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MRI monitoring and quantification of ultrasound-mediated delivery of liposomes dually-labeled with gadolinium and fluorophore through the blood-brain barrier

Muna Aryal¹, Iason Papademetriou², Yong-Zhi Zhang¹, Chanikarn Power¹, Nathan McDannold¹, and Tyrone Porter^{2,3}

¹Department of Radiology, Brigham & Women's Hospital, Harvard Medical School, Boston, USA

²Department of Mechanical Engineering, Boston University

³Department of Biomedical Engineering, Boston University

Abstract

Magnetic Resonance Image-guided Focused Ultrasound (MRgFUS) has emerged as a viable noninvasive technique for the treatment of central nervous system related diseases/disorders. Application of mechanical and thermal effects associated with focused transcranial ultrasound have been studied extensively in preclinical models, which has paved the way for clinical trials. However, *in vivo* treatment evaluation techniques on drug delivery application via blood-brain barrier (BBB) opening have not been fully explored. Current treatment evaluation techniques via MR-imaging are hindered by systemic toxicity resulting from free gadolinium delivery. Here we propose a novel treatment evaluation strategy to overcome limitations by (i) synthesizing liposomes that are dually-labeled with gadolinium, a magnetic resonance imaging contrast agent and with rhodamine, a fluorophore, (ii) applying FUS-mediated BBB opening technique to deliver it across the vascular barriers, achieving the local gadolinium enhancement while reducing the systemic and unwanted regional toxicities that are associated with free gadolinium, and (iii) utilizing MR-imaging modality to confirm the delivery as it is already included in the focused ultrasound treatment in clinic. Liposomes were secondarily labeled with a fluorescent marker to confirm results obtained from MRI quantification postmortem. Two different sizes, 77.5 nm (Group A) and 140 nm (Group B) of gadolinium and fluorescence-labeled liposomes were fabricated using thin-film hydration followed by extrusion methods and determined their stability up to 6 hours under physiological conditions. Gadolinium signal was detected in Contrast-Enhanced T1-weighted MRI in five hours after the delivery of liposomes via BBB opening approach with ultrasound pulse of 0.42 MPa (estimate in water) combined with microbubbles. MRI contrast was enhanced significantly in sonicated regions compared to nonsonicated regions of the brain. This was due to the accumulation of labeled liposomes, which was confirmed by

Corresponding Author: Muna Aryal, 1201 Welch Rd, Stanford, CA 94305, muna01@stanford.edu.

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detection of rhodamine fluorescence in histological sections. The relative increase in MRI signal intensity was greater for smaller liposomes (mean diameter = 77.5 nm) compared to larger liposomes (mean diameter = 140 nm), which suggested greater accumulation of the smaller liposomes in the brain after ultrasound-mediated opening of the BBB. Our findings suggest that the dual-labeled nanocarrier platform can be established, FUS-mediated BBB opening approach can be used to deliver them through vascular barriers, and MRI can be used to evaluate the extent of nanocarrier delivery.

Keywords

Nanocarrier; Focused Ultrasound; Cavitation; Brain Drug Delivery

Introduction

Transcranial image-guided focused ultrasound (FUS) has emerged as a viable noninvasive technique for the treatment of central nervous system related diseases/disorders (Hynynen and Jones 2016). Application of mechanical and thermal effects associated with focused ultrasound have been studied extensively in preclinical models, which has paved the way for their clinical application (Carpentier et al. 2016; Coluccia et al. 2014; Lipsman et al. 2018; Martin et al. 2009; McDannold et al. 2010). In one promising approach, researchers have leveraged mechanical effects for transient and focal opening of the blood-brain barrier (BBB) via microbubble cavitation for brain drug delivery (Hynynen et al. 2001; Poon et al. 2017). Furthermore, dual-modality contrast agent, albumin-shelled Gadolinium-DTPA microbubbles were designed for an ultrasound and magnetic resonance imaging to assist BBB opening (Liao et al. 2012). The use of imaging with this procedure is critical to achieve both a safe and efficacious outcome. Imaging is useful at each stage of the procedure, from treatment planning to determine the target volume and evaluate the skull in the ultrasound beam path for phase aberration correction, to intra-procedural monitoring and feedback control over the acoustic parameters during the ultrasound exposures (sonications).

