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Engineering the Echogenic Properties of Microfluidic Microbubbles Using Mixtures of Recombinant Protein and Amphiphilic Copolymers

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Abstract

Microbubbles are used as ultrasound contrast agents in medical diagnosis and also have shown great promise in ultrasound-mediated therapy. However, short lifetime and broad size distribution of microbubbles limit their applications in therapy and imaging. Moreover, it is challenging to tailor the echogenic response of microbubbles to make them suitable for specific applications. To overcome these challenges, we use microfluidic flow-focusing to prepare monodisperse microbubbles with a mixture of a recombinant amphiphilic protein, oleosin, and a synthetic amphiphilic copolymer, Pluronic. We show that these microbubbles have superior uniformity and stability under ultrasonic stimulation compared to commercial agents. We also demonstrate that by using different Pluronics, the echogenic response of the microbubbles can be tailored. Our work shows the versatility of using the combination of microfluidics and protein/copolymer mixtures as a method of engineering microbubbles. This tunability could potentially be important and powerful in producing microbubble agents for theranostic applications.

Graphical Abstract

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Supporting Information

Sequence of 25–30G(–)–30 oleosin, protein characterization, movies showing the microfluidic generation of OleP microbubbles. The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.langmuir.8b03882](https://doi.org/10.1021/acs.langmuir.8b03882).

Sequence of 25–30G(–)–30 oleosin and protein characterization (PDF).

Video 1: Microfluidic generation of OleP microbubbles using a flow focusing device (AVI).

Video 2: Outlet of the microfluidic device showing stable OleP microbubble generation (AVI).

The authors declare no competing financial interest.

1. INTRODUCTION

Ultrasound imaging is a highly attractive bioimaging modality to visualize the blood circulatory system, muscles, and internal organs because it enables real-time, noninvasive monitoring at low cost without generating potentially hazardous radiation.^{1–4} Contrast agents can significantly improve the sensitivity and resolution of ultrasound imaging for organs and tissues with extremely small vessels or poorly vascularized tumors.^{5–8} Application of contrast agents has also led to the development of various ultrasound techniques to facilitate drug/gas delivery and enable therapeutic strategies such as antivasular ultrasound therapy.⁷ Commercially available ultrasound contrast agents are typically 1–8 μm diameter microbubbles composed of gaseous cores encapsulated in shells of denatured proteins (e.g., Optison) or phospholipid monolayers (e.g., DEFINITY, SonoVue).^{9–11} Despite their useful properties, these shell materials provide limited stability to microbubbles, which results in a relatively short circulation lifetime of less than 10 min,^{12,13} necessitating hastened imaging or multiple injections.

In addition to stability, there are additional factors that must be considered in optimizing the applications of microbubbles in ultrasound. Such factors include their size and size distribution, as well as the chemical composition and properties of the bubble shell. The resonance frequency as well as the scattering intensity of microbubbles under ultrasound depend strongly on the microbubble size. Clearly, uniform size distribution is desirable since such a bubble ensemble responds homogeneously and thus more predictably to the incoming ultrasound with a given frequency characteristic, potentially enhancing the efficacy of therapy and the contrast of imaging.^{14,15} In addition, since all of the bubbles uniformly respond to the incoming ultrasound waves, a smaller dose of uniform bubbles would be required compared to polydisperse bubble suspensions.¹⁶ Moreover, the properties of the shell, which depend on the chemical composition of the stabilizing layer, significantly influence the mechanism by which the incoming ultrasound energy is scattered or dissipated. While it may be desirable to maximize scattering to enhance contrast, it likely will be more advantageous to maximize dissipation to facilitate therapeutic applications of microbubbles. Thus, engineering and tailoring the size and size uniformity of microbubbles as well as the properties of these shells would enable significant advances in the application of microbubbles for various diagnostic, delivery, and therapeutic applications.

