DELIVERY OF LIPOSOMES WITH DIFFERENT SIZES TO MICE BRAIN AFTER SONICATION BY FOCUSED ULTRASOUND IN THE PRESENCE OF MICROBUBBLES

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Abstract—Imaging or therapeutic agents larger than the blood–brain barrier’s (BBB) exclusion threshold of 400 Da could be delivered locally, non-invasively and reversibly by focused ultrasound (FUS) with circulating microbubbles. The size of agents is an important factor to the delivery outcome using this method. Liposomes are important drug carriers with controllable sizes in a range of nanometers. However, discrepancies among deliveries of intact liposomes with different sizes, especially those larger than 50 nm, across the BBB opened by FUS with microbubbles remain unexplored. In the present study, rhodamine-labeled long-circulating pegylated liposomes with diameters of 55 nm, 120 nm and 200 nm were delivered to mice brains after BBB disruption by pulsed FUS with microbubbles. Four groups of peak rarefractional pressure and microbubble dosages were used: 0.53 MPa with 0.1 μL/g (group 1), 0.53 MPa with 0.5 μL/g (group 2), 0.64 MPa with 0.1 μL/g (group 3) and 0.64 MPa with 0.5 μL/g (group 4). The delivery outcome was observed using fluorescence imaging of brain sections. It was found that the delivery of 55-nm liposomes showed higher success rates than 120-nm or 200-nm liposomes from groups 1–3. The result indicated that it may be more difficult to deliver larger liposomes (>120 nm) passively than 55-nm liposomes after BBB opening by FUS with microbubbles. The relative fluorescence area of 55-nm liposomes to the total area of the sonicated region was statistically larger than that of the 120-nm or 200-nm liposomes. Increasing peak rarefractional pressure amplitude or microbubble dose could induce more accumulation of liposomes in the brain using FUS with microbubbles. Moreover, the distribution pattern of delivered liposomes was heterogeneous and characterized by separated fluorescence spots with cloud-like periphery surrounding a bright center, indicating confined diffusion in the extracellular matrix after extravasation from the microvasculature. These findings are expected to provide useful information for developing FUS with microbubbles as an effective trans-BBB liposomal drug delivery strategy. (E-mail: danguo@szu.edu.cn) © 2016 World Federation for Ultrasound in Medicine & Biology.

Key Words: Focused ultrasound, Microbubbles, Blood–brain barrier, Delivery, Liposomes, Different size.

INTRODUCTION

It has been a great challenge to deliver therapeutic drugs to the brain due to the existence of the blood–brain barrier (BBB) (Pardridge 2005). Animal studies have demonstrated that the BBB endothelial lining or tight junctions could be opened locally, non-invasively and reversibly by focused ultrasound (FUS) in the presence of microbubbles in recent years (Choi et al. 2007; Hynynen et al. 2001; Shen et al. 2014). This technique has provided potential availability in targeted drug delivery for brain diseases (Aryal et al. 2014).

There have been many animal studies conducted on the delivery of various imaging molecules or therapeutic agents across the BBB using FUS or unfocused ultrasound with circulating microbubbles, such as Evans bluedye (Beccaria et al. 2013), magnetic resonance...
imaging (MRI) contrast agents (Hynynen et al. 2001), dextran (Chen and Konofagou 2014; Choi et al. 2010b), chemotherapy agents (Aryal et al. 2013; Treat et al. 2007; Treat et al. 2012; Yang et al. 2012), antibodies (Kinoshita et al. 2006), magnetic nanoparticles (Fan et al. 2013), gene therapy vectors (Alonso et al. 2013), stem cells (Burgess et al. 2011) etc. A recent study conducted on rhesus macaques demonstrated that BBB disruption could be achieved with a probability of 50% using 220 kHz FUS with 10-ms bursts of 1 Hz repetition frequency at 0.15 MPa for 70 s without evident histologic or functional damage (McDannold et al. 2012).

Another study conducted on two male rhesus monkeys showed that targeted and safe BBB opening could be achieved by a single-element 500-kHz spherical transducer ultrasound system with a real-time monitoring technique based on cavitation spectral analysis (Marquet et al. 2014). These results supported the feasibility of this unique non-invasive and targeted drug delivery approach of the central nervous system.

