Supplementary Material (ESI) for Soft Matter

Facile One-Pot Synthesis of Polymer-Phospholipid Composite Microbubbles with Enhanced Drug Loading Capacity for Ultrasound-Triggered Therapy

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Electronic Supplementary Information:

Supplementary Figures:

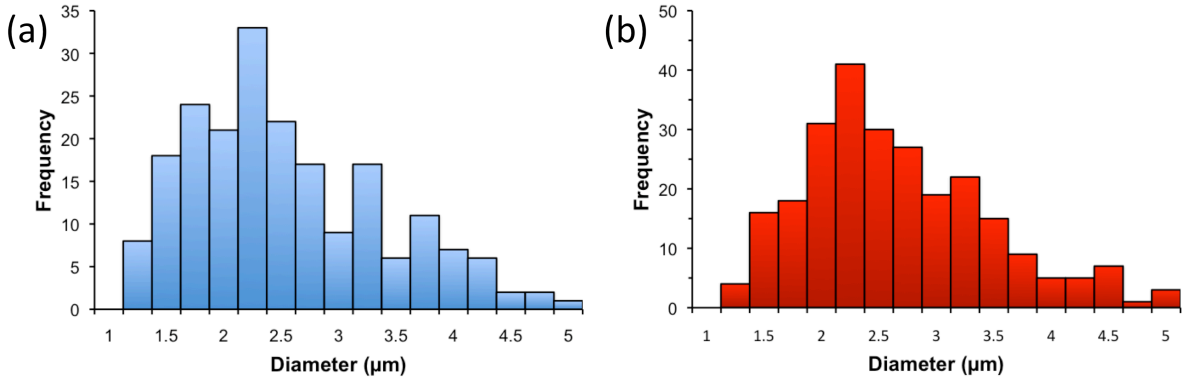

Figure S1: Size histograms for DSPC microbubbles made without (a) and with (b) added PAA from bright field microscopy. There were no bubbles larger than 5 μm found for either sample.

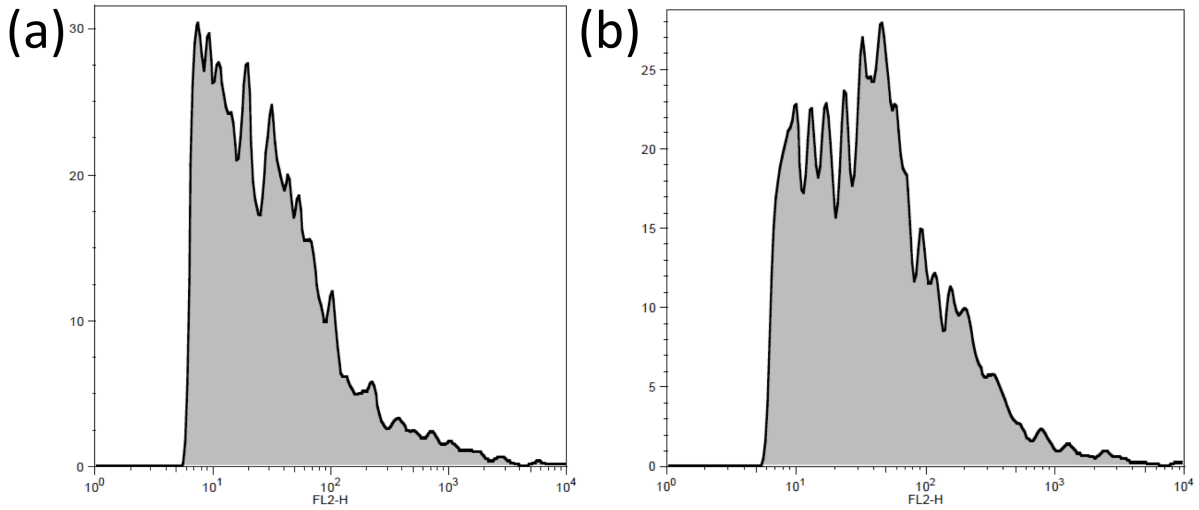

Figure S2: Histogram of Rhodamine B fluorescence encapsulated in DSPC microbubbles synthesized without (a) and with (b) added PAA. The average relative fluorescence is approximately 3-fold greater for bubbles with PAA than without.

