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Targeted Drug Delivery with Focused Ultrasound-Induced Blood-Brain Barrier Opening Using Acoustically-Activated Nanodroplets

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Abstract

Focused ultrasound (FUS) in the presence of systemically administered microbubbles has been shown to locally, transiently and reversibly increase the permeability of the blood-brain barrier (BBB), thus allowing targeted delivery of therapeutic agents in the brain for the treatment of central nervous system diseases. Currently, microbubbles are the only agents that have been used to facilitate the FUS-induced BBB opening. However, they are constrained within the intravascular space due to their micron-size diameters, limiting the delivery effect at or near the microvessels. In the present study, acoustically-activated nanodroplets were used as a new class of contrast agents to mediate FUS-induced BBB opening in order to study the feasibility of utilizing these nanoscale phase-shift particles for targeted drug delivery in the brain. Significant dextran delivery was achieved in the mouse hippocampus using nanodroplets at clinically relevant pressures. Passive cavitation detection was used in the attempt to establish a correlation between the amount of dextran delivered in the brain and the acoustic emission recorded during sonication. Conventional microbubbles with the same lipid shell composition and perfluorobutane core as the nanodroplets were also used to compare the efficiency of FUS-induced dextran delivery. It was found that nanodroplets had a higher BBB opening pressure threshold but a lower stable cavitation threshold than microbubbles, suggesting that contrast agent-dependent acoustic emission monitoring was needed. More homogeneous dextran delivery within the targeted hippocampus was achieved using nanodroplets without inducing inertial cavitation or compromising safety. Our results offered a new means of developing the FUS-induced BBB opening technology for potential extravascular targeted drug delivery in the brain, extending the potential drug delivery region beyond the cerebral vasculature.

1. Introduction

Focused ultrasound (FUS)-induced blood-brain barrier (BBB) opening has been shown to hold great promise for targeted drug delivery in the brain for the treatment of central nervous system diseases, including Alzheimer's [1], Huntington's [2] and Parkinson's [3] diseases

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as well as brain cancers [4, 5]. Various therapeutic agents, including antibodies [6], neural stem cells [7], siRNA [2], chemotherapeutic molecules [8] and neurotrophic factors [3], have been delivered across the BBB and shown capable of inducing therapeutic effects using this technology. The FUS-microbubble-based drug delivery system relies on the capability of systemically-administered and acoustically-activated microbubbles to noninvasively, transiently and reversibly increase the cellular and vascular permeability of the BBB, thus allowing drug molecules that are normally restricted within the vasculature to cross the barrier and be delivered to the brain parenchyma. Previous studies by our group and others have shown that the extent of the BBB opening, as characterized by the opening volume, opening size and opening duration based on magnetic resonance or fluorescence imaging, is directly linked to the acoustic energy applied during sonication [9–12] and the physicochemical properties of the microbubbles used [6, 13, 14]. Informed selections of the acoustic exposure parameters and the type of contrast agent are critical for effective drug delivery while minimizing the possibility of side effects. Not accounting for these factors may result in unintended adverse bioeffects [15] or even undesired brain damage in more severe situations [9].

Real-time passive acoustic emissions from the oscillating microbubbles could offer means to identify signature characteristics that allow for the differentiation of the complex behaviors of microbubble cavitation during sonication [16–19]. During stable cavitation, the magnitude of microbubble oscillation depends on the acoustic pressure applied. Such behavior has been assumed to induce sufficient forces on the endothelium through either radiation or shear forces induced by microstreaming around the microbubbles [19]. Shear stress-induced endocytosis could also be a potential mechanism to increase the transcellular permeability [20]. As the acoustic energy is increased, larger radial microbubble expansion could induce inertial cavitation, leading to microbubble collapse and potentially generating liquid microjets to form pores within the cell membrane improving the cellular uptake of nanoparticles and drugs [21]. The disruption of the tight junction proteins between the adjacent brain endothelial cells during inertial cavitation could also increase the paracellular BBB permeability [22]. Regardless of the exact mechanism, the cavitation emission during sonication could be calculated and correlated with the outcome of the BBB opening, thus suggesting that the acoustic signature of the microbubbles could serve as a predictive indicator for estimating the amount of drug delivered to the targeted region [16, 23].

Currently, FUS-induced BBB opening has only been achieved using commercially available [1, 14, 24] or in-house manufactured [25, 26] gaseous microbubbles. Despite their utility as mediating vehicles, microbubbles can only be used as vascular contrast agents since their size (typically 1–10 μm) prevents their extravasation, limiting the cavitation effects to regions at or near the vessels. Using fluorescence microscopy, our group [27] previously showed that homogeneous distribution of relatively larger dextran molecules (10 kDa and 70 kDa) was more difficult to achieve than with smaller ones (3 kDa), highlighting the need for an agent with greater ability to induce molecular diffusion beyond the cerebral vasculature. In addition, gaseous microbubbles have short *in vivo* circulation half-life, typically on the order of minutes, thus requiring re-administration if repeated sonication is desired.