Extensive attention was paid to investigate delivering various drugs to treat animals with brain diseases using ultrasound with microbubbles, and several studies found that the molecular weight (MW) of an agent had obvious influence on delivery to brain. In the experiment on rhesus macaques, lower-level signal enhancement was observed with gadofosveset trisodium (MW: 67 kDa), an MRI contrast agent that binds to albumin in the blood, when compared to Gd-DPTA, another MRI agent with a much smaller molecular weight (MW: 938 Da) (McDannold et al. 2012). A fluorescence imaging study by Chen and Konofagou (2014) showed that fluorescent dextran with MWs up to 2000 kDa could be delivered to mice brains, but needed a much higher pressure amplitude (0.84 MPa) than that for delivery of 3-kDa dextrans. These results suggested that the MW of particles is an important factor to their extravasation from brain microvasculature into parenchyma using ultrasound with microbubbles. However, the effect of the size of agents, an important characteristic of some drug carrier systems like liposomes, on the delivery outcome using ultrasound with microbubbles has not been widely investigated.

Liposomes (enclosed phospholipid bilayer structures) are proposed as drug carrier systems to deliver many drugs, including anti-cancer, anti-fungal and antibiotic drugs (Allen and Cullis 2013). They have been used to improve the therapeutic efficiency of drugs by enhancing drug accumulation, prolonging biological half-life or reducing toxicity. For example, Doxil is an anti-cancer liposomal drug with a diameter of 80–100 nm that showed enhanced therapeutic effect on extracranial cancers, such as breast and ovarian cancers, over free doxorubicin (Barenholz 2012; Gabizon et al. 1994; Symon et al. 1999). However, liposomal drugs are impeded by the BBB because they are usually too large to cross (Allen and Cullis 2013; Pardridge 2005). Although many studies demonstrated that large molecules could be delivered to the brain by ultrasound with microbubbles, the delivery of intact liposomes, especially those larger than 50 nm, across the BBB opened by ultrasound combined with microbubbles remains uninvestigated. Specifically, the discrepancies among liposomes with different sizes moving across the BBB using this method are unknown.

The main purpose of this study was to investigate delivery of intact liposomes with different sizes after the BBB opening was induced by FUS in the presence of circulating microbubbles. Effects of different pressure amplitudes and microbubble dosages on delivery outcomes were also studied. In particular, fluorescently labeled stabilized long-circulating pegylated (PEG) liposomes with three sets of diameters of 55 nm, 120 nm and 200 nm were prepared and administered intravenously after FUS sonication. Normal mice were sonicated with different peak rarefational pressures and microbubble dosages. The evaluation of delivery outcome was performed using fluorescence imaging of brain slices. Histologic analysis was carried out to examine tissue damaging effects.

MATERIALS AND METHODS

Animal preparation

Experiments were performed on 76 female BALB/c mice (Guangdong Medical Laboratory Animal Center, Foshan, China) (6-wk-old; 20 ± 2 g). They were housed in sterile isolated cages with a 12 h light/dark cycle at constant temperatures (24–26°C) and humidity (30–50%). Animal care and experiments were approved by the Animal Care and Use Committee of School of Medicine in Shenzhen University.

Before sonication, the mice were anesthetized with 1.5% isoflurane (RWD Life Science, Co., Ltd., Shenzhen, China), and placed on a heating pad with a constant temperature of 37°C to maintain their body temperature. Then, the hair on the scalp of each mouse was removed with depilatory cream.

In our preliminary experiment, the BBB could be opened successfully and non-invasively without any hemorrhage and neuron damaging effects at a peak rarefational pressure of 0.53 MPa and with a microbubble dosage of 0.1 μL/g. In order to explore the effects of the parameters on the delivery outcomes of liposomes, four groups of peak rarefational pressures and microbubble dosages were used in the present study: 0.53 MPa with 0.1 μL/g (group 1), 0.53 MPa with 0.5 μL/g (group 2), 0.64 MPa with 0.1 μL/g (group 3) and 0.64 MPa with 0.5 μL/g (group 4). The experiment groups are shown in Table 1. For each group, 18 mice were sonicated transcranially using FUS with
Ningbo, China) with a temperature of 65°C. Then, the dried phospholipid film was hydrated under vacuum (Rotavapor R-210, Buchi, Switzerland) for at least 2 h. The 0.01 M phosphate buffer saline (PBS) mixed with 10 vol% glycerol and 0.22 M propylene glycol was filtered using sterile syringe filters (Merck Millipore, Billerica, USA). The aper- ture diameter used in the measurement was 20 μm with the corresponding measurement range 0.4–12 μm.

