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## **Supplementary Information for**

# Internal Polymer Scaffolding in Lipid-Coated Microbubbles for Control of

# **Inertial Cavitation in Ultrasound Theranostics**

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# **Table of Contents**

# **Supplementary Methods**

- I. Fabrication of lipid-polymer composite microbubbles (LP-MBs)
- II. Morphological characterization of LP-MBs
- III. In vitro stability of LP-MBs
- IV. In vivo ultrasound contrast persistence of LP-MBs
- V. Ultrasound resonance frequency range of LP-MBs
- VI. Stable and inertial cavitation doses of LP-MBs
- VII. Animal preparation

## **Supplementary Tables**

Table S1. Specifications of the ultrasound transducers used in this study

## **Supplementary Figures**

- Fig. S1. Effect of polymerization duration on in vitro stability of LP-MBs
- Fig. S2. Effect of polymerization duration on *in vitro* ultrasound contrast persistence of LP-MBs
- Fig. S3. Ultrasound attenuation meausrement for estimation of resonance frequencies of LP-MBs
- Fig. S4. Passive cavitation detection experiments for evaluation of stable and inertial cavitation behaviors of LP-MBs

### **Supplementary References**

#### I. Fabrication of lipid-polymer composite microbubbles (LP-MBs)

DSPC. Lipids (2-distearoyl-sn-glycero-3-phosphocholine, and methoxypoly(ethylene glycol)distearoyl-phosphatidyl ethanolamine, DSPE-PEG2000), monomers (butyl methacrylate, BMA), and crosslinkers (ethylene glycol dimethacrylate, EGDMA) were used to formulate LP-MBs. They were purchased from Avanti Polar Lipids (AL, USA) and Sigma-Aldrich (Missouri, USA). A lipid-polymer mixture of DSPC, DSPC-PEG2000, EGDMA, and BMA was first prepared in chloroform in the mole ratios of 20 : 1 : 100 : 10 in a 2-mL vial. The chloroform was removed by evaporation for 1 day to forming a thin film on the wall of the vial. To prepare the aqueous solution, 1 mL of degassed phosphate-buffered saline (PBS, pH 7.4) containing 1 wt % glycerol was added in the vial to dissolve the thin film, after which the vial was vacuumed and refilled with perfluoropropane gas  $(C_3F_8)$ . Afterward, the vial was subjected to intense agitation for 45 s to form template LP-MBs. Crosslinking of BMA and EGDMA inside LP-MBs forming polymer networks was triggered by the addition of initiator (ammonium persulfate, APS) and accelerator (tetramethylethylenediamine, TEMED) for induction of radical polymerization. To ensure homogeneity, the polymerization was run under gentle stirring for up to 3 h at room temperature. Polymerized LP-MBs were purified for removal of free lipids and prepolymers that failed to encapsulate C<sub>3</sub>F<sub>8</sub> and excess APS and TEMED to terminate the polymerization process. The purification process was performed by collecting the supernatant cake after the centrifugation and then resuspended with the same amount of clean PBS.

#### II. Morphological characterization of LP-MBs

The size distributions of LP-MBs before and after polymerization were measured with the Coulter counter (Multisizer 3, Beckman Coulter, Miami FL, USA) in the size range of 0.7–18  $\mu$ m. The data were presented in terms of number concentration and total volume fraction, as shown in Fig. S1. The other descriptions of the results are included in the main text.

The structural differences of polymerized and nonpolymerized LP-MBs were visually assessed with a cryogenic transmission electron microscope (Cryo-TEM) (Tecnai G2 F20, FEI, OR, USA). Diluted MBs samples were placed on holey carbon film–covered copper grids (HC300-Cu, PELCO, CA, USA) for blotting humidity and plunge-freezing by Vitrobot (FEI, Hillsboro, OR, USA). Afterward, the copper grids were stored under liquid nitrogen and transferred to the electron microscope for imaging using a cryostage. To further confirm the formation of polymer networks,

negative-stain TEM (HT7700, Hitachi, IL, USA) with 1% of uranyl acetate was conducted for polymerized LP-MBs sample after disruption with 1% Triton X-100 under sonication (2510, Branson, CT, USA). Intact polymer networks not disrupted by sonication were expected to appear as spherical leftovers with diameters similar to those of the initial LP-MBs sample in the TEM images. The descriptions of the results are included in the main text.

