Electronic Supplementary Information

Sink or Float? Characterization of Shell-Stabilized Bulk Nanobubbles Using Resonant Mass Measurement Technique

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Experimental Section

Nanobubble Formulation: A lipid solution was first prepared by dissolving 6.1 mg of DBPC, 2 mg of DPPE, 1 mg of DPPA, and 1 mg of mPEG-DSPE in 0.1 mL of propylene glycol by repeated sonication and heating at 80 °C. A second mixture containing 0.8 mL of phosphate buffered saline (PBS) (pH 7.4) and 0.1 mL of glycerol was brought to 80 °C, added to the lipid solution, and then sonicated at room temperature for 10 minutes. This solution was then transferred to a 3 mL headspace vial and capped with a rubber stopper. The gas inside the vial was then replaced with octafluoropropane gas. To activate the bubbles, the vial was then agitated using a VialMix mechanical shaker for 45 seconds. Nanobubbles were then isolated from the bubble population based on their buoyancy by centrifugation. The terminal velocity (υ_∞) of a bubble was estimated using the force balance between Stokes drag and buoyant forces.[1]

$$\upsilon_\infty = gd^2 (\rho - \rho_b)(18\mu)^{-1} \quad (1)$$

where d is the bubble diameter, g is the gravitational acceleration, μ is the viscosity of the fluid medium (0.001 $N \cdot s \cdot m^{-2}$), ρ and ρ_b are the densities of the fluid medium (1000 $kg \cdot m^{-3}$) and bubble (8.17 $kg \cdot m^{-3}$), respectively. According to equation 1, when centrifuged at $50 \cdot g$ for 5 min, all bubbles larger than 0.7 µm should rise 0.5 cm or greater. Nanobubbles were isolated by collecting below this distance.

RMM Measurement: Particle size and buoyant mass were measured using RMM (Archimedes, Malvern Instruments). In our experiments, we utilized two sensors: a micro sensor provided sizing measurement from 5 µm to 250 nm, and a nanosensor that provided measurement spanning 2 µm to 100 nm for bubbles. Sensors were calibrated using NIST traceable 1.0 µm polystyrene bead standards (ThermoFisher 4010S, Waltham MA, USA). Sensor calibration was finalized after 300 particles were detected. Prior to beginning sample measurements, a 5 minute water blank was run to ensure that the system fluidics and sensor were free of particles. All samples were diluted ~~to~~ 1:1000 with phosphate buffered saline (pH 7.4) to obtain an optimum concentration of 10^8 $particles \cdot mL^{-1}$. This concentration results in an acceptable limit of detection (0.01 Hz) and coincidence (< 10%). Samples were loaded into the system for 140 seconds at 2 psi and analyzed at low pressure (5 psi). Also, between measurements, the sensor and microfluidic tubing were rinsed for 30 seconds with PBS followed by 2 "sneezes" (where the flow is repeatedly reversed in direction in the bypass and sensor channels) for at least 3 cycles. Data was exported from the Archimedes software (version 1.2) and analyzed for positive and negative counts. A density of 0.008 $g \cdot mL^{-1}$ for positively buoyant particles and 1.3 $g \cdot mL^{-1}$ for negatively buoyant particles was inputted into the Archimedes software to convert the measured mass to a particle diameter, which is based on the density of the octafluoropropane gas used to fill the bubbles and the density of

vesicles, which has a similar structure and size as that of non-buoyant particles.[2,3] The sharp cut-off in size observed in Fig. 1c for both buoyant and non-buoyant particles is a result of the LOD of the sensor determined for each measured sample. The cut -off observed in the graph occurs at the smallest size that can detected by the sensor for the measured sample. The LOD is determined at the start of each measurement and accounts for the baseline noise. The authors did not use any threshold or post-processing to reject smaller sized particles.

In Vitro Ultrasound Imaging: The *in vitro* echogenicity of nanobubbles was determined for 6 concentrations ranging from 7.0×10^5 to 7.0×10^{10} bubbles $\cdot$ mL^{-1}. Bubble solutions were placed in a custom-made 1.5% (w/v) agarose mold with a thin channel (L x W x H = 22 x 1 x 10 mm) as described previously.[4] The agarose phantom was fixed over a 12 MHz linear array transducer and imaged using a clinical ultrasound scanner (AplioXG SSA-790A, Toshiba Medical Imaging Systems, Otawara-Shi, Japan). System acquisition parameters were set to contrast harmonic imaging (CHI) with 12.0 MHz harmonic frequency, 0.29 mechanical index (MI), 65 dB dynamic range, and 70 dB gain. The ultrasound signal for each concentration was determined using the pre-loaded quantification software (CHI-Q). Enhancement by nanobubbles was calculated by normalizing the nanobubble signal to the signal to a control phosphate buffered saline solution (n=3).

