Supplementary Information

Microbubble Preparation

Stock solutions of the lipids and the dye were prepared by dissolving the dry powder in chloroform. 1 mg of Distearoyl phosphatidylcholine and 1 mg of distearoyl phosphatidylethanolamine-methyl poly(ethylene glycol) MW5000 (Avanti Polar Lipids, Inc., Alabaster, AL) were combined in a 1.5 mL polypropylene microcentrifuge tube (Eppendorf, Hamburg, Germany). An appropriate amount of 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Biotium, Hayward, CA) was added to the same tube. The solution was vortexed at low speed while applying an argon stream to evaporate all of the chloroform and create a lipid film.

 $500 \ \mu\text{L}$ of Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Bethesda, MD) was added to the lipid film, and the tube was vortexed at high speed for 1 minute. The tube was immediately placed into a heating block which was at 70°C. After 1 hour, the tube was removed and placed directly on ice for 5 minutes. A 3 mL syringe was loaded with 100 μ L perfluorohexane (Sigma-Aldrich, St. Louis, MO). A 1.5" 22 gauge needle was attached and bent at a 60° angle to allow the syringe to be pumped without ejecting liquid. By pumping the syringe, the perfluorohexane was vaporized, and the headspace of the tube was filled with a mixture of air and perfluorohexane vapor.

The XL-2000 probe sonicator (QSonica LLC., Newtown, CT) was operated at the liquid/gas interface for 3 seconds at 25 W to produce MBs. The bubbles were then put back on ice. To purify the bubbles and wash away the excess lipid and dye, the bubbles were centrifuged at 1000 rpm for 3 minutes. The subnatant was removed with a syringe (approximately 450 μ L), and the bubbles were resuspended with 500 μ L DPBS. This was repeated once more. Not all of the perfluorohexane/air mixture in the headspace of the tube was incorporated into microbubbles since some was forced out during probe sonication. Some large (millimeter-sized), relatively unstable bubbles were also produced during sonication, but most of these bubbles were not stable beyond 15 minutes post-sonication and they do not survive the centrifugation of the washing step. For the control samples, MBs containing no DiI were prepared in the same method. In addition, liposomes containing DiI were prepared with fluorescence equivalent to the DiI-labeled MBs as described below.

The perfluorohexane/air mixture used is similar to that of FDA-approved ultrasound contrast agent Imagent®, and the mixture conveys a stabilizing effect on the MBs at atmospheric pressure¹. MBs were stable in PBS for 7 days and size increase could be observed at later time points due to coalescence. Perfluorohexane is the highest molecular weight perfluorocarbon which can exist in the gaseous state, and also has the lowest solubility in water². Therefore, microbubbles had a better chance of persisting after being strongly diluted, as was needed in the acousto-fluorescence experimental setup. No recondensation of perfluorohexane was observed. However, in case there was any recondensation, the purification method (centrifugation) would have removed these particles, since the buoyant microbubbles were collected, and the subnatant was removed. Condensed perfluorohexane (liquid) has a density of 1.68 g/mL³. Since this condensation is accompanied by a ~100-fold volume decrease (~5-fold radial), 1-5 µm bubbles would produce 0.2-1 µm droplets which will pellet easily at the centrifugation parameters.

Microbubble Size Distribution Analysis

MBs were imaged by light microscopy for determination of diameter with a calibrated microscope. MB images were loaded in ImageJ (National Institute of Health, Bethesda, MD) for particle analysis. The mean and standard deviation of 1448 MBs were calculated to be 2.13 µm and 1.20 µm, respectively.



Preparation of Fluorescent Liposomes for Control Experiment

Stock solutions of the lipids and the dye were prepared by dissolving the dry powder in chloroform. 5 mg of Distearoyl phosphatidylcholine and 5 mg of distearoyl phosphatidylethanolamine-methyl poly(ethylene glycol) MW5000 (Avanti Polar Lipids, Inc., Alabaster, AL) were combined in a 4 mL glass vial. Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Biotium, Hayward, CA) was added to the same vial to achieve a final concentration of 0.1 mol% of the liposomes. The solution was vortexed at low speed while applying an argon stream to evaporate all of the chloroform and create a lipid film. 1 mL of Dulbecco's phosphate buffered saline (DPBS) (GIBCO, Bethesda, MD) was added to the lipid film, and the tube was vortexed at high speed for 1 minute to produce a turbid lipid solution. The vial was placed on ice, and the XL-2000 probe sonicator (QSonica LLC., Newtown, CT) was pulsed in the liquid, 3-4 mm from the bottom of the vial for 5 minutes to produce liposomes. Liposomes size was measured to be 226 nm by dynamic light scattering using a Zetasizer Nano ZS (Malvern, Worcestershire, UK).

Prior to ultrasound experiments, fluorescence intensity of microbubbles and liposomes (diluted 50fold in DPBS) were measured using an Infinite 200 PRO (Tecan, Mannedorf, Switzerland) fluorescence microplate reader. The liposomes were then diluted appropriately to equalize the fluorescence with the microbubble sample.

Contrast-Enhanced Ultrasound Imaging of DiI-Loaded Microbubbles

Microbubbles surface-loaded with 2 mol% DiI were diluted 1:1000 into standard disposable transfer pipettes (ThermoFisher Scientific, Waltham, MA) containing DPBS to achieve a total volume of 3 mL. Samples were held in water tank at 22°C while a Logiq E9 (GE Healthcare, Wauwatosa, WI) ultrasound scanner coupled to a ML6-15 probe was used to acquire images of the microbubbles in contrast mode.

