Oscillatory behavior of microbubbles impacts efficacy of cellular drug delivery

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A B S T R A C T

Drug-loaded microbubbles have been proven to be an effective strategy for non-invasive and local drug delivery when combined with ultrasound excitation for targeted drug release. Inertial cavitation is speculated to be a major mechanism for releasing drugs from drug-loaded microbubbles, but it results in lethal cellular pore damage that greatly limits its application. Thus, we investigated the cellular vesicle attachment and uptake to evaluate the efficiency of drug delivery by modulating the behaviors of targeted microbubble oscillation. The efficiency of vesicle attachment on the targeted cell membrane was 36.5 ± 15.9% and 3.8 ± 2.3% under stable and inertial cavitation, respectively. Further, stable cavitation enhanced cell permeability (26.8 ± 3.2%), maintained cell viability (90.8 ± 2.1%), and showed 7.9 ± 1.9-fold enhancement of in vivo vesicle release on tumor vessels. Therefore, our results reveal the ability to improve drug delivery via stable cavitation induced by targeted microbubbles. We propose that this strategy might be suitable for tissue repair or neuromodulation.

1. Introduction

Ultrasound (US) contrast agent microbubbles (MBs) can carry drugs, therapeutic gases, or reporter genes to enable simultaneous diagnostic and therapeutic functions in tumor therapy, tissue repair, and neuromodulation [1–3]. During US stimulation, MBs undergo stable and inertial cavitation to release nano-sized vesicles and produce different mechanical effects that modulate cell metabolism [4–6]. The inertial cavitation of MBs under high-intensity US sonication presents non-linear MB oscillation and disruption, which provides violent mechanical forces that disperse vesicles and stimulate cells [7]. The enhanced cell permeability promotes cellular uptake of vesicles, which assists with drug delivery and gene transfection to increase treatment outcome [8]. However, MB inertial cavitation might induce cell death due to the direct cell disruption or unrecoverable sonoporation [9]. Optimizing the balance between vesicle release, cellular uptake, and cell viability is important for developing MB-based strategies for tissue repair and neuromodulation [10,11].

Stable cavitation of MBs under low-intensity US stimulation results in periodical MB oscillation that releases vesicles and causes adjacent liquid flow [4,12]. Luan et al. used a high-speed camera to observe the vesicle release during MB stable cavitation [13]. The trajectory of vesicle release was parallel to the optical focal plane and coverage was restricted to the adjacent area. According to the features of MB stable cavitation, this in-plane shedding event might be suitable for local vesicle release to targeted cells. The microstreaming produced by MB stable cavitation increases the shear stress on cells, which enhances cellular permeability without causing unrecoverable damage [6,14,15]. De Cock et al. investigated the interactions between cells and free MBs under different acoustic pressures, and reported that MB stable cavitation caused cell membrane deformation that triggers endocytosis [16].

The modification of MBs with specific antibodies, peptides, or...
ligands can target MBs to tumor vessels, atherosclerotic plaques, or inflammation for precise theranostics [17–19]. US molecular imaging is an emerging technique that uses surface-modified MBs to detect intravascular targets [20,21]. Further, targeting MBs loaded with drug can facilitate local drug delivery and release for enhanced efficiency of targeted therapy [19,22,23]. Our previous study used folate-conjugated DNA-loaded cationic MBs (FCMBs) to enhance gene delivery and transfection in a rat glioma model [23]. The home-made FCMBs contained a phospholipid shell and C2F3 gas with the mean size of 3.2 ± 0.1 μm. The threshold of stable and inertial cavitation of FCMBs stimulated by a 1-MHz focused US was 300 and 500 kPa (peak-negative pressure), respectively. Although the optimal acoustic pressure of 700 kPa (mechanical index of 0.7) significantly enhanced blood-brain barrier opening and gene transfection, slight erythrocyte extravasation was visualized in the sonication area. Van Rooij et al. compared the sonoporation and cell viability between free and targeted MBs on the effect of long acoustic pulses (500–50,000 cycles) [24]. The limited displacement of targeted MBs might improve drug delivery and maintain cell viability due to the reversible sonoporation. Although various studies have shown the interaction between drug delivery and cellular bioeffects under MB cavitation, the associations between free/targeted MB with stable/inertial cavitation in drug delivery and bioeffects were not compared. The behaviors of free/targeted MBs would be limited by the attached cells to impact the MB oscillation property and efficacy of cellular drug delivery. Therefore, in this study, we used DiI-loaded folate-targeted MBs (Dil-ftMBs) and Dil-MBs to compare the effects of MB oscillation on the efficiency of vesicle release, cellular drug attachment, and cellular bioeffects. The red fluorescence dye DiI was used to simulate drugs and view the location of MBs and vesicles under a fluorescence microscope. Since folate receptors are overexpressed in tumor cell membranes, folate is a powerful modifier of drug carriers in targeted tumor therapy [23,25]. By adjusting the peak-negative acoustic pressure (200, 400 and 600 kPa), we were able to compare cellular drug transportation with Dil-ftMB under stable cavitation and inertial cavitation. The thresholds of stable and inertial cavitation were defined to compare the acoustic characteristics between free and targeted MBs. The behavior of vesicle release to cells via stable or inertial cavitation was evaluated under a high-speed acoustic-optical system. Moreover, the cell viability, membrane permeability, and vesicle attachment were estimated to demonstrate the biosafety of Dil-ftMB stable cavitation. Finally, the in vivo vesicle release with Dil-ftMB stable cavitation was observed using an intravital imaging system.