Conventional methods of bulk preparations of microbubbles, including high shear stirring,¹⁷ ultrasonic emulsification,^{18–20} pressurized gyration,^{10,11} coaxial electrohydrodynamic atomization,^{9,21,22} or membrane emulsification^{23–25} lead to poly-disperse populations and potentially to batch-to-batch variations.²⁶ While it should be possible to control the echogenic response of microbubbles by changing the shell composition, few studies have systematically demonstrated such a strategy, to our best knowledge. A possible reason for the lack of such studies is that the polydispersity of microbubbles produced using conventional techniques often make it challenging to decouple the effect of shell composition versus size on the echogenic response of microbubbles.

There is a growing interest in using microfluidics to engineer microbubbles.²⁷ The advent of droplet-based microfluidics^{28–30} has enabled the generation of microbubbles or microdroplets with high size uniformity and precision. Xu et al.³¹ investigated the nature and mechanism of monodisperse microbubble formation in a T-junction microfluidic device using a cross flow shear-rupturing technique. Reduction of channel dimensions has led to the production of microbubbles that have appropriate sizes for ultrasound applications.^{33–36} Kanaka et al. achieved the generation of monodisperse micron-sized lipid shell-based perfluorocarbon (PFC) gas microbubbles through microfluidic flow-focusing.³² A microfluidic device that allows for production of monodisperse, sub-10 μm microbubbles has been developed by taking advantage of an air-actuated membrane valve.³⁷ Moreover, recent work demonstrates the large-scale production of uniform microbubbles via parallelization of bubble formation or application of an external electric field.^{38–41} These results show that microbubbles can be produced at the rate that is relevant for commercialization. These breakthroughs will play an important role in the translation of the microfluidic technologies for biomedical applications.

In this study, we study and engineer the stability and echogenic properties of microfluidic microbubbles that are stabilized by a mixture of a recombinant amphiphilic protein, oleosin, and a series of amphiphilic triblock copolymers, Pluronics.^{42–44} In our recent work, we have shown that highly uniform and stable microbubbles can be produced by using a combination of oleosin/Pluronic (henceforth, also referred to simply as “OleP”) and microfluidics.³⁷ We also showed that the mechanical properties of the bubble shell could be systematically controlled by varying the composition of the Pluronic.⁴² When Pluronics with longer hydrophilic domains are used along with the oleosin, the shell becomes stiffer. In this report, we compare the uniformity and stability of OleP microbubbles with and without clinical ultrasound stimulation to those of commercial agents. We also show that the composition of the shell and in turn the echogenic response of these OleP microbubbles can be systematically varied by using a homologous series of amphiphilic triblock copolymers. Our work demonstrates that engineering the size of the bubble and composition of the bubble shell via microfluidics allows for the optimal design of microbubbles for specific applications in diagnostics and therapy.

2. MATERIALS AND METHODS

2.1. Materials.

(PEO)₇₈-(PPO)₃₀-(PEO)₇₈ (Pluronic F68), (PEO)₅₁-(PPO)₃₁-(PEO)₅₁ (Pluronic F77), (PEO)₃₇-(PPO)₅₆-(PEO)₃₇ (Pluronic P105), and (PEO)₂₅-(PPO)₃₀-(PEO)₂₅ (Pluronic P65), phosphate-buffered saline (PBS), lysozyme, Luria–Bertani (LB), and HisTrap Ni-NTA columns are purchased from Sigma-Aldrich (St. Louis, MO). Kanamycin sulfate and B-PER Bacterial Protein Extraction Reagent are purchased from Thermo Fisher Scientific (Waltham, MA). *E. coli* BL21(DE3) competent cells are obtained from New England Biolabs Inc. (Ipswich, MA). Nitrogen gas (N₂) is obtained from Airgas. Aqueous solutions are all prepared in Milli-Q deionized water (18 M Ω cm⁻¹ deionized water, Millipore CO., Milli-Q system).