Liposome preparation
Rhodamine-labeled stabilized long-circulating PEG liposomes were prepared by the lipid film hydration and extrusion method (Shen et al. 2008). Briefly, a chloroform solution of egg phosphocholine, cholesterol, DSPE-PEG2000 (Lipoid) and rhodamine-labeled DHPE (Life Technologies Corporation, Eugene, USA) in the molar ratio of 10:5:0.8:0.1 was dried on a rotary evaporator (Buchi Labortechnik AG) under high vacuum for at least 2 h to form a thin lipid film on the side of a round-bottom flask. Large and multilamellar liposomes were formed by hydrating the lipid film with 0.01 M filtered PBS. The final lipid concentration was 10 mg/mL. Then, the lipid suspension was forced through a polycarbonate (PC) membrane with a defined pore size using a Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster, USA). The homogenization of liposomes could be achieved by at least 20 passes through the PC membrane. In the present study, PC membranes with pore sizes of 0.05 μm, 0.1 μm and 0.2 μm were used to obtain liposomes of three specific sizes.

The size distribution and polydispersity index (a measure of the width of the particle size distribution) of liposomes was measured by the dynamic light scattering method using a Zetasizer Nano-ZS90 (Malvern Instruments, Malvern, UK). Liposomes with a polydispersity index less than 0.1 were used in this study and typically referred to as monodispersed particles.

Experimental setup
The experimental setup is illustrated in Figure 1a. The FUS beam was generated by a single-element spherical transducer (center frequency: 1.282 MHz; focal length: 51.3 mm). The transducer was immersed in a cone filled with degassed water. The tip of the cone was capped with a thin polyurethane membrane providing an acoustic window that allows the ultrasound beam to pass through. The focal zone of the ultrasound beam was 2.5 mm beneath the cone tip. The transducer with the cone was mounted on a 3-D motor-controlled positioning system (Zolix Instruments Co., Ltd., Beijing, China) and driven by a 50-dB power amplifier (325LA, Electronics & Innovation, Ltd., Rochester, USA). The excitation waveform was generated by a function generator (AFG3102C, Tektronix, Inc., Beaverton, USA).

The dimensions of the beam generated by the transducer were measured using a needle hydrophone (HGL-0200, Onda Corporation, Sunnyvale, USA) in an Acoustic Intensity Measurement System (Onda

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Lipo = liposome.
* All mice were sonicated with the same time bursts, repetition frequency and duration, which was 7.8 ms, 1 Hz and 60 s, respectively.

Microbubble generation
Microbubbles with a lipid shell and a perfluoropropane gas core were prepared in-house according to the protocol described by a previous study (Feshitan et al. 2009). The microbubbles were coated with DSPC/DSPE-PEG2000 (Lipoid, Ludwigshafen, Germany) at a molar ratio of 9:1. The phospholipids were dissolved in chloroform (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and the organic solvent was evaporated under vacuum (Rotavapor R-210, BUCHI Labortechnik AG, Flawil, Switzerland) for at least 2 h. The 0.01 M phosphate buffer saline (PBS) mixed with 10 vol% glycerol and 0 vol% propylene glycol was filtered using 0.22 μm sterile syringe filters (Merck Millipore, Billerica, USA). Then, the dried phospholipid film was hydrated with the PBS mixture to a final total concentration of 3 mg/mL. The lipid suspension was sonicated in a water bath (SB120 DT, NingBo Scientz Biotechnology Co., Ltd., Ningbo, China) with a temperature of 65°C to disperse the lipid aggregates. After sealing the lipid suspension within a 3-mL glass serum vial, the air headspace was exchanged with perfluoropropane gas of 99.999% purity (Suzhou Xundong gas technology, Suzhou, China). Before sonication, the microbubbles were generated by agitating the vial mechanically at a speed of 4500 oscillations/min for 20 s using an amalgamator (Hangzhou ZhongRun Medical Instrument Co., Ltd., Hangzhou, China).

The concentration and particle size distribution were measured with the Coulter Counter Multisizer 4 (Beckman Coulter, Inc., Miami, USA) after dilution with Isoton II diluent (Beckman Coulter, Inc., Brea, USA). The aperture diameter used in the measurement was 20 μm with the corresponding measurement range 0.4–12 μm.
Corporation). The lateral and axial full-width at half maximum (FWHM) intensity of the beam were 1.35 mm and 8.60 mm, respectively, as shown in Figure 1b.