Phase-shift perfluorocarbon droplets may potentially offer the solution for such a demand [28, 29]. Several groups have demonstrated the therapeutic utility of this alternative class of ultrasound contrast agents for applications such as targeted vessel occlusion [30], ablation enhancement [31], and drug delivery to solid tumors [32, 33]. Initially in the liquid state, nanoscale droplets show high stability in circulation (i.e., on the order of hours) and can be generated in sizes small enough to extravasate through leaky vasculature; but once exposed to sufficient rarefactional pressures they expand to form microbubbles capable of oscillation in a similar fashion as traditional microbubbles - ideal for ultrasound-mediated imaging and

therapy. For the purposes of BBB opening, these agents may present a unique opportunity. Because droplet activation is dependent on the local rarefactional pressure, the microbubbles will only be generated within the narrow focal region where acoustic properties are the strongest, ensuring the therapeutic effect is limited to the desired treatment areas [34, 35]. Once initial BBB permeabilization is achieved, the droplets remaining will be small enough for potential extravasation during subsequent passes through the vasculature. Once entered into the interstitial space behind the barrier, the droplets could then be acoustically activated to form vaporized gaseous bubbles outside the constraints of the cerebral microvessels. Therefore, the possibility exists for an extravascularly activated contrast agent for enhanced drug delivery at sites that are located deeply in the brain tissue or at regions with relatively low vasculature density. Activation of typical nanodroplet formulations requires acoustic pressures much higher than those used for FUS-induced BBB opening [36, 37], but recent studies have shown that it is possible to generate nanodroplets from highly volatile perfluorocarbons by pressurizing preformed microbubbles and condensing the gas core into liquid phase during slow cooling [23, 38]. This methodology can produce uniform nanodroplet size distributions with peak diameters near 200–300 nm that vaporize at acoustic pressures on the order of those required for BBB opening with microbubbles.

The main objective of the present study was to show the feasibility of utilizing phase-shift nanodroplets as the mediating agents for targeted drug delivery using FUS-induced BBB opening *in vivo*. Conventional in-house generated microbubbles with the same lipid shell composition and perfluorocarbon gaseous core were also used as the “standard” for comparing the efficiency of BBB opening based on targeted delivery of fluorescent dextran molecules. A series of clinically relevant acoustic exposure conditions were used to further demonstrate the safety of this technology for drug delivery in the brain.

2. Materials and methods

2.1 Contrast agent generation

Nanodroplets and microbubbles were formulated using the same lipid composition containing 90 mol% 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 10 mol% 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000] (DSPE-PEG2000) (Avanti Polar Lipids, Alabaster, AL). The perfluorobutane gas (PFB, 99 wt% purity) used for contrast agent generation was obtained from FluoroMed (Round Rock, TX). All chemicals were used as purchased without further purification.

Microbubbles were generated via mechanical agitation using a Vialmix shaker (Bristol-Myers-Squibb, New York, NY). A Multisizer III particle counter (Beckman Coulter, Opa Locka, FL) with a 30 μ m aperture was used to measure the microbubble suspension size distribution and concentration. Nanodroplets were generated via microbubble condensation, in which pre-formed microbubbles of volatile compounds were reverted to the liquid state by application of reduced temperature and increased ambient pressure [23, 38]. Briefly, PFB microbubbles were generated as described above and allowed to cool to room temperature. The vial containing the microbubbles was then immersed in a CO₂/isopropanol bath maintained at a temperature between –7 °C and –10 °C for approximately 1 min. The vial was subsequently connected to an adjustable air-pressure supply, and the headspace pressure inside the microbubble vial increased 30 – 70 kPa for approximately 30 s to facilitate condensation. A Malvern Nano Zetasizer (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.) was used to measure the size distribution of the droplet emulsion generated.

2.2 *In vitro* acoustic nanodroplet vaporization setup

Acoustic nanodroplet vaporization was investigated *in vitro* using an experimental setup described previously [39]. An ultra-high-speed framing camera with a 24-frame buffer (SIMD24; Specialised Imaging, Simi Valley, CA) was interfaced with an inverted microscope (IX71; Olympus, Center Valley, PA) with a 100× (NA=1.0) water immersion objective. An acrylic-lined, continuously degassed water bath was mounted to the microscope and maintained at 37 °C. The optical resolution of the system allowed observation of particles larger than approximately 500 nm. A 1 MHz spherically-focused piston transducer with a 2.2 cm diameter and a focal length of 3.75 cm (IL0106HP; Valpey Fisher Corp., Hopkinton, MA) was aligned with the optical focus by use of a calibrated needle hydrophone (HNA-0400; Onda Corp., Sunnyvale, CA). The transducer was driven by sinusoidal 20-cycle pulses generated by a manually triggered arbitrary waveform generator (AFG 3101; Tektronix, Inc., Beaverton, OR) amplified 60 dB (A500; ENI, Rochester, NY). The sinusoid amplitude was adjusted to change the peak rarefactional pressure experienced by the droplets in focus. The manual trigger was synchronized with the input of the high-speed camera in order to simultaneously capture video of droplet vaporization. Droplet emulsions were diluted 50% in phosphate-buffered saline (PBS) and pumped through a nearly optically and acoustically transparent microcellulose tube (Spectrum Laboratories, Inc., Greensboro, NC). The focal plane of the tube was controlled via a 3-axis micropositioner (MMO-203; Narishige Group, East Meadow, NY). The ultra-high-speed camera was set to operate at 20 million frames per second with a 30 ns exposure such that many frames could be captured within a single cycle of the ultrasound pulse. The transducer was calibrated at focus by the needle hydrophone (HNA-0400).

2.3 Animal preparation

All experiments were performed in accordance with procedures approved by the Columbia University Institutional Animal Care and Use Committee. A total of 80 male C57BL/6 mice (Harlan Laboratories; Indianapolis, IN) weighing 20–25 g were used for this study. The animals were divided into two experimental groups using either nanodroplets (group #1) or microbubbles (group #2) as the contrast agents for FUS-induced BBB opening. The groups were further divided into 14 cohorts based on the experimental protocol as listed in Table 1. Before sonication, each mouse was anesthetized using 1–2% isoflurane-oxygen mixture (SurgiVet, Smiths Medical PM; Norwell, MA) and its hair on the scalp was removed with an electric clipper and a depilatory cream. A modified 27-gauge 1½ butterfly catheter (Terumo Medical; Somerset, NJ) was inserted into the tail vein for contrast agent injection. The animal body temperature was maintained throughout the experiment using a heated pad.

