Temperature-Dependent Biphasic Shrinkage of Lipid-Coated Bubbles in Ultrasound

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ABSTRACT: Lipid-coated microbubbles and emulsions are of interest as possible ultrasound-mediated drug delivery vehicles and for their interesting behaviors and fundamental properties. We and others have noted that bubbles coated with the long chain saturated phospholipid distearoylphosphatidylcholine (DSPC) rapidly shrink to a quasistable size when repeatedly insonated with short ultrasound pulses; such stability may adversely affect the bubble’s subsequent ability to deliver its pharmacological cargo. Bubbles coated with the unsaturated lipid dioleoylphosphatidylcholine (DOPC) did not show stability but did undergo an abrupt change from rapid initial shrinkage to a slow persistent shrinkage, leading ultimately to dissolution or dispersion. As DSPC and DOPC differ not only in chain saturation but also phase behavior, we performed additional studies using dimyristoyl PC (DMPC) as a coat lipid and controlled the solution temperature to study bubble behavior on exposure to repeated ultrasound pulses for the same coat, in both fluid and gel phases. We find, first, that essentially all bubbles show an initially rapid shrinkage, in which gas loss exceeds the limit imposed by gas diffusion into the surrounding medium; this rapid shrinkage may be evidence of nanoscopic bubble fragmentation. Second, upon reaching a fraction of their initial size, bubbles begin a slower shrinkage with a shrinkage rate that depends on the resting phase state of the coat lipid: fluid DMPC monolayers give a more rapid shrinkage than gel phase. DOPC-coated bubbles showed no temperature-dependent responses in the same temperature range. The results are especially interesting in that bubble compression during the pulse is likely to adiabatically heat the bubble and fluidize the coat, regardless of its initial phase state; thus, some structural feature of the resting coat, such as defect lines in the gel phase, may be important in the subsequent response to the ~3 μs ultrasound pulse.

1. INTRODUCTION

There is growing interest in the application of lipid-coated bubbles or emulsions to ultrasound-targeted drug delivery.1,2 An attractive feature of these vehicles is that they can be disrupted or dispersed by ultrasound levels that are not damaging to healthy tissues. Disruption may enhance the local efficacy of pharmacologically active compounds via a number of proposed mechanisms including repartitioning,3 chemical cleavage,4 or enhanced fragment uptake by cells.5 Because of the role of bubble or droplet disruption in delivery, it is important to elucidate the role of the lipid coat in determining the fate of bubbles or droplets exposed to ultrasound.

For lipid-coated gas bubbles, the selection of the appropriate lipid coat is in part determined by the desirability of stabilizing the (resting) bubbles, so as to provide a relatively large time window in which they can be used effectively after their preparation. Micron-sized uncoated gas (nitrogen or oxygen) bubbles rapidly dissolve in the blood, in a few seconds.6 For targeted delivery applications, it is essential to have stability that exceeds the circulation time, about 1 min, and desirable to have bubbles remain stable for perhaps an order of magnitude longer, to minimize untargeted release. Stabilization is aided by using a very poorly soluble gas, such as perfluorobutane (PFB), but even with PFB, Laplace pressure arising from bubble surface tension tends to drive the gas into the surrounding solution.6 For the best stability in ultrasound imaging, long chain saturated lipids (e.g., distearoylphosphatidylcholine) are often used; long chain saturated lipids are an important component of Definity, a commercial bubble formulation for ultrasound imaging. We7,8 and others9,10 have observed that DSPC-coated PFB bubbles typically shrink rapidly when exposed to repeated pulses of megahertz ultrasound but that these bubbles usually reach a stable size and are subsequently unresponsive, at least to the extent that no further size changes are microscopically observable with the same pulse amplitude and duration. Such stability may well be undesirable from a delivery perspective, as the remaining bubble may sequester a large fraction of the drug to be delivered. It is generally possible to destabilize the bubbles by using stronger insonation,9 but such changes in the insonation protocol may be undesirable and add uncertainty as to the extent of release.

