Comparing Strategies for Magnetic Functionalization of Microbubbles

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Supporting Information

ABSTRACT: The advancement of ultrasound-mediated therapy has stimulated the development of drug-loaded microbubble agents that can be targeted to a region of interest through an applied magnetic field prior to ultrasound activation. However, the need to incorporate therapeutic molecules while optimizing the responsiveness to both magnetic and acoustic fields and maintaining adequate stability poses a considerable challenge for microbubble synthesis. The aim of this study was to evaluate three different methods for incorporating iron oxide nanoparticles (IONPs) into phospholipid-coated microbubbles using (1) hydrophilic IONPs within an oil layer below the microbubble shell, (2) phospholipid-stabilized IONPs within the shell, or (3) hydrophilic IONPs noncovalently bound to the surface of the microbubble. All microbubbles exhibited similar acoustic response at both 1 and 7 MHz. The half-life of the microbubbles was more than doubled by the addition of IONPs by using both surface and phospholipid-mediated loading methods, provided the lipid used to coat the IONPs was the same as that constituting the microbubble shell. The highest loading of IONPs per microbubble was also achieved with the surface loading method, and these microbubbles were the most responsive to an applied magnetic field, showing a 3-fold increase in the number of retained microbubbles compared to other groups. For the purpose of drug delivery, surface loading of IONPs could restrict the attachment of hydrophilic drugs to the microbubble shell, but hydrophobic drugs could still be incorporated. In contrast, although the incorporation of phospholipid IONPs produced more weakly magnetic microbubbles, it would not interfere with hydrophilic drug loading on the surface of the microbubble.

KEYWORDS: magnetic targeting, microbubble, ultrasound, drug delivery, contrast agents

INTRODUCTION

Gas microbubbles (MBs) stabilized by a surfactant or a polymer shell were initially developed as contrast agents to enhance ultrasound imaging of the vasculature. The highly nonlinear response of the MBs to moderate ultrasound pressures produces acoustic radiation over a range of frequencies that can be readily distinguished from those due to the surrounding tissue. At higher pressures, the large amplitude oscillations exhibited by the MBs can produce substantial biological effects and these are being actively investigated for their potential application in ultrasound-mediated therapy.

A further complementary application of MBs is as therapeutic carriers of highly potent drugs. By encapsulating drugs within a MB and releasing them “on demand” using ultrasound, the risk of toxic side effects can potentially be reduced. Numerous formulations have been proposed to facilitate the loading of drugs within the MB coating and/or on its surface in the form of liposomes, nanoparticles, or single molecules. Extending the functionality of MBs, however, generates a series of potentially conflicting requirements for their design. In particular, MBs must have adequate stability both in storage and in circulation. They must be sufficiently responsive to ultrasound to promote therapy and ideal to be imaged during treatment. They must be able to carry sufficient quantities of drug to be effective; and it is also desirable to be able to target them to specific sites, for example, tumor endothelium.

With respect to the last of these requirements, there are a number of different approaches that can be applied either alone or in combination. The attachment of targeting species, such as antibodies, to the MB surface enables specific binding to target regions. The short half-life of MBs in circulation, however, can hinder the efficiency of this approach. To address this, acoustic radiation force has been used to concentrate and slow down the MBs at a site of interest to increase binding. A further possibility is to incorporate magnetic material into the MB, enabling localization at a target site using an externally applied magnetic field. The aim of this study was to investigate different methods previously reported for incorporating iron...
oxide nanoparticles (IONPs) and compare them to new formulations. The different types of magnetic MBs were assessed in terms of their stability, acoustic response, and delivery of genes or thrombolytic drugs,27 magnetic lipid-coated MBs have incorporated hydrophobic IONPs in an oil phase (OilMB), phospholipid-stabilized IONPs with hydrophobic cores (LipMB), and surface-bound hydrophilic IONPs (SurfMB). Biotin–dextran IONPs: Biotinylated carboxymethyl dextran-coated IONPs.

### EXPERIMENTAL SECTION

#### Materials and Methods. MB Formulations.

Five different MB formulations were tested. The first formulation (OilMB) consisted of phospholipid MBs loaded with hydrophobic IONPs suspended in an isoparaffin oil phase that partitions into the bubbles upon sonication. The OilMB method, previously presented,27 is similar to the acoustically activated lipospheres reported by others, where a layer of soybean oil is embedded in the bubbles to act as a reservoir for a hydrophobic drug.40,41 The IONPs used for this formulation have a diameter of 10 nm and are suspended in isoparaffin oil. The ferrite particles are reported to take approximately 10% by volume of the solution, therefore providing a magnetic flux density of 400 gauss, according to the manufacturer, corresponding to a saturation magnetization of 6.3 emu/g.

Two further hydrophobic IONP formulations (LipMB-DBPC and LipMB-LapPC, collectively referred to as LipMB hereafter) were prepared using phospholipid-coated IONPs directly incorporated into the bubble coating. LipMB-LapPC has been previously described,42 and LipMB-DBPC was chosen to test the impact of the IONP lipid coating on magnetic MBs. The method relies on the phospholipid coating to transfer the IONPs to the MB shell upon sonication and allows loading of hydrophobic IONPs into MBs without introducing an oil phase. The lipid-coated IONPs had a hydrodynamic diameter of 50 nm with a crystal core of 30 nm made of magnetite.

In the fourth formulation (SurfMB), hydrophilic dextran IONPs were biotinylated by reacting the carboxylic acids on the

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**Table 1. Properties of Magnetic MBs**

| MB Formulations | Concentration (MB/mL) | Diameter (μm) | Iron Loading (pg/MB) | Magnetic Retention (%) | Half-life in flow (h) |
|-----------------|----------------------|--------------|---------------------|------------------------|-----------------------|
| OilMB | (1.0 ± 0.7) × 10^6 | 5.1 ± 1.4 | 0.61 | 19 ± 7 | 0.1 |
| LipMB-DBPC | (1.4 ± 0.4) × 10^6 | 1.9 ± 0.3 | 0.036 | 15 ± 6 | 2.0 |
| LipMB-LapPC | (1.4 ± 0.6) × 10^6 | 1.9 ± 0.2 | 0.014 | 10 ± 8 | 0.3 |
| SurfMB | (1.1 ± 0.6) × 10^6 | 2.1 ± 0.4 | 0.71 | 61 ± 11 | 0.9 |
| unloaded | (0.9 ± 0.5) × 10^6 | 2.2 ± 0.4 | - | - | - |

“Concentration measured immediately after production, prior to cleaning. bPercentage difference in the B-mode image intensity of a channel with flowing MBs ± 1 application of a magnetic field. cObtained by fitting an exponential curve to the measured change in the MB concentration over time at 37 °C.