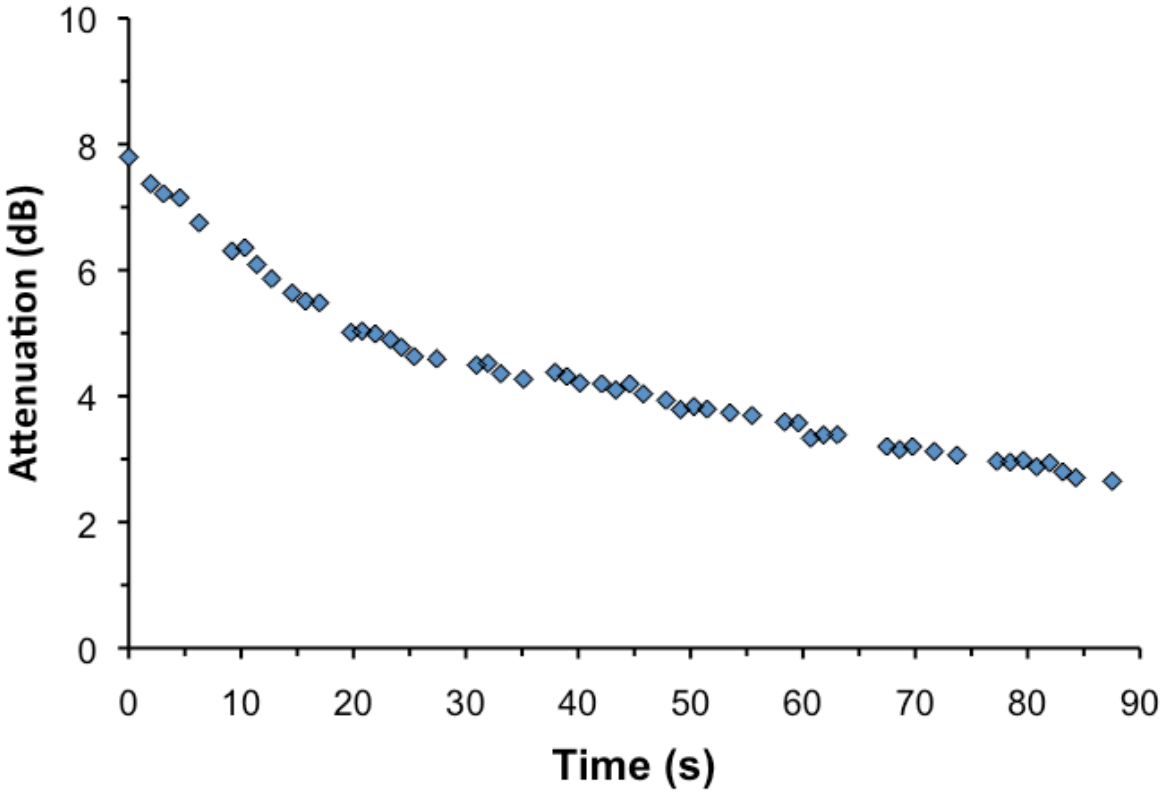

Figure S3: Attenuation of PAA-microbubbles at 2.25 MHz while insonated continuously at 357 kPa at 100 Hz with a center frequency of 2.25 MHz. The attenuation was measured by needle hydrophone and compared to PBS blank as with backscatter studies.

Experimental Methods:

Synthesis of Partially Thiolated Poly(acrylic acid) (PAA-SH). This synthesis was adapted from a literature procedure.[9] 200 mg poly(acrylic acid) (PAA, 2.77 mmol per monomer unit, M_w ~ 5000, 50% wt% in water, Aldrich) was mixed with 14 mL deionized water and 46 mg sodium hydroxide (Fisher). 192 mg N-hydroxysuccinimide (NHS, 1.662 mmol, Pierce) and 159 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 0.831 mmol, Pierce) were dissolved and agitated 20 min. 95 mg cysteamine hydrochloride (0831 mmol, Aldrich) was added in one portion, and the mixture was agitated at RT overnight. The mixture was transferred to a regenerated cellulose dialysis tube (MWCO ~ 3500) and dialyzed 2 d against several changes of distilled water, followed by removal of water through lyophilisation (116 mg, 42.2%). ^{1}H NMR (500 MHz, D_2O): δ 1.34-1.89 (m, 138H), 2.05-2.60 (m, 69H), 2.63-2.81 (m, 18H), 2.91-3.10 (m, 18H).