In an earlier study,7 we observed that bubbles coated primarily with the long chain unsaturated phospholipid dioleoylphosphatidylcholine (DOPC) also showed a rapid initial shrinkage but that, rather than reach a fully stable size, they show a very slow shrinkage and ultimately do vanish through complete dissolution or nanoscopic (i.e., microscopically invisible) fragmentation. DOPC has both chemical (bond...
unsaturation) and physical (monolayer phase) differences, compared with DSPC; in principle, either or both differences could be responsible for different responses to insonation, especially given that ultrasound can often enhance chemical reactivity via the generation of radicals.\textsuperscript{11,12}

To gain further insight into the role of the lipid coat in ultrasound responsivity, we undertook a study of the behavior of dimyristoylphosphatidylcholine (DMPC)-coated PFB bubbles, with the sample temperature varied between 4 and 26 °C. The rationale is that if lipid phase (gel vs fluid) can play an important role in bubble stability, then DMPC-coated bubbles should show a temperature-dependent response to insonation, with more stable bubbles obtained at lower temperatures. DMPC was chosen because DOPC lipid bilayers and monolayers solidify below 0 °C, and thus solid or gel phase DOPC coats are unrealizable in aqueous buffers.

2. MATERIALS AND METHODS

2.1. Materials. Perfluorobutane (PFB) was purchased from SynQuest (Alachua, FL). Phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1,2-distearyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). Phosphate buffered saline (PBS) was prepared with 100 mM NaCl and 40 mM NaHPO\textsubscript{4} in nanopure water (D13321, Barnstead, Dubuque, IA) and pH adjusted to 7.4 using HCl (measured with a UB-10 Denver Instrument pH meter (Arvada, CO)).

2.2. Bubble Preparation. Lipid-coated PFB bubbles were prepared by entrainment of gas bubbles in a lipid suspension via probe sonication at the gas/fluid interface, as described elsewhere.\textsuperscript{3,13,14} Lipid coats consisted of 100% DMPC or 100% DOPC. (As the bubbles were used shortly after preparation, stabilizing PEG-lipids were not needed or included. Bubbles without PEG will more easily coalesce, but these bubbles were diluted such that there was only one or two in each field of view (140 μm across), so they were unlikely to encounter another bubble.) The lipid suspension was prepared as follows: the appropriate amount of lipid (dissolved in chloroform) was measured out into a glass vial, and chloroform was removed by nitrogen evaporation followed by vacuum desiccation for 1 h. The lipid, which now appears as a thin, opaque film in the vial, was suspended at 5 g/L in PBS by vortex mixing (VM-3000, VWR) at room temperature until the lipid film was removed from the vial wall. The lipid suspension was then probe sonicated (VC 130PB, Sonics & Materials, Newton, CT), with the sonicator tip near the vial, at 30/100 power (∼1 W) for 15 min.

To make lipid-coated bubbles, a 0.25 mL aliquot of the lipid suspension was put in a 2 mL glass vial capped with a septum with a hole for the probe sonicator tip to go through. The sonicator tip was positioned at the gas/fluid interface, the vial headspace was flushed with PFB, and probe sonicator was turned on at maximum power (∼10 W) for 10 s. The vial was immediately cooled in an ice bath after sonication. After about 15 s on ice, bubbles were diluted 1:100 in PBS and put in the sample chamber (a specially designed 3 mL rectangular cuvette made out of 5 mil PVC, chosen for its high optical and acoustic (−99%) transmissivity).

The PBS was either air saturated (where no particular effort was made to change the dissolved gas) or PFB saturated. To saturate PBS with PFB, 4 mL of PBS was put in a glass vial capped with a septum, PFB was bubbled through the PBS for 30 s, and the vial was left at room temperature for at least 24 h. To get the PFB saturated PBS into the sample chamber without exposing it to air, the cuvette cap on the sample chamber was replaced with a cuvette cap with a hole cut in it and a septum glued to it, and the sample chamber was filled with PFB gas. PFB saturated PBS was retrieved from the vial with a syringe and injected into the sample chamber, and then bubbles were added (also by injection with a syringe).