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**Figure 1.** Schematics and optical images of magnetic MB formulations. Scale bar indicates 10 μm. Abbreviations: phospholipid MBs loaded with hydrophobic IONPs (OilMB), phospholipid-stabilized IONPs with hydrophobic cores (LipMB), and surface-bound hydrophilic IONPs (SurfMB). Biotin–dextran IONP: Biotinylated carboxymethyl dextran-coated IONPs.
carboxymethyl dextran coating with biotin—PEG—NH₂ and attached to the surface of biotinylated MBs via an avidin bridge. The motivation behind this method was to produce IONPs with a surface chemistry similar to that of the clinical product Feraheme,⁶ which is made of IONPs coated with a carboxymethyl dextran sorbitol ether. The carboxylic acids on Feraheme could be used as handles for biotinyla tion to enable loading onto the external surface of biotinylated MBs. The dextran IONPs used for this formulation had a hydrodynamic diameter of 50 nm and crystal core of 30 nm made of magnetite. The fifth formulation represented the control population and consisted of phospholipid MBs without IONPs (unloaded).

The properties of the different MB formulations are summarized in Table 1.

The different IONPs were chosen to have a hydrodynamic diameter of 50 nm for all formulations except for OilMB, which used IONPs of 10 nm in diameter in isoparaffin to allow a comparison with the method used earlier.⁷ Surfactants were used to promote the formation of phospholipid MBs. To ensure the biocompatibility of the final product, they were chosen from the ingredients of clinically approved MBs.⁴⁴,⁴⁵

The different magnetic MBs shown in Figure 1 were compared in terms of their physical stability and acoustic and magnetic responses. The physical stability of MBs was quantified in terms of their concentration decay over time at 37 °C and under flow. It should be noted, however, that this method does not reflect all considerations for in vivo stability as it does not incorporate clearance mechanisms found in living organisms. Langmuir trough measurements were used to monitor the mechanical properties of the lipid monolayer systems at a gas/water interface corresponding to the MB shell.⁴⁶ In particular, these measurements were conducted to capture the changes in the surface tension of lipid-mix monolayers upon compression and expansion, and the findings were compared with the MB physical stability results. The acoustic properties of the different MB formulations were assessed at both 1 MHz, typical of therapeutic ultrasound applications, and 7 MHz to evaluate them for the purpose of diagnostic ultrasound imaging. The iron content of MBs was measured using inductively coupled plasma optical emission spectrometry (ICP–OES) to determine the quantity of IONPs still incorporated into the MBs after cleaning of the suspension. The quantity of MBs retained under flow under the influence of a magnetic field was also measured. Finally, the toxicity of the formulations was examined by measuring the levels of hemolysis recorded in horse blood following incubation with MBs at 37 °C.

Materials. The formulations considered in this study are summarized in Figure 1. 1,2-Diheptanoyl-sn-glycero-3-phosphocholine (DBPC) and 1,2-distearyl-sn-glycero-3-phosphoholine (DBS) were purchased from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt was purchased from Thermo Fisher Scientific. Polyoxyethylene (40) stea rate (PEG40S), chloroform, Dulbecco’s phosphate-buffered saline (PBS), and avidin–biotin were all purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK). Finally, sulphur hexafluoride (SF₆) gas was purchased from The BOC Group (Guildford, Surrey, UK).

Biotinylated carboxymethyl dextran magnetic nanoparticles (bio tin–dextran IONPs) were prepared in-house (50 nm hydrodynamic diameter, Figure S1). Other IONPs used for this study were L-α-phosphatidylcholine coated IONPs (LαPC IONPs) purchased as FluidMAG-lipid (50 nm hydrodynamic diameter) from Chemicell GmbH (Berlin, Germany). DBPC-coated IONPs (DBPC IONPs, 50 nm hydrodynamic diameter) were manufactured by Ocean NanoTech (San Diego, CA, USA). IONPs dispersed in isoparaffin (isoparaffin IONPs) were purchased as WHJS1-B (10 nm diameter) from Liquids Research Ltd. (Bangor, North Wales, UK).

To assess the hemolytic potential of the different formulations, horse blood in acid-citrate-phosphate was used. A 100 mL sample was purchased (ref: HB046, Lot 34701900) from TCS Biosciences Ltd. (Botolph Claydon, Buckingham, United Kingdom). Positive controls were achieved by adding a solution of Triton X-100 purchased from Sigma-Aldrich Ltd. (Gillingham, Dorset, UK), and they were diluted in deionized water.

**MB Manufacture. General Protocol.** A mixture of lipids and surfactants dissolved in chloroform was added to a glass vial to produce a 5 mL batch of MBs at a total concentration 4 mg/mL. The sample was covered with a pierced parafilm and set on a hot plate at 50 °C for 12 h to evaporate the chloroform. Once all of the solvent evaporated, the dried lipid film was suspended in 5 mL of Dulbecco’s PBS for 1 h at 80 °C under constant magnetic stirring. The magnetic stir bar was then removed.

The lipid mix solution was sonicated at a low intensity (QSonica Q125, 20 kHz, 3 mm probe tip, amplitude: 20%, 1 min) with the sonicator probe tip immersed in the solution. The sonicator probe tip was removed to move the air and water interface, and a light flow of SF₆ gas was added to fill the headspace of the sample vial. The sonicator was then turned on at high intensity (amplitude: 80%; 20 s). The samples were capped and cooled on ice for 10 min after which a layer of foam was visible at the top of the sample as well as a thick layer of densely packed MBs underneath the foam.

Cleaning of MBs was done through centrifugation using 10 mL syringes modified to fit into 50 mL centrifuge tubes. The sample was aspirated into the syringe using a blunt 18-G needle. The syringe was capped, placed into a 50 mL centrifuge tube, and centrifuged at 100g for 5 min at 4 °C. The subnatant was removed, the bubbles were resuspended in the original volume of PBS at 4 °C, and the washing procedure was repeated three times.

**Preparation of Unloaded MBs.** Unloaded MBs were prepared following the general protocol using a 9:1 molar ratio mixture of DBPC/PEG40S to prepare the lipid mix.

**Preparation of LipMB (Phospholipid-Mediated Loading).** To make magnetic MBs through phospholipid-mediated loading, the lipid mix was prepared according to the protocol for unloaded MBs. A volume of LaPC or DBPC IONPs corresponding to 3.75 mg of iron oxide was added to the lipid mix between the low-intensity and high-intensity sonication. Cleaning was carried out according to the general protocol.

**Preparation of OilMB (Oil Loading).** To make magnetic MBs through oil loading, the lipid mix was prepared according to the protocol for unloaded MBs. A volume of isoparaffin IONPs corresponding to 3.75 mg of iron oxide was added after the two sonication steps. The vial was capped and cooled on ice for 5 min before repeating the two sonication steps to form magnetic MBs. Cleaning was carried out according to the general protocol.

**Preparation of SurfMB (Surface Loading).** To make magnetic MBs through surface loading, the lipid mix was prepared with a 82:9:9 mixture of DBPC/PEG40S/DSPE-PEG2000-biotin. The general protocol was followed until the cleaning step. The suspension was first cleaned to remove excess lipids in solution via centrifugation. A solution of avidin (2 mL, 2.5 mg/mL) in PBS was added to the resuspended biotinylated MBs and left to mix on ice on a rotary shaker for 5 min. The MBs were then centrifuged again before adding 300 μL of biotin–dextran IONPs (12.5 mg/mL) to the resuspended avidin-loaded MBs. The sample was left to mix on ice on a rotary shaker for 5 min again. The magnetic MBs were centrifuged once more and finally resuspended with PBS at 4 °C.

