

Calcein, a water-soluble polyanionic fluorescein derivative with photosensitizing properties, is used as a fluorescent indicator in drug release studies as well as a contrast agent in retinal angiography.^{14,15} Papaverine, a spasmolytic lipophilic opium alkaloid, is administered into cerebral arteries through a microcatheter for clinical treatment of posthemorrhagic cerebral vasospasm.^{16,17} In this study, calcein-loaded ELIPs (C-ELIPs) and papaverine-loaded ELIPs (P-ELIPs) were circulated in a flow model and exposed to pulsed color Doppler ultrasound to trigger calcein or papaverine release from C-ELIPs or P-ELIPs. The objective of this study was to quantify the release of calcein and papaverine from C-ELIPs and P-ELIPs, respectively. Dynamic changes in echogenicity were also assessed with B-mode ultrasound before, during, and after color Doppler ultrasound exposure.

Materials and Methods

Solutions of C-ELIP and P-ELIP in 0.5% bovine serum albumin (Sigma-Aldrich Co, St Louis, MO) were circulated through a latex flow model and imaged with a clinical duplex ultrasound scanner (HDI 5000; Philips Medical Systems, Bothell, WA) to assess echogenicity over 3 minutes. Circulating C-ELIP and P-ELIP solutions were either treated with duplex color Doppler ultrasound ($n = 5$) or 0.05% Triton X-100, a nonionic detergent (positive control; $n = 5$), or left untreated (control; $n = 5$). Treated and untreated solutions collected from the flow model were analyzed by spectrofluorimetry or absorbance spectrophotometry to quantify the amount of calcein or papaverine associated with the liposome and free in the solution. Quantitative differences in free calcein or papaverine determined before and after treatment were used to calculate calcein release from C-ELIPs and papaverine release from P-ELIPs.

Preparation of Echogenic Liposomes

Calcein- and papaverine-loaded ELIPs were prepared at the University of Texas Health Science Center as described previously and shipped to the University of Cincinnati in lyophilized powder form.^{4,18} Calcein-loaded ELIPs were composed of egg yolk phosphatidylcholine/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine/dipalmitoylphosphatidyl-glycero/cholesterol at a molar ratio of 69:8:8:15, and P-ELIPs were formulated from egg yolk phosphatidylcholine/1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine/cholesterol at a molar ratio of 69:8:15. The C-ELIPs and P-ELIPs were reconstituted at a concentration of 10 mg/mL in 0.2- μ m filtered, air-saturated, deionized water and diluted in 0.5% bovine serum albumin such that final concentrations of 3- μ g/mL calcein and 80- μ g/mL papaverine were achieved. These corresponded to estimated liposome number densities of 9.9×10^8 liposomes/mL for C-ELIPs and 1.32×10^8 liposomes/mL for P-ELIPs.⁹ A calcein concentration of 3 μ g/mL is easily detected by spectrofluorimetric methods, and a papaverine concentration of 80 μ g/mL is the estimated therapeutic intra-arterial concentration achieved during clinical transcatheter infusions. Clinically, a solution of papaverine is infused through a microcatheter directly into a cerebral artery. Assuming an average cerebral arterial flow rate of 120 mL/min and a microcatheter infusion rate of 3-mg/mL papaverine at 3.3 mL/min, the therapeutic intra-arterial concentration of papaverine is approximately 80 μ g/mL.¹⁹

Exposure Flow Model

Samples of reconstituted C-ELIP or P-ELIP solutions were pumped through 100 cm of latex tubing (1/8-in inner diameter, 3/16-in outer diameter, and 1/32-in wall; McMaster-Carr Supply Company, Atlanta, GA) using a peristaltic pump (Rabbit; Rainin Instrument, LLC, Oakland, CA) at 2 mL/min (Figure 1). The total volume of C-ELIPs or P-ELIPs in the flow model was 7 mL. A 10-cm segment of the tubing was submersed in a tank of deionized water, and the face of a Philips CL15-7 compact linear array ultrasound transducer was

immersed 1.5 cm above the model. Acoustic absorbing material (Precision Acoustics, Dorchester, England) was placed below the model to prevent reflection of ultrasound. After the solutions were circulated through the flow model for 3 minutes, 2-mL aliquots of C-ELIP or P-ELIP solutions were collected for analysis with a 3-way valve.