2. Materials and methods

2.1. Materials

The lipid materials 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearyloxy-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)]-2000 (DSPE-PEG2000), and 1,2-distearyloxy-sn-glycerol-3-phosphoethanolamine-N-[amino(polyethylene glycol)]-2000 (DSPE-PEG2000-Amine) were purchased from Avanti Polar Lipids (AL, USA). Perfluoropropane (C2F6) was purchased from ABCR GmbH & Co. KG (Karlsruhe, Germany). Folate, fluorescent dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), and 1,2-distearyloxy-sn-glycerol-3-phosphoethanolamine-N-[7-amido-3-hydroxy-5-carboxy-3,5-dihydro-1H-pyrrole-1-carboxamide]-5-carboxylate (DSPE-NBD) were purchased from Sigma Aldrich (St Louis, USA). Doxorubicin was purchased from SeederChem Company PTY LTD (Vic Melbourne, Australia). The in vitro cellular experiments utilized Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), fetal bovine serum (FBS), and 1% penicillin-streptomycin obtained from Gibco (NY, USA).

2.2. Preparation of MBs

In the present study, different types of MBs were prepared as follows. The Dil-ftMBs contained red fluorescence dye, which was used to indicate MB position and simulate drug release during MB cavitation. The Dil-MBs were fabricated to be free MBs for comparison with Dil-ftMBs. To observe the structural composition of drug vesicles released from MBs, the Dil-NBD-ftMBs were fabricated using green fluorescent lipids of DSPE-NBD to reveal the relative position between drugs (Dil) and lipids (DSPE-NBD). In the cell viability and cell membrane permeability experiments, the ftMBs were used to prevent fluorescence interference under flow cytometry analysis. The Dox-ftMBs were prepared to evaluate cellular drug uptake and the inhibition of tumor cell proliferation. Further, the efficiency of cellular drug uptake using Dox-ftMBs and liposomal Dox (Lipo-Dox) was compared to estimate the advantage of drug-loaded ftMBs.

The fabrication of ftMBs is described below. The folate solution was prepared in 350 μL DMSO with 1.5 mg folate and 6.7 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at 60 °C for 1 h. The lipid solution was prepared in 200 μL phosphate-buffered saline (PBS) with 2.3 mg DSPE-PEG2000-Amine. Then, 340 μL folate solution was mixed with 200 μL lipid solution by sonicator at 60 °C for 10 min. For ftMB film preparation, DPPC and DSPE-PEG2000 (weighted ratio of 10:4) were dissolved in chloroform. The chloroform was volatilized in a water bath at 65 °C for 1 h and then removed via an evaporator overnight (R-210, Büchi Labortechnik AG, Flawil, Switzerland). The ftMB lipid film was then mixed homogeneously with 30 μL folate-lipid solution and 770 μL glycerol-PBS. The C2F6 gas was refilled into the degassed ftMB mixture solution. Finally, the ftMBs were fabricated via violent shaking for 45 s by an agitator (VIALMIX, Bristol-Myers Squibb Medical Imaging, New York, NY, USA), and then the free folate-lipid was removed by centrifugation at 2000 rcf for 2 min.

For Dil-MB preparation, the Dil-MB lipid film contained DPPC, DSPE-PEG2000, and Dil with a weighted ratio of 10:4:0.25. The Dil-MB lipid film was mixed homogeneously with 800 μL glycerol-PBS. For Dil-ftMB preparation, the Dil-MB lipid film was mixed homogeneously with 30 μL folate-lipid solution and 770 μL glycerol-PBS. For Dil-NBD-ftMB preparation, the 10 μL DSPE-NBD (2 mg/mL dissolved in chloroform) was added in the Dil-loaded lipid solution to form Dil-NBD-ftMB lipid film. For Dox-ftMB preparation, 1.5 mg Dox was added to the ftMB mixture and mixed homogeneously at 65 °C for 60 min. The Dox was embedded into the lipid shell of ftMBs via electrostatic attraction with a negative charge lipid of DSPG [26]. The fabrication procedure of MBs was followed according to the above description.

For Lipo-Dox preparation, the lipid film (DPPC: DSPE-PEG2000: cholesterol = 7: 6: 2.5 weight ratio) was added to 2 mL ammonium sulfate buffer (350 mM) and homogenized by sonicator. The lipid solution was dialyzed (Cellu-Sep T4 Tubular Membrane, 12,000–14,000,000 MW, Seguin, TX, USA) for 24 h to remove free ammonium sulfate. The liposome suspensions were sequentially passed through the 200, 100, and 80 nm polycarbonate membrane to obtain a homogeneously size distribution of liposomes by an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). The Lipo-Dox was made by mixing the liposome suspensions and Dox solution (10 mg/mL) with a volume ratio of 6:4 for 24 h. Finally, Lipo-Dox was separated from free Dox by centrifugation of 1000 rcf for 4 min in the P-6 column (Bio-Rad Laboratories, California, CA, USA). The Dox payload was measured by a plate reader (Synergy 2, Tecan Infinite M200, Tecan Trading AG, Männedorf, Switzerland) at an absorbance wavelength of 490 nm. The size distribution of Lipo-Dox was analyzed using dynamic light scattering (Nanosizer-S, Malvern, Worcestershire, UK).

2.3. Characteristic of MBs

The size distribution of Dil-ftMBs was analyzed using a coulter counter (Model Multisizer 3, Beckman Coulter Inc., CA, USA). The
morphology of Dil-ftMBs was observed under an inverted microscope (IX71, Olympus, Tokyo, Japan). To evaluate the folate loading, the free folate, Dil-MBs, and Dil-ftMBs were disrupted by sonicator to measure the absorbance wavelength from 230 to 430 nm using a plate reader system. Folate has an absorption peak at 280 nm. The loading efficiency of folate on Dil-ftMBs was also evaluated. The stability of Dil-ftMBs was estimated by the image enhancement of US B-mode images at 37 °C. A 2% agarose phantom (UltraPure™ AGarose, Invitrogen, CA, USA) was made to contain a cylindrical hollow chamber. Dil-ftMBs (5 × 10^8 MBs/mL) were added into the hollow chamber. A commercial US imaging system (central frequency: 5–12 MHz, model t3000, Terson, MA, USA) was used to record B-mode images at 0, 10, 20, 30, 40, 50, and 60 min, respectively. After that, the signal-to-noise ratio of US B-mode images was analyzed by MATLAB software (MathWorks, Natick, MA, USA) to show the stability of Dil-ftMBs.