**MB Characterization. MB Size and Concentration.** MB size and concentration were determined through the analysis of optical images as previous studies have confirmed the reliability of this method compared to other options available.⁴⁸,⁴⁹ For this, the MB suspension was diluted 1:20 in PBS, and 10 μL of this was loaded onto a hemocytometer with a cover slide. Thirty microscope images were acquired through an optical microscope (Leica DM500 optical microscope, Larch House, Milton Keynes, MK14 6FG, UK) with a 40x objective lens at room temperature. The images were then analyzed using purposely written MATLAB code (R2016b, The MathWorks, Natick, MA, USA) to determine the MB mean size and concentration.

**Stability Studies.** The stability of the different MB formulations was investigated by measuring the changes in the MB concentration.
over time under flow at 37 °C in PBS. Flow was induced by a peristaltic pump used with a 1.6 mm inner diameter latex tube producing a flow rate of 10 mL/min. Measurements were made at t = 15, 30, and 45 min and 1, 1.5, 2, 2.5, and 3 h. Each formulation was tested four times, and the collected data was fitted to a one-phase exponential decay nonlinear curve model using GraphPad Prism (Version 6, GraphPad Software Inc., La Jolla, CA, USA), which showed randomness of residuals.

Langmuir Trough. Langmuir trough experiments were conducted at room temperature and at 37 ± 1 °C on a MicroTrough XL model from Kibron (Kenilworth, Warwickshire, UK) connected to a recirculating chiller-heater for temperature control. A lipid monolayer was formed at the gas/water interface of 75 mL of deionized water by adding 1 μL of lipids dissolved in chloroform (25 mg/mL) and letting it equilibrate for 5 min, after which it was compressed (56.1 mm2/s) from 11,000 to 2000 mm2. The average area per lipid molecule was calculated using the average lipid molecular weight. The surface pressure (Π) of the monolayer was then measured over the molecular area (Å2/molecule).

The effect of phospholipid-mediated loading of IONPs on a lipid monolayer was studied by adding IONPs to the deionized water subphase at a concentration of 20 μg/mL before forming the lipid monolayer. Isoparaflavin IONPs were added in corresponding amounts on top of the lipid monolayer before compression.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was used to visualize the incorporation of IONPs onto the MBs. To obtain dried MB samples for TEM imaging, a previously described method was used. Briefly, a carbon-coated copper grid (C267/050 thin clean films of carbon 300 mesh 3 mm copper grid, TAAB Laboratories Equipment Ltd, Berks, RG7 8NA, UK) was exposed to a glow-discharge cycle, and the carbon side of the copper grid was placed onto a 10 μL drop of the diluted MB sample on a piece of parafilm for 2 min. The TEM grid was then lifted and placed onto a 20 μL drop of 2% w/v uranyl acetate for 10 s. The grid was again lifted, carefully dried with a piece of filter paper, and left to dry for 10 min under an aluminum cover for reduced light exposure. The samples were imaged through a FEI Tecnai 12 transmission electron microscope operated at 120 kV, and the images were acquired using a Gatan OneView CMOS camera.

MB Acoustic Properties. At 1 MHz. The acoustic emissions from MBs exposed to 1 MHz ultrasound were recorded using a passive cavitation detector (PCD, 7.5 MHz centre frequency, 12.7 mm diameter, 75 mm focal distance, Olympus NDT, Essex, UK). A 2 MHz high-pass filter was used to remove the drive frequency from the recorded PCD traces before preamplifying (SR445A, SRS, Sunnyvale, CA, USA), digitising (Handyscope HS3, TiePie Engineering, Sneek, Netherlands), and saving on a computer drive. Acquired PCD traces were analyzed by fast Fourier transform using MATLAB, and harmonics (multiples of the drive frequency ±100 kHz, ±2 MHz), ultraharmonics (half-integer harmonics of the drive frequency ±50 kHz, ±2 MHz), and broadband (remaining signal >2 MHz) components were extracted for each acquisition. The power in these frequency subsets was calculated for each acquisition over the exposure time.

At 7 MHz. The magnetic MBs were then tested at 7 MHz to give an indication of their behavior while being exposed to diagnostic ultrasound. Deionized water was used as a diluent and flown through a 1.6 mm diameter channel in a 2.5% agar phantom at a rate of 0.2 mL/min. A bolus of 200 μL of cleaned (9 ± 1) × 106 MB/mL was injected through a three-way valve and imaged using a commercially available ultrasound system (ii22, Philips, Bothell, WA, USA) and a linear array (L12-5, 7 MHz). A mechanical index (MI) of 0.04 was found to minimize MB destruction for the least stable formulation and used for all experimental runs. Each MB formulation was injected into the phantom and imaged three times. Commercially available SonoVue MBs (Bracco Imaging, Milan, Italy) were also imaged to provide a comparison. Images were analyzed with MATLAB (R2016b, the MathWorks, Natick, USA) by computing grayscale intensities in predefined regions of interest (ROIs) for all acquisitions to determine the point at which the injected MBs reached a constant concentration. Sets of 100 frames at constant MB concentration were examined to measure the average mean intensity within the ROIs.

Magnetic Properties. ICP–OES. The iron content of the different MB formulations was detected using ICP–OES (PerkinElmer, Optima 8400). These measurements were used to determine the amount of IONPs loaded onto MBs to compare with MB magnetic retention results. Iron measurements were performed at a wavelength of 238.204 nm, and a 7-point calibration curve was completed for iron in 2% nitric acid solution (0–1 mg/L) with a R2 value of 0.9999 prior to sample measurements.

Magnetic Retention under Flow. The same setup as for the acoustic characterization of MBs at 7 MHz was used to study the magnetic retention of MBs under flow when exposed to a magnetic field. The magnetic field was applied using a five-element Halbach array magnet placed 1 cm below the channel. The magnet was previously described by Stride et al. and consisted of five rectangular N52 grade NdFeB permanent magnets (25 × 10 × 10 mm, supplied by NeoTex, Berlin, Germany) with transversal magnetizations of 1.50 T, oriented at an angle of 90° from one another, and held by an aluminum frame. A bolus of 100 μL of 5 × 107 MB/mL was injected through a three-way valve, and MBs were left to flow for 1 min above the magnet before acquiring ultrasound images with a linear array (L12-5, 7 MHz) angled perpendicular to the magnet. An ultrasound MI of 0.04 was used to minimize MB destruction. Tests were completed for cleaned magnetic MBs, and flashes of increased acoustic drive level were used to destroy and confirm the retention of MB material. As grayscale intensities of magnetic MBs flowing through the system without magnet have been acquired for their acoustic characterization, quantification of magnetic retention was performed through analysis of image intensity within the ROIs with and without magnet over 10 frames and compared to background measurements. ROIs were automatically segmented from the bottom of the channel with the first one-third of the channel being without magnet, and the remaining two-third being directly 1 cm above the Halbach array magnet (Figure 2).