2.3. Methods. The sample chamber was positioned in a water bath at the focus of an ultrasound transducer (H-101, Sonic Concepts, Bothell, WA), and the bubbles were sonicated with short pulses of ultrasound and imaged after each pulse (Figure 1A). Ultrasound pulses were generated as described previously.\textsuperscript{7} 1.1 MHz ultrasound was chosen because it provides a more biomedical relevant system than lower frequencies. The sound is focussable to mm dimensions and to high intensity. Three types of pulses with various amplitudes and lengths were used: 3 cycle 200 kPa amplitude, 7 cycle 200 kPa amplitude, and 3 cycle 300 kPa amplitude. All pulses had a frequency of 1.1 MHz and a pulse repetition rate of 25 Hz. When collecting data, we rotated through the three ultrasound pulse types so that each batch of bubbles would be treated in the same way. Bubbles 1–10 μm in diameter were exposed to pulsed ultrasound; bubbles outside this range may be highly nonresonant at 1.1 MHz (5.3 μm is the resonant size).\textsuperscript{17}

Bright-field images were taken using a CCD camera (DFK 3B2U03, The Imaging Source, Charlotte, NC) and a water immersion microscope objective (LUMPLFLN 40XW NA 0.8, Olympus, Tokyo, Japan), giving a measured resolution of 0.6 μm. The objective was focused on bubbles resting buoyantly against the top surface of the sample chamber and near the edge closest to the ultrasound transducer to minimize effects of attenuation and scattering by other bubbles in the path of the ultrasound pulse. During insonation, images were first collected after each pulse (∼25 Hz); after shrinkage slowed, images were collected every second or half second. The resulting series of images was analyzed using NIH ImageJ. ImageJ automatically thresholds the images, and the area of the bubble in the thresholded image was measured and converted to an effective radius (Figure 1B).

The ambient temperature was varied by putting ice in the ultrasound transducer and a water-immersion microscope objective. Bubbles are illuminated from below to form bright-field images. (B) Images of a typical DMPC bubble shrinking in pulsed ultrasound. The scale bar is 5 μm. The number of pulses and the time since the onset of insonation are indicated. The diameter vs time for this bubble is plotted in Figure 2. Bubble diameter was measured by thresholding the images in ImageJ (thresholded images are shown below the raw images), finding the area, and converting that to a diameter (assuming the bubble is circular).

Figure 1. (A) Experimental setup. The sample chamber containing the bubbles is placed in a water tank at the focus of an ultrasound transducer and a water-immersion microscope objective. Bubbles are illuminated from below to form bright-field images. (B) Images of a typical DMPC bubble shrinking in pulsed ultrasound. The scale bar is 5 μm. The number of pulses and the time since the onset of insonation are indicated. The diameter vs time for this bubble is plotted in Figure 2. Bubble diameter was measured by thresholding the images in ImageJ (thresholded images are shown below the raw images), finding the area, and converting that to a diameter (assuming the bubble is circular).
Bubble shrinkage must necessarily involve the loss of gas into the surrounding medium. We have also observed rapid fragmentation of DMPC bubbles exposed to 200 kPa 3 cycle pulses ($p < 0.006$); otherwise, temperature had no effect on the qualitative manner of bubble dissolution.

Figure 3. Fraction of bubbles showing various modes of dissolution, for different coat lipids (DMPC, top, and DOPC, bottom) and different pulse durations and amplitudes. Most DMPC-coated bubbles exhibited biphasic shrinkage (Figure 2), some fragmented into a small number of microscopically visible parts, a few underwent approximately linear “steady shrinkage” (monophasic). There was some evidence of increased fragmentation for DMPC bubbles exposed to 200 kPa 3 cycle pulses ($p < 0.006$); otherwise, temperature had no effect on the qualitative manner of bubble dissolution.

\[
\frac{dR}{dr} = -\frac{Dk_BT}{R} \left[ 1 - \frac{\rho g \Delta \sigma}{\rho BTR} \right] \frac{\Delta \sigma}{\rho BTR}
\]

(1)

using parameter values that maximize shrinkage rate: zero dissolved PFB (at infinity in the bulk solution) and a bubble surface tension equal to that of bare water, 72 dyn/cm. The use of the steady-state EP equation is justified by the fact that the bubbles are resting in buffer for several minutes prior to insonation, allowing for local equilibration. During the rapid initial phase, we observed here that both DMPC and DOPC bubbles shrink faster than can be accounted for by diffusion of gas into the surrounding medium. We have also observed rapid shrinkage with DOPC and DSPC-coated PFB bubbles, as reported previously.\(^8\)

Diffusional limitations on bubble shrinkage can also be measured by observing the shrinkage of uncoated bubbles, in the absence of insonation. As reported previously,\(^6\) these bubbles shrink much more slowly than insonated bubbles, in spite of their high Laplace pressures, and fall within the EP limit. Additional and compelling evidence of rapid loss of bubble volume was recently obtained by Thomas et al.,\(^7\) who found significant shrinkage of Definity microbubbles during, but not between, 1.6 MHz ultrasound pulses, using a high-speed (13 Mfps) video camera.