The stability of Dox loading on ftMBs was evaluated by the leakage of Dox under the various time points at 37 °C. Dox-ftMBs were diluted with culture medium to simulate the in vivo bloodstream condition. At each time point, diluted Dox-ftMBs (5 × 10^8 MBs/mL) were centrifuged at 500 rcf for 2 min to separate free Dox/vesicles and Dox-ftMBs. The DOX leakage at various time points was quantified using the plate reader and normalized to the zero time point.

### 2.4. Tumor cell targeting capability of Dil-ftMBs

C6 glioma cells were chosen as the targeting cell in this study due to the high expression of folate receptors on the glioma cell surface [27]. Cells were maintained routinely in culture medium of DMEM/F12 supplemented with 10% FBS and 1% penicillin-streptomycin. During the experimental processes, the cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

The tumor cell targeting capability of Dil-ftMBs was estimated by flow cytometry (BD FACSCanto™, BD Biosciences, NJ, USA). We added 3 × 10^4 C6 cells to each well of a 24-well plate. After 24 h, 5 × 10^8 Dil-ftMBs were added in each well for co-incubation of 10 min at 37 °C. Then, wells were washed with PBS to remove the non-targeted Dil-ftMBs. The targeting efficiency of Dil-ftMBs to the cells was assessed by estimating the percentage of red fluorescence on the cells.

### 2.5. Measurement of the acoustic threshold of stable cavitation dose (SCD) and inertial cavitation dose (ICD) of Dil-ftMBs

Since Dil-ftMBs were targeted to the tumor cell membrane to influence the MB cavitation behavior, we investigated the acoustic characteristics of free MBs and targeted MBs using the threshold stable cavitation dose (SCD) and inertial cavitation dose (ICD). We seeded 3 × 10^5 C6 cells in each well of a 24-well plate. After 24 h, 5 × 10^8 Dil-ftMBs were added to each well for 10-min co-incubation at 37 °C. Then, wells were washed with PBS to remove the non-targeted Dil-ftMBs. The cells with Dil-ftMB targeting were collected as the Dil-ftMB group for the SCD and ICD measurements. For the Dil-MB group, Dil-MBs were mixed with cells without washing. Samples were infused into a 200-μm cellulose tube (Spectrum Labs, CA, USA) using a flow rate of 0.92 μL/s controlled by a syringe pump (KDS120, KD Scientific, New Hope, PA, USA). A homemade annular 2-MHz high-intensity focused ultrasound (HIFU) transducer was driven by a waveform generator (AWG 2041, Tektronix, CT, USA) and a radiofrequency power amplifier (model A150, E&I, NY, USA) was used to generate acoustic pulses (500-cycle, pulse repetition frequency of 19.53 Hz). The parameters of HIFU were regulated to automatically capture the high-speed images (Fig. 1B). The HSC transducer was fixed perpendicularly to the HIFU transducer. During 2-MHz HIFU stimulation, the oscillating Dil-ftMBs produced scattered US signals for detecting by the received transducers. The received signals were amplified by a broadband receiver (Model 5072PR, Panametrics-NDT, Waltham, MA, USA) and then digitized by an oscilloscope (model LT322, LeCory, Chestnut Ridge, NY, USA) for statistical analysis using MATLAB software. The 100 RF data lines were recorded and repeated three times in each experimental group. The signal was converted from time domain into frequency domain spectrum by fast Fourier transform. SCD was calculated using the area under the subharmonic peak, which is the specific signal of the MB resonance. The broadband signals in the spectrum were detected by a 15-MHz received transducer when Dil-ftMBs started to do the inertial cavitation. The area under the curve of harmonic peaks from 14.5 to 15.5 MHz was quantified to be the inertial cavitation doses. The quantified frequency range was used to avoid the interference by multiple frequency signals of 2-MHz HIFU. The cell only group was measured to provide a baseline for normalizing the SCD and ICD.

### 2.6. Characteristics of Dil-ftMB vesicles

The structure of Dil-ftMB vesicles/fragments disrupted by HIFU sonication was investigated to evaluate the acoustic characteristics. The diluted MBs (5 × 10^8 MBs/mL) were infused into a 200-μm cellulose tube with a flow rate of 0.92 μL/s controlled by a syringe pump. The 2-MHz HIFU transducer transmitted various acoustic pressures with a 500-cycle pulse. The pulse repetition frequency was regulated to stimulate Dil-ftMBs once and ten times to observe the variation in vesicle size distribution by dynamic light scattering. The Dil-NBD-ftMBs were used to discuss the composition of vesicles after HIFU sonication. The relative positions of drugs (Dil) and lipids (NBD) on vesicles were observed under an inverted microscope. Moreover, the structure of the vesicles after US stimulation was observed using a cryo-electron microscope (Tecnai G2 F20 TWIN, FEI, Netherlands). In order to evaluate the probability of having gas within vesicles (i.e., nanobubbles), the acoustic characteristics of the vesicles were evaluated by detecting ICD.

### 2.7. High-speed imaging for monitoring vesicle release

In order to observe the correlation between cells, Dil-ftMB cavitation, and vesicle release, a high-speed acoustic-optical system was designed to provide high-speed fluorescence and bright-field microscopic imaging during HIFU sonication. The high-speed acoustic-optical system consisted of an inverted microscope, a high-speed camera (HSC; model FASTCAMSA4, Photon Ltd., Tokyo, Japan), a high-power laser (532 nm, 0.32 mW/μm², SDL-532-1000 T, Dream Lasers Technology, Shanghai, China), and a HIFU sonication system (Fig. 1A). An annular 2-MHz HIFU transducer was conically aligned with a 60 × water-immersion objective (LUMPLFLN60XW, Olympus Ltd., Tokyo, Japan).