#### III. In vitro stability of LP-MBs

To determine whether the formation of polymer networks reduced the stability of LP-MBs, the *in vitro* stability of polymerized and nonpolymerized LP-MBs was assessed under conditions mimicking the steady-state *in vivo* scenario. The samples were diluted to a volume fraction of 10 nL/mL in isotonic saline close to the clinically acceptable dose of commercial ultrasound contrast agents such as Definity. Afterward, they were maintained at 37°C for up to 3 h, during which the total volume fraction of MBs was measured with the Coulter counter to quantify the change in MBs surviving fraction as a function of time. Each experimental case comprised the results of five independent tests for statistical analysis. An *in vitro* half-life was defined for each sample as the time taken for a 50% reduction in the total volume fraction. The results are shown in Fig. S1. The descriptions of the results are included in the main text.

#### IV. In vivo ultrasound contrast persistence of LP-MBs

The *in vivo* stability of polymerized and nonpolymerized LP-MBs was assessed by the ultrasound contrast persistence of MBs in ultrasound brain imaging in rats. A bolus of two hundred microliters of MBs dilution in saline with the same total MB volume fraction of 10 nL/mL (approximately corresponding to  $8 \times 10^6$  bubbles/mL) was intravenously injected via the jugular vein. Serial B-mode imaging was conducted at 2 fps after MB injection with the homemade 25-MHz high-frequency ultrasound imaging system established in the previous study [1]. The contrast enhancement of flowing MBs in the dorsal sagittal sinus in each image was plotted as a function of time for assessment of *in vivo* clearance of different MB samples.

Fig. S2a shows typical examples of B-mode images of a rat brain at 0 and 20 s after the bolus injection of LP-MBs polymerized for 3 h. Dotted enhancement in contrast intensity represented the presence of MB particles flowing by the imaging plane. Fig. S2b shows the time versus contrast enhancement curves analyzed in the dorsal sagittal sinus for polymerized (3 h) and nonpolymerized (0 h) LP-MBs. The rapid rise rate and slow decay rate represented the wash-in and wash-out characteristics,

respectively, of MBs due to the bolus injection; these were found to be similar for both the polymerized and nonpolymerized LP-MBs. A similar maximum level of contrast enhancement was achieved for both the samples to be about 20 dB. These results suggest polymerized and nonpolymerized LP-MBs provide identical *in vivo* ultrasound contrast persistence. The other descriptions of the results are included in the main text.

#### V. Ultrasound resonance frequency range of LP-MBs

The influences of polymer network formation on the acoustic properties of LP-MBs were assessed in two separate experiments. First, the range of LP-MBs' ultrasound resonance frequencies was estimated by taking advantage of the frequency-dependent attenuation of through-transmission ultrasound signals due to MBs oscillation. The frequency range presenting high spectral attenuation revealed the resonance frequencies of MBs [2-4]. The experimental setup is illustrated in Fig. S3a. Three ultrasound transducers with overlapped the frequency bandwidths from 3.5 to 42 MHz were used for ultrasound transmission. The specifications are summarized in Table S1. An arbitrary waveform generator (AWG 2040, Tektronix, CA, USA) and a 50-dB power amplifier (325LA, E&I, NY, USA) were used to drive these transducers. Single one-cycle ultrasound pulses with a peak negative pressure of 50 kPa were used to insonate diluted MBs solution (total volume fraction: 2 nL/mL) in a plastic chamber through the attached acoustically transparent membranes. After traveling across the MB solution for a distance of 17 mm, the ultrasound pulses were attenuated due to the scattering and absorption of ultrasound energy by oscillating MBs. The attenuated pulses were received on the opposite side by a broadband hydrophone (model HGL-0085, Onda Corp., CA, USA). The received signals (N = 50) were digitized by an oscilloscope (LT322, LeCory, Chestnut Ridge, NY, USA) for off-line analysis using MATLAB software (MathWorks, MA, USA). The attenuation spectrum for each MB sample was obtained by comparing the Fourier spectra of the received signals in the absence and presence of MBs. Only the spectral values within the -6-dB bandwidth of each transducer were included in order to ensure the reliability of the results.

Fig. S3b shows that LP-MBs polymerized for different durations of 0–3 h exhibited a similar attenuation spectrum in the range of 10–15 MHz with a maximum attenuation coefficient of about 0.45 dB/mm at 12 MHz. In the following experiments we used the frequency of 10 MHz for quantification of stable and inertial cavitation doses, since it has been widely used for contrast-enhanced ultrasound research and medical imaging. The other descriptions and discussion of the results are included in the main text.