2.2. Protein Preparation.

In our prior studies, we employed an oleosin variant called 42–30G–63 with 42 and 63 amino acid residues in its N-terminus and C-terminus hydrophilic arms respectively, and 30 residues in its hydrophobic arm, not including five extra, evenly distributed glycines (G) to impart flexibility.⁴³ The current study uses a truncated and anionic variant of the above, accordingly named as 25–30G(–)–30 (also referred to simply as “oleosin”). The (–) indicates that all positively charged amino acids in the hydrophilic arms have been mutated to negatively charged amino acids. We have found that the latter variant produces more stable microbubbles, likely due to the electrostatic repulsion produced by the negative charges between its shorter hydrophilic arms. Recombinant oleosin protein variant 25–30G(–)–30 is created from a gene obtained by carrying out multiple rounds of PCR on the original sunflower seed oleosin gene. The mutation in the gene is confirmed through DNA sequencing (see Supporting Information). The protein is expressed in *E. coli* strain BL21(DE3) (New England Biolabs), controlled by a T7 promoter. The protein has a 6-Histidine tag for purification using immobilized metal affinity chromatography (IMAC). Bacterial cultures are grown in 1 L Luria–Bertani (LB) broth with kanamycin (50 μ g/mL) at 37 °C. At an optical density of ~0.8, the cultures are induced with IPTG (1 mM final concentration) and incubated for an additional 3 h. Cells are isolated by centrifugation and stored at –20 °C until lysis and purification are performed.

To lyse the cells obtained from each 1 L original culture, B-PER, lysozyme, protease inhibitor, and DNase are added, as detailed previously.⁴⁴ Following cell lysis, the supernatant containing the protein is incubated with an equilibrated Ni-NTA column and purified using fast protein liquid chromatography (FPLC) by eluting with 500 mM imidazole and 500 mM NaCl in Tris Buffer. The eluted fractions are then dialyzed overnight against 1 $\times$ PBS; the purified protein is stored at –80 °C for characterization (see Supporting Information and Figure S1) and use in bubble formulation.

2.3. Fabrication of Microfluidic Device.

The microfluidic device with an air-actuated membrane valve is prepared by single layer soft lithography in poly(dimethylsiloxane) (PDMS; Figure 1a). First, a clean silicon wafer is spin-coated with the mixture of SU-8 developer and SU-8 2010 photoresist in a 1:3 ratio and

then patterned to UV light through a transparency photomask by Karl Suss MA4Mask Aligner (SUSS MicroTec Inc., Sunnyvale, CA). Second, Sylgard 184 PDMS (Dow Corning, Midland, MI), mixed with the cross-linker at a ratio of 12:1 homogeneously, is degassed for 1 h, and then poured onto the patterned silicon wafer. Next, they are cured at 70 °C for about 1 h ensuring the membrane is completely compliant. Finally, the PDMS replica is peeled off from the wafer with the pattern, and then after oxygen plasma activation of both surfaces, the replica is bonded to a PDMS membrane which is prepared by spin-coating PDMS on a clean glass slide. The chamber used for collecting the microbubbles from the outlet of microfluidic device and taking the ultrasound images consists of two layers of PDMS slabs. The top layer (8 mm thickness) was first punched for several vials and then bonded to the bottom PDMS slab (2 mm) after oxygen plasma activation of both surfaces.

2.4. Microbubble Preparation and Characterization.

The continuous phase is prepared with oleosin protein 25–30G(–)-30 and Pluronic diluted in PBS, to a concentration of 10 mg/mL and 1.2 mM, respectively (which translates to 1% by weight of Pluronic F68). This continuous phase with optimal concentration is injected into the microfluidic device at the flow rate of 500 μ L/h using a syringe pump, and the inner phase made of 99.999% N₂ is provided to the microfluidic device at a pressure between 173 and 207 kpa using a pressure regulator. The expanding nozzle is controlled by a dual-valve pressure regulator with the pressure ranging from 0 to 138 kpa.