For cellular experiments, C6 cells (3 × 10^5 cells in 500 μL culture medium) were added to a round glass coverslip (diameter of 25 mm, Marienfeld-Superior, Lauda-Konigshofen, Germany) and placed into a 24-well plate for 1 h. After cell seeding on the coverslip, 2 mL culture medium was added to immerse the coverslips for 12 h incubation. 5 × 10^6 Dil-ftMBs were added in each well and the plate was inverted 10 min for cell targeting. Then, wells were washed with PBS to remove the non-targeted Dil-ftMBs. Finally, the coverslip was put into an Attolfluor™ cell chamber (Thermo Fisher Scientific, Waltham, MA, USA), which was fixed on the microscope. The HIFU transducer was placed to immerse into the cell chamber, which was filled with PBS. For the Dil-MB group, 5 × 10^6 Dil-MBs were directly added into the cell chamber and then filled with PBS.

The time-scale of HSC, HIFU, laser, and light operation were regulated to automatically capture the high-speed images (Fig. 1B). The HSC (frame rate of 40 kfps) recorded high-speed images before, during, and after HIFU sonication (2-MHz, 500-cycle, single pulse, 200–600 kPa). Simultaneously, the laser and light were switched on to record the fluorescence and bright-field images, respectively. During HIFU sonication, the high-speed fluorescence images were recorded under both conditions.
The high-speed fluorescence images were analyzed to manually define the region of interest (ROI) at MB and cell locations by MATLAB software. The events of vesicle release from DiI-ftMBs after HIFU sonication were separated into four types (Fig. 1C): (1) MBs that remained in the original location; (2) MBs that moved to the other site; (3) MBs that remained in the original location, but the shape was covered by the vesicles; (4) MBs that were disrupted. The efficiency of vesicle release from MBs was defined by the percentage decrease in fluorescence intensity (FI) between the pre-HIFU MBs and post-HIFU MBs. For type 3 without post-HIFU MBs, the FI at the MB original location was measured to assume the FI of post-HIFU MBs. For type 4, the efficiency of vesicle release was defined as 100% due to the disruption of MBs. In contrast, the efficiency of vesicle release on cell membranes was defined by the percentage increase in FI on the cell membrane relative to the FI of pre-HIFU MBs.

2.8. Cellular bioeffects of ftMB cavitation

The cellular viability, cellular permeability, and vesicle release on cell membranes after ftMB sonication were evaluated by flow cytometry (BD FACS Canto™, BD Biosciences, NJ, USA). The cellular experiments were performed as shown in Fig. 2A. 3 × 10^4 C6 cells were seeded in each well of a 24-well plate. After 24 h, 5 × 10^5 ftMBs were added to each well for 10-min co-incubation at 37 °C. Then, wells were washed with PBS to remove the untargeted ftMBs. The 0.25 μg/mL calcein acetoxymethyl (calcein AM; C1429, Invitrogen, Carlsbad, CA, USA) was co-incubated with cells for 10 min and washed with PBS to estimate the cell viability. Before HIFU sonication, 25 μg/mL propidium iodide (PI; Invitrogen, Carlsbad, CA, USA) was added to evaluate the permeability of the cell membrane. A 2-MHz HIFU with an acoustic pressure of 200–800 kPa for 500 cycles was applied for cellular sonication, and controlled to receive one US pulse at each focal spot area. After sonication, cells were washed 3 times and collected for centrifugation at 1500 rcf for 1 min. The FIs of calcein AM and PI were measured by flow cytometry to evaluate the cellular viability and permeability, respectively. Moreover, the efficiency of vesicle release on cell membrane was also estimated based on the FI of DiI on cells. As shown in Fig. 2B, 5 × 10^6 DiI-ftMBs were added to each well for 10-min co-incubation at 37 °C. Then, wells were washed with PBS to remove untargeted DiI-ftMBs. Cellular sonication was performed according to the above parameters. After sonication, cells were washed 3 times and collected by centrifugation at 1500 rcf for 1 min. The FI of DiI on cells was measured by flow cytometry to evaluate the efficiency of drug delivery.

2.9. Drug uptake and cytotoxicity of Dox-ftMBs

2 × 10^4 C6 cells were seeded in each well of a 24-well plate (Fig. 2C). After 24 h, Dox-ftMBs (LD_{50} = 5 μg Dox/mL) were added to each well for 10-min co-incubation at 37 °C. The cellular sonication (2-MHz, 400 kPa, 500 cycles) was performed to promote drug release and uptake and cells were washed with PBS after 2 h co-incubation. The cell images were recorded by an inverted microscope to evaluate the efficiency of cellular drug uptake. After 24 h, 0.25 μg/mL calcein AM was added to the cells for 10 min and the cells were washed with PBS prior to flow cytometry to estimate cell viability.