Quantification of Static Fluorescence & Calculation of Intermolecular Distance

Microscope images were acquired through MetaMorph® Microscopy Automation & Image Analysis Software. Fluorescence was imaged using a Cy3/Cy5 filter. Unscaled images were acquired for each dye concentration and processed in ImageJ. For each dye concentration, the pixel intensity per unit area from 20 representative bubbles was integrated and the standard deviation was calculated. For the calculation of quenching efficiency, the lowest dye concentration (.25 mol%) was taken as a completely unquenched state, for simplicity. Quenching efficiency at a given dye concentration was calculated as the percent decrease of fluorescence per mole of dye from that at .25 mol%.

Intermolecular distance was calculated using an average lipid head diameter of 7 Å⁴. The linear density was taken as the square root of the surface density. The theoretical quenching efficiency was computed using the Förster resonance energy transfer (FRET) efficiency equation

$$E = \frac{1}{1 + (r/R_0)^6}$$

where R_0 is the Förster radius of 45 Å, as cited for the DiO-DiI distance⁵. DiO is a lipophilic carbocyanine dye structurally similar to DiI and the DiO-DiI Forster radius was assumed similar to that of DiI-DiI, since there is a similar degree of spectral overlap.

Calculation of Signal-to-Noise Ratio (SNR)

To determine the signal intensity, the mean of all positive peaks was calculated, as well as the mean of negative peaks. To determine the noise, the standard deviation of positive peaks was calculated, as well as the standard deviation of the negative peaks. The SNR of individual readings was calculated as the difference of means divided by the sum of standard deviations, or,

$$SNR = \frac{\mu}{\sigma} = \frac{\mu^+ - \mu^-}{\sigma^+ + \sigma^-}$$

Experimental Setup

An acousto-fluorescence detection setup is shown in Figure A. A two liter black water bath was used for a clean sound field with transparent optical windows for light delivery and collection. A Verdi-V5 continuous-wave 532nm laser source (Coherent Inc., Santa Clara, CA, USA) was focused with a 20x objective with a power density greater than 5μ W/cm² at the laser focus. A 2.25MHz focused singleelement Panametrics V306 ultrasound transducer (Olympus NDT Inc., Waltham, MA, USA) was aligned with its focus confocal with the laser focus. The US was pulsed at a 1Hz repetition rate, with 15 cycles per pulse at 2.25MHz, which was within the resonance frequency range of the 1-5 µm MBs. Transducer positioning and US pressure were calibrated with a HNC-0200 needle hydrophone (Onda Corp., Sunnyvale, CA, USA).

Light was collected at 75° to 95° and split into two paths with a beam splitter with two photomultiplier tubes (PMTs) used simultaneously to monitor both the fluorescence signal and the optical scatter of excitation light to correlate MB size changes to fluorescence modulation intensities. Both optical signals were recorded simultaneously with LabVIEW. The scattered light was intense enough to be detected with a PMT operating at low gain. At this gain, MB fluorescence was too weak to be detected. To detect the fluorescent signal, light was passed through two optical filters onto a low dark current R6095 PMT optical detector (Hamamatsu Corp., Bridgewater, NJ, USA) with a 10MHz bandwidth, operated at high gain. Two filters (a 546 nm long pass and 570 nm band pass) were used for a high rejection ratio of the excitation light. Because the filters are interference filters, normal incidence of photons is necessary for high rejection ratios. Therefore, two long black optical barrels were used to ensure only straight photons could pass through the filters and reach the optical detector. The PMT signal was then passed through a low noise M7279 amplifier (Hamamatsu Corp., Bridgewater, NJ, USA), held at constant gain, to a high-speed digital TDS2020 sampling oscilloscope (Tektronix Inc., Beaverton, OR, USA) which was controlled using LabVIEW software on a computer. MBs were delivered using a PHD 2000 syringe pump (Harvard Apparatus, Holliston, MA, USA) and a capillary tube placed in-line with the laser optical axis and outside the US interaction region. The presence of flowing MBs was confirmed by looking at the unfiltered scattered light with a standard video-rate 1322 CCD camera (Cohu Inc., San Diego, CA, USA).

To load a sample, a 60 mL syringe was filled with 40 mL of DI water, to which 100 μ L of the MB suspension was added. For the control samples, after 100 μ L of undyed MBs was added, 100 μ L of fluorescent liposomes was then added to achieve a bulk fluorescence equal to that of the fluorescent MBs. The syringe was then rotated gently to completely disperse the MBs and liposomes in the entire volume. It was chosen to have the sample flow directly into the water tank, since water is an excellent medium both for optics and acoustics. This prevented the need for any tube which could potentially scatter light and/or sound. In between samples, the water tank was drained, washed, and refilled to prevent contamination.



Figure A - Experimental setup for fluorescence modulation detection. An US transducer, laser-focusing lens, and collection objective are all confocal and mutually orthogonal. The collected light is split into two paths, one of which is filtered to look at fluorescence. Two independent PMTs collect the different signals, and their output is connected to an oscilloscope. Data is acquired and preprocessed through LabVIEW.

- 1. E. G. Schutt, T. J. Pelura and R. M. Hopkins, *Academic radiology*, 1996, **3 Suppl 2**, S188-190.
- M. G. Freire, L. Gomes, L. M. N. B. F. Santos, I. M. Marrucho and J. A. P. Coutinho, *The Journal of Physical Chemistry B*, 2006, 110, 22923-22929.
- 3. M. M. Piñeiro, D. Bessières, J. M. Gacio, H. Saint-Guirons and J. L. Legido, *Fluid Phase Equilibria*, 2004, **220**, 125-134.
- 4. T. Heimburg, *Biophys J*, 2000, **78**, 1154-1165.
- S. L. Yefimova, G. Y. Gural'chuk, A. V. Sorokin, Y. V. Malyukin, I. A. Borovoy and A. S. Lubyanaya, *Journal of Applied Spectroscopy*, 2008, 75, 658-663.