Post-sonication imaging is particularly important for evaluating whether unanticipated tissue damage was produced, to confirm that BBB disruption occurred throughout the targeted volume, and to characterize the level of permeabilization that was achieved. BBB disruption (BBBD) has typically been evaluated using contrast-enhanced T1-weighted magnetic resonance imaging MRI (Hynynen et al. 2001). Quantitative estimates of contrast agent delivery and permeability changes can be performed using T1 mapping and pharmacokinetic modeling (Park et al. 2012; Vlachos et al. 2010). Several groups have investigated the use of these methods to estimate the tissue concentration of a drug or tracer agent after sonication (Chu et al. 2013; Fite et al. 2017). In this approach, a water-soluble MRI contrast agent, such as chelated gadolinium, acts as a drug surrogate, and the relationship between the tissue concentrations of the MRI contrast agent and the therapeutic agent is derived in experimental animal studies. Presumably, these experimentally-derived relationships could be used in subsequent clinical studies. Being able to estimate local drug concentrations could be an important way to ensure that the treatment was effectively and uniformly applied. It is appropriate to use a MRI contrast agent as a surrogate for a drug of comparable

physicochemical properties for monitoring and estimating the amount delivered to brain tissue through openings created by transcranial ultrasound. However, many drugs have poor solubility in water or are substrates for circulating enzymes and thus must be packaged within nanocarriers before systemic administration. Therefore, it is important to monitor and confirm delivery of the nanocarrier through ultrasound-induced openings in the BBB. At present, FUS-mediated BBBD has been utilized to deliver several types of nanocarriers to the brain, including PEGylated liposomes (Kovacs et al. 2015), PEGylated polymer nanoparticles (Nance et al. 2014), gold nanoparticles (Diaz et al. 2014; Etame et al. 2012), and viral vectors (Hsu et al. 2013; Thévenot et al. 2012; Wang et al. 2015). These studies have established that combining FUS-mediated BBBD with nanocarriers can be used to enhance delivery of chemotherapy, nucleic acids, and contrast agents across the BBB as well as improve subsequent penetration in brain tissue (Nance et al. 2014). Previously, we investigated the relationship between MRI-derived tumor permeability estimates and the resulting tissue concentration in rats that received liposomal doxorubicin (Aryal et al. 2015a). We found that the relationship between FUS-induced changes in tumor permeability to the MRI contrast agent gadolinium Gd-DTPA depended on the stage of the tumor. Importantly, at later stages of tumor growth, we did not observe an increase in permeability for the small molecule contrast agent, but we did find a significant increase in liposomal drug delivery. This discrepancy was most likely due to the difference in physicochemical properties between Gd-DTPA and liposomal DOX.

Because of these issues, to map concentrations of large therapeutic agents and nanocarriers one would need to either develop new imaging agents with similar physical properties or to directly label the drug itself. The latter approach has been demonstrated with FUS-induced blood-brain barrier disruption (BBBD), radiolabeled drugs, and SPECT and PET imaging (Yang et al. 2012; Ye et al. 2018). It is also possible to use gadolinium as a drug label for MRI (Frenzel et al. 2008). This approach would be advantageous due to the higher spatial resolution that can be achieved with MRI and because standard MRI sequences can be used to confirm that no unexpected tissue damage occurred during sonication. Furthermore, recent clinical studies reported that the gadolinium accumulates in the brain, most notably in the dentate nuclei and globus pallidus (Gulani et al. 2017; Kanda et al. 2015), which has raised concerns in the scientific and clinical communities. If we succeed in designing a liposome-based MR-contrast agent, it will reduce the systemic toxicity as well as unwanted regional toxicity that is associated with free gadolinium. Therefore, we are envisioning on using Gd-labeled liposomes primarily for fundamental, preclinical studies, and perhaps in the clinic, investigating the pharmacokinetics and efficiency of FUS-mediated brain drug delivery. So our long-term goal is to develop MR-detectable nanoparticles, particularly for finding an MR-based imaging method to noninvasively predict how the pharmacodistribution of a given therapeutic agent would change following ultrasound-mediated BBBO. The work described in this manuscript is the first step towards that goal. Here, we designed a liposome-based nanoparticle labeled with an MR-contrast agent and fluorophore and investigated nanoparticle extravasation across the BBB after the transcranial focused ultrasound in vivo via MRI and post-mortem via spectrofluorometric in this study. We also incorporate passive cavitation technique to monitor cavitation activities during BBBO. The study is designed to be a proof-of-concept, and the findings can be used to guide

the design of subsequent experiments in which changes in BBB penetration of drug-loaded nanoparticles as a function of acoustic parameters and/or cavitation activity can be evaluated.