Treatment Methods

Five samples of C-ELIP or P-ELIP solutions each were treated with duplex color Doppler ultrasound for 1 second while circulating in the flow model with a calibrated CL15-7 compact linear array transducer (center frequency, 6 MHz; peak rarefactional pressure, 2 MPa; in situ mechanical index [MI], 0.8; and pulse repetition frequency, 150 Hz) with a 3 × 12-mm color window placed within the lumen of the flow model (Figure 1). The in situ rarefactional pressure within the latex tubing was measured with a 0.2-mm-diameter needle hydrophone (Precision Acoustics) mounted on a 3-dimensional motor-driven positioning system (Velmex, Inc, Bloomfield, NY). An on-screen MI output setting of 1.3 at a depth of 1.5 cm yielded an in situ peak rarefactional pressure of 2 MPa within the latex tubing (in situ MI, 0.8). Five different samples of C-ELIP or P-ELIP solutions were exposed to 0.05% vol/vol Triton X-100 (Sigma-Aldrich Co) before introduction into the flow model, which solubilized the lipid and released the drug, serving as a positive control.^{4,12} In addition, 5 C-ELIP and P-ELIP solutions each were circulated through the flow model to measure echogenicity but were left untreated. This last sham treatment group was used to assess inadvertent drug release caused by the flow model independent of color Doppler ultrasound exposure.

Quantitative Spectrophotometric Techniques

The calcein concentration in the solution was quantified by spectrofluorimetry using an RF-5301PC spectrofluorimeter (Shimadzu Corporation, Kyoto, Japan) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The papaverine concentration was quantified by absorbance spectrophotometry using a UV-1700 spectrophotometer (Shimadzu Corporation) with a wavelength of 326 nm. Papaverine fluorescence was determined on the spectrofluorimeter with an excitation wavelength of 326 nm and an emission wavelength of 366 nm. The excitation wavelengths were chosen at the peak absorbance in the spectra for calcein and papaverine.

Fluorescence intensity is shown as a function of concentration for calcein and papaverine (Figure 2, A and B). The fluorescence of each compound increases with the concentration until self-quenching occurs, causing the amount of fluorescence to decrease at higher concentrations. The self-quenching effect appears as a decrease in fluorescence at concentrations of greater than 6 µg/mL for calcein and greater than 20 µg/mL for papaverine. The effect of the papaverine concentration on absorbance is shown in Figure 2C. It should be noted that the papaverine absorbance varied linearly with the concentration.

The fluorescence of sample C-ELIP solutions was measured immediately after liposome reconstitution and after treatment (color Doppler ultrasound, Triton X-100, or sham). Additionally, 0.3-mL aliquots of C-ELIP solutions were removed after treatment (or sham treatment), combined with 20 µL of 10-mmol/L cobalt chloride to quench the calcein fluorescence in the free solution, and the fluorescence was measured. Similarly, the fluorescence and absorbance of sample P-ELIP solutions were measured immediately after liposome reconstitution and after treatment (color Doppler ultrasound, Triton X-100, or sham). After treatment or sham treatment, 0.3-mL aliquots of P-ELIP solutions were passed through a spin column to filter papaverine in the free solution, and the spectrophotometric absorbance and fluorescence were measured. Thereafter, each sample was diluted by a factor of 10, and the fluorescence measurement was repeated to determine whether self-

quenching effects were present and to quantify absolute calcein and papaverine concentrations.

Echogenicity Measurements

Each sample C-ELIP or P-ELIP solution was exposed to low-MI B-mode ultrasound (MI, 0.04) in the flow model to assess echogenicity over a 3-minute period. Identical gain settings and the same gray scale map were used for all ultrasound image acquisitions. Previous studies with ELIPs have shown that this B-mode exposure level is well below the ELIP acoustic pressure destruction threshold.²⁰ Echogenicity was assessed by acquiring still images at 30-second intervals of C-ELIPs or P-ELIPs circulating in the flow model and transferring the image data via a magneto-optical disk to a desktop computer (OptiPlex GX260; Dell, Round Rock, TX). Two 2×2 -mm regions of interest (ROIs) indicating echogenicity before and after treatment (Figure 3) were selected on each image, and the mean gray scale value was measured with ImageJ software (National Institutes of Health, Bethesda, MD). Mean gray scale value measurements were converted to mean digital intensity (MDI) with a previously described calibration procedure.²⁰

Spin Column Filtration Technique

Papaverine not associated with the liposomes in the free solution was filtered by a spin column technique. Sephadex G-50 medium (50–150 μ m; Sigma-Aldrich Co) was hydrated in 0.9% saline, loaded into 3-mL syringes stopped with a 0.25-mL plug of glass wool, and centrifuged at 1000g for 2.5 minutes. Spin columns were equilibrated with 0.9% saline and centrifuged again at 1000g for 2.5 minutes. Each P-ELIP sample was loaded onto a spin column and then centrifuged a third time at 1000g for 2.5 minutes, and the eluant was collected in a glass tube for spectrophotometric or spectrofluorimetric analysis.