2.10. In vivo vesicle release by DiI-ftMB cavitation

The in vivo vesicle release by Dil-ftMB cavitation was evaluated using the dorsal skinfold window chamber mouse model. Male nude mice (BALB/cAnN.Cg-Foxn1nu/CrlNarl, aged 8 weeks-old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All the animal experiments were approved and performed according to the guidelines by Chang Gung University Institutional Animal Care and Use Committee (approval number: CGU107–153). Mice were subcutaneously implanted with 1 × 10^6 C6 cells at the dorsal skin, and 5 days were allowed for tumor growth. The window chamber kits (SM100, APJ Trading, Ventura, CA, USA) were mounted on the mouse dorsal skin to display the tumor within the window location. An acoustic-optical system was used to record the intravital imaging during DiI-ftMB sonication. Mice received 5 × 10^6 Dil-ftMBs via retro-orbital injection, followed
by 10 min to allow for Dil-ftMB targeting. The 2-MHz HIFU transducer was triggered to transmit 500-cycle pulses with an acoustic pressure of 400 kPa. The pulse repetition frequency was 18 Hz and the sonication time was 1 min. The protocol of HIFU sonication including waiting for Dil-ftMB targeting and release of Dil by HIFU was repeated 3 times to accumulate more Dil-ftMBs/vesicles on the tumor vessels. The intravital images were collected to observe the in vivo feasibility of Dil-ftMB targeting, vascular bioeffect during MB cavitation, and vesicle release in the intra- and extra-vascular regions. The vessel pattern and extra-vascular tissue were defined by subtracting the post- and pre-Dil-ftMB injection images. The threshold of vessel pattern was set by the mean fluorescence intensity (FI) of post-Dil-ftMB injection images, and the opposite area was defined as extravascular tissue. Afterward, the FIs of post-HIFU images in the intra- and extra-vascular regions were calculated as the percentage increase in FI relative to the pre-HIFU images. The Dil-MBs were used to represent free MBs for comparison with Dil-ftMBs.

2.11. Statistical evaluation

Each experimental group included more than three independent samples to calculate the mean and standard deviation. The standard deviation of each group is shown as an error bar in the graph. The statistically significant differences (p value < 0.05) between experimental groups were analyzed using the paired two-tailed Student’s t-test for two individual groups and the one-way ANOVA and Bonferroni post-hoc test for multiple groups by using SPSS 13.0 (SPSS Inc., IBM, Armonk, NY, USA).

3. Results

3.1. Characteristics of Dil-ftMBs

The size and volume distributions of the Dil-ftMBs are shown in Fig. 3A. The mean size of the Dil-ftMBs was 1.5 ± 0.7 μm with a concentration of (7.8 ± 0.5) × 10^9 MBs/mL. The bright-field image shows the morphology of the Dil-ftMBs and demonstrates that Dil was distributed on the lipid shell in the red fluorescence image (Fig. 3B). The folate loading on the Dil-ftMBs was established by detecting the absorption peak (Fig. 3C). There was an absorption peak at 280 nm in the folate and Dil-ftMB groups, but there was no significant peak in the Dil-MB group. The folate loading efficiency of Dil-ftMBs was 11.8 ± 1.4% (1.8 × 10^-10 nmol folate per Dil-ftMB). The surface density of folate on Dil-ftMBs was 0.57 mmol of folate per mol of lipid shell, and the interval distance of folates on the surface of Dil-ftMB was 8.1 nm. The US imaging maintained high-contrast enhancement indicating the stability of Dil-ftMBs (Fig. 3D). The acoustic stability of Dil-ftMBs was 100.0 ± 0.0, 97.3 ± 1.6, and 97.2 ± 1.5% at 0, 30, and 60 min, respectively (each p > 0.05). In Fig. 3E, the microscopic image reveals that Dil-ftMBs was targeted to the C6 cells. The targeting capability of Dil-ftMBs on C6 cells was 47.8 ± 7.4% estimated by flow cytometry.

After evaluating the size distribution, folate loading, stability, and C6 cell targeting ability of Dil-ftMBs, the cavitation signals of Dil-ftMBs were detected to confirm the threshold of acoustic pressures for SCD and ICD. The DiI-MB group showed a significant decrease at an acoustic pressure of 300 kPa in the SCD analysis, and a significant increase at 600 kPa in the ICD analysis (Fig. 3F). Further, we investigated the oscillatory behavior of Dil-ftMBs targeted to the C6 cells. With SCD, Dil-ftMBs revealed no significant difference in comparison to Dil-MBs. However,
with ICD, there was a 1.61 ± 0.19-fold enhancement in the acoustic pressures of 500 to 1000 kPa in the DiI-ftMB group relative to the DiI-MB group. When DiI-ftMBs targeted the cells, the asymmetric oscillation intensified with a higher ICD than observed with DiI-MBs due to the partial anchoring of MBs on cell membrane. Since the size of MBs is associated with the cavitation dose, the polydisperse size distribution of DiI-ftMBs would influence the precision of the SCD and ICD thresholds [28]. Thus, the acoustic pressures of SCD and ICD were defined by relative weighting in the present study. In order to slightly amplify the effects of stable and inertial cavitation, we chose acoustic pressures of 400 and 600 kPa to induce MB stable and inertial cavitation in the following experiments, respectively.

3.2. Characteristics of vesicles produced by DiI-ftMB cavitation

After evaluating the characteristics of DiI-ftMBs, we further investigated how different types of DiI-ftMB cavitation would affect the size distribution of released drugs from DiI-ftMB. The mean size of vesicles was 1791 ± 424, 2223 ± 402, 101 ± 23, 134 ± 24 nm in the DiI-ftMB, 200 kPa, 400 kPa, 600 kPa, and 800 kPa group, respectively (Fig. 4A). The results revealed that stimulating DiI-ftMBs by HIFU with acoustic pressure of 200 kPa might not produce vesicles. In the 400 kPa group, there were two peaks with 98.7% at 125 nm and 1.3% at 819 nm. The results indicated that DiI-ftMB stable cavitation might release numerous nano-sized vesicles with a small amount of DiI-ftMB remaining. The 600 and 800 kPa groups displayed a single peak to demonstrate that all the DiI-ftMBs were disrupted after HIFU sonication. The correlation between vesicle size and multiple HIFU sonication was also investigated. In Fig. 4B, the size distribution of vesicles under 400 kPa with ten times pulse stimulation showed a single peak and sharper profile than that under once pulse stimulation. The mean diameters of the vesicles significantly increased to 274 ± 36, 251 ± 38, and 275 ± 52 nm in the 400, 600, and 800 kPa groups, respectively, with ten times pulse stimulation. The mean sizes of the vesicles were not significantly different among the different acoustic pressures after multiple HIFU sonication.