Materials and methods

Liposomes preparation

Fluorescent- and gadolinium-labeled liposomes were prepared by the thin film hydration method. A rhodamine-labeled lipid (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt), DPPE-Rhodamine) and a gadolinium labeled lipid ((DTPA-bis(stearylamide) (gadolinium salt), Gd-DTPA-BSA) was used to incorporate rhodamine and gadolinium on the head groups of the lipid bilayer. The lipid composition was a molar ratio of 58.4-59.5 mol percent DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), 35 mol percent Gd-DTPA-BSA, 5 mol percent DSPE-PEG2k (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt)), and 0.5-1.6 mol percent DPPE-Rhodamine. Lipids were dissolved in a 2:1 volume ratio of chloroform:methanol which was evaporated over a stream of argon gas and left in a vacuum desiccator overnight to remove residual solvent. The lipid thin film was rehydrated with PBS for 2 hours at 66°C with occasional stirring and pipetting. The lipid solution was sonicated at 20% power for 2 min with a probe sonicator, and extruded sequentially through membrane filters with 1.0 μm pores (4 $\times$), 0.4 μm pores (6 $\times$), and 0.2 μm pores (8 $\times$) to prepare liposomes with a mean diameter of 130-150 nm (Group B). To prepare liposomes with a mean diameter of 70-85 nm (Group A), the lipid solution was extruded in the same manner with additional extrusion through membrane filters with 0.1 μm pores (8 $\times$), and 0.05 μm pores (12 $\times$). The size of the liposomes was measured by dynamic light scattering. The administered dose was similar for group A (0.56 ± 0.03 mmol total lipid/kg body weight) and group B (0.51 ± 0.08 mmol total lipid/kg body weight). The calculated administered dose assumes no loss of lipid due to extrusion.

Liposome stability

To assess liposome stability in physiological condition, liposomes were incubated at 37°C with 15% serum for 6 and 24 hrs. Following incubation, liposome size was measured via dynamic light scattering to assess aggregation. Disassociation of rhodamine from liposomes was assessed by filtration of liposomes through a 30 kDa centrifugal filter (Amicon Ultra, Sigma Aldrich) to collect solvent containing released rhodamine. The fluorescence of liposomes and liposome filtrate was measured in methanol with a spectrophotometer (Shimadzu, ex/em 560/595 nm, slit widths 1.5/5 nm. Fluorescence of methanol, PBS, and PBS with 15% serum were measured as negative controls.

Animals

The Institutional Animal Care and Use Committees of Brigham and Women's Hospital approved all animal experiments. Tests were performed in 16 Male mice (Charles River Laboratories; ~35 g). Animals were divided into two groups based on the size of PEGylated liposomal particles used for the study: group A, 70-85 nm ($n = 9$) and group B, 130-150 nm ($n = 7$). Two animals from group A and one animal from group B were excluded because of

errors that occurred during fluorescence measurement or during sonication. Before each procedure, the animals were anesthetized via intraperitoneal injections of ketamine (80 mg/kg/h) and xylazine (10 mg/kg/h). A catheter was placed in the tail vein, and the hair on the scalp was removed with clippers and depilatory cream. The body temperature was maintained throughout the experiment with a heated water pad.

Sonication system

An air-backed, single element, 690-kHz focused piezoelectric transducer (diameter/radius of curvature: 40/30 mm) generated the ultrasound field. The transducer was assembled in-house. It was driven by an arbitrary waveform generator (33220A, Agilent, Santa Clara, CA, USA) and radiofrequency (RF) amplifier (240L, E&I, Rochester, NY, USA); electric power was measured with a power meter (E4419B, Agilent, Santa Clara, CA, USA) and dual-directional RF coupler (C5948-10, Werlatone, Cranberry Township, PA, USA). A radiation force balance (Mettler Toledo, Columbus, OH, USA) was used to measure the acoustic power and a 0.2-mm diameter needle hydrophone (HNC-0200, Onda, Sunnyvale, CA) was used to calibrate the transmitted pressure and map the acoustic field; its half-maximum diameter and length were 2.3 and 10 mm, respectively. These calibrations were used to estimate the peak negative pressure amplitude at the focus in water (Hynynen 1990).