Calculation of Calcein and Papaverine Release

The change in the free calcein concentration in the solution due to treatment, ΔCn , can be written as

$$\Delta Cn = |[Cn_{\text{after}}] - [Cn_{\text{before}}]|, \quad (1)$$

where $[Cn_{\text{after}}]$ is the calcein concentration after treatment (color Doppler ultrasound, Triton X-100, or sham), and $[Cn_{\text{before}}]$ is the calcein concentration before treatment. The change in the free papaverine concentration in the solution due to treatment, ΔPap , can be written as

$$\Delta Pap = |([Pap_{\text{after}}] - [Pap_{\text{SA}}]) - ([Pap_{\text{before}}] - [Pap_{\text{SB}}])|, \quad (2)$$

where $[Pap_{\text{after}}]$ is the total papaverine concentration after treatment (color Doppler ultrasound, Triton X-100, or sham); $[Pap_{\text{SA}}]$ is the papaverine concentration after treatment and after spin column filtration; $[Pap_{\text{before}}]$ is the total papaverine concentration before treatment; and $[Pap_{\text{SB}}]$ is the papaverine concentration before treatment and after spin column filtration. The free papaverine concentration in the solution was determined by subtracting the liposome-associated papaverine concentration from the total papaverine concentration. The liposome-associated papaverine concentration was determined by spectrophotometric analysis of spin column–filtered samples before and after treatment.

The percentage of encapsulated calcein released by color Doppler ultrasound treatment, Cn_{released} , was determined by subtracting the change in the calcein concentration after sham treatment, ΔCn_{sham} , from the change in the calcein concentration after ultrasound treatment, ΔCn_{US} , and normalizing by the change in the calcein concentration after Triton X-100 treatment, $\Delta Cn_{\text{TritonX}}$:

$$Cn_{\text{released}} = 100 * \frac{(\Delta Cn_{\text{US}} - \Delta Cn_{\text{sham}})}{\Delta Cn_{\text{TritonX}}}. \quad (3)$$

Similarly, the percentage of liposome-associated papaverine released by color Doppler ultrasound treatment, Pap_{released} , was determined by subtracting the change in the papaverine concentration after sham treatment, ΔPap_{sham} , from the change in the papaverine concentration after ultrasound treatment, ΔPap_{US} , and normalizing by the change in the papaverine concentration after Triton X-100 treatment, $\Delta Pap_{\text{TritonX}}$:

$$Pap_{\text{released}} = 100 * \frac{(\Delta Pap_{\text{US}} - \Delta Pap_{\text{sham}})}{\Delta Pap_{\text{TritonX}}}. \quad (4)$$

Equations 3 and 4 were used to determine the efficacy of ultrasound-triggered calcein and papaverine release from the ELIPs.

Statistical Analysis

Statistical analysis was performed by a 2-tailed Student *t* test with OpenEPI software (Emory University, Atlanta, GA). The calcein and papaverine concentrations after ultrasound or Triton X-100 treatment, determined spectrofluorimetrically and spectrophotometrically, were compared with those after sham treatment (flow alone). The MDI of the C-ELIP and P-ELIP solutions within the ROI after ultrasound or Triton X-100 treatment was also compared with that after sham treatment (flow alone). $P \leq .05$ indicated a significant difference between the treatments.

Results

Treatment-Induced Calcein Release

The concentration of calcein in the C-ELIP solution, determined from fluorescence measurements, is shown in Figure 4. The concentration of calcein in the solution increased by 0.97 ± 0.4 $\mu\text{g/mL}$ (mean $\pm$ SD) after color Doppler ultrasound treatment. Triton X-100 treatment increased the calcein concentration by 1.16 ± 0.2 $\mu\text{g/mL}$. Sham treatment with flow alone caused an increase in the calcein concentration of 0.41 ± 0.1 $\mu\text{g/mL}$. The difference in the calcein concentration after color Doppler ultrasound or Triton X-100 treatment was greater than that after sham treatment ($P = .02$ for ultrasound treatment; $P = .001$ for Triton X-100 treatment). Cobalt chloride completely quenched calcein fluorescence after each treatment or sham treatment, indicating that the encapsulated calcein, which was not exposed to cobalt chloride, was self-quenched. Exposing C-ELIPs to color Doppler ultrasound (on-screen MI, 1.3) released the encapsulated hydrophilic fluorescent indicator. The percentage of calcein released, Cn_{released} , was $47.5\% \pm 33\%$. The variability was due to a low sample size, but statistical analysis indicates that color Doppler ultrasound successfully released calcein from C-ELIPs ($P < .01$ compared with control).