2.4 *In vivo* BBB opening ultrasound setup

The experimental setup, as shown in Fig. 1, was used as previously described [26, 27]. A single-element, spherical FUS transducer (center frequency: 1.5 MHz; focal depth: 60 mm; Imasonic, Besancon, France) was driven by a function generator (Agilent Technologies, Palo Alto, CA) through a 50 dB power amplifier (E&I, Rochester, NY). A confocal pulse-echo transducer (center frequency: 10 MHz; focal length: 60 mm; Olympus NDT, Waltham, MA), which was attached to a computer-controlled 3D positioning system (Velmex, Lachine, QC, Canada), was used to target specific brain structures following procedures published previously [14]. The pulse-echo transducer, which was driven by a pulser-receiver system with a 20 dB amplification in the receive-only mode (Model 5800; Olympus NDT), was connected to a digitizer (Gage Applied Technologies, Lachine, QC, Canada) to passively acquire acoustic emissions from activated nanodroplets and microbubbles within ± 1 V input range.

The peak-rarefactional pressure profile used in the present study was estimated based on previously performed calibrations done in degassed water with a 0.2 mm diameter needle hydrophone (Precision Acoustics; Dorchester, Dorset, UK) [14]. The pressure amplitude values were corrected to account for 18.1% attenuation through the murine skull [14], whereas the axial and lateral full-widths at half-maximum intensities of the beam were 7.5 mm and 1 mm, respectively. The acoustic exposure reported throughout the present study is given in terms of the peak-rarefactional pressure amplitudes.

2.5 *In vivo* BBB opening protocol

Pulsed FUS (pulse length: 6.7 ms; pulse repetition frequency: 5 Hz; duration: 5 min) at acoustic pressures ranging between 0.15 and 0.60 MPa was applied transcranially to the targeted left hippocampus of the mouse brain while the right hippocampus served as the control. Prior to any contrast agent administration, a 30-s sonication using the same acoustic parameters was applied in order to measure the baseline background signal needed in the acoustic emission analysis, as described below. The injected contrast agent samples were freshly diluted before each animal injection. For group #1, a 60 μ L nanodroplet suspension was first diluted at 50 vol% using PBS to an estimated final concentration of 5×10^9 #/mL based on 100% condensation during production. It was then co-administered, via bolus injection, with 60 μ L 3 kDa dextran solution (Life Technologies; Carlsbad, CA) at 2 mg/mL concentration through the tail vein 10 s prior to each sonication. For group #2, the microbubble suspension was diluted in PBS to a final concentration of 8×10^8 #/mL, and a 60 μ L of the diluted microbubble sample, together with 60 μ L dextran solution was bolus injected following the same protocol. In addition, two sham cohorts to which no ultrasound was applied were also injected with either nanodroplets or microbubbles and fluorescent dextrans to serve as the basis for comparison in the fluorescence imaging analysis (see description below).

A 1-h period was allowed after sonication to enable the dextran to circulate throughout the vasculature and diffuse into the brain parenchyma. At the end of the allotted time, the animal was sacrificed by transcardiac perfusion using 30 mL PBS for 5 min followed by 60 mL 4% paraformaldehyde for 8 min. The mouse brain was extracted from the skull, post-fixed in 4% paraformaldehyde overnight, and then prepared for either frozen (60 μ m) or paraffin (6 μ m) sections. The frozen sections were used to analyze fluorescence intensity in order to determine the BBB opening magnitude, while the paraffin sections were used to study the safety of the procedure through histological examinations of representative brain samples from each treatment cohort via hematoxylin and eosin (H&E) staining. The histology image interpretation was performed blindly, i.e., without knowledge of the FUS exposure parameters.

2.6 Fluorescence imaging analysis

Bright-field and epi-fluorescence images of the brain sections were captured using an Olympus DP30BW digital camera mounted on an upright Olympus BX61 microscope. The extent of BBB opening was determined based on the quantification of dextran delivery into the targeted hippocampus. The fluorescence intensity measurement was similarly performed following our previously published protocol [40]. Briefly, a section representing the ventralsdorsal midline, as determined by anatomical landmarks, was first selected, and 4 adjacent sections were then selected on either the dorsal or the ventral side of the midline. The sonicated (left) and the control (right) hippocampus was manually outlined using MATLAB (The Mathworks; Natick MA), and the spatial average of fluorescence intensity in the region of interest (ROI) was calculated using ImageJ (National Institutes of Health, Bethesda, MD). The relative fluorescence enhancement was calculated by dividing the difference in fluorescence intensity between the left and right ROIs by the spatial average of

the right hippocampus. A fluorescence intensity threshold, defined as twice that of the standard deviation of the control ROI, was applied on each image in order to separate the fluorescence signal from dextran molecules from the background tissue autofluorescence. For each brain, the reported fluorescence enhancement was thus approximated as the sum of the relative fluorescence intensity from all 9 sections. Successful dextran delivery for an individual brain was concluded if the fluorescence enhancement was higher by two standard deviations relative to the average of the corresponding sham cohort. The fluorescence enhancement value for each of the 12 experimental conditions was obtained by averaging all mice sonicated under the same acoustic exposure. 100% opening efficiency was determined when all the mice evaluated in each cohort showed significant dextran delivery.