The experiments were performed under MRI guidance (Figure 1A). The transducer was mounted on a manually operated, MRI-compatible positioning system and coupled to the mouse's head with degassed, deionized water. A transmit/receive surface coil (diameter/radius of curvature: 4/3 cm) was placed under the mouse head, and the system was placed in an animal 7T MRI (Biospec, Bruker). T1-weighted RARE images (parameters: TR/TE: 600/18 ms; ETL: 4; matrix size: 128×128; slice thickness/spacing: 1 mm/interleaved; FOV: 4 cm; NEX: 4, bandwidth: 64.1 kHz) were used to detect signal enhancement from the injected liposomes at different time points ($t = 0.5$ hr, 5 hrs and 24 hrs). T2*-weighted imaging (parameters: TR/TE: 494.5/15ms; matrix size: 128×128; slice thickness: 0.8 mm; FOV: 3.5cm; bandwidth: 50 kHz, NEX: 2) was used to plan the sonication and ensure petechiae, which can result from excessive FUS exposures (Hynynen et al. 2001), did not occur (Figure 2B).

Sonications

Peak negative pressure amplitude of 0.42 MPa (estimate in water) was applied in burst mode (burst length: 10 ms, pulse repetition frequency: 1 Hz, duration, 60s) for a single target. Sonication was applied in multiple points, two or three minimally overlapping targets with 1.5 or 2 mm spacing. Each sonication was combined with an intravenous injection of a microbubbles (Optison; Princeton, NJ, USA) administered at 20 μ l/kg. The time interval between each sonication was 1-2 min, which allowed residual microbubbles from the previous sonication to mostly clear. To facilitate the injections of such a small volume, the agent was diluted in PBS to 0.1 times its normal concentration. It was injected as a bolus approximately 9 s before each sonication, followed by a 0.1 ml saline flush. MR images were obtained with a transmit/receive MRI surface coil, which was placed under the rat's head. The MRI coil and positioning system were assembled in-house. Before the mouse

experiments, we visualized heating in a silicone phantom using temperature-sensitive MRI (T1-weighted RARE) to localize the acoustic focal point in the MRI space.

Passive cavitation detection system

The acoustic emissions from cavitating microbubbles were recorded using a custom-built piezoelectric transducer (center frequency: 1.5 MHz, bandwidth ~ 30%, diameter: 8 mm) operated as a passive cavitation detector (PCD). The procedure for measurement and analysis of the emissions have been described previously (Arvanitis et al. 2012; Aryal et al. 2017). Briefly, the PCD was mounted adjacent to the focused transducer and aimed at the focal region to monitor the acoustic emission produced during sonication. The detector was connected to a Notch filter (Allen Avionic INC, Mineola, NY, USA) with central frequency of 650 kHz. Filtered signals were routed through an amplifier (Standard Research System INC, Sunnyvale, CA, USA) and recorded with a computer using a highspeed digitizing card (PXI-5124; National Instruments, Austin, Texas, USA). The time signal, frequency spectra, and magnitude of the emission at different harmonics were displayed in real-time during each sonication using software developed with Matlab (Mathworks, Natick, MA, USA) and stored for later analysis. Emissions recorded during sonication with microbubbles were normalized to data obtained beforehand at the same locations and exposure levels without microbubbles. Frequency spectra were created from the time signals via fast Fourier transform. The strength of the relative integrated power spectra (in dB-Hz) was calculated over 20 Hz bins for the harmonics (FUS frequency $\times$ 2, 3), at the 1/2 subharmonic (FUS frequency $\times$ 1/2) and first, second ultraharmonic. For each animal, the mean signal for all bursts and all sonications was calculated.

Fluorometric quantification of brain homogenates

To mark the targeted areas for tissue extraction, trypan blue (MP Biomedicals, Solon, OH, USA) was administered after the sonications. The agent was prepared for each experiment. The agent was dissolved in 0.45% NaCl and then boiled and filtered to avoid formation of microcrystals. The solution was administered IV at a dose of 0.1 g trypan blue per kg of body weight.