Treatment-Induced Papaverine Release

The concentration of papaverine in the P-ELIP solution was determined from absorbance measurements and is shown in Figure 5. Color Doppler ultrasound treatment induced a decrease in papaverine concentration, ΔPap , of $10.5 \pm 6.4 \mu\text{g/mL}$, measured after spin column filtration. Triton X-100 treatment decreased the papaverine concentration by $16.4 \pm 5.2 \mu\text{g/mL}$ after spin column filtration. Sham treatment with flow alone caused a decrease in the papaverine concentration of $7.2 \pm 2.4 \mu\text{g/mL}$. The difference in the papaverine concentration after color Doppler ultrasound was not different from that after sham treatment with flow alone ($P = .33$). However, the difference in the papaverine concentration after Triton X-100 treatment was different from that after sham treatment with flow alone ($P = .01$). Exposing P-ELIPs to color Doppler ultrasound (on-screen MI, 1.3) did not result in a large amount of lipophilic papaverine release compared with sham treatment with circulation alone. The percentage of papaverine released, Pap_{released} , was $20.1\% \pm 42.4\%$. The variability was due to a low sample size, but statistical analysis indicated that color Doppler ultrasound did not trigger release of papaverine from P-ELIPs ($P = .33$ compared with control).

Echogenicity of C-ELIPs and P-ELIPs

Echogenicity, expressed as MDI, of the C-ELIP and P-ELIP solutions over time is plotted in Figures 6 and 7, respectively. The echogenicity of C-ELIP suspensions immediately after reconstitution was $10.2 \pm 1.5 \text{ dB}$ higher than that of P-ELIP suspensions, which reflects the difference in number density of the liposomes in the two solutions (9.9×10^8 liposomes/mL for C-ELIPs and 1.32×10^8 liposomes/mL for P-ELIPs) and potentially the difference in gas encapsulation efficiency on reconstitution of the two liposomal formulations. Color Doppler ultrasound and Triton X-100 treatments both resulted in a decrease in the C-ELIP echogenicity (12.3 dB) compared with circulation in the flow model alone after 3 minutes ($P = .006$). The decrease in the P-ELIP echogenicity was different after color Doppler ultrasound or Triton X-100 treatment compared with circulation alone ($P < .01$). Circulation alone decreased the C-ELIP echogenicity by $2.7 \pm 2.5 \text{ dB}$ and reduced the P-ELIP echogenicity by $0.4 \pm 2.2 \text{ dB}$ after 3 minutes of circulation in the flow model.

Discussion

We have shown the release of calcein, a hydrophilic agent, from ELIPs with color Doppler ultrasound. We also found that a significant release of papaverine, a lipophilic drug, from ELIPs was not achieved with ultrasound. Our discussion will focus on the differences observed between ultrasound-triggered release of a hydrophilic agent (calcein) and a lipophilic agent (papaverine) from ELIPs.

Ultrasound-Triggered Release of Calcein From C-ELIPs

In this study, pulsed color Doppler ultrasound released $47.5\% \pm 33.0\%$ of calcein encapsulated in ELIPs. A similar release of $47\% \pm 15\%$ of encapsulated calcein was previously achieved by Huang and MacDonald⁴ with continuous wave ultrasound. Although Lin and Thomas¹¹ achieved high-efficiency calcein release from C-ELIPs with low-frequency ultrasound (20 kHz), this frequency is not attractive for clinical applications because of bioeffects concerns. Moreover, the calcein release in this study was achieved as C-ELIPs circulated through the latex tubing, albeit at a low flow rate (2 mL/min), which in humans is only observed in small peripheral vessels such as the dorsalis pedis artery.²¹

The CL15-7 transducer, which was used in this study, enables higher acoustic outputs in the color Doppler mode than in the B-mode at an image depth of 1.5 cm. In addition, color Doppler is a scanned mode, so a large volume of ELIPs is exposed to the ultrasound field.