Synthesis of 5(6)-Fluoresceinyl-2-Maleimidoethyl-Thiourea (FITC-Mal). Dissolved 31 mg 2-maleimidoethylammonium triflate (0.124 mmol) in 0.4 mL dimethyl sulfoxide (EMD). Added 29 μL triethylamine (0.206 mmol, Alfa Aesar) and mixed. Added fluorescein isothiocyanate (FITC, 0.103 mmol, Pierce) and stirred in dark 2 d. Added aqueous 1 M HCl until pH ~ 3. Centrifuged at 5000 g for 5 min to obtain pellet and removed supernatant. Added 2 mL methanol to dissolve solid, then reprecipitated into 15 mL water. Centrifuged again, removed supernatant, and removed remaining water via lyophilisation to obtain orange fluffy solid (43 mg, 80.7%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 3.59-3.80 (m, 4H), 6.42-6.78 (m, 6H), 7.01-7.25 (m, 2H), 7.60-7.78 (m, 2H), 7.82-7.94 (m, 1H), 8.00-8.21 (m, 2H), 10.05-10.21 (m, 2H).

Synthesis of Fluorescein-Labeled Partially Thiolated Poly(acrylic acid) (PAA-SH-FITC). Dissolved 53 mg PAA-SH (0.00883 mmol; See Supporting Information) 2 mL deionized water in vial with

stirbar. In separate tube, dissolved 4.3 mg FITC-Mal (0.00883 mmol; See Supporting Information) in 3 mL methanol (Fisher). Combined mixtures, stirred overnight in dark. Added to regenerated cellulose dialysis tubing (MWCO ~ 1000), dialyzed against several changes of distilled water 2 d. Lyophilized to obtain an orange fluffy solid (25 mg, 43.6%). ^{1}H NMR (500 MHz, D_2O): δ 1.32-1.89 (m, 138H), 2.05-2.40 (m, 69H), 2.63-2.81 (m, 18H), 2.91-3.10 (m, 20H), 6.42-6.78 (m, 4H), 7.01-7.25 (m, 2H), 7.60-7.78 (m, 2H), 7.82-7.94 (m, 1H), 8.00-8.21 (m, 2H), 10.05-10.21 (m, 2H).

Preparation of DSPC Suspension. 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti Polar Lipids) was dissolved in chloroform (EMD) at a concentration of 20 mg/mL. Under air flow at room temperature, chloroform was evaporated to form a thin film; this film was then placed under vacuum for at least 12 h to ensure total removal of solvent. The film was then suspended in distilled water at a concentration of 4 mg/mL and heated to 80°C for 30 min while stirring. The suspension was allowed to cool back to RT prior to use in microbubble suspension. For Rhodamine-B loaded microbubbles, 2 μL/mg DSPC of 0.05 mg/mL Rhodamine-B (Aldrich) in methanol (Fisher) was added prior to evaporation of the chloroform.

Preparation of Uncrosslinked PAA-SH-FITC Solution. Prior to microbubble formation, PAA-SH-FITC (see Supporting Information) was dissolved in 10 mM phosphate buffer at a concentration of 0.25 mg/mL. Dithiothreitol (DTT, Pierce) was added at a concentration of 2.5 mg/mL, and solution was mixed and allowed to sit overnight.