Hemolytic Potential. Horse blood was used to assess the hemolytic potential of the different magnetic MB formulations. The blood was stored at 5 °C and used the day following delivery at room temperature (21 °C). The blood (200 μL) was diluted with 163 μL of MBs at 1.8 × 107 MB/mL or with PBS for positive and negative controls. The samples were then incubated at 37 °C for 5 min. An aliquot of 80 μL of each blood sample was added to 1.12 mL of PBS. The positive control was further diluted with 120 μL of 1% Triton X-100 in water as a 100% hemolysis reference, whereas the other samples were further diluted with 120 μL of PBS. All samples were then centrifuged at 500g and 4 °C for 5 min. The absorbance of the
supernatants was measured in triplicate (120 μL per well, in a Greiner UV-Star 96-well plate) at 541 nm. This procedure was performed twice.

Statistical Analysis. Statistical analyses were performed using Microsoft Excel and GraphPad Prism with significance defined as $p < 0.05$. One-way analysis of variance was used for experiments investigating the difference between more than two groups with post-hoc Tukey honestly significant difference test. Data is presented as mean ± standard deviation.

■ RESULTS AND DISCUSSION

MB Size Distribution and Concentration. The concentration and mean diameter of MBs ($n = 3$) for the different formulations are shown in Figure 3. OilMB exhibited a significantly lower yield and larger mean diameter compared to all other groups, with approximately 10 times fewer bubbles produced of double the size. This effect could be due to the excess surfactant present in the isoparaфин IONPs suspension disrupting the phospholipid coating or due to the oil phase itself reducing the coating integrity. The remaining MB formulations tested presented comparable concentrations and sizes.

Centrifugal cleaning of MBs enabled removal of excess starting materials in solution, although the final yield was lower after cleaning (Figure S2), indicating that some MBs were also lost during processing. An average final concentration of $(4 ± 1) \times 10^8$ MB/mL was obtained for unloaded MBs, LipMB, and SurfMB, whereas OilMB presented a lower final yield of $(4 ± 0.5) \times 10^7$ MB/mL.

MB Stability under Flow. The results of the stability studies on MBs exposed to flow at 37 °C are shown in Figure 4. One-phase exponential decay nonlinear regression models were fitted to the datasets, which indicate that LipMB-DBPC and SurfMB both showed significantly improved stability by more than doubling their half-lives to 2 and 0.9 h, respectively, compared to 0.4 h for unloaded bubbles. In contrast, the OilMB exhibited a significant decrease in stability (half-life of 0.1 h).

MBs were observed for over 3 h circulating in a 2 mL flow loop at 37 °C, which is a longer timeframe than that reported in the in vivo models. This difference in timescale is explained by the absence of clearance mechanisms that exist in vivo and by the fact that the MBs were undiluted in the flow loop.31

The incorporation of nanoparticles into MBs has been shown previously to prevent gas diffusion and MB coalescence through a form of Pickering stabilization, which might explain the stability of LipMB-DBPC and SurfMB. The solid nanoparticles reduce the surface area available for gas diffusion, and physical jamming of the particles can inhibit MB shrinkage. The addition of LαPC IONPs had a destabilizing effect, which could be due to the presence of acyl chains of different lengths in LαPC (approximately 33% 16:0, 13% 18:0, 31% 18:1, and 15% 18:2 PC), reducing the molecular packing on the MB surface and hence the resistance to gas diffusion. Isoparaфин IONPs also had a destabilizing effect on MBs, which was probably the reason for the low production yield of this formulation (Figure 3A). This may similarly have been due to the effect of isoparaфин on the packing of the phospholipid coating. The Langmuir trough measurements discussed in the next section support this.

The relative instability of this formulation was further indicated by the results shown in Figure 5. Additional measurements were made at 37 °C and under flow of the number of magnetic droplets settled at the bottom of the hemocytometer and the number of buoyant magnetic MBs at the top of the sample underneath the coverslip. Figure 5 indicates a decrease in the buoyant OilMB over time, while the number of magnetic droplets sunken at the bottom of the haemocytometer increased. The size of the droplets suggests that this might have been caused by diffusion of the encapsulated gas out of OilMB, forming magnetic droplets that then sank because of the absence of the gas core.

Langmuir Trough. Surface pressure—area ($II−a$) isotherms of DBPC/PEG40S monolayers without IONPs were characterized and imaged at 18 °C for different pressure ranges.
Thus, the lipid packing increases, squeezing the emulsion formation of MBs, the lipid-mix coating is compressed because of the addition of L-α-surfactant. These results suggest that PEG40S in the monolayer beyond 35 mN/m, and led to a squeeze-out of the coating until the remaining condensed-phase lipids reach the monolayer. Following PEG40S elimination, the slope of the remaining DBPC monolayer, which finally collapses at ~60 mN/m at 18 °C. A broadening in the hysteresis was observed beyond 35 mN/m, indicating a loss of material into the water phase, which can be due to the formation of the bilayer, lipid budding, and vesicles from the remaining DBPC (Figure S3C1, C2).

The effect of adding isoparaffin (Figure 6A) and DBPC IONPs (Figure 6B) to the monolayer was then tested at 37 °C, and isotherms similar to those for the control were obtained. The addition of isoparaffin IONPs did, however, lower the final collapse pressure of the system, and the DBPC IONPs suppressed the “squeeze-out” plateau to some extent. The addition of LotPC IONPs (Figure 6C) also reduced the surfactant “squeeze-out” plateau, indicating retention of PEG40S in the monolayer beyond 35 mN/m, and led to a lower monolayer collapse pressure. These results suggest that the addition of LotPC IONPs to DBPC/PEG40S MBs may reduce the loss of PEG40S during compression. Upon formation of MBs, the lipid-mix coating is compressed because of the Laplace pressure driving the system toward dissolution. Thus, the lipid packing increases, squeezing the emulsifier out of the coating until the remaining condensed-phase lipids reach a metastable state with a very low surface tension enabling bubble stabilization. Any remaining PEG40S within the coating would hinder the system reaching this metastable state.

**MB Acoustic Properties.** The acoustic emissions from MBs exposed to 1 MHz ultrasound were investigated, as this frequency is relevant for therapeutic ultrasound drug delivery, and the energy and duration of emissions have been shown to be correlated with the therapeutic response. The acoustic emissions from each magnetic MB formulation were measured for suspensions [(1 ± 0.5) × 10^6 MB/mL] exposed to 1 MHz ultrasound, 0.85 MPa pk-pk, and 30% duty cycle for 30 s (Figure 7A). The signals were analyzed for harmonics, ultraharmonics, and broadband emissions separately over the exposure time, and the results in Figure 7B–D indicate that there was no significant difference between the formulations.

The MBs were also examined at 7 MHz through analysis of B-mode images of the MBs [200 μL (9 ± 1) × 10^6 MB/mL] under 0.2 mL/min flow at room temperature. The results shown in Figure 8A indicate that all magnetic formulations behaved similarly at an MI of 0.04. Although all MBs were clearly visible, the image intensity was lower than that for the clinical agent SonoVue for the same concentration of MB injected and comparable size distribution (Figure 8B). This was attributed to the stiffer shell of DBPC/PEG40S MBs preventing bubble expansion compared to softer systems made of 1,2-distearoyl-sn-glycero-3-phosphocholine phospholipid as for SonoVue. Contrastingly, no acoustic difference was observed between the magnetic MB formulations and the unloaded systems, indicating that the lipid composition governed the acoustic oscillations of the system more than the presence of the nanoparticles.