2.7 Acoustic emission analysis

The acoustic emission analysis was performed similarly to that described previously [26]. In order to quantify the acoustic responses of the vaporized nanodroplets or microbubbles, two cavitation parameters were calculated: stable cavitation dose (SCD) and inertial cavitation dose (ICD). The SCD, which was associated with the stable nonlinear oscillation of the contrast agents, was quantified based on the peak amplitude of the spectra around each harmonic frequency of each pulse in the range between 3 and 9 MHz over the first minute sonication duration. The ICD, which was associated with the inertial energy of the collapsing microbubbles, was quantified based on the broadband emission after filtering the harmonic and ultraharmonic signals using a comb filter with rectangular rejection bands centered around the harmonic and ultraharmonic frequencies (rejection bandwidths of 350 kHz and 100 kHz, respectively) across the same frequency range over the same sonication duration. The net emission from the contrast agents could then be determined by subtracting the background signal measured using the same acoustic exposure prior to nanodroplet or microbubble administration.

2.8 Statistical analysis

An unpaired two-tailed Student's t-test was performed to evaluate the significance of the fluorescence enhancement between the sonicated and the control hippocampus under each of the acoustic exposures. In addition, unpaired two-tailed Student's t-tests were used to determine the significance of the acoustic emission responses from the contrast agents across different sonication pressures. All statistical analysis was performed using GraphPad Prism (La Jolla, CA).

3. Results

3.1 Contrast agent generation

The PFB phase-shift nanodroplet emulsions appeared partially translucent by visual inspection and were similar in stability and size to previously reported results [38]. Fig. 2A shows a representative number-weighted size distribution obtained by averaging 3 separate samples (3 measurements per sample). The size distributions measured in the present *in vivo* study were similar across all samples, and fell within the instrumental uncertainty of the Zetasizer. The averaged number-weighted mean, median and mode diameters across all samples were 204 ± 10 nm, 209 ± 29 nm and 180 ± 25 nm, respectively.

The mechanical agitation method produced opaque milky microbubble suspensions that were stable during the experimental timeframe. Fig. 2B shows a representative number-weighted size distribution of the microbubble sample as measured by Multisizer III. The size distributions among all microbubble suspensions used throughout the study were found to be statistically the same, and the averaged number-weighted mean, median and mode diameters were 1.36 ± 0.32 μ m, 1.08 ± 0.23 μ m and 0.86 ± 0.35 μ m, respectively.

3.2 *In vitro* acoustic nanodroplet vaporization

In order to confirm the nanodroplets generated via microbubble condensation were acoustically vaporizable, high-speed optical microscopy was used to visually verify the vaporization of individual stationary droplets at pressures relevant to our *in vivo* experiment. Fig. 3 shows that at peak-rarefactional pressure of approximately 0.35 MPa, no detectable number of vaporized bubbles appeared within the focal plane. However, when the pressure amplitude was increased to 0.45 MPa, vaporized bubbles were observed almost immediately upon exposure to the ultrasound beam. These acoustically activated bubbles were within the micrometer size range and appeared to be acoustically responsive (i.e., they could expand and contract according to the pressure change during a single pulse). The activation of sub-micron droplets was also repeatedly observed at higher pressures (up to 1.1 MPa tested in the current study) using the *in vitro* setup, complementing our finding of the *in vivo* pressure threshold of BBB opening using nanodroplets (see discussion below).

3.3. *In vivo* FUS-induced BBB opening

Using fluorescently-labeled 3 kDa dextran as a model drug molecule, the extent of the FUS-induced BBB opening could be quantified as the relative fluorescence enhancement in the sonicated hippocampus over the control. The two sham cohorts, for which no ultrasound was applied, did not show any change in fluorescence intensity between the two hemispheres (Fig. S1). Quantified fluorescence enhancement results confirmed this observation as no detectable increase in fluorescence intensity between the two ROIs was calculated (Table 2). For the rest of the 12 experimental conditions, the measured fluorescence enhancement was compared to their corresponding sham cohort in order to determine whether sufficient amount of dextran molecules were delivered into the targeted region.

Following the systemic administration of nanodroplets and subsequent BBB opening, a significant increase in dextran delivery in the targeted ROI was observed at 0.45 and 0.60 MPa (60% opening efficiency with $P=0.047$ and 100% opening efficiency with $P=0.0002$, respectively) while only up to 33% of the animals evaluated showed a significant fluorescence enhancement when sonicated at pressures below 0.45 MPa (Table 2). Due to the inconsistency across animals, there was no statistically significant ($P>0.56$) increase in fluorescence enhancement to clearly indicate BBB opening with dextran delivery for sonication pressures ranging between 0.15 and 0.30 MPa (Fig. 4A). At pressures above 0.45 MPa, fluorescence was observed not only within or near large vessels, but also diffusely distributed across the hippocampi (Figs. 5D and 5E).

Following the systemic administration of microbubbles and subsequent BBB opening, a significant fluorescence enhancement was detected for all mice when sonicated at pressures including and above 0.30 MPa ($P<0.0050$). A similar inconsistency of dextran delivery across animals was seen at 0.225 MPa that only 3 out of 5 mice showed significant fluorescence enhancement in the targeted ROI (Table 2). Thus, no sufficient statistical difference ($P>0.16$) could be obtained to unequivocally show BBB opening at pressures below 0.30 MPa (Fig. 4B). The detectable fluorescence signal was predominately contained within vessels at 0.30 MPa but more diffusely distributed throughout the targeted region at 0.45 MPa, indicating a more homogenous distribution of dextran molecules (Figs. 5H and 5I). At 0.60 MPa, heterogeneous spots of particularly high levels of fluorescence in combination with diffusely distributed fluorescence was observed (Fig. 5J).