Thus, both direct and indirect observations suggest that the initial, rapid shrinkage of these bubbles must be accompanied by the loss of gas into aqueous solution during the 3 μs pulse. This gas loss, in turn, is presumed to occur via diffusion into the aqueous buffer in between the brief insonation pulses. (The pulses would still enhance shrinkage via coat shedding, resulting in increased internal pressure.) However, the initial rapid phase of bubble shrinkage is actually faster than can be accomplished via diffusion alone (Figure 4). The diffusion-limited shrinkage of a bubble may be computed from the Epstein–Plesset equation

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by loss of gas by a mechanism other than dissolution into the surrounding medium. We have suggested that these bubbles are fragmenting, with (gas-entrapping) fragments smaller than the optical resolution limit being shed from the bubble surface.\(^8\)

### 3.1. Transition Diameter and Bubble Lifetime

Figure 5A shows the bubble diameter at which ultrasound-induced shrinkage changed from the rapid phase to the slow phase, plotted against the initial (preinsonation) bubble diameter. The lines show the theoretically maximal (diffusion limited) rates of shrinkage for a completely uncoated, quiescent perfluorobutane bubble (eq 1), in an air-saturated buffer (\(f = 0\)) (black line), or PFB-saturated buffer (\(f = 1\)) (gray line), calculated for \(T = 25\, ^{\circ}\)C. The rapid shrinkage is faster than the diffusion limit for gas efflux, while the slow shrinkage is not.

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Figure 5A shows the bubble diameter at which ultrasound-induced shrinkage changed from the rapid phase to the slow phase, plotted against the initial (preinsonation) bubble diameter. There is a strong correlation: shrinkage slows when the bubble diameter is \(\sim 60\%\) of the initial diameter or when the surface area is about a third of the initial area. This “memory” of initial bubble size could arise from initial shedding of uncoated bubble fragments (and dissolution of gas into the medium), leaving the coat molecules in place. (Indeed, it is difficult to imagine a mechanism for memory in which coat molecules are not retained.) This suggests that the density of the coat is an important parameter controlling the rate of bubble shrinkage and that whatever mechanism is responsible for rapid initial shrinkage is inactive when the coat density becomes sufficiently high. It is noteworthy, however, that previous studies by us\(^7\) and others\(^8\) have found no correlation between initial size and transition size, when using coats containing stabilizing PEG-lipids. It unclear how the presence of a small fraction (typically 10 mol %) of PEG-lipid decouples these parameters.

The initial rapid shrinkage rate is somewhat faster for larger bubbles (Figure 5B), and this may partly account for the fact that the overall bubble lifetime is essentially uncorrelated with the initial bubble size (Figure 5C). (The rate of slow phase shrinkage was uncorrelated with initial size. In addition, a significant fraction of bubbles show a very rapid final shrinkage,
like the DOPC bubble in Figure 2; this could also weaken the correlation of lifetime with initial size.)