**MB Magnetic Properties.** Individual MBs produced via different loading methods were examined using electron microscopy (Figure 9). The images suggest that hydrophobic isoparaffin and LotPC IONPs remain in clusters on MBs (A1–3, B1–3), unlike DBPC and hydrophilic dextran IONPs (C1–3, D1–3). Additionally, there appeared to be larger quantities of dextran IONPs based upon visual inspection of images D1–3 compared to other formulations.

Quantification of iron loading in the different formulations is presented in Figure 10A. These data indicate a significantly higher iron loading in OilMB (0.61 pg/MB) and SurfMB (0.71 pg/MB) compared to that in LipMB (~0.025 pg/MB) and the background measurement in the unloaded control (0.006 pg/MB). The lower loading of LotPC and DBPC IONPs in MBs could be due to the low incorporation of IONPs during MB production or the removal of IONPs from the MB coating during the washing procedure. Nevertheless,
the LipMB-DBPC still exhibited a detectable response to magnetic fields, as shown in Figure 10B.

All magnetic MB formulations were tested for their ability to be retained under flow at a rate of 0.2 mL/min in a 1.6 mm channel in a tissue-mimicking phantom and imaged using a commercially available diagnostic ultrasound device. The channel size was chosen to fit the human physiological range for small arteries and veins that surround the tumor lesions with a flow rate of 0.2 mL/min.58

The concentration of MBs injected into the system was kept fixed with 100 μL, 9 × 10^6 MB/mL tested for each formulation. MBs were left to circulate at 0.2 mL/min for 1 min with the Halbach array magnet situated 1 cm underneath the channel. The diagnostic ultrasound probe was kept off during this 1 min period to avoid MB destruction. After 1 min of circulation, a series of B-mode images were captured during which a “flash” of increased ultrasound intensity was used to destroy the retained material.
and obtain background images. As the magnetic MBs produced similar grayscale intensities while exposed to 7 MHz diagnostic ultrasound and an MI of 0.04, their magnetic retention was compared by analyzing the intensity of a sequence of 10 frames for each formulation. Average image intensities of selected ROIs were compared to the control regions within the same frame. The regions chosen for analysis were located at the bottom of the channel to avoid noise from the remaining MBs flowing through the system (Figure 2).

The results in Figure 11 confirm the results from the ICP–OES measurements, with SurfMB showing significantly higher retention (61% increase in the B-mode image intensity) than the other MBs tested (10–19% increase under the same conditions). OilMB did not exhibit a significant difference in retention compared to LipMB, which might be due to their lower stability compared to the other formulations. In fact, OilMB produced a retained bolus that was comparable to...
SurfMB only on the first imaging frame, after which the bolus was destroyed due to the imaging ultrasound. No bolus of retained MBs was observed when using LipMB but only a downward movement of the MBs toward the magnet.

**Hemolytic Potential.** The toxicity of the different formulations was assessed in horse blood, and the results (Figure 12) showed no hemolysis following the addition of different magnetic MBs. The samples were incubated at 37 °C for 5 min, reflecting the short half-life of MBs in circulation.\(^{59,60}\) Additionally, several in vivo studies using OilMB\(^{27,28}\) and LipMB-LatPC\(^{42,61,62}\) have been performed with no reported toxicity.

**Evaluation.** A summary of the findings is presented in Table 2. Different loading methods were compared for producing magnetic MBs, with the aim of prolonging their stability and enabling strong magnetic and acoustic responses. Although, OilMB enabled high loading of iron, this method produced unstable MBs with a substantially reduced half-life at 37 °C and under flow compared to unloaded MBs. In contrast, SurfMB was found to be more stable than unloaded systems and magnetically more responsive than all other formulations. Whereas LipMB-DBPC presented a lower retention efficiency during magnetic targeting, this system still demonstrated a significant improvement in stability compared to unloaded MBs. It should also be noted that magnetic retention could be affected by the different magnetic properties of the IONPs used in different formulations, and further optimization should be carried out to investigate the effect of IONP size, charge, and magnetic properties.

For drug delivery purposes, the most versatile way of loading MBs with hydrophilic drugs or liposomes relies on the postfunctionalization of MB shell phospholipids. SurfMB could present a challenge for this type of additional surface functionalization, although it would not interfere with the loading of hydrophobic drugs within the shell. Contrastingly, LipMB-DBPC remains a versatile method to produce stable magnetic MBs, which allows the attachment of hydrophilic drugs to shell phospholipids but challenges the loading of hydrophobic drugs.\(^5\)

Although the incorporation of IONPs can potentiate oxidative stress because of the release of free iron from the particles followed by iron-catalyzed Fenton reactions,\(^63–65\) the iron content after washing was found to be low, with a maximum of 0.7 mg/mL for a MB concentration of \(10^9\) MB/mL. Additionally, considering the recommended dose of SonoVue MBs of 2–2.4 mL in adults,\(^66\) which equates to approximately \(10^8\) MB administered, the corresponding dose of iron would be 0.7 mg. Compared to clinical intravenous injections of Feraheme (ferumoxytol) recommending two doses of \(510\) mg of iron over 8 days in adults with a chronic kidney disorder,\(^63\) the administered dose of iron within magnetic MBs is orders of magnitude lower than the currently approved dose for anemia. Nevertheless, the composition of material used is important to ensure minimal toxicity even at low doses. The phospholipids used to make the MB shell and to coat phospholipid-stabilized IONPs were chosen to be saturated to minimize the chance of lipid peroxidation during ultrasound-induced MB collapse in the presence of IONPs.\(^67–69\) The externally conjugated dextran IONPs were chosen to mimic Feraheme, but additional improvements in biocompatibility could be implemented to inhibit macrophage clearance and improve the efficiency of this method in vivo.

### CONCLUSIONS

Magnetic MBs produced using different IONP loading methods were investigated to compare their stability, acoustic emissions, and magnetic retention. External binding of IONPs to the MB coating was found to produce a significant improvement in MB stability compared to unloaded systems while preserving their acoustic behavior. This method also produced the most magnetically responsive MBs. The disadvantage of this method is that although it allows for hydrophobic drug loading within the MB coating, it renders further attachment of hydrophilic drugs to MB shell phospholipids challenging. The phospholipid-mediated loading

Table 2. Summary Table of Findings Comparing Different Loading Methods of IONPs To Produce Magnetic MBs and the Downstream Consequences on MB Properties\(^{49}\)

| considerations for DBPC-based MBs | OilMB | LipMB-LatPC | LipMB-DBPC | SurfMB |
|----------------------------------|-------|--------------|-------------|--------|
| stability enhancement in vitro, 10 mL/min, 37°C | unstable | none | very good | good |
| caviation signal 1 × 10^7 MB/mL suspension, 1 MHz, 30% dc, 100 Hz PRF, 0.85 MPaL−1⋅s−1 | comparable | comparable | comparable | comparable |
| diagnostic imaging 0.2 mL/min, 9 × 10^7 MB/mL, 0.04 MI | comparable | comparable | comparable | comparable |
| magnetic retention 0.2 mL/min, 5 × 10^7 MB/mL, 0.04 MI | bolus observed but unstable | no bolus observed | no bolus observed | bolus observed |
| hydrophobic drug loading | ✓ | ✓ | ✓ | ✓ |
| hydrophilic drug loading | ✓ | ✓ | ✓ | |

\(✓\): possible; \(✗\): challenging.
of DBPC IONPs within the coating of MBs, while offering a weak magnetic response, may provide a more favorable compromise between targeting efficiency, stability, acoustic response, and surface loading potential for hydrophobic drugs.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b18418.