For each acoustic pressure, microbubbles produced greater fluorescence enhancement compared to nanodroplets. The normalized fluorescence enhancement with pressure amplitude followed a linear relationship with correlation coefficients at 0.76 and 0.94 for nanodroplets and microbubbles, respectively. The pressure threshold, at which significant

fluorescence enhancement was detected in comparison to the sham animals, was higher for the nanodroplets (0.60 MPa) than that for the microbubbles (0.30 MPa), although the percent enhancement values at the threshold pressures were not statistically different ($P=0.56$).

3.4 Contrast agent-dependent BBB opening threshold

Fig. 6 shows the quantitative acoustic emission results detected before and after the contrast agent administrations at various sonication pressures. For nanodroplets, the quantified SCD showed significant increase ($P<0.0001$) at 0.60 MPa (Fig. 6A), corresponding to the significant dextran delivery detected based on fluorescence microscopy. However, no statistical difference was determined at 0.45 MPa ($P=0.21$) despite a 90% mean signal increase after nanodroplet administration owing to the large variations among different mice. The SCD increase followed a linear relationship with the sonication pressure ($R^2=0.99$). The quantified ICD, on the other hand, showed no detectable inertial cavitation dose across all pressures (Fig. 6B), implying that no significant vaporized nanodroplet fragmentation was detected during sonication.

The acoustic emission results for the microbubble group are shown in Figs. 6C and 6D. Interestingly, the SCD showed significant increase ($P<0.0005$) for all pressure levels after microbubble injections regardless the outcome of the BBB opening (Fig. 6C). Similar to the nanodroplet group, the SCD increase followed a linear correlation with the FUS pressure amplitude ($R^2=0.93$). The ICD measurement showed significant signal increase after microbubble injection at 0.60 MPa ($P=0.017$) but not at other pressure levels, indicating microbubbles underwent inertial cavitation during sonication only at the highest acoustic energy exposure (Fig. 6D).

In order to test whether acoustic emissions could be used to predict the magnitude of the BBB opening, the relative fluorescence enhancement was plotted against the SCD for all sonications using each contrast agent (Fig. 7). Both agents showed relatively good linear correlations between these two parameters ($R^2=0.74$ for nanodroplets and $R^2=0.67$ for microbubbles). A cavitation dose opening threshold can also be derived when SCD was grouped based on whether significant dextran delivery was detected based on fluorescence enhancement [16] (Fig. 8). For the mice sonicated in the presence of nanodroplets, a statistically higher ($P<0.0001$) stable cavitation emission was detected for cases where significant fluorescence enhancement was measured. The highest SCD value, at which no evidence of dextran delivery was detected, was 74 V·s. Out of the 10 animals that showed significant fluorescence increase, 3 mice (30%) had their SCD lower than 74 V·s, suggesting that this level could be used as the threshold for predicting successful FUS-induced BBB opening using nanodroplets as the contrast agents.

Interestingly, the acoustic threshold of BBB opening appeared to be contrast agent dependent. For the microbubble group, the highest SCD value, for which no detectable dextran delivery was observed, was 1.4 kV·s, significantly higher ($P<0.0001$) than the SCD threshold found for the nanodroplet group. A much more prominent threshold was held for group #2, for 1 out of 15 mice (6.7%) with significant fluorescence enhancement had a SCD lower than 1.4 kV·s. Despite the wider range of the calculated SCDs, in which successful BBB opening was detected, a statistical difference was obtained ($P=0.04$) between cases with or without significant dextran delivery. The threshold for predicting FUS-induced BBB opening in the presence of microbubbles was therefore concluded to be 1.4 kV·s (Fig. 8).

3.5 Safety

Histological evaluation was performed in order to assess for potential tissue damage caused by the procedure. Fig. 9 shows the bright-field microscopic images taken of representative brain samples at 0.45 and 0.60 MPa. These two pressure amplitudes were chosen since relatively consistent BBB opening was detected only at these pressure levels for both nanodroplet and microbubble groups. Close examinations did not reveal any discrete damage sites, such as clusters of dark neurons, small erythrocyte extravasations, hemorrhage or microvacuolations at 0.45 MPa for either the nanodroplet or the microbubble group (Fig. 9 top). A few (<10) dark neurons were identified from 3 nonadjacent sections of the representative brain sample that was sonicated at 0.30 MPa using nanodroplets (Fig. S2). No other tissue damage indicators were observed, indicating that this could be an artifact due to inadequate perfusion/fixation [41]. However, small clusters of extravasated erythrocytes in addition to a few dark neurons were observed for a sample from the microbubble/0.60 MPa cohort (Fig. 9O). Less than 10 clusters were found throughout the sonicated region across all sections, indicating minor tissue damage. The larger portion of the targeted hippocampus appeared to be normal and the neurons appeared to be unaffected (Fig. 9M).