**Liposome delivery procedure**

Each mouse was placed in the prone position and anesthetized with 1.5% isoflurane. The head was immobilized with a stereotaxic apparatus (RWD Life Science, Co., Ltd.) as shown in Figure 1a. The body temperature of each mouse was maintained through a heating pad. The transducer with the cone was moved to the top of the mouse head by the positioning system. A thin layer of ultrasound gel was used between the polyurethane membrane and mouse scalp skin as a coupling medium to allow ultrasonic waves to transmit to the mouse brain with minimal attenuation.

Before sonication, a tail vein catheter was inserted using a 27-gauge needle. Then, a 0.1 mL bolus of diluted microbubble solution was injected and circulated for about 15 s. Next, pulsed FUS was applied on the left hemisphere of each mouse for 60 s with a burst length of 7.8 ms (10 000 cycles) and a repetition frequency of 1 Hz. The right hemisphere was not sonicated and was used as a control. The peak rarefational pressure amplitude presented in this study was not corrected due to skull attenuation effects.

In the present study, liposomes were administered after sonication because they could be disrupted during

Fig. 1. (a) Experimental setup; (b) beam profile of the focused ultrasound beam at its focal zone.
sonication (Geers et al. 2011). Thus, after sonication, a 0.1-mL liposomes solution was administered through the tail vein catheter. For the mice injected with Evans blue, 2% solution was administered in a dose of 5 μL/g.

**Histology**

Four h after liposomes or Evans blue injection, all the mice were anesthetized and perfused with saline through the left ventricle until clear perfusion fluid effused from the right atrium. Then, 4% of freshly prepared paraformaldehyde solution (PFA) was perfused. After heart perfusion, the brain tissues were extracted from the skull and post-fixed in 4% PFA at 4°C overnight. For cryoprotection, the brains were put into 10%, 18% and 30% sucrose solution until they sank. Then they were quickly frozen in −70°C isopentane (Sinopharm Chemical Reagent Co., Ltd.). The brains were embedded in an optimum cutting temperature compound (Sakura Finetek, Zoeterwoude, The Netherlands) for 1 h at −20°C and sectioned serially in the coronal direction into 30-μm slices using a cryostat (CM1860, Leica Biosystems, Wetzlar, Germany). To assess tissue damaging effects, several slices of 10-μm thickness of each mouse brain were sectioned and stained with hematoxylin and eosin and examined under brightfield using a microscope (BX53, Olympus, Ina, Nagano-ken, Japan) with a 40× objective.

**Image analysis**

To detect the delivery outcome of rhodamine-labeled liposomes, brain slices were imaged using a fluorescence microscope (Olympus) equipped with a motorized XY scanning stage and a filter set (excitation: 530–550 nm; broadband emission filter: >575 nm). Multiple images were acquired using the motorized stage and an DP72 digital camera (Olympus). These images were then automatically aligned and stitched together using cellSens Dimension software (Olympus) into a single high-resolution image. In this way, the image of the whole brain section could be obtained. One image of the unsonicated right hemisphere was taken first as the background and all images were recorded under the same fluorescence imaging parameters. Delivery of liposomes was considered to be successful if there was enhanced fluorescence signal in the sonicated region compared with the image of the unsonicated region.

Fluorescence images were analyzed using cellSens Dimension software (Olympus). First, the sonicated and unsonicated areas were manually outlined. The spatial average fluorescence intensity in the unsonicated region was calculated as background auto-fluorescence. Fluorescence signals in the sonicated region were then segmented above the background fluorescence intensity. Three sets of values were then quantified to analyze the trans-BBB delivery outcomes of liposomes with different sizes: the relative area of fluorescence to the total area of the sonicated region, the total fluorescence intensity over the sonicated region and the number of fluorescent spots with areas larger than 100 μm². The quantitative results in each group across three sets of liposomes were compared using one-way analysis of variance. The least significant difference method was used to compare multiple pairs within each group, and p < 0.05 was considered to be statistically significant.