#### VI. Stable and inertial cavitation doses of LP-MBs

The cavitation behaviors (i.e., stable and inertial cavitation) of polymerized and nonpolymerized LP-MBs under 10-MHz ultrasound exposure were tested in a passive cavitation detection setting as described as follows. The experimental setup is shown in Fig. S4a. Diluted MB solutions (total volume fraction: 10 nL/mL) were injected into an agar-based (2% w/v) vessel phantom with 1-mm diameter using a syringe pump (KDS100, KD Scientific, New Hope, PA, USA). A 10-MHz ultrasound transducer (V322, Olympus, MA, USA), as specified in Table S1, was used to transmit ultrasound pulses (100 cycles with a Hanning window) with different pressure amplitudes to insonate flowing MBs. An arbitrary waveform generator (AWG 2040, Tektronix, CA, USA) and a radio-frequency power amplifier (325LA, E&I, NY, USA) were used to drive this transducer. The flow velocity of MBs and pulse repetition rate were regulated so that MBs were exposed to only one single US pulse within the focal zone. Ultrasound signals scattered from oscillating MBs contained distinct spectral characteristics that can be used to quantify the doses of stable and inertial cavitation. Hence, a 5-MHz transducer (model V308, Olympus, MA, USA) was placed perpendicularly to the 10 MHz transducer to received scattered ultrasound signals. The received signals were amplified by a broadband receiver (model BR-640A, Ritec, RI, USA) and then digitized by an oscilloscope (model LT322, LeCory, NY, USA) for statistical analysis using MATLAB software. Each data set comprised 50 signals, and each experimental case comprised five data sets acquired in independent tests for statistical analysis. For each case, a stable cavitation dose was quantified with the area under the curve of the subharmonic peak that has been suggested to be the exclusive characteristic of MBs undergoing resonance (Fig. S4b) [5]. At increasing pressure amplitudes, MBs might start to undergo inertial collapse and give rise to shock wave emissions that resulted in the spectral broadening of harmonic peaks. An inertial cavitation dose was thus computed with the neighboring area under the curve clear of the contribution of fundamental and subharmonic peaks. Both the calculated doses were normalized to those obtained for pure PBS in the absence of MBs under the same conditions. The results obtained for LP-MBs with different polymerization durations from 0 h to 3 h were compared. The descriptions of the results are included in the main text.

# VII. Animal preparation

Sprague–Dawley rats were obtained from the National Laboratory Animal Center of Taiwan. Animal research protocols were in accordance with institutional animal use and care regulations approved by the Animal Care & Utilization Committee of National Tsing Hua University. The rats were anesthetized IP with chloral hydrate (400 mg/kg) before intubation and craniotomy. For intravenous injection of MBs, a catheter (PE-50, BD, NJ, USA) was inserted into the jugular vein. A cranial window of approximately  $1 \times 1$  cm<sup>2</sup> was fashioned with a high-speed drill before applying FUS irradiation to reduce the distortion of the imaging ultrasound beam. All experiments were performed in accordance with institutional guidelines for animal research under a protocol approved bythe National Tsing Hua University animal experiment committee.

Table S1. Specifications of the ultrasound transducers used in the passive cavitation detection (5 and 10 MHz) for evaluation of stable and inertial cavitation doses of LP-MBs, and in the through transmission broadband attenuation measurements (7.5, 15, and 30 MHz) for evaluation of MB resonance frequency range.

	Transduce	Focal	Anorturo	Center	Frequency
	r	length	(inch)	frequency	bandwidth (–6 dB)
	model	(inch)	(men)	(MHz)	(MHz)
1.	V307	2	1	5	3.5-6.5
2	V321	2	0.75	7.5	5-10
3.	V322	2	1	10	6.5–13.5
4.	V319	2	0.5	15	10–22
5.	V375	0.75	0.25	30	18–42



Fig. S1. Effect of polymerization duration on *in vitro* stability of LP-MBs. Total volume fraction and surviving fraction of LP-MBs were calculated as a function of time for different polymerization durations. All the tested LP-MBs were diluted to an identical volume fraction of 10 nL/mL and maintained at 37°C for up to 3 h throughout the experiments.



Fig. S2. Effect of polymerization duration on *in vitro* ultrasound contrast persistence of LP-MBs. (a) Ultrasound B-mode images of a rat' brain after bolus injection of LP-MBs polymerized for 3 h. (b) Contrat enhancenets of LP-MBs in the region of interest in the dorsal sagittal sinus as a function of time for polymerization durations of 0 h and 3 h.



Fig. S3. Ultrasound through-transmission attenuation meausrement for estimation of resonance frequencies of LP-MBs. (a) Schematic diagram of the experimental setup. (b) Calculated attenutaion coefficinet as a function of ultraosund freuqency for LP-MBs with different polymerization durations.



Fig. S4. Passive cavitation detection experiments for evaluation of stable and inertial cavitation behaviors of LP-MBs. (a) Schematic diagram of the experimental setup. (b) Data processing for calculation of stable and inertial cavitation doses (abbreviated as SCD and ICD, respectively).

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