First, a small pressure (~35 kpa) is applied to the gas inlet, and the continuous phase is injected to the microfluidic device at a flow rate of 500 μ L/h immediately. The gas pressure can then be increased slowly until stable microbubble formation is achieved (see Videos 1 and 2 in the Supporting Information). Images can be captured during the generation of microbubbles by an inverted microscope (Nikon Diaphot 300), which is connecting with a high-speed Phantom V7 camera. Microbubbles are stored in PDMS wells at 4 °C in a covered Petri dish for long-term use. For the characterization of long-term stability, microbubbles are collected in a 35 mm Petri dish, and then the images are acquired with an upright microscope (Carl Zeiss Axio Plan II). The images over time can be captured and the diameter as well as size distribution are analyzed using ImageJ (v 1.47v, NIH).

2.5. Ultrasound Characterization of Microbubbles.

OleP microbubbles are collected and imaged in a PDMS multiwell chamber (Figure 1b) for ultrasonic imaging. The prepared microbubbles are imaged using a broadband transducer (L38, 5–10 MHz, Titan Sonosite Ultrasound System) to investigate the effect of concentration, size, and shell properties of microbubbles on their echogenicity. Unless otherwise noted, all experiments are conducted at room temperature, at a mechanical index (MI) of 0.4. The concentration of microbubbles is measured using a hemocytometer (Levy Counting Chamber, Hausser Scientific). Microbubbles at an approximate concentration of 6×10^8 microbubbles/mL are used for all experiments, unless otherwise specified. The effects of MI and irradiation time are analyzed with a clinical ultrasound scanner Zonare since a wider adjustment of MI value(0.37–1.30) can be achieved, and the transducer L14–5w (frequency = 12 MHz) is placed below the multiwell for sending and receiving the signals. Grayscale B mode images are acquired and the brightness of ultrasound images is quantified

based on the gray value. Once the images are captured, they are analyzed with ImageJ (v 1.47v, NIH) to obtain gray value. For the region of interest, the size and shape of the region are kept fixed for all measurements. The relative gray value is calculated by dividing the sample gray value by that of the reference.

3. RESULTS AND DISCUSSION

3.1. Size Uniformity of Microbubbles.

As mentioned above, the size uniformity of microbubbles significantly influences their efficacy as contrast and therapeutic agents. Thus, we first evaluate the size uniformity of OleP F68 microbubbles and compare their uniformity with that of a commercial contrast agent, DEFINITY, which unlike OleP F68 microbubbles is prepared using the conventional method of high shear agitation. For OleP F68 microbubbles produced using the flow focusing microfluidic device, we find that the bubbles are uniform in size with a very small number (typically less than 1%) larger than 10 μm . Since no major coalescence in the flow-focusing device is observed (see Videos 1 and 2 in the Supporting Information), we believe these large microbubbles likely formed when bubbles were flowing through various interconnections between the microfluidic device and polyethylene tubing and between the tubing and the collection chamber. The dimensions of the conduits as well as the flow rate of fluids change abruptly when suspensions flow from the flow-focusing device to the tubing, which could cause collision and coalescence between microbubbles to form large microbubbles. These larger microbubbles, however, disappear in approximately 2–3 h after collection, leaving highly uniform and monodisperse microbubbles (Figure 2a). As for the commercial microbubbles, many large microbubbles are observed when they are produced by the conventional method of high shear agitation. Unlike OleP F68 microbubbles, a substantial number of commercial microbubbles with diameter over 6 μm remain even after 2 h (Figure 2b). Overall, microfluidic microbubbles made with oleosin and Pluronic F68 have a much narrower size distribution compared to the commercial microbubbles. Over 90% of OleP F68 microbubbles have a diameter between 2 and 4 μm , with only a few between 4 and 6 μm (Figure 2c). In contrast, about 45% of the commercial microbubbles are between 1 and 2 μm , and over 50% of them are widely dispersed in the diameter ranges of 0–1 μm and 2–10 μm (Figure 2c). The standard deviation in microbubble sizes for OleP F68 and commercial microbubbles are 0.37 and 1.72 μm , respectively. The size distribution of Optison, another commercial ultrasound contrast agent, is also very broad, with a standard deviation of 1.85 μm .⁴⁵