**RESULTS**

**Size distributions of microbubbles and liposomes**

Microbubbles prepared in this study were coated with a lipid shell. The size distribution and concentration were measured using a Coulter Counter Multisizer 4 (Beckman Coulter, Inc.). Figure 2 shows the size distribution and micrograph of prepared microbubbles. The mean diameter of the freshly prepared microbubbles was 1.34 μm. Microbubbles with a diameter less than 2.06 μm accounted for 90% in total. The concentration was 2.0×10⁸ per mL. In contrast to the concentration of the commercial SonoVue microbubbles (5.0–8.0×10⁸ per mL, Bracco, Milan, Italy), it was enough for animal study.

The size of liposomes used in the present study was controlled through an extrusion method. The lipid suspension was forced through a PC membrane with pore sizes of 0.05 μm, 0.1 μm or 0.2 μm. The liposomes were labeled with rhodamine so the extravasation from brain microvasculature could be detected using fluorescence imaging. Figure 3 shows the size distribution of the three sets of liposomes used in this study. After at least 20 passes through the PC membranes, the mean diameters of liposomes were 55.9 nm, 121.4 nm and 204.0 nm, respectively. The polydispersity index was 0.074, 0.025 and 0.054 for the 55-nm, 120-nm and 200-nm liposomes, indicating narrow size distributions around the mean diameter.

**BBB opening confirmed by Evans blue extravasation**

In each group, one mouse was injected intravenously with Evans blue, which could bind to albumin (MW: 67 kDa) once entering circulation. Evans blue-tagged albumin extravasated from vasculature into the brain parenchyma only when the BBB was opened. Thus, blue coloration of the brain indicated successful BBB opening by FUS with microbubbles. In this study, the FUS was applied to the left hemisphere. As shown in Figure 4, Evans blue extravasation was observed only on the left hemisphere, while it was invisible on the unsonicated right hemisphere. Moreover, the degree and area of Evans blue dispersion increased when the acoustic pressure was raised from 0.53 MPa to 0.64 MPa, as shown in Figures 4a and 4c. When the microbubble dose was increased from
0.1 μL/g to 0.5 μL/g, the Evans blue extravasation was also enhanced, as shown in Figure 4.

**Delivery of liposomes with different sizes across the BBB**

In this study, rhodamine-labeled liposomes were systemically administered to mice after sonication. Undelivered liposomes were washed away from the circulation by cardiac perfusion before brain extraction and sectioning. Trans-BBB delivery of liposomes was observed using fluorescence microscopy. Enhanced fluorescence signals could be detected only when liposomes penetrated across the BBB. The numbers of mice with successful delivery of liposomes in four groups are summarized in Table 1. For the mice that were administered 55-nm liposomes, the deliveries were achieved in all mice from the four groups. However, in group 1 (0.53 MPa; 0.1 μL/g), one out of six mice injected with 120-nm and two out of six mice injected with 200-nm liposomes did not show significant fluorescence signals. Fewer numbers of mice with successful delivery outcomes of 120-nm or 200-nm liposomes were obtained than in the case of 55-nm liposomes in group 2 (0.53 MPa; 0.5 μL/g) or group 3 (0.64 MPa; 0.1 μL/g). In group 4 (0.64 MPa; 0.5 μL/g), deliveries of liposomes were successful in all the mice administered liposomes with three sizes. It suggested that it may be more difficult to deliver liposomes with diameters >120 nm than the 55-nm liposomes after BBB disruption induced by FUS with microbubbles.

Fluorescence images of coronal brain slices were captured to observe distribution characteristics of liposomes penetrating across the BBB, as shown in Figure 5. In general, separated spots with fluorescent signals scattered spatially. The distribution pattern of extravasated liposomes was heterogeneous rather than homogeneous. For group 1 (0.53 MPa; 0.1 μL/g), in the brain sections of mice injected with 200-nm liposomes, fewer fluorescent spots were observed under microscope compared with the other two smaller-size liposomes (Fig. 5a–c). When the microbubble dose increased to 0.5 μL/g, more spots appeared within the field of view for all three sets of liposomes (Fig. 5d–f). Moreover, the 55-nm liposomes case showed more spots compared with the 120-nm or 200-nm liposomes cases. In group 3, at a higher pressure of 0.64 MPa with a microbubble dose of 0.1 μL/g, fluorescence spots with larger areas were visible compared with the cases of pressure 0.53 MPa (Fig. 5g–i). Moreover, with increasing size of liposomes, areas of fluorescence decreased. In group 4, with a higher pressure (0.64 MPa) and microbubble dosage (0.5 μL/g), distribution of 55-nm liposomes across the BBB was significantly more diffusive than the other two larger-size liposomes (Fig. 5j–l). The extravasation of three sets of liposomes from the microvasculature was enhanced compared with group 3. In general, the extent of extravasation of liposomes through the BBB opening by FUS and microbubbles was related to the size of the liposomes.