4. Discussion

The current study utilized acoustically-activated nanodroplets as a new class of contrast agents to facilitate targeted drug delivery in the brain after FUS-induced BBB opening in mice. Though similar agents have been used in other therapeutic applications, to the best of our knowledge, this is the first study that has explored the benefits of a nanodroplet-based approach to FUS-induced BBB opening. Using fluorescent dextran as a model drug, the extent of the BBB opening was quantified and compared between nanodroplets and the conventional contrast agent, i.e., microbubbles (Figs. 4 and 5). The two agents were compositionally the same – having lipid-encapsulated perfluorobutane cores – but the nanodroplet approach afforded taking advantage of the benefits of both the liquid and gaseous state of the cores. The acoustic emission generated from the contrast agents during sonication was recorded and analyzed in order to gain insights to their cavitation characteristics (Fig. 6). A linear correlation between BBB permeabilization, as indicated by the relative fluorescence enhancement within the targeted hippocampus, and the acoustic emission, as characterized by the SCD, was found for both nanodroplets ($R^2 = 0.74$) and microbubbles ($R^2 = 0.67$) (Fig. 7). Interestingly, the acoustic threshold, at which significant dextran delivery was observed, appeared to be contrast agent dependent (Fig. 8). Our results suggested future contrast agent-specific monitoring during FUS-induced BBB opening might be needed.

The high-speed microscopy results confirmed that the nanodroplet samples were acoustically vaporizable at exposure conditions similar to that used during BBB opening *in vivo* (Fig. 3). The vaporization pressure threshold detected *in vitro* corresponded to our *in vivo* estimation remarkably well. While it was possible that nanodroplet vaporization at pressures lower than 0.45 MPa could still occur, it was more likely that the number of activated nanodroplets was too low to cause any detectable fluorescence enhancement. Thus, nanodroplet-mediated dextran delivery to the targeted hippocampus could not be consistently achieved until the sonication pressure was higher than 0.45 MPa (Table 2). For the purpose of direct comparison, the droplets used in this study were formed from perfluorobutane. While the pressures used were sufficient to cause some droplet vaporization, other studies have shown that highly efficient vaporization at frequencies near 1 MHz required pressures on the order of 1 MPa or greater [23, 42]. Further studies are needed to develop different nanodroplet formulations in order to decrease the *in vivo* vaporization threshold by incorporating more volatile perfluorocarbons, such as perfluoropropane [38]. Additionally,

the not-yet-optimized droplet formulation could also explain the lower fluorescence enhancement observed using nanodroplets than that of microbubbles even though the injection amount of the former was much greater (Fig. 4).

Previous work reported by our group and others have been focusing on increasing the delivered dose and distribution by optimizing ultrasonic parameters and microbubble characteristics, including peak-rarefactional pressure [10], pulse repetition frequency [40], pulse length [27] and microbubble size [25, 26]. Choi et al. [27] showed that homogenous distribution of 3 kDa dextran molecules was possible by incorporating short ultrasonic pulses to allow sufficient microbubble replenishment at the focus during sonication. The utilization of acoustically vaporized nanodroplets in this paper produced a similar homogenous dextran distribution throughout the targeted volume and was more prominent when compared to microbubbles under the same acoustic exposure settings (Figs. 5D–5J). Similar to our previous studies, fluorescence was not only detected within or near large vessels but also diffusely distributed within the hippocampus, thus showing greater promise for delivery of therapeutic agents to a wider range of cellular targets outside the microvasculature. As a preliminary feasibility study, the sonication parameters used in the current paper were simply adapted based on our previous FUS-induced BBB opening experiments using microbubbles. Therefore, it is possible that nanodroplet-specific pulse sequence could be custom designed to further enhance the delivery of larger drug molecules. Since short pulse lengths appeared to be adequate to activate nanodroplets at clinically relevant pressures [39] (Fig. 3), it is possible that higher delivery doses and a more homogenous distribution could be achieved using short pulses with nanodroplets as well [27]. In addition, it is possible that a higher but safer sonication pressure could be achieved with nanodroplets since the threshold for inducing inertial cavitation was higher than that of microbubbles (Fig. 6).

Initial histological examinations confirmed the safety for the nanodroplet-mediated FUS-induced BBB opening technology. The case where a few dark neurons were identified for the nanodroplet/0.30 MPa brain sample was believed to be an isolated incident that was more likely due to inadequate perfusion-fixation [41] (Fig. S2). In comparison to nanodroplets, microbubble-mediated BBB opening resulted minor tissue damage at the targeted region for both brain samples evaluated from the 0.60 MPa cohort (Fig. 9O). Based on the H&E staining results, it is reasonable to conclude that the significantly increased dextran delivery at 0.60 MPa was to be caused by the increased magnitude of disruption at the focal region. However, such molecular delivery enhancement with compromised safety may not be desirable in the clinic.

Passively detected acoustic emissions originated from the oscillating contrast agents could be used to characterize the type of cavitation events occurred during sonication, providing insights to the mechanism of the FUS-induced BBB opening. Only an increase in the harmonics and ultraharmonics signals detected after nanodroplet administration at all pressures, suggesting that the vaporized nanodroplet underwent mainly nonlinear stable cavitation during sonication. The quantified SCD showed significant increase at the highest pressure amplitude (0.60 MPa), indicating that a minimum SCD threshold was needed to induce BBB opening. Interestingly, when microbubbles were used as the contrast agent, significant SCD increase was seen across all sonication pressures regardless of the outcome of BBB opening. The SCD value for the microbubble group at the lowest pressure (902 V·s at 0.15 MPa) was much higher than that for the nanodroplet group even at the highest pressure (353 V·s at 0.60 MPa), suggesting that the SCD threshold was contrast agent-dependent. This result was further demonstrated in Fig. 8, in which significant difference was calculated between threshold baselines (i.e., negative cases for which no dextran delivery was observed). This finding was very important for acoustic emission based BBB

opening monitoring since contrast agent-specific thresholds would then need to be investigated separately. The significant increase in ICD for microbubbles at 0.60 MPa in combination with the minor tissue damage observed based on histological evaluation also suggested that inertial cavitation could be used as a safety indicator to avoid undesired bioeffects.