As a result, color Doppler pulses at an MI of 1.3 may be more effective at liberating pharmaceuticals from ELIPs than B-mode pulses. The rarefactional acoustic pressure used for our color Doppler treatment was 2.0 MPa in situ. At this level, extravasation of Evans blue dye into skeletal muscle, capillary hemorrhage, and cardiomyocyte injury bioeffects have been observed.^{22,23} Ultrasound-enhanced vascular permeability, although generally considered undesirable, can have a positive effect on delivering drugs across blood vessel walls, perhaps resulting in more effective transmural drug delivery.

Color Doppler ultrasound exposure resulted in an immediate and near complete loss of echogenicity, consistent with acoustic destruction and rapid fragmentation of liposomes. Ultrasound exposure of C-ELIPs induced a loss of echogenicity that was commensurate with calcein release, but ultrasound exposure of P-ELIPs did not release papaverine even though a loss of echogenicity was observed. Thus, echogenicity alone cannot be used to determine drug release.

Ultrasound-Triggered Release of Papaverine From P-ELIPs

Significant papaverine release from P-ELIPs with color Doppler ultrasound was not observed in this study using spectrophotometric analysis techniques. Although P-ELIP samples also showed an immediate and near complete loss of echogenicity on exposure to color Doppler ultrasound, there was not a commensurate release of the liposome-associated pharmaceutical as observed for calcein. In addition, the low association efficiency noted in P-ELIPs is possibly due to the limited volume within the lipid bilayer housing the papaverine as opposed to the larger volume within the aqueous core housing the calcein in C-ELIPs.

In our study, papaverine fluorescence was observed only when albumin was also present in the solution. Because papaverine is known to bind strongly to albumin, our results indicate that papaverine only fluoresces when it is bound to albumin.²⁴ Consequently, in our experimental model, papaverine bound to the lipid membrane would escape detection by fluorescence spectrophotometry. Because the ratio of albumin-bound papaverine to lipid-bound papaverine was unknown, fluorescence measurements were not useful as indicators of papaverine release. Consequently, absorbance spectrophotometry was used to analyze the ultrasound-triggered papaverine release.

Differential Behavior of Lipophilic and Hydrophilic Agents

The failure of Doppler ultrasound to release papaverine effectively from P-ELIPs markedly contrasted with the robust release of calcein from C-ELIPs by Doppler ultrasound under identical conditions. These differential release behaviors may be related to differences in chemical structure and water solubility. The results of our study suggest that water-soluble compounds might be efficiently released from ELIPs by ultrasound. Indeed, rt-PA release from ELIPs by ultrasound has been shown.^{12,13} Hydrophilic compounds such as calcein are likely to be trapped within the aqueous core of liposomes. When liposomal membranes are disrupted by ultrasound or by other means, pharmaceuticals contained in the water-soluble compartment are released into the aqueous extraliposomal space. In contrast, lipophilic compounds such as papaverine are likely to be strongly associated with the phospholipid membrane of liposomes. When liposomal membranes are disrupted, these agents may remain associated with phospholipids and may become rapidly incorporated into micelles or other macromolecular lipid complexes but nevertheless remain excluded from the aqueous space.

The amount of calcein or papaverine respectively released from untreated C-ELIPs or P-ELIPs circulating in our flow model was minimal. The amount of echogenicity lost by C-

ELIPs or P-ELIPs circulating in our flow model for 3 minutes was also very small. Further studies are needed to determine the effects of different flow models on ELIP echogenicity loss and the release of ELIP contents.

The number density of our experimental C-ELIP solutions was higher than that of our P-ELIP solutions (9.9×10^8 and 1.32×10^8 liposomes/mL, respectively). This difference could partially account for the higher echogenicity observed for C-ELIPs compared with P-ELIPs. The degree of ultrasound backscattering from liposomal solutions depends on the ELIP number density, which suggests that the C-ELIP samples had more encapsulated nanobubbles than the P-ELIP samples.⁹ The interaction of ultrasound with these bubbles is a requirement for the release of the contents. Thus, having a higher number of bubbles may lead to the potential for a more efficient release of drugs from ELIPs. Given the lipophilic nature of papaverine, differences in nanobubble contents between C-ELIPs and P-ELIPs are plausible and may be related to modification of liposomal membrane stability by papaverine-phospholipid interactions.