The significance of differences among delivery outcomes of liposomes with three sizes was supported by quantified fluorescence analysis as shown in Figure 6. The relative areas of fluorescence to the total area of the sonicated region were calculated to elucidate the extent of liposomes delivered (Fig. 6a). In group 1 (0.53 MPa; 0.1 μL/g), 55-nm liposomes delivered to brain
tissue were extremely confined, accounting for only 1.2% of the sonicated region, although it was significantly larger than the 200-nm liposomes. From group 2 to group 4, the relative areas of fluorescence of 120-nm and 200-nm liposomes were statistically smaller than the 55-nm liposomes ($p < 0.05$). The total fluorescence intensities in the sonicated regions were also measured to represent the relative amounts of liposomes delivered across the BBB (Fig. 6b). The total fluorescence intensities of 55-nm liposomes delivered across the BBB were 1.83-fold, 3.27-fold, 2.53-fold and 1.41-fold of those of the 200-nm liposomes under the experimental settings in group 1, 2, 3 and 4. These results indicated that extravasation of liposomes across the BBB by sonication with microbubbles varied with the size of the liposomes.

The study also investigated the effects of increasing peak rarefational pressure or microbubble dosage on the delivery outcomes of liposomes. Increasing acoustic pressure or microbubble dosage or both produced a statistically significant increase in the relative area of...
fluorescence for the three sets of liposomes ($p < 0.05$). In group 4 (0.64 MPa; 0.5 μL/g), the relative areas of 55-nm, 120-nm or 200-nm liposomes extravasated across the BBB were 7.17-fold, 5.72-fold and 4.03-fold of the ones in group 1 (0.53 MPa; 0.1 μL/g). In addition, the total fluorescence intensities in group 4 increased to 4.09-fold, 4.90-fold and 5.29-fold, respectively, of those in group 1 for liposomes with three sizes. These results indicated that increasing peak rarefactional pressure amplitude or microbubble dose could induce more accumulation of liposomes in the brain using FUS with microbubbles.

Once liposomes crossed the BBB, they would diffuse within the brain’s extracellular space. In the present study, it was observed that most of the fluorescence spots were characterized by cloud-like periphery surrounding a bright center. For the two larger-size liposomes, the periphery area was relatively smaller than that of the 55-nm liposomes, indicating confined diffusion. Thus, the spots with areas larger than 100 μm$^2$ were counted, and are illustrated in Figure 6c. Within each group under the same parametric condition, the number of fluorescence spots with areas larger than 100 μm$^2$ for the 55-nm liposome was significantly greater than in the case of the 200-nm liposome, which was consistent with the fluorescence observation. The numerical values for the 55-nm liposomes across the four groups were 67, 210, 100 and 280, respectively, and were 2.14-fold, 1.98-fold, 2.05-fold and 1.27-fold of those of the 200-nm liposomes. Since rhodamine was labeled on the lipid shell, the variance of fluorescence spot areas indicated discrepancy of physical diffusion capability of liposomes with different sizes. The findings suggested that it was easier for 55-nm liposomes to diffuse through the brain’s extracellular space than it was for larger liposomes.

Fig. 5. Fluorescence images of coronal mice brain sections for 55-nm liposomes (a, d, g, j), 120-nm liposomes (b, e, h, k) and 200-nm liposomes (c, f, i, l). The mice were sonicated with different peak rarefational pressures and microbubble dosages. Scale bar: 500 μm.
Histologic examinations

Mouse brains were harvested after cardiac perfusion and sectioned coronally. Hematoxylin and eosin staining was used to detect hemorrhage and tissue damaging effects. Figure 7 shows histologic images of the sonicated left hemisphere and the contralateral unsonicated right hemisphere. When mice brains were exposed to a larger acoustic pressure of 0.64 MPa with 0.1 μL/g microbubbles, no obvious neuron damage or tissue vacuolation was visible, despite erythrocyte extravasation, as indicated in Figure 7c. However, punctate hemorrhages occurred when the mice brains were sonicated at a peak rarefractional pressure of 0.64 MPa with a microbubble dosage of 0.5 μL/g. At the site of the hemorrhages there appeared damaging effects to neurons as shown in Figure 7g.