The linear correlation between the calculated SCD and the relative fluorescence enhancement was slightly weaker for microbubbles than that for nanodroplets (Fig. 7). Two factors could be contributing to the dissociation between these two parameters for microbubble-mediated BBB opening. First, our passive cavitation detection was sensitive to signals arising from any microbubbles in the beam path of the transducers, including those that were outside the focal region. For those microbubbles, it was possible that their contribution to the nonlinear acoustic emission signal was not proportionate to their contribution to the fluorescence enhancement, and therefore weakening the correlation between these two parameters. On the other hand, the acoustic emission signal during nanodroplet-mediated BBB opening only came from those nanodroplets vaporized within the focal region, as opposed to the inherent signal from the off-targeted microbubbles. This could also explain the significantly lower BBB opening threshold determined based on acoustic emission for nanodroplets (Fig. 8). Secondly, in the present study, the extent of the BBB opening was determined based on intensity measured using fluorescence microscopy. Despite the fact that precautions were taken during image capturing to minimize over-exposure, it might be possible that signal saturation could still occur after large amount of dextran delivery. As a result, the level of the BBB opening might be underestimated at higher pressures, especially for the microbubble group (Fig. 5J).

Based on optical microscopy, Sheeran et al. [23] previously reported that it was possible to have microbubbles formed from acoustically vaporized nanodroplets larger than those in the original parent microbubble suspension. One possible explanation for such an increase in size was bubble fusion or Ostwald ripening resulted from vaporization of nanodroplets in very close proximity at high concentration. The resulting increased size distribution of the activated nanodroplets could attribute to the lower SCD threshold for BBB opening using nanodroplets than that with microbubbles (Fig. 8) [26]. In addition, the larger bubbles formed from vaporized nanodroplets could amplify the shear stress induced during stable cavitation to other neighboring cells along the microvessels [13] at higher efficiency than traditional smaller microbubbles, thus increasing the number of BBB disrupted sites to facilitate a more homogenous dextran delivery distribution without the concern of causing undesired inertial cavitation at higher pressures (Figs. 5D and 5E). Finally, it is also possible that the vaporization of the volatile perfluorocarbon droplets used here introduced additional mechanical effects beyond the standard microbubble cavitation effects, aiding permeabilization without being registered as ICD or SCD. Recent studies have shown that the expansion of less volatile perfluorocarbon droplets occurs on a microsecond timescale [43], but little is known on the magnitude of the shear stresses introduced by the rapid expansion of more highly volatile droplets, such as those formed from perfluorobutane.

Our initial results utilizing nanodroplets as the mediating agent for FUS-induced BBB opening offer a new means of developing this technology for potential extravascular targeted drug delivery in the brain parenchyma. Our findings indicate that a potential advantage for employing nanodroplets as the contrast agent is that they would only be activated within the transducer focal zone when sufficient acoustic energy is reached. This yields the possibility of enhancing specificity through focusing of the ultrasound beam, thus promising more spatial and temporal control of the targeting procedure to minimize undesired thermal and cavitation-based bioeffects. Moreover, it eliminates the “noise” signal generated from non-activated nanodroplets, allowing more accurate monitoring of BBB

opening based on passive acoustic emission detection (Fig. 7). Additionally, the nanodroplets could potentially be conjugated with targeting ligands to allow for more specific active-targeting applications on the molecular and cellular level [44]. The previously described post-labeling technique [45] for generating targeted microbubbles could easily be translated to produce targeted nanodroplets. This “shake and bake” methodology offers the possibility of developing the nanodroplet-mediated FUS-induced BBB opening technology for potentially a large number of molecular targets for future personalized nanomedicine.

5. Conclusion

In this paper, we have performed an initial feasibility study to explore the utilization of phase-shift nanodroplets for targeted drug delivery in the brain with FUS-induced BBB opening. Significant dextran delivery was achieved in the mouse hippocampus using acoustically-activated nanodroplets at clinically relevant pressure amplitudes. Passive cavitation detection was used in the attempt to establish a correlation between the amount of dextran delivered in the brain and the acoustic emission recorded during sonication. Conventional microbubbles with the same lipid shell composition and perfluorobutane core as the nanodroplets were also used to compare the efficiency of FUS-induced dextran delivery. It was found that nanodroplets had a higher BBB opening pressure threshold but a lower stable cavitation threshold than the microbubbles. More homogeneous dextran delivery within the targeted hippocampus was achieved using nanodroplets without inducing inertial cavitation or compromising safety. Our results offered a new means of developing the FUS-induced BBB opening technology for potential extravascular targeted drug delivery in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Experimental setup of *in vivo* FUS-induced BBB opening and transcranial cavitation detection.

Fig. 2.

Representative size distributions of nanodroplet (A) and microbubble (B) suspensions as measured by Malvern Zetasizer and Coulter Multisizer, respectively. (A) The averaged number-weighted mean, median and mode diameters for the nanodroplet sample shown were 206 ± 93 nm, 190 nm and 164 nm, respectively. All nanodroplet samples were diluted by 50 vol% prior to injection. (B) The averaged number-weighted mean, median and mode diameters for the microbubble sample shown were 1.36 ± 0.02 μm , 1.10 μm and 0.79 μm , respectively. All microbubble samples were diluted to 8×10^8 particle/mL prior to injection.

0.35 MPa

0.45 MPa

Fig. 3.