Twenty-four hours after the sonication, animals were deeply anesthetized with ketamine/xylazine and sacrificed. A 30-90 mg volume of brain tissue was harvested using the trypan blue to differentiate sonicated and unsonicated brain regions. The brain tissue was homogenized, resuspended in 100% methanol, centrifuged to pellet debris, and fluorescence measured using a spectrofluorophotometer (Shimadzu, 560/585 nm excitation/emission wavelengths, 3/5 nm slit widths, High sensitivity setting). Background fluorescence was assessed using brain homogenates from mice that did not receive liposome injections and the background was subtracted from the measurements. A calibration curve was used to convert fluorescence signal intensity to lipid mass (Supplemental Figure S1). The mass of lipid was divided by the weight of brain tissue to normalize for differences in mass of brain tissue harvested, and the final data output was expressed as nmol lipid per gram of brain tissue.

Histology

One mouse each from each group A and group B was used to evaluate the histological effects. After transcardial perfusion with normal saline (0.9% NaCl, 250 ml) to flush unabsorbed Gd-labeled liposomal particles from the cerebral vasculature, the brain was removed and flash-frozen. Frozen brains were sectioned into 8- μ m slices in the axial plane (perpendicular to the direction of ultrasound beam propagation). Every 20th section (20 μ m apart) was stained with hematoxylin and eosin (H & E). In each animal, the sections with the greatest tissue effects were identified and examined in detail under high magnification; the dimensions of any damaged area were measured.

Data analysis

Differences in signal intensity between sonicated and non-sonicated brain were calculated in T1-weighted MRI acquired 5 and 24 h after the delivery of the liposomes. The mean and the standard deviation (SD) of the signal intensity were measured in regions of interest (ROIs) centered on the sonicated target and in the same structure in the contralateral hemisphere. Similarly, mean $\pm$ SD of lipid concentrations on sonicated and non-sonicated tissue were determined based on the fluorescence measurements. For comparing the gadolinium signal intensity and lipid concentrations between sonicated and non-sonicated tissue in each group, a two-tailed paired Student's t-test was used. Two-tailed unpaired student's t-tests were used to compare gadolinium signal intensities, lipid concentrations and mean harmonic strength between group A and group B. Values of $p < 0.05$ were considered statistically significant.

Results

Liposome stability

To assess stability of liposomes in physiological conditions, liposomes were incubated at 37°C with 15% serum. No significant differences in the size of Group A or B liposomes was observed after 6 hours of incubation (Supplemental Figure S2). After 24 hours incubation, the mean diameter of the Group A liposomes increased to ~300 nm, while no significant change was observed with the Group B liposomes. Following ultrafiltration of the liposomes with a 30 kDa filter, no rhodamine was detectable in the filtrate of either Group A or B liposomes after incubation for 6 and 24 hours (Supplemental Figure S2). The stability of the Gd-DTPA chelate bound to liposomes was not measured in the current study. In a previous report, less than 1% of Gd was released from Gd-DTPA chelate after 24 hours incubation at 37°C in the presence of serum (Frenzel et al. 2008).

MRI Findings

The liposomes were labelled with Gd and Rho, thus enabling detection of extravasated liposomes *in vivo* via MRI and *post mortem* via fluorometry. CE-T1WI image had no changes on signal intensity at 0.5 hr after the sonication. At 5 and 24 hrs after FUS, CE-T1WI images were appeared with hyperintense area in sonicated tissue. Figure 2A and 3A show representative examples of CE-T1WI images with hyperintense area in sonicated tissue (red square in the Figure). Measurement of signal enhancement on CE-T1WI images for sonicated and control tissue are shown in Figure 2B, 3B. Mean gadolinium signal

intensity in the sonicated hemisphere was 7634.5 ± 720 and 5216.9 ± 492 on groups A ($n = 4$) and B ($n = 2$), respectively. These values were higher than the control volumes, where the mean values were 6053.8 ± 210 and 4190.3 ± 98 in these groups, respectively. The increased signal intensity (26% and 24% in group A and B, respectively) represented statistically significant difference in Group A ($p = 0.017$). The number of samples was not sufficient for statistical analysis in Group B.

Fluorescent labeled lipid concentrations

In both experimental groups, the concentration of rhodamine in the sonicated and non-sonicated (control) tissues was measured 24 hours after FUS-BBBD (Figure 4). Mean fluorescence signal intensity in the sonicated hemisphere were 0.83 ± 0.22 and 2.37 ± 1.29 in groups A ($n = 3$) and B ($n = 3$), respectively. These values were higher than the control groups, where the mean values were 0.51 ± 0.3 and 1.69 ± 1.03 , respectively. The increased signal intensity (62% and 40% in group A and B respectively) represented statistically significant difference in-group A ($p = 0.02$) but not in-group B ($p = 0.34$). The mean ratio of fluorescence signal intensities between the sonicated and control hemispheres was 1.9 ± 0.2 and 1.5 ± 0.56 in groups A and B, respectively. The difference in this ratio for the two groups was not statically significant ($p = 0.48$).