DISCUSSION

A growing number of animal studies showed that imaging or therapeutic agents larger than the BBB’s exclusion threshold of 400 Da could be successfully delivered by FUS with microbubbles (Alonso et al. 2013; Choi et al. 2007; Choi et al. 2010a; Hynynen et al. 2001; Kinoshita et al. 2006; Marquet et al. 2014; Shen et al. 2014). Several studies demonstrated that the molecular weight of agents is an important factor to the delivery outcome using this method (Chen and Konofagou 2014; Choi et al. 2010b). However, the influence of the size of particles has rarely been studied. Based on the fact that liposomes are an important drug delivery system with controllable sizes (Allen and Cullis 2013), stabilized long-circulating PEG liposomes with three different diameters (55 nm, 120 nm and 200 nm) were delivered to mice brains by FUS with microbubbles in the present study. It was found that 55-nm liposomes could be delivered at higher success rates with larger extravasation areas than 120-nm or 200-nm liposomes. The discrepancy among deliveries of these three sets of liposomes suggests that it may be more difficult for larger liposomes (diameters >120 nm) to penetrate into brain parenchyma from BBB disruption sites induced by FUS with microbubbles. There have been two studies using other agents with a comparable size of 55 nm that were delivered to normal animals (Chen and Konofagou 2014; Marty et al. 2012). Using dextrans with different molecular weights, it was found that 2000-kDa dextrans (D₄₅: ~54 nm) could not be delivered to mice brains until the acoustic pressure was raised to 0.84 MPa (Chen and Konofagou 2014). In their experiment, Definity microbubbles (1.1–3.3 μm, Lantheus Medical Imaging, Billerica, Massachusetts) were used at a dose of 0.05 μL/g. In another experiment performed on Sprague Dawley rats, it was found that a super-paramagnetic MR contrast agent with a hydrodynamic diameter 65 nm could penetrate across the BBB.
at 0.45 MPa in situ using SonoVue microbubbles (2–6 µm; 2 µL/g) (Marty et al. 2012).

Previous studies showed that the magnitude of BBB disruption increased as acoustic pressure amplitude increased, but vascular damage would be induced at high pressures (Chopra et al. 2010; McDannold et al. 2008). BBB disruption is more likely related to inertial cavitation when increasing acoustic pressure above 0.51 MPa (Chen and Konofagou 2014). Since acoustic pressures used in this study were above 0.51 MPa, the delivery of liposomes may probably be associated with inertial cavitation effects. Noticeably, increasing acoustic pressure could enhance delivery of liposomes. Meanwhile, red blood cell extravasation occurred by histologic examination, although there was no obvious neuron damage when increasing peak rarefractional pressure or microbubble dosage alone. Moreover, hemorrhages and neuron damage were induced when increasing peak rarefractional pressure and microbubble dosage simultaneously. This may be due to the vascular wall structure or tight junction disruption due to inertial cavitation enhancement.

Other than the acoustic pressure, microbubble concentration and size distribution are important factors influencing BBB opening induced by ultrasound combined with microbubbles (Choi et al. 2010a; Samiotaki et al. 2012; Tung et al. 2011; Yang et al. 2007; Yang...
Previous studies showed that the magnitude of BBB disruption increased with microbubble doses (Yang et al. 2007; Yang et al. 2009). Thus, the effect of microbubble doses on delivery of liposomes was investigated in our study. It was noted that increasing the microbubble dose from 0.1 μL/g to 0.5 μL/g, equaling 2×10⁵ per g to 1×10⁶ per g, respectively, could significantly enhance liposome extravasation from brain microvasculature. Furthermore, the numbers of fluorescence spots were significantly increased. Although the exact mechanism of BBB disruption induced by ultrasound with microbubbles has not been fully understood, it is widely believed that the magnitude of BBB disruption is strongly related with the degree of the microbubble-vessel interaction in an ultrasonic field (Aryal et al. 2014; Cho et al. 2011; Tung et al. 2011). Increasing microbubble doses would increase the number of sites on which microbubbles interact with vessel walls and enhance the tight junctions opening. Thus, the position of fluorescence spots related to the vessels needs to be further investigated to elucidate the relationship between liposome extravasation and the interaction effect. In addition, animal studies indicated that the acoustic pressure threshold inducing BBB disruption was lower with microbubbles with diameters of 4–5 μm than with those of 1–2 μm diameters (Tung et al. 2011). The magnitude of BBB disruption was also enhanced when using larger microbubbles (Choi et al. 2010a). The numerical analysis found that the acoustic pressure threshold of microbubble collapse decreased significantly when bubble size increased using a bubble-elastic vessel coupled model (Hosseinkhah et al. 2015). The stresses on the vessel wall were much larger during bubble collapse and may induce vascular wall disruption (Hosseinkhah et al. 2013). In our study, polydispersed microbubbles were used, and 90% of prepared microbubbles were smaller than 2 μm. Therefore, the effect of different microbubble sizes on liposome delivery needs to be explored in a further study.