Still-frame images showing nanodroplet activation within the first two cycles of the pulse as a function of sonication pressure. Frames A–D and G–J were taken in the first rarefactional half-cycle, while E–F and K–L were taken in the following compressional cycle. No nanodroplet vaporization was detected at 0.35 MPa (left) while micron-scale acoustically-responsive bubbles were seen expanding in the rarefactional cycle at 0.45 MPa (G–J). When transitioned to the next positive pressure cycle (K, L), the newly formed bubbles were observed to respond to the acoustic pressure by compressing until no longer visible in the field of view. Times shown are relative to the start of the pulse, and the scale bars indicate 5 μm .

Fig. 4.

Normalized fluorescence enhancement between the targeted and the control hippocampi using either nanodroplets (A) or microbubbles (B) to mediate BBB opening at distinct sonication pressures. The normalized fluorescence enhancement with pressure followed a linear relationship with correlation coefficient R^2 being 0.76 and 0.94 for nanodroplets and microbubbles, respectively. Significant dextran delivery was observed at pressures higher than 0.45 MPa using nanodroplets while the BBB opening threshold was decreased down to 0.30 MPa when microbubbles were administered. All numbers are reported as mean $\pm$ standard deviation. (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$)

Fig. 5.

Representative fluorescence images comparing the targeted and the control (insets) hippocampi when nanodroplets (top) or microbubbles (bottom) were used to mediate BBB opening at various sonication pressures. The left hippocampus was sonicated in the presence of either nanodroplets or microbubbles and fluorescently-labeled 3-kDa dextran. Sonications were achieved at distinct peak-rarefactional pressures: 0.15 MPa (A&F), 0.225 MPa (B&G), 0.30 MPa (C&H), 0.45 MPa (D&I) and 0.60 MPa (E&J). The scale bar in A depicts 1 mm.

Fig. 6.

Quantified acoustic emission detected during BBB opening at various sonication pressures. Stable cavitation implied that vaporized nanodroplets or microbubbles underwent stable nonlinear oscillation during sonication, while inertial cavitation signified particle fragmentation. Significant SCD increase was detected after nanodroplet injection at the highest pressure level (A), but no significant ICD increase was measured (B). On the other hand, significant increase of SCD was detected for all pressures after microbubble administration (C), and ICD increase was detected at the highest acoustic exposure level (D). All numbers are reported as mean $\pm$ standard deviation. (ns: not significant; *: $P < 0.05$; ***: $P < 0.001$)

Fig. 7.

Correlation between differential stable cavitation dose (SCD) and fluorescence enhancement. The SCD was normalized based on the background emission signal measured prior to contrast agent injection. Linear correlations were obtained for both (A) nanodroplets ($R^2=0.74$) and (B) microbubbles ($R^2=0.67$).

Fig. 8.

Stable cavitation dose (SCD)-indicated BBB opening threshold. The SCD was grouped based on whether significant dextran delivery was detected. Each individual brain was concluded to have significant BBB opening when the difference in fluorescence intensity between the sonicated and the control hippocampi was two standard deviations greater than the average value obtained for the corresponding sham group. Sonication using microbubbles as the contrast agents produced a much wider range of SCD values comparing to when nanodroplets were used. The SCD threshold for predicting BBB opening appeared to be contrast agent dependent. 7 out of 10 mice showed significant dextran delivery with SCD values greater than 74 V·s using nanodroplets while 92.3% of the animals showed significant dextran delivery at SCD above 1.4 kV·s using microbubbles. The slightly weaker threshold for the nanodroplet group indicated secondary mechanism other than cavitation could induce the BBB opening. (*: $P < 0.05$; ***: $P < 0.001$)

Fig. 9.

Representative histological images of the targeted and control hippocampi. The animals were sacrificed 1 h after sonication. (Left) No erythrocyte extravasations, dark neurons, gross hemorrhage or microvacuolations were observed when nanodroplets were used to mediate BBB opening at either 0.45 MPa or 0.60 MPa. (Right) When microbubbles were used, erythrocyte extravasations or dark neurons were not seen at 0.45 MPa. However, small clusters of erythrocyte extravasations and a few dark neurons were observed (arrow heads in O), indicating minor damage, after sonication at 0.60 MPa. The boxed regions in all 4 $\times$ images are further zoomed into 10 $\times$. The scale bars in A and C depict 1 mm and 100 μ m, respectively.

Table 1

Summary of the experimental groups.

Group	Contrast agent	Number of mice per experimental condition ^{<i>I</i>}				
		Sham	Acoustic pressure (MPa)			
			0.15	0.225	0.30	0.45 0.60
1	Nanodroplet	6	6	6	7	7 8
2	Microbubble	5	7	7	7	7 7

^{*I*} Number shown including up to 2 mice per experimental condition used for histology examination.

Table 2

Summary of the fluorescence imaging analysis.

Contrast agent	Acoustic pressure (MPa)	Fluorescence enhancement $\pm$ S.D. (%)	Number of mice with significant dextran delivery/total number of mice evaluated using fluorescence microscopy
Nanodroplet	Sham	21.41 $\pm$ 25.97	0/6
	0.15	11.04 $\pm$ 30.75	0/5
	0.225	12.73 $\pm$ 38.81	0/5
	0.30	18.70 $\pm$ 36.07	1/6
	0.45	91.84 $\pm$ 67.91	3/5
	0.60	155.09 $\pm$ 55.03	7/7
Microbubble	Sham	-7.67 $\pm$ 15.41	0/5
	0.15	5.95 $\pm$ 17.04	0/5
	0.225	72.26 $\pm$ 114.28	3/5
	0.30	184.10 $\pm$ 110.77	5/5
	0.45	353.64 $\pm$ 46.23	5/5
	0.60	487.82 $\pm$ 271.31	5/5