3.2. Microbubble Stability at Normal Hydrostatic Pressure.

The stability of microbubbles at normal hydrostatic pressure in the absence of ultrasound measures their shelf life, whereas their stability under ultrasound indicates their survival time during ultrasound imaging and therapy. First, we compare the stability of the OleP F68 microbubbles to that of the commercial agent by storing them in liquid at room temperature without ultrasound insonation. Despite the differences in specific physical properties, such as microbubble diameter, shell properties of the two microbubble groups, it is reasonable to compare them for meaningful assessment of the duration the microbubbles survive in practice. As shown in Figure 3a, OleP F68 microbubbles are remarkably stable. When these

microbubbles are stored in the PBS solution with 1% F68, the mean diameter decreases from 2 to 1.8 μm in the first week. These OleP F68 microbubbles remain stable with little change in their size over 2–3 weeks (Figure 3b). Even after 2 weeks, the majority of the microbubbles have a diameter of around 2 μm , which indicates that these microbubbles do not undergo significant dissolution or coalescence within this time span. Figure 2c indicates that Definity microbubbles are more polydisperse than the OleP F68 microbubbles. Thus, it could be expected that the stability profile of the Definity microbubbles would be heterogeneous (i.e., some bubbles will last longer than others). Despite such heterogeneity, commercial microbubbles disappear within 48 h, as shown in Figure 3c, indicating superior stability of OleP F68 microbubbles. The stability of OleP F68 microbubbles is especially remarkable considering that these bubbles are produced using nitrogen, whereas many commercial microbubbles are made with highly insoluble fluorinated gases such as octafluoropropane.

3.3. Microbubble Stability during Ultrasound Imaging.

To test whether the enhanced stability of OleP F68 microbubbles can be translated to their stability under ultrasound, we investigate their stability under ultrasound exposure during imaging. The ultrasound images of OleP F68 microbubbles and the commercial agent are acquired at increasing time of insonation at a MI of 0.4 and microbubble concentration of 6×10^8 microbubbles/mL. Figure 4a shows that for the same microbubble concentration and ultrasound MI the image brightness (gray value) of commercial microbubbles is initially slightly higher than that of the OleP F68 microbubbles at the same concentration. As will be discussed in more detail later, we believe the initial high values of ultrasound signal from the commercial agent compared to that of OleP F68 bubbles are likely due to the softer shell of commercial microbubbles compared to that of the OleP F68 bubbles.^{47–49} After ~7 min of ultrasound exposure, the ultrasound signal from the commercial microbubble decreases sharply to ~94% of its initial intensity, whereas the acoustic response from the OleP F68 microbubbles shows a mere 6% decline from its initial acoustic intensity after 20 min of ultrasound insonation (Figure 4a). The decay constants, τ , of these two microbubbles calculated using eq 1 are 0.78 and 0.41, respectively, for commercial agent and OleP F68 microbubbles. The half-life, $t_{1/2}$, of the commercial and OleP F68 microbubbles measured using eq 2 are 0.88 and 1.68, respectively.

$$BS(t) = BS_0 e^{-t/\tau} \quad (1)$$

$$t_{1/2} = \ln 2 / \tau \quad (2)$$

where $BS(t)$ is the echogenicity at time t and BS_0 is the initial echogenicity.

Remarkably, over 90% of the OleP F68 microbubbles remain intact after ultrasonic irradiation, which is confirmed by optical microscopy (Figure 4b). In comparison, only ~60% of the commercial microbubbles survive after ultrasound exposure (Figure 4b). It has

been previously demonstrated that close packing of the surface monolayer at the bubble interface reduces the diffusion of the encapsulated gas into the surrounding, enhancing the bubble stability.^{50,51} The high stability of OleP F68 microbubbles suggests that the shell made from the mixture of oleosin and Pluronic F68 limits the diffusion of the encapsulated gas (N₂) into the surrounding much more effectively than the phospholipids that are used for the commercial agent. The high stability of the OleP F68 microbubbles bodes well for prolonged ultrasound imaging and also may have great potential in delivering therapeutic gases that exhibit high solubility in water, for example, oxygen.