Ultrasound-mediated Nanobubble Destruction: The custom agarose phantom was fixed above the ultrasound transducer, and the isolated nanobubbles were diluted to 7.0×10^{10} bubbles $\cdot$ mL^{-1} in PBS and transferred to the trough. Bubbles were destroyed using the flash/replenish feature on the clinical ultrasound (20 flash cycles, 12 MHz harmonic frequency, 1.52 MI). Bubble contrast was

monitored using an AplioXG SSA-790A clinical ultrasound scanner (Toshiba Medical Imaging Systems, Otawara-Shi, Japan) equipped with a 12 MHz linear array transducer. The ultrasound signal was determined using the pre-loaded quantification software (CHI-Q) (n=5).

In Vitro Nanobubble Stability: *In vitro* US stability of the nanobubbles were determined by performing US imaging (AplioXG SSA-790A, Toshiba Medical Imaging Systems, Otawara-Shi, Japan) of the nanobubbles from a period of 0 -5 h every hour using a custom-made 1.5% (w/v) agarose mold with a thin channel (L x W x H = 22 x 1 x 10 mm) (n=3).[2] System acquisition parameters were set to contrast harmonic imaging (CHI) with 12.0 MHz harmonic frequency, 0.29 mechanical index (MI), 65 dB dynamic range, and 70 dB gain. The ultrasound signal was determined using the pre-loaded quantification software (CHI-Q). Initial signal enhancement was compared for each hour time point. Simultaneous RMM (Archimedes, Malvern Panalytical) measurements were performed for each trial at each time point to determine bubble concentration and size distribution. All samples were diluted to 1:1000 with phosphate buffered saline (pH 7.4) prior to measurement.

In Vivo Ultrasound Imaging: Animal experiments were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University. In all procedures, the animals were anesthetized with 3% isoflurane with 1 L/min oxygen. Both NB solutions and Lumason (sulfur hexafluoride lipid-type A microspheres, Bracco Diagnostics Inc.) were tested *in vivo*. Tumors were inoculated in the flank of male nude mice by injection of PC3 prostate cancer cells in Matrigel®. Two weeks after inoculation, mice with tumor diameters of 0.8 cm or larger were selected for bubble injection. The US probe was placed in an orientation to allow

visualization of the subcutaneous tumor image. Contrast-enhanced US images were acquired with a Vevo 3100 (FUJIFILM VisualSonics) at 24 fps, 18 MHz, and 4 % power following tail vein injections of 200 µL of undiluted NBs (~7 x 10^{11} particles mL^{-1}) or Lumason (~3.5 x 10^{8} particles mL^{-1}). The images were acquired for approximately 5 min post-injection and the bubble distribution in the tumor over time was compared for both NB and Lumason.

Statistical Analyses: Statistical analyses were performed using one-way ANOVA followed by a Tukey test to determine differences between the groups. Statistics with a value of at least $p < 0.05$ were considered to be significant.

References

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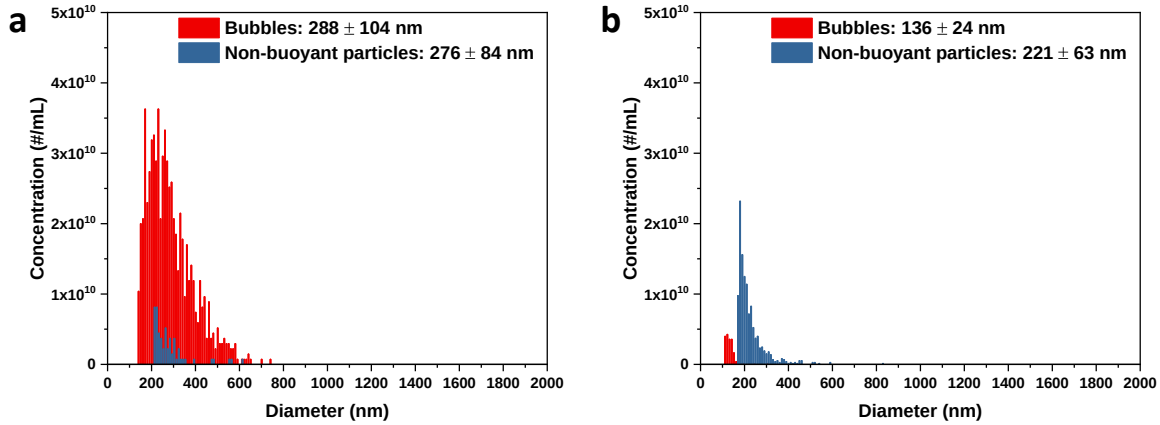