Preparation of Microbubbles. In a 1.6 mL centrifuge tube, the following stock solutions were combined, in order: 259.4 μL deionized water, 50 μL 100 mM aqueous acetic acid solution, 50 μL 1.5 M NaCl solution, 15.6 μL PAA-SH-FITC solution, and 125 μL of DSPC suspension to make 500 μL

total volume in a buffer containing 10 mM acetic acid and 150 mM NaCl (acetate buffered saline at pH 3.4, ABS). Perfluorobutane (PFB, Synquest Labs) was flowed twice through the solution and into the headspace above the solution twice, and the mixture was sonicated at the gas-liquid interface continuously for 10 s at 70% amplitude (Fisher Scientific Sonic Dismembrator 150T). The opaque suspension was washed once by centrifuging at 300 g for 3 min at RT to cause the bubbles to float, followed by removal of the subnatant via syringe and needle and replacement with ABS. Hydrogen peroxide (Fisher) was added to a final concentration of 10 mM, and the solution was mixed and allowed to sit without agitation. After 20 minutes, bubbles were centrifuge washed three time as above, replacing each time with PBS (Gibco) instead of ABS. The bubble solution was then diluted 2X with PBS, drawn slowly into a syringe, and allowed to sit for 15 min, plunger upwards, to let large bubbles float to the surface. The middle 80% of the solution was retained for analysis.

Fluorescence Microscopy. Bright field and fluorescence images were obtained on a Nikon Eclipse TE200 inverted microscope with a Spot Idea 5Mp Color Mosaic camera. High magnification images were obtained with a Nikon 100X objective with oil immersion. Green and red fluorescence were obtained using appropriate filter cubes.

Ultrasound backscatter measurements. An agar phantom measuring 7x7x4.5 cm with a central rectangular cavity measuring 2.5x2.5x3.5 cm was weighed down in a fish tank filled with water to the top of the phantom. A 2.25 MHz ultrasound transducer (Panametrics V306) was placed on a flat side of the phantom and aligned with the center of the sample cavity. Ultrasound signal was provided as an impulse via a Panametrics Computer Controlled Pulser/Receiver Model 5800 connected to and synchronized with an oscilloscope (Tektronix TDS 3032), which was connected to a PC (Dell) running Labview (National Instruments).

First, PBS was placed into the cavity of the phantom and the backscatter was recorded. Next, microbubbles were added to the PBS to a final dilution of 100 fM, mixed, and allowed to settle at least 5 min to prevent scattering due to sample currents. At least 100 consecutive impulses were sent into the sample at 2.25 MHz center frequency at 100 kHz repetition rate, receiving at 40 dB gain with a 300 kHz and 10 MHz high pass and low pass filter, respectively. Backscatter power was calculated as the square of the voltage difference between the signal and the baseline of the AC coupled signal, integrated over the entire time corresponding to the length of the sample, not including the walls of the chamber.[10] This was converted to dB, setting 1 V^2 equal to 0 dB. Net backscatter was calculated by subtracting the backscatter from the sample of PBS prior to microbubble addition. The phantom was washed at least three times between runs to remove any microbubbles adhered to the sidewall. Pulse peak pressure was measured with a calibrated needle hydrophone (Onda HND-0200, amplified by Onda AH-2020-DCBSW) placed on the opposite side of the phantom.

Ultrasound Attenuation Experiments. A different ultrasound setup was used to reach insonation peark pressures beyond 110 kPa. Experiments were set up as with backscatter experiments (see Main Text) except signal was generated as a single sine wave at 2.25 MHz (HP 8116A Pulse/Function Generator), amplified (T&C Power Conversion AG Serires Amplifier), and transmitted through the agar block and received by a needle hydrophone (Onda HND-0200, amplified by Onda AH-2020-DCBSW). The pulse was found to have a peak pressure of 357 kPa through calibrated hydrophone measurements. Attenuation was taken as the difference between the signal at 2.25 MHz measured with blank PBS and with added sample.

Microbubble Counting and Sizing. At least ten images per sample were taken at 20X in bright field at random locations in the sample. The microbubbles were counted and sized using ImageJ (NIH), using

a polystyrene bead standard (Duke Scientific) to calibrate pixels/μm. The concentration was calculated by determining the area of spot size against known volumes of buffer and was checked against silica microparticle standards (Bangs Labs). Microbubbles were also counted in an Invitrogen Countess Automated Cell Counter to confirm the accuracy of microbubble counting.

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