3.4. Microbubble Characteristics and Echogenicity.

As discussed above, microfluidic microbubbles stabilized with the oleosin and Pluronic F68 have superior stability and size uniformity compared to the commercial agent. Microbubbles as contrast agents are designed to enhance echogenicity of the images. Several factors that may affect echogenicity include microbubble concentration, size, and shell properties. The combination of microfluidics with the use of oleosin and Pluronic mixtures allows us to control these factors precisely and thus enables a detailed study of the relationship between echogenicity and these factors. It is often difficult to perform such an analysis using commercial agents due to their polydispersity and inability to control shell composition and their mechanical properties.

3.4.1. Concentration-Dependent Microbubble Echogenicity.—The effect of the microbubble concentration on ultrasound echogenicity is studied using 3.2 μm OleP F68 microbubbles, which falls within the optimal range of 2–5 μm for ultra-sonography. The brightness of the B-mode images, normalized by the background noise from PBS solution, increases with the concentration of microbubbles. Since the echo signal in the ultrasound images is logarithmically compressed, the normalized brightness (representing log of echo signal) is plotted as a function of the log concentration (Figure 5). A log plot with a slope of 1.1 and $R^2 = 0.9902$ is observed, indicating the echo signal increases linearly with the microbubble concentration in the concentration range studied. Such a linear relationship has also been previously observed for phospholipid-shell microbubbles,⁵² and this trend is also consistent with theory⁵³ that backscatter (BS) from an ensemble of scatterers is proportional to the concentration of scatterers at low concentrations:

$$\text{BS} = n \frac{\sigma_s(a, \omega)}{4\pi} \quad (3)$$

where n represents the total number of microbubbles per unit volume, a is the radius of microbubbles, and σ_s is the acoustic power scattered in all directions per unit incident intensity by individual bubbles.

3.4.2. Effect of Bubble Size on Microbubble Echogenicity.—Equations 4 and 5 show that the scattering intensity of microbubbles depends on the size. The echogenicity of the microbubbles with different diameters ranging from 2.50 to 4.75 μm are examined. At a fixed concentration of approximately 6×10^8 microbubbles/mL, a marked increase in

echogenicity is observed with the increase in the bubble size (Figure 6). Scattering from larger microbubbles of diameter 4.75 μm is as much as 1 order of magnitude greater than the scattering from 2.50 μm microbubbles (Figure 6).

According to an oscillation model (eq 4), ultrasound scattering exhibits a complex dependence on the size of microbubbles:^{54–56}

$$\sigma_s(a, \omega) = 4\pi a^2 \frac{\Omega^4}{(1 - \Omega^2)^2 + (\Omega\delta)^2}, \quad \Omega = \frac{\omega}{\omega_0} \quad (4)$$

where δ is the damping constant and ω and ω_0 are the driving frequency and the resonant frequency, respectively. For radius larger than resonance radius, the scattering cross section increases as the geometric cross-section of the microbubble, $\sigma_s \propto a^2$.⁵⁴ For radii smaller than the resonance radius, the oscillation model converges to Rayleigh model and scattering cross section increases with the sixth power of the diameter, $\sigma_s \propto a^6$.⁵⁴ The measurements show echogenicity with OleP F68 microbubbles increase as $a^{4.5}$, which is in between the two limiting cases described above. The reason for the difference is not fully understood; it could be related to at least two factors. First, the theory (eq 4) applies to the measurements made at a single frequency, whereas imaging is performed using short bursts of ultrasound pulses with a broad band of frequencies. Second, the difference may be related to the unique shell properties of oleosin. It has previously been reported that both the viscosity and the elasticity of the microbubble shell play an important role in determining the characteristics of the acoustic signal.⁵⁵ Increased shell stiffness causes the resonance frequency to increase, whereas increased damping from shell viscosity broadens the resonance peak. Viscoelastic polymer shells have been shown to increase the resonance frequency and to broaden the resonance peak to the point where the peak almost disappears.⁵⁵ Similar to this prior work, we do not observe any resonance peaks, which indicates that the shell of OleP F68 microbubbles may be too stiff to undergo resonance oscillation. Thus, it is conceivable that the difference in the rheological properties of the shell, which are not taken into consideration in the mathematical models, may also affect the relationship between scattering intensity and bubble size.