Protocol of the conjugation of biotin—dextran IONPs and characterization, characterization of MB size distribution before and after centrifugal cleaning, and optical acquisition of Langmuir trough measurements for lipid-mix monolayer without the addition of IONPs at 18 °C (PDF)

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**Notes**

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**ADDITIONAL NOTES**

The isoparaffin IONPs used in this work are stabilized with a surfactant. We unfortunately could not obtain information on the nature of the surfactant from the manufacturer.

Preliminary experiments showed that a lower loading of paclitaxel was obtained in LipMB compared to unloaded MBs (data not shown).

**REFERENCES**

(1) Feinstein, S. B.; Ten Cate, F. J.; Zwehl, W.; Ong, K.; Maurer, G.; Tei, C.; Shah, P. M.; MeerbauM, S.; Corday, E. Two-Dimensional Contrast Echocardiography. I. In Vitro Development and Quantitative Analysis of Echo Contrast Agents. J. Am. Coll. Cardiol. 1994, 3, 14–20.

(2) Lindner, J. R. Microbubbles in Medical Imaging: Current Applications and Future Directions. Nat. Rev. Drug Discovery 2004, 3, 527–533.

(3) Ferrara, K.; Pollard, R.; Borden, M. Ultrasound Microbubble Contrast Agents: Fundamentals and Application to Gene and Drug Delivery. Annu. Rev. Biomed. Eng. 2007, 9, 415–447.

(4) Meijering, B. D. M.; Juffermans, L. J. M.; van WameL, A.; Henning, R. H.; Zuhorn, I. S.; Emmer, M.; VersteiLEN, A. M. G.; Paulus, W. J.; van Gilst, W. H.; KooiMan, K.; et al. Ultrasound and Microbubble-Targeted Delivery of Macromolecules Is Regulated by Induction of Endocytosis and Pore Formation. Circ. Res. 2009, 104, 679–687.

(5) KashiAn, R.; Bevan, P. D.; Williams, R.; Samac, S.; Burns, P. N. Sonoporation by Ultrasound-Activated Microbubble Contrast Agents: Effect of Acoustic Exposure Parameters on Cell Membrane Permeability and Cell Viability. Ultrasound Med. Biol. 2009, 35, 847–860.

(6) Juffermans, L. J. M.; van Dijk, A.; Jongenelen, C. A. M.; Drukarch, B.; Reijerkerk, A.; de Vries, H. E.; Kamp, O.; Musters, R. J. P. Ultrasound and Microbubble-Induced Intra- and Intercellular Bioeffects in Primary Endothelial Cells. Ultrasound Med. Biol. 2009, 35, 1917–1927.

(7) Kooiman, K.; Van Der Steen, A. F. W.; Jong, N. Role of Intracellular Calcium and Reactive Oxygen Species in Microbubble-Mediated Alterations of Endothelial Layer Permeability. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 2013, 60, 1811–1815.

(8) Lentacker, L.; De Smedt, S. C.; Sanders, N. N. Drug Loaded Microbubble Design for Ultrasound Triggered Delivery. Soft Matter 2009, 5, 2161.

(9) Borden, M. A.; Sarantos, M. R.; Stieger, S. M.; Simon, S. I.; Ferrara, K. W.; Dayton, P. A. Ultrasound Radiation Force Modulates Ligand Availability on Targeted Contrast Agents. Mol. Imag. 2006, 5, 139–147.

(10) Dayton, P.; Klibanov, A.; Brandenburger, G.; Ferrara, K. Acoustic Radiation Force in Vivo: A Mechanism to Assist Targeting of Microbubbles. Ultrasound Med. Biol. 1999, 25, 1195–1201.

(11) Owen, J.; Pankhurst, Q.; Stride, E. Magnetic Targeting and Ultrasound Mediated Drug Delivery: Benefits, Limitations and Combination. Int. J. Hyperther. 2012, 28, 362–373.

(12) Yang, F.; Gu, Z. X.; Jin, X.; Wang, H. Y.; Gu, N. Magnetic Microbubble: A Biomedical Platform Co-Constructed from Magnetics and Acoustics. Chin. Phys. B 2013, 22, 104301.

(13) Yang, F.; Li, L.; Li, Y.; Chen, Z.; Wu, J.; Gu, N. Superparamagnetic Nanoparticle-Inclusion Microbubbles for Ultrasound Contrast Agents. Phys. Med. Biol. 2008, 53, 6129–6141.

(14) Liu, Z.; Lammers, T.; Ehling, J.; Fokong, S.; Bornemann, J.; Kiessling, F.; Gätjens, J. Iron Oxide Nanoparticle-Containing Microbubble Composites as Contrast Agents for MR and Ultrasound Dual-Modality Imaging. Biomaterials 2011, 32, 6155–6163.

(15) Bismar, T. B.; Grishenkov, D.; Gustafsson, B.; Härmark, J.; Barrefelt, Å.; Kothapalli, S. V. V. N.; Margheritelli, S.; Oddo, L.; Caidahl, K.; Hebert, H.; et al. Magnete Nanoparticles Can Be Coupled to Microbubbles to Support Multimodal Imaging. Biomacromolecules 2012, 13, 1390–1399.

(16) Gun, S.; Edirisinghe, M.; Stride, E. Encapsulation of Superparamagnetic Iron Oxide Nanoparticles in Poly-(Lactide-Co-Glycolic Acid) Microspheres for Biomedical Applications. Mater. Sci. Eng., C 2013, 33, 3129–3137.

(17) Poehlmann, M.; Grishenkov, D.; Kothapalli, S. V. V. N.; Härmark, J.; Hebert, H.; Philipp, A.; Hoeller, R.; Seuss, M.; Kuttner, C.; Margheritelli, S.; et al. On the Interplay of Shell Structure with Ligand Availability on Targeted Contrast Agents. Biomater. 2012, 33(12), 3733–3740.

(18) Song, W.; Luo, Y.; Zhao, Y.; Liu, X.; Zhao, J.; Luo, J.; Zhang, Q.; Ran, H.; Wang, Z.; Guo, D. Magnetic Nanobubbles with Potential for Targeted Drug Delivery and Trimodal Imaging in Breast Cancer: An in Vitro Study. Nanomedicine 2017, 12, 991–1009.