We next investigated the structure and morphology of released drugs from DiI-ftMB. The fluorescent images revealed the overlap between DiI and NBD in the post-HIFU group to demonstrate that DiI-NBD-ftMB cavitation released drugs and lipids to form vesicles (Fig. 4C). In the cryo-electron microscopic images, the structure of the vesicles showed a similar pattern to that with DiI-NBD-ftMBs, where the central region of the particles was transparent due to the potential existence of gas (Fig. 4D). However, the spectra revealed no broadband signal under acoustic pressures of 400, 600, and 1000 kPa (Fig. 4E).

3.3. High-speed imaging for vesicle release

For the high-speed imaging study, the bright-field images were captured to indicate the positions of MBs and cells (Fig. 5). The red fluorescent high-speed images were recorded to observe DiI-vesicle distribution on cells after DiI-ftMB cavitation. In the control group without HIFU sonication, the morphology and fluorescence intensity of MBs were unchanged. Since DiI-MBs were unable to anchor to the cell membrane, the images showed distance between the DiI-MBs and cells. The acoustic pressures for HIFU sonication were 200, 400, and 600 kPa to estimate the efficiency of vesicle release induced by MBs with non, stable, and inertial cavitation, respectively. There was no morphological
change of DiI-MBs/DiI-ftMBs and no DiI release with an acoustic pressure of 200 kPa. The images revealed that stable cavitation was sufficient to induce MB size shrinking and trigger DiI release at 400 kPa. When the acoustic pressure was increased to 600 kPa, the inertial cavitation generated from MBs disrupted the MBs, resulting in a release of DiI to a distant region. Compared to the DiI-MB group, the DiI-ftMB group showed more DiI deposition on cells. In addition, no visible changes in cell shape were observed under any experimental conditions.

The high-speed imaging revealed that the events of vesicle release from DiI-ftMBs after HIFU sonication could be separated into four types (Fig. 1C): (1) MBs that stayed in the original location; (2) MBs that moved to the other site; (3) MBs that stayed in the original location, but the shape was covered by vesicles; (4) MBs that were disrupted. The incidences of each type of vesicle release under different acoustic pressures were counted to compare the modes of vesicle release from DiI-MBs and DiI-ftMBs (Fig. 6A). For the DiI-MB group, there was 100.0% type 1 MBs, indicating that DiI-MBs did not move under an acoustic pressure of 200 kPa. Since acoustic pressure of 400 kPa can induce DiI-MB stable cavitation, the free DiI-MBs easily moved during oscillation to show 100.0% type 2 MBs (Fig. 6B). Under acoustic pressure of 600 kPa, DiI-MBs revealed 50.0% type 3 MBs and 50.0% type 4 MBs. The DiI-MBs remained in the original location, but were covered by a vesicle cloud (Fig. 6B) or were disrupted after inertial cavitation. For the DiI-ftMB group, the incidence of type 1 MBs increased to 62.5% under acoustic pressure of 400 kPa due to the tumor cell targeting capability of DiI-ftMBs. Under an acoustic pressure of 600 kPa, the incidence of DiI-ftMBs was 61.5, 7.7, and 30.8% for type 2, 3, and 4, respectively. Fig. 6C reveals the quantification of vesicle release under different acoustic pressures. The relative FI of vesicle release from DiI-MB showed 5.9 ± 2.5, 97.7 ± 3.0, and 65.6 ± 37.2% under acoustic pressure of 200, 400, and 600 kPa, respectively (each p < 0.05). However, the efficiency of vesicle release on cell membranes in the DiI-MBs group was zero, because the distance between the DiI-MBs and cells was longer than the vesicle release distance. The non-fixed DiI-MBs were easily pushed away from cells during US sonication, and vesicles might be released along the track of the moving DiI-MBs. The efficiency of vesicle release from DiI-ftMBs was directly proportional to the acoustic pressures (each p < 0.05). The efficiencies of vesicle release on the cell membrane were 0.0 ± 0.0, 36.5 ± 15.9, and 3.8 ± 2.3% under acoustic pressures of 200, 400, and 600 kPa, respectively (each p < 0.05). The results indicate that DiI-ftMBs with stable cavitation could induce more vesicle release on cell membranes for enhanced drug delivery to cells.

3.4. Cellular bioeffects between ftMB stable and inertial cavitation

In order to evaluate the cellular bioeffects induced by ftMB stable and inertial cavitation, the cell viability and permeability were measured under different acoustic pressures. The cell viabilities were 94.2 ± 2.4, 91.1 ± 0.8, 90.8 ± 2.1, 82.5 ± 1.8, and 68.3 ± 5.2% in the cell only, for 200 kPa, 400 kPa, 600 kPa, and 800 kPa groups,
respectively (Fig. 7A). Because ftMB inertial cavitation can disrupt cells, the acoustic pressures of 600 and 800 kPa revealed a significant reduction in cell viability. The cell permeability after HIFU sonication was detected based on fluorescence enhancement of PI staining. The percentage of PI-positive cells showed a significant increase under acoustic pressures of 200 and 400 kPa (each \( p<0.05 \)), and was not significantly different from that observed at 400 to 800 kPa (each \( p>0.05 \)).

The efficiency of vesicle release on cells was also analyzed by flow cytometry to compare with the results of high-speed imaging. The percentage of DiI-positive cells was 5.3 ± 0.4, 47.8 ± 7.4, 48.5 ± 7.4, 76.1 ± 7.1, 87.2 ± 5.1, and 82.7 ± 5.3% in the cell only, 0 kPa, 200 kPa, 400 kPa, 600 kPa, and 800 kPa groups, respectively (Fig. 7B). The high number of DiI-positive cells in the 0 kPa group was likely caused by the natural targeting of DiI-ftMBs to C6 cells. The percentage of DiI-positive cells was significantly increased at an acoustic pressure of 400 kPa, which was consistent with the results of high-speed imaging analysis. The stable cavitation induced by DiI-ftMBs under an acoustic pressure of 400 kPa can enhance cell permeability without influencing cell viability and delivery of DiI to the cell membrane.