It was found that the distribution pattern of liposomes penetrating from brain vasculature was scattered spots with cloud-like periphery. This finding was consistent with the results of Chen and Konofagou’s study (2014). The spatial distribution of fluorescent 500-kDa dextrans (Dₜ: 30.6 nm) was characterized by separated spots, while it was homogeneous for 3-kDa dextrans (Dₜ: 2.3 nm). The phenomenon is likely associated with slow diffusion of large molecules in brain tissue interstitium. The width of brain extracellular space was found to be nearly 64 nm, and the brain extracellular space limits the diffusion of therapeutic agents, especially for large molecules (Thorne and Nicholson 2006). In this study, the number of fluorescence spots with areas larger than 100 μm² of 55-nm liposomes was greater than those of 120-nm or 200-nm liposomes. Thus, it is an issue that extracellular matrix may hinder the diffusion of large therapeutic agents (>120 nm), even after successful delivery across the BBB.

Several experiments were performed on brain tumor animal models to explore the anti-tumoral effect of liposome drugs (90–120 nm) delivered by FUS with microbubbles (Aryal et al. 2013; Treat et al. 2012; Yang et al. 2012). The accumulation of a chemotherapy drug in tumor tissue was augmented and the tumor growth was delayed. However, the degree of 120-nm liposome delivery was quite limited at 0.53 MPa in our study. One important reason explaining the discrepancy was that in those studies, liposome drugs were administered before sonication and doxorubicin was released from the liposomes, which were disrupted during sonication (Geers et al. 2011). Additionally, FUS was applied at 0.55–1.2 MPa in those studies, which was higher than that used in our study. The tumor microvasculature is immature and defective, and thus, may be more vulnerable to mechanical stresses induced by microbubble-vessel interaction in an ultrasound field (Hardee and Zagzag 2012). Therefore, it is necessary to perform parametric studies when delivering liposome drugs to animal models with brain diseases, especially tumors.

This study had some limitations. Due to lack of MRI or passive cavitation detection systems, real-time monitoring of BBB disruption was not realized in the present study. Improvement of the experimental setup on BBB disruption monitoring will be focused on and developed in further investigation. In addition, histology results showed that erythrocyte extravasations were detected when increasing microbubble doses or acoustic pressure, and that long-term effects need to be investigated in further studies. Also, the proportion of delivered liposomes accounting for the total injection dose was not quantified due to the fluorescence imaging method limitations. Nevertheless, the present study showed preliminary findings on delivery of liposomes larger than 50 nm using FUS with microbubbles, with the expectation to provide useful information for this attractive brain-targeted drug delivery strategy toward clinical use.

**CONCLUSIONS**

This study investigated the discrepancies among deliveries of 55-nm, 120-nm and 200-nm stabilized long-circulating liposomes to mice brains after FUS sonication with microbubbles. The results showed that 55-nm liposomes could be delivered at higher success rates than 120-nm or 200-nm liposomes. The delivery of 55-nm liposomes exhibited larger extravasation areas than the other two larger-size liposomes. Increasing peak rarefractional...
pressure amplitude or microbubble doses could exert significant influence on the extent of the three sets of liposomes passively delivered after BBB opening. Moreover, the distribution pattern of delivered liposomes was characterized by separated spots with cloud-like periphery surrounding a bright center, indicating confined diffusion in the extracellular matrix after extravasation from microvasculature. These results are expected to provide useful information for designing a delivery strategy of liposomal drugs larger than 50 nm to brain parenchyma using the method of FUS with microbubbles.

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