(19) He, W.; Yang, F.; Wu, Y.; Wen, S.; Chen, P.; Zhang, Y.; Gu, N. Microbubbles with Surface Coated by Superparamagnetic Iron Oxide Nanoparticles. Mater. Lett. 2012, 68, 64–67.
(20) Sciallero, C.; Trucco, A. Ultrasound Assessment of Polymer-Shell Nanoparticle-Loaded Microbubbles as Dual Contrast Agents. J. Acoust. Soc. Am. 2013, 133, EL478–EL484.

(21) Qiao, W.; Yang, F.; Song, L.; Fang, K.; Tian, J.; Liang, Y.; Xi, M.; Xu, N.; Chen, Z.; Zhang, Y.; et al. Controlled Assembly of Magnetic Nanoparticles on Microbubbles for Multimodal Imaging. Soft Matter 2015, 11, 5492–5500.

(22) Ahmed, M.; Cerroni, B.; Razzavaev, A.; Härmark, J.; Paradossi, G.; Caidahl, K.; Gustafsson, B. Cellular Uptake of Plain and SPION-Modified Microbubbles for Potential Use in Molecular Imaging. Cell. Mol. Bioeng. 2017, 10, 537–548.

(23) McEwan, C.; Fowell, C.; Nomikou, N.; McCaughan, B.; McHale, A. P.; Callan, J. F. Polymeric Microbubbles as Delivery Vehicles for Sensitzers in Sonodynamic Therapy. Langmuir 2014, 30, 14926–14930.

(24) Siris, S. R.; Borden, M. A. Microbubble Compositions, Properties and Biomedical Applications. Bubble Sci. Eng. Technol. 2009, 1, 3–17.

(25) Appis, A. W.; Tracy, M. J.; Feinstein, S. B. Update on the Safety and Efficacy of Commercial Ultrasound Contrast Agents in Cardiac Applications. Echo Res. Pract. 2015, 2, R55–R62.

(26) Ninh, B. W.; Larsson, K.; Lo Nostro, P. Two Sides of the Coin Part 1. Lipid and Surfactant Self-Assembly Revisited. Colloids Surf. B 2017, 152, 326–338.

(27) Stride, E.; Porter, C.; Prieto, A. G.; Pankhurst, Q. Enhancement of Microbubble Mediated Gene Delivery by Simultaneous Exposure to Ultrasonic and Magnetic Fields. Ultrasound Med. Biol. 2009, 35, 861–868.

(28) Mulvana, H.; Eckersley, R. J.; Browning, R.; Hajnal, J. V.; Stride, E.; Barrack, T.; Tang, M.; Pankhurst, Q.; Wells, D. Enhanced Gene Transfection in Vivo Using Magnetic Localisation of Ultrasound Contrast Agents: Preliminary Results. 2010 IEEE International Ultrasonics Symposium, 2010; pp 670–673.

(29) de Saint Victor, M.; Carugo, D.; Barnsley, L. C.; Owen, J.; Cossius, C.-C.; Stride, E. Magnetic Targeting to Enhance Microbubble Delivery in an Occluded Microarterial Bifurcation. Phys. Med. Biol. 2017, 62, 7451–7470.

(30) Vlaskou, D.; Mykhaylyk, O.; Krötz, F.; Hellwig, N.; Renner, R.; Schillinger, U.; Gleich, B.; Heidieck, A.; Schmitz, G.; Hensel, K.; et al. Magnetic and Acoustically Active Lipospheres for Magnetically Targeted Nucleic Acid Delivery. Adv. Funct. Mater. 2010, 20, 3881–3894.

(31) Manwell, H.; Pircher, J.; Fochler, F.; Stampnik, Y.; Räthel, T.; Gleich, B.; Plank, C.; Mykhaylyk, O.; Dahmani, C.; Wörnle, M.; et al. Site Directed Vascular Gene Delivery in Vivo by Ultrasonic Destruction of Magnetic Nanoparticle Coated Microbubbles. Nanomed. Nanotechnol. Biol. Med. 2012, 8, 1309–1318.

(32) Heun, Y.; Hildebrand, S.; Heidieck, A.; Gleich, B.; Anton, M.; Ribeiro, A.; Mykhaylyk, O.; Eberbeck, D.; Wenzel, D.; Pfeifer, A. Targeting of Magnetic Nanoparticle-Coated Microbubbles to the Vascular Wall Empowers Site-Specific Lentiviral Gene Delivery in Vivo. Theranostics 2017, 7, 295–307.

(33) Räthel, T.; Manwell, H.; Pircher, J.; Gleich, B.; Pohl, U.; Krötz, F. Magnetic Stents Retain Nanoparticle-Bound Antibiotesten Drugs Transported by Lipid Microbubbles. Pharm. Res. 2011, 29, 1295–1307.

(34) Dove, J. D.; Murray, T. W.; Borden, M. A. Enhanced Photoacoustic Response with Plasmonic Nanoparticle-Templated Microbubbles. Soft Matter 2013, 9, 7743.

(35) Dove, J. D.; Borden, M. A.; Murray, T. W. Optically Induced Resonance of Nanoparticle-Loaded Microbubbles. Opt. Lett. 2014, 39, 3732–3735.

(36) Chertok, B.; Langer, R. Circulating Magnetic Microbubbles for Localized Real-Time Control of Drug Delivery by Ultrasound-Driven Magnetic Targeting and Ultrasound. Theranostics 2018, 8, 341–357.

(37) Soetanto, K.; Watarai, H. Development of Magnetic Microparticles for Drug Delivery System (DDS). Jpn. J. Appl. Phys. 2000, 39, 3230–3232.

(38) Kovalenko, A.; Jouhannaud, J.; Polavarapu, P.; Kraft, M. P.; Water, G.; Pourry, G. Hollow Magnetic Microspheres Obtained by Nanoparticle Adsorption on Surfactant Stabilized Microbubbles. Soft Matter 2014, 10, 5147–5156.

(39) Gao, X.; Qiu, G.; Song, L.; Liang, Y.; Xie, D.; Cai, J.; Yeo, D. C. L.; Alsema, A. M.; Arora, M.; Chong, M. S. K.; Shi, P.; et al. Controlled Nanoparticle Release from Stable Magnetic Microbubble Oscillations. NPG Asia Mater. 2016, 8, e260.

(40) Unger, E. C.; McCreery, T. P.; Sweitzer, R. H.; Caldwell, V. E.; Wu, Y. Acoustically Active Lipospheres Containing Paclitaxel. Invest. Radiol. 1998, 33, 886–892.

(41) Tartis, M. S.; McCahan, J.; Lum, A. F. H.; LaBell, R.; Steiger, S. M.; Matsunaga, T. O.; Ferrara, K. W. Therapeutic Effects of Paclitaxel: Preliminary Results from Fluorescently Tagged Magnetic Resonance-Visible Magnetic Microbubbles in Vivo. Ultrasound Med. Biol. 2016, 42, 3022–3036.

(42) FDA. Ferahe (ferumoxytol) Injection Label. https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/022180bl.pdf (accessed May 9, 2018).