3.2. Slow Phase Shrinkage and Temperature Dependence. Prior work has shown that the slow phase depends strongly on the species of lipid used in the coat. In particular, bubbles with coats consisting (primarily) of the long chain saturated lipid DSPC exhibited such slow shrinkage that they could be considered “stable” for the duration of the measurement, many hundreds of ultrasound pulses. In contrast, bubbles coated with the unsaturated lipid DOPC showed persistent (slow) shrinkage and typically vanished entirely after 100–200 pulses. These two lipid coats differ in both chemical (bond saturation) and physical (lipid phase) properties. To better understand what factors control bubble stability and slow shrinkage, we prepared bubbles with DMPC or DOPC coats and studied their behavior as a function of temperature. In Figure 6, the mean lifetime of bubbles is plotted vs buffer temperature for each coat lipid. Although there is large variability in the data, there is a clear trend toward lower temperature for each coat lipid. Even when large temperature fluctuations are present (such as with DOPC), bubbles in a DMPC coat generally show higher stability (Figure 6) so that even with the shorter pulses, there remains a clear temperature dependence, which is a much larger fraction of bubbles show visible fragmentation (Figure 3). Clearly, the additional stability provided by the saturated DMPC coat lipid can, at least in part, be overcome by using a longer pulse duration or, even more effectively, stronger pulses (though even with the stronger pulses, there remains a clear temperature dependence, Figure 6.) We speculate that longer or stronger pulses may lead to increased temperature in the vicinity of the bubble, through either viscous heating or stronger adiabatic heating. (Adiabatic heating on compression of the bubble should always increase the temperature above the DMPC melting transition, however, as discussed below. Thus, if the diminished temperature response with longer or stronger pulses is due to heating, it must be local heating of the solution, not the oscillatory temperature changes in the bubble gas.)

3.3. Collapse Pressures. The pressure amplitude of the ultrasound pulse is 200–300 kPa, which, in a spherical shell with a radius on the order of micrometers, would give surface pressures of many hundreds of dyn/cm—far above the collapse pressures of lipid monolayers (55 dyn/cm for DMPC\(^1\)), regardless of phase state. The fact that the (initial) lipid phase state appears to contribute to bubble stability may seem
remarkable, in consideration of the very high surface pressures reached. In addition, of course, adiabatic heating of the bubble during compression should give rise to highly elevated temperatures (ca. 100 °C, for an initially 3 μm bubble compressed to 100 nm \(^{7,20}\)), regardless of the bath temperature.

If lipid monolayer phase plays a role in bubble stability, as our observations imply, it must do so by affecting the initial distribution of lipids on the surface and thus creating spatially varying surface properties that greatly modify the subsequent bubble behavior in the presence of the strong ultrasonic field. We suggest that condensed or crystalline/gel phases, present in DMPC and DSPC monolayers, contain defects between domains that direct the bubble compression in a highly anisotropic manner, for example by leading to ‘pancaking’. Although such modes of compression are speculative, they offer a mechanism by which lipid coats or shells would remain associated with the bubble throughout the collapse, while avoiding extensive corrugations or invaginations that would more readily micellize or otherwise be shed from the bubble.

4. CONCLUSIONS

We have observed micrometer-scale perfluorobutane bubbles coated with DMPC or DOPC in pulsed ultrasound at temperatures from 4 to 26 °C and found that most bubbles exhibit biphasic shrinkage with an initial rapid phase followed by a much slower phase. The rate of the initially rapid shrinkage was much greater than that allowed by diffusional gas loss, as given by the Epstein–Plesset equation. The bubble size at the transition from one phase to the other was correlated with initial size; this “memory” of the initial size may be dependent on the initial coat density, if the coat remains associated with the bubble during the initial rapid bubble shrinkage.

The lifetime of bubbles coated with DMPC was strongly correlated with temperature, with increased stability at lower temperatures. No such correlation was observed for DOPC bubbles. This suggests that decreased stability in bubbles is due to the fluid phase, rather than the chemical bond saturation, of the lipid coat. This does not rule out a role for the chemical bond unsaturation, of course, and experiments with a very long chain unsaturated lipid, or chemical studies to look for lipid degradation or oxidation, are certainly warranted. The most significant temperature dependence was found with the slow phase shrinkage, which became progressively slower as temperature was reduced.

These experimental results indicate that the resting phase of the lipid coat plays an important role in bubble stability in response to pulsed insonation. Controlling coat phase, and in particular using coats that may show phase changes near body temperature (as has been done with liposomes \(^{21}\)), is thus an attractive design strategy for responsive bubble-based vehicles for ultrasound-mediated drug delivery.

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**Notes**
The authors declare no competing financial interest.

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■ ABBREVIATIONS

PFB, perfluorobutane; DSPC, distearoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; PBS, phosphate buffered saline; PEG, poly(ethylene glycol); PVC, poly(vinyl chloride); EP, Epstein–Plesset.

■ REFERENCES