3.5. Tumor cell cytotoxicity induced by Dox-ftMB stable cavitation

In the present study, we determined that an acoustic pressure of 400 kPa was optimal for drug release on cells without causing cellular damage. The Dox-ftMBs were fabricated to measure the Dox-vesicle penetration and tumor cell cytotoxicity under stable cavitation. The drug payload of Dox-ftMBs was 1.26 ± 0.10 mg/mL with the loading efficiency of 83.7 ± 6.8%. The level of Dox loading per ftMB was 0.04 ± 0.01 pg. The stability of Dox loading on ftMBs was 62 ± 0, 59 ± 7, 55 ± 7, and 39 ± 2% at 0, 30, 60, and 120 min, respectively. Although the results showed immediate Dox leakage after dilution (at 0 min), the subsequent Dox loading on Dox-ftMBs was maintained at higher than 50% during 60 min. In Fig. 7C, the bright-field images reveal the intact morphology of cells after HIFU sonication. The red fluorescence images show the enhancement of PI at cell nuclei, which proves that Dox-vesicles were released from Dox-ftMBs and penetrated into the cells.

The cell viability was 99.9 ± 0.0, 86.0 ± 0.2, 84.7 ± 0.8, 70.5 ± 5.1, and 80.2 ± 2.7% in the cell only, LipoDox, Dox-ftMBs, Dox-ftMBs + US, and LipoDox + ftMBs + US groups, respectively (Fig. 7D). These data indicate that the DOX-vesicles released from Dox-ftMB stable cavitation retain their cytotoxicity and can kill tumor cells.

3.6. Evaluation of vesicle release under intravital imaging

The mouse dorsal skinfold window chamber model provided clear intravital imaging under an acoustic-optical system (Fig. 8A). The morphology and vessel pattern of tumors were visualized in the bright-field images. The intravital images revealed intact and unchanged vessel patterns after HIFU sonication in the DiI-MB and DiI-ftMB group (Fig. 8B), demonstrating that MB stable cavitation does not influence nor damage tumor vessels. For the quantification process, the pre- and post-HIFU images showed the fluorescent intensity in the intravascular and extravascular regions and the difference images were obtained by subtracting the post- and pre-HIFU images. In the pre-HIFU intravital images, the fluorescent enhancement in the extravascular regions might be caused from the intra-capillary DiI-ftMB projection at depth of field of optics system. In the DiI-MB group, the intravital images showed intravascular red fluorescent speckle disappearing after HIFU sonication to indicate the MB disruption and vesicle release. Note that the post-
HIFU image revealed non-visualized fluorescent enhancement in the extravascular regions. In contrast, the DiI-ftMB case showed the fluorescent enhancement in the intravascular and extravascular regions with FI% of $26.9 \pm 7.0$ and $36.4 \pm 3.2$, respectively, after HIFU sonication (Fig. 8C). The difference image of DiI-ftMB case showed that some of the vessel contours with fluorescence increasing at intravascular regions might be the adhesion of vesicles on the vessel wall or targeting on the vascular-adjacent tumor cells. The enhancement of FI% in the extravascular regions demonstrated that DiI-ftMBs could be converted into nano-sized vesicles for increasing vesicles penetrating and tumor cells targeting. Intravital imaging demonstrated that in vivo DiI-ftMB stable cavitation was sufficient for transporting drug onto the tumor vessel walls, without damaging the vessels.

4. Discussion

Modulation of MB oscillation using stable or inertial cavitation provides dynamic strategies for different therapeutic applications. For tumor therapy, MB inertial cavitation induces highly efficient drug release and violent mechanical force for combined chemical and physical therapies [5,29]. The anti-vascular effect and direct tumor cell disruption induced by MB inertial cavitation can assist the inhibition of tumor growth [30]. However, for other therapeutic applications, like ischemia-reperfusion injury, thrombolysis, and neurodegenerative diseases, preventing cellular bioeffects is an important issue during treatment [10,31,32]. Datta et al. demonstrated that the enhancement of thrombolysis by MB stable cavitation was greater than that achieved from combining MB stable and inertial cavitation [33]. The enhanced thrombolytic effect in the stable cavitation regime, as opposed to the inertial cavitation regime, may simply be attributed to the fact that this stable cavitation activity was sustained for a longer period of time. Thus, using MB stable cavitation is potentially a safe way to release drugs and maintain the efficiency of targeted drug delivery for tissue repair and neuromodulation.

In the present study, we used an acoustic pressure of 400 kPa to induce stable cavitation for free MBs and ftMBs. Although ftMB inertial cavitation showed a 1.79 $\pm$ 0.62-fold better efficiency of vesicle release than stable cavitation, the vesicle attachment on cells decreased 6.4 $\pm$ 1.5-fold. The out-of-plane shedding of vesicle released under MB inertial cavitation results in dispersion of vesicles to distant areas [13], reducing the number of vesicles on the targeted-cell membrane. In contrast, MB stable cavitation limits vesicle dispersion to the local area to significantly increase the efficiency of vesicle release on the targeted-cell membrane. The cell permeability was enhanced without significantly influencing cell viability, which proves that Dox-vesicle uptake is improved using ftMB stable cavitation. The intravital imaging showed a

![Figures and Tables](image-url)
7.2 and 8.0-fold enhancement of vesicle release in the intra- and extra-vascular regions, respectively, induced by DiI-ftMBs relative to DiI-MBs. Because of the specific vascular morphology in tumors, the submicron-sized DiI-ftMBs and nano-sized vesicles might target or stick on the gaps of vessel wall to enhance the intravascular FI [34]. On the other hand, the nano-sized vesicles released by DiI-ftMB cavitation could penetrate the tumor tissue to increase the extravascular FI (i.e., the enhanced permeability and retention effect [35]). These results indicate that stable cavitation could be a suitable way to improve the efficiency of vesicle release from targeted MBs to cells without inducing undesired bioeffects.