(43) FDA. DEFINITY Injection Label, 2001; pp 1–18.

(44) European Medicines Agency. Sonovue—Annex I Summary of Product Characteristics, 2007.

(45) Borden, M. A.; Pu, G.; Runner, G. J.; Longo, M. L. Surface Phase Behavior and Microstructure of Lipid/PEG-Emulsifier Monolayer-Coated Microbubbles. Colloids Surf. B 2004, 35, 209–223.

(46) Sennogn, C. A.; Mahue, V.; Loughran, J.; Casey, J.; Seddon, J. M.; Tang, M.; Eckersley, R. J. On Sizing and Counting of Microbubbles Using Optical Microscopy. Ultrasound Med. Biol. 2010, 36, 2093–2096.

(47) Saitinov, S. J.; Dove, J. D.; Borden, M. A. Single-Particle Optical Sizing of Microbubbles. Ultrasound Med. Biol. 2014, 40, 138–147.

(48) Mulvana, H.; Browning, R. J.; Luan, Y.; de Jong, N.; Tang, M.; G; Eckersley, R. J.; Stride, E. Characterization of Contrast Agent Microbubbles for Ultrasonic Imaging and Therapy Research. IEEE Trans. Ultrason. Ferroelectrics Freq. Contr. 2017, 64, 232–251.

(49) Agrg, G.; Thomas, A. A.; Borden, M. A. The effect of lipid monolayer in-plane rigidity on in vivo microbubble circulation persistence. Biomaterials 2013, 34, 6862–6870.

(50) Mohamed, G.; Azmin, M.; Pastoriza-Santos, I.; Huang, V.; Perez-Juste, J.; Liz-Marzán, L. M.; Edirisinghe, M.; Stride, E. Effects of Gold Nanoparticles on the Stability of Microbubbles. Langmuir 2012, 28, 13808–13815.

(51) Kilic, S. Quantification of PEG 40 St squeeze out from DSPC/PEG 40 St monolayers at higher molar ratios. Colloids Surf., A 2018, 551, 58–64.

(52) McDannold, N.; Vykhotseva, N.; Hynynen, K. Targeted Disruption of the Blood-Brain Barrier with Focused Ultrasound: Association with Cavitation Activity. Phys. Med. Biol. 2006, 51, 793–807.

(53) Hwang, J. H.; Tu, J.; Brayman, A. A.; Matula, T. J.; Crum, L. A. Correlation between Inertial Cavitation Dose and Endothelial Cell Damage in Vivo. Ultrasound Med. Biol. 2006, 32, 1611–1619.

(54) Qiu, Y.; Lu, Y.; Zhang, Y.; Cui, W.; Zhang, D.; Wu, J.; Zhang, J.; Tu, J. The Correlation between Acoustic Cavitation and Sonoporation Involved in Ultrasound-Mediated DNA Transfection with Polyethylenimine (PEI) in Vitro. J. Controlled Release 2010, 145, 40–48.

(55) Kwan, J. J.; Borden, M. A. Lipid Monolayer Dilatational Mechanics during Microbubble Gas Exchange. Soft Matter 2012, 8, 4756–4766.
(58) Greger, R.; Windhorst, U. Peripheral Circulation: Fundamental Concepts, Comparative Aspects of the Control in Specific Vascular Sections, and Lymph Flow. *Comprehensive Human Physiology*; Springer-Verlag Berlin Heidelberg, 1996; pp 1865–1873.
(59) Mullin, L.; Gessner, R.; Kwan, J.; Kaya, M.; Borden, M. A.; Dayton, P. A. Effect of Anesthesia Carrier Gas on in Vivo Circulation Times of Ultrasound Microbubble Contrast Agents in Rats. *Contrast Media Mol. Imaging* **2011**, *6*, 126–131.
(60) Schneider, M. Characteristics of SonoVue. *Echocardiography* **1999**, *16*, 743–746.
(61) Owen, J.; Crake, C.; Lee, J. Y.; Carugo, D.; Beguin, E.; Khrapitchev, A. A.; Browning, R. J.; Siberon, N.; Stride, E. A Versatile Method for the Preparation of Particle-Loaded Microbubbles for Multimodality Imaging and Targeted Drug Delivery. *Drug Delivery Transl. Res.* **2017**, *8*, 342–356.
(62) Sheng, Y.; Beguin, E.; Nesbitt, H.; Kamila, S.; Owen, J.; Barnesley, L. C.; Callan, B.; O’Kane, C.; Nomikou, N.; Hamoudi, R.; et al. Magnetically Responsive Microbubbles as Delivery Vehicles for Targeted Sonodynamic and Antimetabolite Therapy of Pancreatic Cancer. *J. Controlled Release* **2017**, *262*, 192–200.
(63) Tietze, R.; Unterweger, H.; Alexiou, C. Magnetic Nanoparticles for Drug Delivery. *Handbook of Nanobiomedical Research: Fundamentals, Applications, and Recent Developments*, 2014; Vol. I, pp 595–620.
(64) Huang, C.-C.; Liao, Z.-X.; Lu, H.-M.; Pan, W.-Y.; Wan, W.-L.; Chen, C.-C.; Sung, H.-W. Cellular Organelle-Dependent Cytotoxicity of Iron Oxide Nanoparticles and Its Implications for Cancer Diagnosis and Treatment: A Mechanistic Investigation. *Chem. Mater.* **2016**, *28*, 9017–9025.
(65) Patil, U. S.; Adireddy, S.; Jaiswal, A.; Mandava, S.; Lee, B. R.; Chrisey, D. B. In Vitro/in Vivo Toxicity Evaluation and Quantification of Iron Oxide Nanoparticles. *Int. J. Mol. Sci.* **2015**, *16*, 24417–24450.
(66) FDA. LUMASON/Sonovue Injection Label; [https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/203684s002lbl.pdf](https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/203684s002lbl.pdf), accessed 11/20/2018.
(67) Hristov, P. K.; Petrov, L. A.; Russanov, E. M. Lipid Peroxidation Induced by Ultrasound in Ehrlich Ascitic Tumor Cells. *Cancer Lett.* **1997**, *121*, 7–10.
(68) Vyšniauskas, A.; Qurashi, M.; Kuimova, M. K. A Molecular Rotor That Measures Dynamic Changes of Lipid Bilayer Viscosity Caused by Oxidative Stress. *Chem.—Eur. J.* **2016**, *22*, 13210–13217.
(69) Rokitskaya, T. I.; Kotova, E. A.; Agapov, I. I.; Moisenovich, M. M.; Antonenko, Y. N. Unsaturated Lipids Protect the Integral Membrane Peptide Gramicidin A from Singlet Oxygen. *FEBS Lett.* **2014**, *588*, 1590–1595.
(70) Lassenberger, A.; Scheberl, A.; Stadlbauer, A.; Stiglbauer, A.; Helbich, T.; Reinhardt, E. Individually Stabilized, Superparamagnetic Nanoparticles with Controlled Shell and Size Leading to Exceptional Stealth Properties and High Relaxivities. *ACS Appl. Mater. Interfaces* **2017**, *9*, 3343–3353.