Acoustic emission

A typical example of an acoustic emissions spectrum is shown in Supplemental S3. The data were obtained with a passive cavitation detector with a center frequency of 1.5 MHz and normalized to recordings acquired without an injection of microbubbles. After normalization, only harmonic emissions (FUS frequency $\times 2, 3$) and occasionally sub- or ultra-harmonic emission (FUS frequency $\times 1/2$) were observed. A correlation between liposome concentrations in brain tissue after FUS-mediated BBBD and strength of microbubble cavitation during FUS-mediated BBBD was noted in Supplemental Figure S3 (D-E), particularly on Group A. Each data point represents an individual subject, and the data points generates by averaging acoustic strengths from multiple sonication sites (2-3) of an individual subject. Lipid concentrations have good correlation with harmonics ($R^2 = 0.67$) and wideband ($R^2 = 0.535$) strengths. There was no significant difference in the strength harmonic (Supplemental Figure S3. B) or wideband (Figure Supplemental S3.C) emissions for the two experimental groups. However, 5/12 of the sonicated locations in the Group B animals produced wideband emissions that were clearly elevated above the noise floor. Such emissions were only produced in 2/32 locations targeted in the Group A animals.

Histological findings

Histological analysis was performed on two mice, one from each group at twenty-four hours after Gd-Rho labeled liposomes delivery via FUS-BBBD. Figure 5 shows example H&E stained sections from these animals. The sonicated and control locations both appeared unaffected.

Discussion

The results of this study demonstrate that delivery of Gd-labeled liposomes to the brain after FUS-BBBD can be monitored with MRI. Being able to directly image drug-loaded nanoparticles with MRI can potentially ensure that one achieves an efficacious and reliable concentration of nanoparticles. We anticipate that this ability will be useful in preclinical studies, providing valuable information that can guide the selection of acoustic parameters chosen for clinical trials on ultrasound-mediated liposomal drug delivery to the brain. Predicting the extent and “magnitude” of ultrasound-mediated BBBD can be challenging, and depends on a number of factors including the amount of acoustic attenuation produced by the skull, the underlying vascular density, and the pharmacokinetics of the circulating microbubbles. These factors are expected to be particularly challenging in the heterogeneous vasculature in tumors. Having a means to visualize and potentially quantify the concentration of drug-loaded nanoparticles *in vivo* can provide important information that can be related to treatment outcomes in future studies.

The use of MRI for this purpose has advantages over other modalities. Others have shown that radiolabeled nanocarriers delivered across the BBB with FUS can be imaged with PET or SPECT imaging (Yang et al. 2012; Ye et al. 2018). While these methods excel in their sensitivity to small concentrations, MRI has a higher spatial resolution and can be used to ensure that tissue damage did not occur as a result of the sonications. Importantly, the use of Gd-labeling offers the potential of serial imaging over extended periods of time. Even though the distribution phase and elimination phase of free Gd-DTPA is 12 min and 94 min respectively (Weinmann et al. 1984), it is still detectable up to 5 days after FUS-mediated vascular manipulation (Sun et al. 2015). So, one could expect that the customized liposomal-based Gd would have more extensive distribution, diffusion and elimination phase as it was detectable 5 hours after the BBB disruption in our current finding. We are excited to explore the detailed characteristics of it in our future work. This study was designed to test a proof of concept on generating liposomal-based Gd-nanoparticles, delivering them via FUS-BBBD and confirming its delivery via two different imaging modalities, fluorescence, and MRI. We succeeded in achieving our primary endpoints and the findings generated several points for discussion. First, it is possible that the majority of injected liposomes were intact following delivery to brain tissue via FUS and microbubbles. This is supported by the previous findings where colocalization of fluorophores was evident after liposome delivery, suggesting that the entire liposomal package crossed the BBB (Kovacs et al. 2015). The circulation half-life of PEGylated liposomes has been reported to range between 2-24 hours in mice (Allen 1994; Moghimi and Szebeni 2003). Thus a significant fraction of liposomes may have reached brain tissue intact, particularly since accumulation was detected in sonicated tissue at 5 hours post FUS BBBD.