3.4.3. Effect of Shell Composition on Microbubble Echogenicity.—The scattering cross section from a single bubble described by eq 4 can be simplified to describe the relationship between bubble stiffness and ultrasound scattering. For small microbubbles below resonance, there is a direct relationship between ultrasound scatter and the compressibility of the bubble (eq 5), which in turn depends strongly on the shell property.^{54–56}

$$\sigma_s = \left[\frac{4}{9} \pi a^2 \left(\frac{2\pi}{\lambda} a^4 \right) \right] \left\{ \left[\frac{K_g - K_1}{K_1} \right]^2 + \frac{1}{3} \left[\frac{3(\rho_g - \rho_1)}{2\rho_g + \rho_1} \right]^2 \right\} \quad (5)$$

where λ is ultrasonic wavelength, K_g is compressibility of gas bubble, K_l is compressibility of liquid, ρ_g is density of microbubble gas, and ρ_l is density of liquid. Although the relationship between scatter cross-section and scatterer compressibility is simple (eq 5), it has been challenging to study the relationship between the shell stiffness and echogenic response experimentally and quantitatively. This is primarily due to the unavailability of uniform bubbles with controlled shell stiffness. The combination of microfluidics and OleP shell provides an excellent platform to establish this relationship. In our prior work, we have shown that changing the concentration or composition of the Pluronic surfactant is effective in changing the shell elasticity.⁴² Increasing the concentration of Pluronic F68 increases the stiffness of the microbubbles. Pluronics with longer hydrophilic poly(ethylene oxide) domains also make microbubbles stiffer.

To test the effect of bubble stiffness on the microbubble echogenicity, OleP F68 microbubbles with different concentrations of Pluronic F68 are studied. It was previously observed that as the concentration of Pluronic F68 is increased from 0 to 20 mg/mL, the areal expansion modulus of the stabilizing layer becomes three times higher (increasing from 2000 to 6500 dyn/cm) compared to that with no F68. Based on eq 5 it is to be anticipated that echogenicity should decrease with increased F68 concentration. Consistent with this expectation, as the concentration of F68 in the stabilizing media is increased, the ultrasound signal is reduced, as seen in Figure 7. The increase in the concentration of F68 in the stabilizing layer increases the stiffness of the shell and in turn decreases the compressibility of the bubble, reducing their ability to oscillate under ultrasound.

Intrigued by the effect of Pluronic F68 concentration on the shell elasticity, microbubbles with mixtures of oleosin protein 25–30G(–)–30 and different Pluronic triblock copolymers are prepared, and their effect on echogenicity is studied. Pluronic F77, Pluronic P105, and Pluronic P65 are employed as they are commercially available, and they are capable of stabilizing microbubbles effectively when they are mixed with the oleosin. The lengths of the hydrophobic domains of these Pluronics are approximately the same, whereas those of the hydrophilic domains of the Pluronics increase in the order of P65, P105, F77, and F68. Since the oleosin used in this study has a similar structure as the one used in our prior work, we believe the elasticity of OleP microbubbles will increase with the length of the hydrophilic domain of Pluronic.⁴²