Fig. S1. Representative RMM measurement of nanobubbles before (a) and after (b) nanobubble destruction. A drastic decrease in bubble population was observed following exposure of nanobubbles to strong US power and a concomitant increase in concentration of non-buoyant particles.

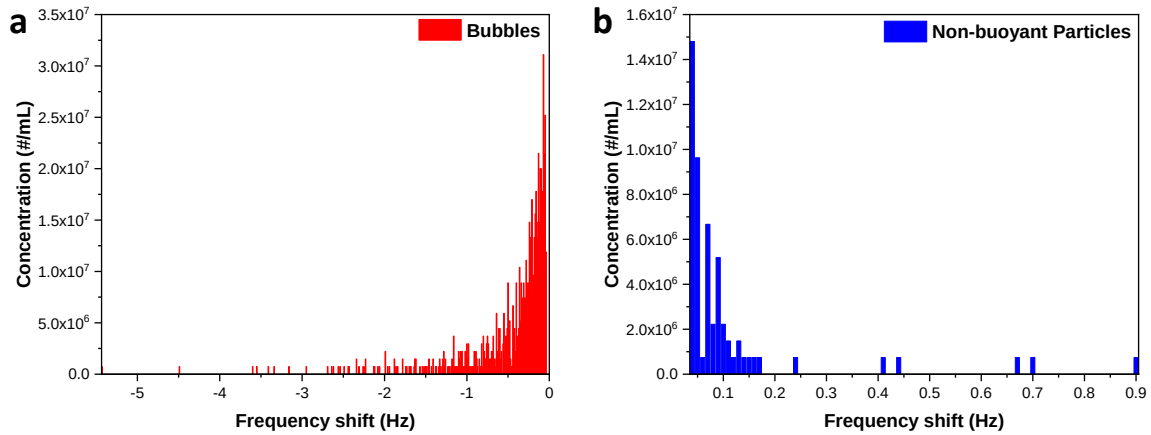

Fig. S2. RMM plots of concentration vs. frequency shift for both (a) buoyant and (b) non-buoyant (right) particles with respect to PBS solution.

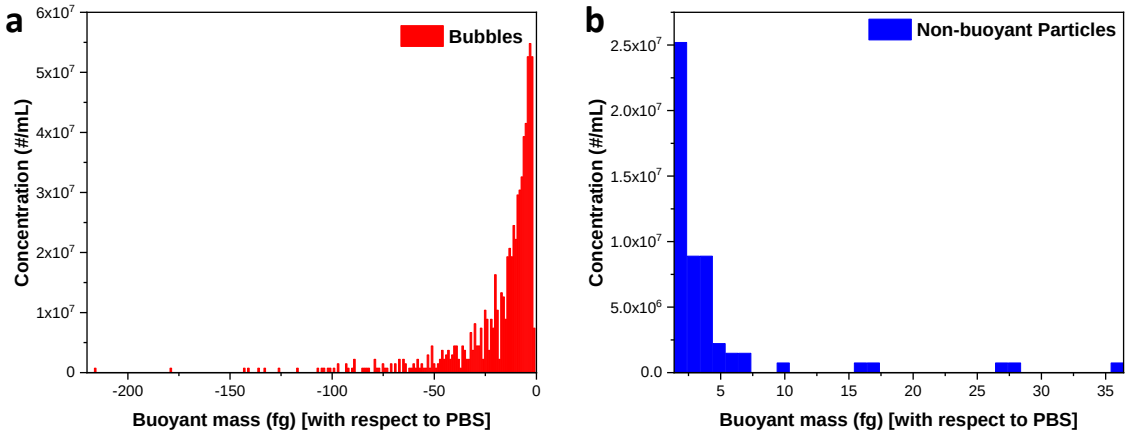

Fig. S3. RMM plots of concentration vs. buoyant mass for both (a) buoyant and (b) non-buoyant particles with respect to PBS solution.