A direct comparison between group A and B was difficult, perhaps due to the relatively low sample numbers ($n = 2-4$ mice per group), but also due to other factors that we observed during nanoparticle formulation of two different sizes and fluorescence signal quantification in similar groups as described below. The signal intensity from Gd-labeled liposomes has been reported to decrease with increasing liposome size (Fossheim et al. 1999; Ghaghada et al. 2008). This is implied in our current data as the MR signal in unsonicated brain tissue

was higher on average in Group A than in Group B. Further, although an equivalent dose of liposomes was administered for Group A and Group B (based on mass of lipid per kg bodyweight), the dose of Group A liposomes may have been lower than estimated due to more extensive liposome extrusion. This is implied by the higher fluorescent signal in control brain tissue for Group B than Group A. Nevertheless, Group A liposomes and Group B liposomes could be compared directly in sonicated vs unsonicated brain tissue in a single mouse brain, as these factors are controlled in this circumstance. Another strategy to avoid this issue was to compare the ratio of signal in sonicated vs unsonicated brain tissue. The ratio of fluorescence signal intensity was slightly elevated for Group A (1.9) compared to Group B (1.5), but the ratio was similar when detected via MRI (1.26 for Group A vs 1.24 for Group B). Thus, it is difficult to compare Group A and Group B liposomes directly.

Others have investigated the time window for BBB penetration of MR contrast agents ranging from < 1 nm to 65 nm following FUS and reported that the time window decreases with increasing agent size (Marty et al. 2012). A separate study has shown that there is a direct relation between cavitation activity with different applied pressure during BBBD (Chen and Konofagou 2014). In the case of nanocarrier delivery following FUS BBBD, rhodamine-labeled liposomes of 55 nm, 120 nm, and 200 nm were delivered across the BBB via FUS BBBD in a size-dependent manner with 55 nm liposomes exhibiting greater brain accumulation than liposomes 120 nm or greater (Shen et al. 2016). Polymer nanoparticles have been delivered to brain via FUS BBBD, and it was shown that smaller nanoparticles penetrated deeper into brain tissue than larger nanoparticles (Nance et al. 2014). Our results compare favorably with the findings of these studies, as collectively Group A liposomes appeared to accumulate in brain tissue to a greater extent than Group B liposomes following FUS BBBD. Group A liposomes resulted in a more intense MR signal than group B. In addition, the accumulation of Group A liposomes in sonicated brain tissue was significantly greater than in unsonicated brain tissue, both via MRI and fluorescence quantification. While group B liposomes were observed visually to accumulate in sonicated brain tissue, accumulation levels were not statistically significant compared to unsonicated brain tissue either via MRI- or fluorescence-based quantification. At present, there has not been a single report available on making liposomes that are labeled with MRI-contrast agent to confirm BBB penetration and validate the MR-signal with a different imaging modality, such as fluorescence microscopy. Different studies on “sonoporation” have shown that different size agents can be delivered across the cell membrane through the creation of pores that are present for a short time (Inoue et al. 2014; Lentacker et al. 2014; Rychak and Klivanov 2014; Sato et al. 2014; Tomizawa et al. 2013; Yamatomo et al. 2013). This implies that at a given pressure, agents of different size can have different drug-penetration depth and different acoustic signature during BBBD with microbubbles.

Nevertheless, we do not know whether the gadolinium and fluorophore detected in the brain were still encapsulated in the liposomes or how far from the blood vessels it penetrated into the brain parenchyma over time. Further work is needed to evaluate the dynamics of liposome delivery into brain tissue. For example, it would be interesting to investigate the kinetics and extent of penetration into brain tissue of Gd-labeled liposomes compared with free Gd-DTPA following FUS BBBD.

Given that earlier work with doxorubicin found higher concentrations in the brain when Lipo-DOX was injected before sonication (Aryal et al. 2015b), we suspect that ultrasound could respond differently when it encounter bigger molecules, 100 nm in this case, on its way for BBB disruption. Even though, Lipo-Dox was not echogenic, one could expect that the behaviors of microbubble oscillation, radiation force, microstreaming etc would change if the bigger molecules present on the blood stream. Moreover, our finding on different acoustic signatures with two different sizes liposomes also suggests that this could be one of the mechanisms but further work is needed to support the given hypothesis.