As shown in Figure 8a, highly uniform and monodisperse microbubbles can be formed with different Pluronics. These microbubbles are stable and can be stored over 2 weeks with little change in size distribution (Figure 8b). The enhanced stability of OleP microbubbles also translated to their stability under ultrasound, as shown in Figure 8c; regardless of the type of Pluronics used, OleP microbubbles remain highly echogenic even after 25 min of insonation (Figure 8c). The echogenic response of OleP microbubbles, however, depends strongly on the composition of the shell as shown in Figure 8c,d. The ultrasound signal indeed becomes stronger as the hydrophilic arms of the Pluronic become shorter. That is, microbubbles with softer shells give stronger echogenic response consistent with eq 5. Such tunability would be especially important and critical for applications that require very specific bubble size with specific shell properties.

4. CONCLUSIONS

In this study, we have prepared uniform microbubbles using a mixture of recombinant protein, oleosin, and amphiphilic copolymer surfactants, Pluronic, and showed the superiority of their uniformity and stability to those of a commercial agent. Unlike other lipid/polymer/protein shell materials that can stabilize nitrogen-based microbubbles for only a few hours,⁵⁷ OleP is capable of keeping nitrogen microbubbles stable for days. Such a high stability indicates that hydrophobic and more expensive gases like perfluorocarbons that are typically used for microbubble applications are not necessary when OleP is used for microbubble stabilization. The OleP microbubbles also have long shelf time and are stable when exposed to ultrasound. The high stability of the OleP microbubbles bodes well for prolonged ultrasound imaging and also may have great potential in delivering therapeutic gases, which exhibit good solubility in water. We have demonstrated that the ultrasound response of OleP microbubbles can be engineered by changing the concentration or composition of Pluronic. This study shows the feasibility of tuning echogenic response of uniform microbubbles for ultrasound imaging applications by engineering microbubble shell properties. While this work focused on imaging, this tunability could potentially be important and powerful in producing microbubble agents that would be used for theranostic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Pluronic F68	(PEO) ₇₈ -(PPO) ₃₀ -(PEO) ₇₈
Pluronic F77	(PEO) ₅₁ -(PPO) ₃₁ -(PEO) ₅₁
Pluronic P105	(PEO) ₃₇ -(PPO) ₅₆ -(PEO) ₃₇
Pluronic P65	(PEO) ₂₅ -(PPO) ₃₀ -(PEO) ₂₅
PBS	phosphate-buffered saline
OleP	Oleosin/Pluronic

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Figure 1.

(a) Schematic illustration of a microfluidic flow focusing device and (b) a multiwell chamber used for ultrasound characterization of microbubbles. (c) Photographs of a multiwell chamber. The width of each chamber is approximately 6 mm.

Figure 2. Microscopic images of (a) OleP F68 microbubbles and (b) commercial microbubbles. (c) Size distribution of OleP F68 microbubbles (black line), commercial microbubbles (red line, the result is consistent with ref 46), and Optison microbubbles (blue line). Size distribution data of Optison microbubbles is adapted from ref 45.

(a)

(b)

(c)

Figure 3.

(a) Change in the size of microbubbles over 7 days. (b) Microscopic image of OleP F68 microbubbles after 7 days. (c) Microscope image of commercial microbubbles after 2 days.

Figure 4.

(a) Echogenicity of OleP F68 microbubbles and commercial microbubbles as a function of time. (b) Microscope images of OleP F68 microbubbles and commercial microbubbles before and after 25 min irradiation. Some microbubbles appear lighter, especially in the case of Definity microbubbles, because they are not in the same focal plane as the ones that appear dark.

Figure 5.
Effect of the bubble concentration on their echogenicity.

Figure 6.
Effect of the bubble diameter on their echogenicity.

Figure 7.
Echogenicity of OleP F68 microbubbles made with varying concentrations of Pluronic F68.

Figure 8.

(a) Size distribution of OleP microbubbles. (b) Size of OleP microbubbles over 7 days. (c) Echogenicity of OleP microbubbles as a function of time. (d) Echogenicity of microbubbles with different Pluronics (Pr denotes bubbles made with oleosin without Pluronic).