In our study, $20.1\% \pm 42.4\%$ of papaverine was released from P-ELIPs by pulsed ultrasound, or roughly 80% of papaverine in ultrasound-treated P-ELIPs remained associated with liposomes. Using our paradigm of ultrasound-triggered drug release, in which ultrasound is intended to liberate a sequestered drug payload into the circulation, this showed that a lack of drug release may be disadvantageous. However, it remains possible that ultrasound treatment could alter the bioavailability of the drug. The bioavailability of papaverine was not explored, and this was a limitation of our study. Recently, Tartis et al²⁵ examined the cellular effects of ultrasound-mediated delivery of paclitaxel, a lipophilic drug, from acoustically activated lipospheres in vitro but did not measure the release of paclitaxel directly. In addition, Chen et al^{26,27} showed that ultrasound can deliver water-soluble genes from liposomes into cardiac muscle and pancreatic islet cells in vivo.

Conclusions

In summary, color Doppler ultrasound-triggered release of calcein from circulating ELIPs has been shown with a clinical diagnostic ultrasound scanner. The robust release of a hydrophilic agent (calcein) markedly contrasts with the lack of release observed for a lipophilic pharmaceutical (papaverine). These results suggest that water-soluble drugs might be better candidates for the design and development of ELIP-based ultrasound-controlled drug delivery systems than lipophilic drugs. The differential release of hydrophilic versus lipophilic compounds from ELIP deserves future study.

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Abbreviations

C-ELIP	calcein-loaded echogenic liposome
ELIP	echogenic liposome
MDI	mean digital intensity
MI	mechanical index

P-ELIP	papaverine-loaded echogenic liposome
ROI	region of interest
rt-PA	recombinant tissue plasminogen activator

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Figure 1.

Schematic of the flow chamber. Samples of reconstituted C-ELIP and P-ELIP solutions were pumped through latex tubing with a peristaltic pump at 2 mL/min. A segment of the tubing was submerged in a tank of deionized water, and the face of a CL15-7 compact linear array ultrasound transducer was immersed 1.5 cm above the model. Acoustic absorber material was placed below the model to prevent reflection of ultrasound. After circulating through the flow model for 3 minutes, aliquots of C-ELIP or P-ELIP solutions were collected for analysis with a 3-way valve.

Figure 2.

A, The fluorescence of C-ELIPs increases with the concentration until self-quenching occurs, causing the amount of fluorescence to decrease at calcein concentrations of greater than 6 μg/mL. **B**, The fluorescence of P-ELIPs increases with the concentration until self-quenching is observed at papaverine concentrations of greater than 20 μg/mL. **C**, The absorbance of P-ELIPs varies linearly as a function of the papaverine concentration. A.U. indicates arbitrary units; and bars, SD.

Figure 3. Screen shot of P-ELIP acquired during color Doppler ultrasound treatment. The color Doppler window highlights the region exposed to color Doppler pulses. The red boxes were added to indicate ROIs for echogenicity analysis.

Figure 4.

Concentration of calcein in the C-ELIP solution determined from fluorescence measurements. An increase in the calcein concentration indicates calcein release from C-ELIPs. The concentration increased after color Doppler ultrasound treatment ($n = 5$; $P = .02$) and after Triton X-100 treatment ($n = 5$; $P = .001$) compared with sham treatment. Bars indicate SD.

Figure 5.

Concentration of papaverine determined from absorbance measurements. A decrease in the papaverine concentration after spin column filtration indicates papaverine release from P-ELIPs. After spin column filtration, the concentration decreased for the Triton X-100-treated P-ELIPs ($n = 5$; $P = .01$) compared with sham treatment. However, the decrease in the papaverine concentration after spin column filtration was not different for the ultrasound-treated P-ELIPs ($n = 5$; $P = .33$) compared with sham treatment. Bars indicate SD.

Figure 6.

Echogenicity of C-ELIPs, expressed as MDI, over time. The loss of echogenicity was higher for ultrasound- and Triton X-100-treated C-ELIPs ($n = 5$; $P < .01$). Circulation alone did not induce a loss of echogenicity in C-ELIPs ($n = 5$; $P > .05$). Bars indicate SD.

Figure 7.

Echogenicity of P-ELIPs, expressed as MDI, over time. The loss of echogenicity was higher for ultrasound- and Triton X-100-treated P-ELIPs ($n = 5$; $P < .05$). Circulation alone did not induce a loss of echogenicity in P-ELIPs ($n = 5$; $P > .05$). Bars indicate SD.