Lastly, nanocarrier features (e.g. size, shape, surface functionality) may have a significant effect on nanocarrier delivery across the BBB via FUS BBB. A systematic characterization of these parameters and their impact on nanoparticle penetration through openings created by FUS and microbubbles is in a nascent stage, as only a few studies have examined the effect of nanocarrier size whereas none have explored shape, and two studies have examined nanocarriers functionalized with ligands intended for markers present in the brain parenchyma (Diaz et al. 2014; Etame et al. 2012). The impact of these parameters on nanoparticle diffusion through openings created by FUS and microbubbles and subsequent penetration depth into brain tissue warrant further investigation.

Conclusions

Overall, this work demonstrates the proof of concept of making liposomes that are labeled with MR-contrast agent and fluorophore, delivering them into the brain parenchyma via ultrasound-mediated blood-brain barrier opening and quantifying the delivered amount via MRI and fluorescence microscopy. Since the liposomes can be visualized noninvasively with MRI and postmortem via fluorescence, they provide a useful tool to estimate the extent of nanocarrier extravasation through the blood-brain barrier.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1:

A. Schematic of the magnetic resonance imaging-guided focused ultrasound (MRIgFUS) system used in this work. The function generator, amplifier, and power meter were located outside the MRI room. A passive cavitation detector (PCD) was used to monitor the acoustic emissions. B. Sonication plan, monitoring, and assessment of FUS-induced blood-brain barrier disruption (BBBD) after microbubble-enhanced sonication. Axial MRI acquired during FUS-BBBD. Pre-FUS T2*-weighted imaging (T2*WI) was used for treatment planning and post-FUS T2*-weighted imaging (T2*WI) to detect tissue damage. Post-FUS, contrast-enhanced T1-weighted imaging (CE-T1WI) was taken over time 5-24 hours to observed contrast-enhancement after the delivery of liposomes via ultrasound mediated BBBD. Circles indicate the targeted spots; the yellow curves outline the extent of gadolinium enhancement due to BBB permeabilization.

Figure 2:

Contrast-enhancement on brain tissue after focused ultrasound-induced blood-brain barrier disruption (FUS-BBBD) combined with microbubbles and its corresponding signal intensities. A. Axial MRI was acquired 24 hours FUS-BBBD. Contrast-enhanced T1-weighted (CE-T1WI) was used to verify contrast-enhancement on sonicated location. Sonicated and control locations are indicated by red and blue squares respectively. B. Measurement of gadolinium signal intensity on CE-T1WI images for sonicated and non-sonicated (control) tissue. Gadolinium signal intensity on sonicated location was increased by 26% as compared with control and the comparison was statistically significant ($p < 0.05$). Scale bar: 5 mm

Figure 3:

Contrast-enhancement on brain tissue after focused ultrasound-induced blood-brain barrier disruption (FUS-BBBD) combined with microbubbles and its corresponding signal intensities. A. Axial magnetic resonance image (MRI) was acquired 24 hours FUS-BBBD. Contrast-enhanced T1-weighted (CE-T1WI) was used to verify contrast-enhancement on sonicated location. Sonicated and control locations are indicated by red and blue squares respectively. B. Measurement of gadolinium signal intensity on CE-T1WI images for sonicated and non-sonicated (control) tissue. Gadolinium signal intensity on sonicated location was increased by 24% as compared with control Scale bar: 5 mm

Figure 4:

Lipid concentrations in brain tissue corresponding to Group A and Group B liposomes.

Liposomes were labeled with gadolinium (Gd) and fluorophore called rhodamine (Rho).

Fluorescence measurement was done 24 hours after focused ultrasound-induced blood- brain barrier disruption (FUS-BBBD). A. For group A, liposomes, a concentration of 0.83 ± 0.22

nmole/g (mean $\pm$ standard deviation) of lipid was measured in sonicated brain tissue (n = 3 mice), an increase of 62% over unsonicated brain tissue, and was statically significant (p < 0.05, two-tailed, paired t-test) B. For Group B liposomes, a concentration of 2.37 ± 1.29

nmole/g (mean $\pm$ standard deviation) of lipid was measured in sonicated brain tissue (n = 3 mice), an increase of 40% over unsonicated brain tissue, but was not stastically significant (p = 0.34, two-tailed, paired t-test).

Figure 5: Histological appearance of the brain after sonication of Group A or group B liposomes. The sonicated area is indicated in by the red square; the corresponding non-sonicated region in the contralateral hemisphere (black square) served as a control. The sonicated and control locations both appeared unaffected. H&E; scale bars: 5 mm in a – b.