Since the nano-sized vesicles can carry drugs and penetrate cells for various treatments, the characteristics of vesicles released from DiI-ftMBs were assessed to evaluate the influence of cellular uptake and drug penetration [16,36]. The size of vesicles released from DiI-ftMBs was influenced by stable or inertial cavitation. Under stable cavitation, MBs released nano-sized vesicles and the MB volume decreased. The remaining MBs and vesicles showed two peaks at nano and submicron scales in the size distribution profile. In contrast, under inertial cavitation, MBs were disrupted to generate vesicles and showed a single peak in the vesicle size distribution profile. Interestingly, the mean size of vesicles increased after multiple HIFU sonication, which might represent the probability of liposome forming due to vesicle reorganization during sonication. Nevertheless, the structure of these large vesicles was not presented in this study. The assumption of the vesicle reorganization should be demonstrated to further evaluate the correlation between vesicle structure and drug release in the future.

In addition, the forms of drug release from Dox-ftMB with stable cavitation may comprise not only vesicles, but also free Dox. Following the procedures of SCD/ICD detection, the diluted Dox-ftMB \((5 \times 10^8 \text{ MBs/mL})\) was insonated by 2-MHz HIFU with acoustic pressure of 400 kPa under the flow condition. Then, the samples were collected and separated to vesicles and free Dox by P-6 column. The Dox amount in vesicles was measured and compared with the total Dox amount release by a plate reader system. The percentage of Dox in vesicles, free Dox, and total amount was about 43 ± 4, 34 ± 4, and 77 ± 2% with HIFU sonication, respectively, and 37 ± 8, 17 ± 8, and 54 ± 6% without HIFU sonication. Based on the pathway of cellular uptake, the diffusion of free drugs might be easier for cellular uptake than endocytosis of vesicles. However, gene delivery needs vesicle structure to protect from lysosomal degradation during the intracellular delivery. Therefore, the correlations between MB cavitation and release forms should be further investigated to improve the efficient cellular uptake.

For tumor therapy, the conversion of MBs to vesicles improved drug penetration within tumors through the enhanced permeability and retention effect. Previous studies have been demonstrated that ftMB with US cavitation could release nano-sized vesicles, and then penetrate into tumor tissue for cell targeting [23,37]. Huynh, et al. investigated the conversion of MBs to nanoparticles (vesicles), and also observed the same optical properties of nanoparticles as those of the original MBs [38]. In our previous study, liposomes show obvious double-layer structure with the membrane thickness of 7.8 nm, and the inner core reveals gray color caused by the diffraction signals of frozen liquid [39]. Thus, we considered that vesicles released by DiI-ftMB cavitation contained single-layer shell and gaseous core in the cryo-electron microscopic images, just like the nano-sized bubbles. However, the frequency...
spectrum did not show the broadband signal induced by vesicle inertial cavitation. Since the acoustic resonance frequency of bubbles is inversely proportional to the bubble size, the acoustic frequency of 2-MHz HIFU might be too low to induce vesicle cavitation [40]. The additional evidence of gas presence inside the vesicles might be confirmed by investigating the change of gas volume in vesicles under various hydrostatic pressures. Further, finding the optimal acoustic resonance frequency of vesicles might increase the possibility of detecting the broadband signal for ensuring vesicle cavitation.

Since MB oscillation can promote vesicle production, the efficiency of vesicle release might be proportional to the intensity of MB cavitation [7,41]. However, our results suggest that vesicle release from Dil-MBs via stable cavitation (400 kPa) is higher than that via inertial cavitation (600 kPa), because the efficiencies of vesicle release in type 3 (31.3 ± 9.2%) and 4 (100.0 ± 0.0%) were significantly different. Although the visualized vesicle release appeared large during high-speed imaging, the quantification of type 3 could be underestimated. The FI of post-HIFU MBs contained the vesicle cloud, so the relative FI of actual vesicle release might be smaller. In the Dil-fltMB group, the type 1 and 2 induced 37.9 ± 13.5 and 80.6 ± 8.4% vesicle release from MBs, respectively, to enlarge the variance of mean efficiency under acoustic pressure of 400 kPa (p < 0.05). The Dil-fltMB stable cavitation presented 53.9 ± 24.7% vesicle release from MBs, and then maintained 36.5 ± 15.9% drugs on the cell membrane. For the inertial cavitation, although the efficiency of vesicle release from MBs was higher than that with stable cavitation, the violent oscillation of MBs produced strong mechanical forces to push Dil to distant regions away from cells (out-of-plane shedding [41]). Roovers et al. also investigated the events of drug release by sonoprinting under stable cavitation of nanoparticle-loaded MBs (filled with C$_{60}$ gas) [42]. Under the low acoustic pressure (100 kPa), the released vesicles and nanoparticles were transported away from cells due to the microstreaming around the MBs. In contrast, the significant microstreaming under high acoustic pressure (300 kPa) induced sonoprinting to deposit vesicles and nanoparticles onto the cell membrane. In the present study, the events of vesicle release on the cell membrane under Dil-fltMB stable cavitation were also induced by sonoprinting.

5. Conclusion

Recently, therapeutic applications of MBs have been developed for ischemia-reperfusion injury, thrombolysis, and neurodegenerative diseases. In contrast to tumor therapy applications, these applications require the prevention of cell damage during US-stimulated MB cavitation. Therefore, the non-damaging effects of vesicle release and cell permeable enhancement improve the applied value of targeted-MB stable cavitation. In this study, we demonstrated the ability to release vesicles via targeted-MB stable cavitation, which might be suitable for tissue repair and neomodulation.

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