Multi-time scale characterization of acoustic droplet vaporization and payload release of phase-shift emulsions using high-speed microscopy

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ABSTRACT

Acoustic droplet vaporization (ADV) is the phase-transitioning of perfluorocarbon emulsions, termed phase-shift emulsions, into bubbles using focused ultrasound. ADV has been utilized in many biomedical applications. For localized drug release, phase-shift emulsions with a bioactive payload can be incorporated within a hydrogel to yield an acoustically-responsive scaffold (ARS). The dynamics of ADV and associated drug release within hydrogels are not well understood. Additionally, emulsions used in ARSs often contain high molecular weight perfluoroalkyls, which is unique relative to other ADV applications. In this study, we used ultra-high-speed brightfield and fluorescence microscopy, at frame rates up to 30 million and 0.5 million frames per second, respectively, to elucidate ADV dynamics and payload release kinetics in fibrin-based ARSs containing phase-shift emulsions with three different perfluorocarbons: perfluoropentane (PFP), perfluorohexane (PFH), and perfluorooctane (PFO). At an ultrasound excitation frequency of 2.5 MHz, the maximum expansion ratio, defined as the maximum bubble diameter during ADV normalized by the initial emulsion diameter, was 4.3 ± 0.8, 4.1 ± 0.6, and 3.6 ± 0.4, for PFP, PFH, and PFO emulsions, respectively. ADV yielded stable bubble formation in PFP and PFO emulsions, though the bubble growth rate post-ADV was three orders of magnitudes slower in the latter emulsion. Comparatively, ADV generated bubbles in PFH emulsions underwent repeated vaporization/recondensation or fragmentation. Different ADV-generated bubble dynamics resulted in distinct release kinetics in phase-shift emulsions carrying fluorescently-labeled payloads. The results provide physical insight enabling the modulation of bubble dynamics with ADV and hence release kinetics, which can be used for both diagnostic and therapeutic applications of ultrasound.

1. Introduction

Phase-shift emulsions, composed of perfluorocarbon (PFC) liquids, are versatile particles used in diagnostic and therapeutic applications of ultrasound. The volatility (i.e., high vapor pressure) of PFC liquids provides a thermodynamic driving force for the liquid to phase-transition into a gas. Ultrasound can trigger a phase transition in a PFC liquid, without the generation of heat, in a process termed acoustic droplet vaporization (ADV) [1]. Specifically, the rarefactional component of the ultrasound wave reduces the local pressure below the saturation vapor pressure of the PFC liquid, thereby making vaporization thermodynamically favorable [2,3]. PFC emulsions vaporize into bubbles above a threshold value of the rarefractional pressure, namely, the ADV threshold which depends significantly on physical properties of the phase-shift emulsion (e.g., molecular weight of the PFC species) as well as acoustic parameters (e.g., frequency) [4,5].

Since ADV enables on-demand production of bubbles, both non-invasively and locally, it has been widely studied in ultrasound-based diagnostic and therapeutic applications. For example, ADV-triggered payload release can help overcome limitations within the field of drug delivery. Payload release in conventional hydrogels, which are typically used for local drug delivery, is dominated by passive mechanisms such as diffusion or matrix degradation [6]. Therefore, these approaches inherently lack on-demand modulation of release, both spatially as well

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as temporally. We have developed a smart hydrogel platform, termed an acoustically-responsive scaffold (ARS), that enables precise spatiotemporal control of release, and thereby patient specific therapy. ARSs are composed of fibrin and a payload-carrying, phase-shift emulsion [7]. Since PFC liquids are hydrophobic and lipophic, therapeutic agents cannot be dissolved directly in the PFC phase. For payload-carrying PFC emulsions, we have developed phase-shift double emulsions (PSDEs) with the structure of water-(W) in-PFC-in-water (W2) [8]. The deliverable payload is contained within the innermost water phase (i.e., W1). ADV disrupts the morphology of the PSDE, resulting in release of the payload.

Prior studies have frequently explored submicron and micron-size PFC emulsions with bulk boiling points below physiological temperature such as perfluorobutane and perfluoropentane (PF2C3F8) [9]. However, PSDEs in ARSs are typically formulated with higher bulk boiling point PFC liquids, like perfluorohexane (PFH, C6F14) and perfluorooctane (PFO, C8F18). These higher molecular weight PFCs are more thermally stable, thus eliminating the potential for spontaneous bubble formation that can occur in implanted ARSs over the course of days or weeks [10]. Although we have utilized higher bulk boiling point PFCs in our prior in vitro and in vivo studies to control release of single payloads [7], sequential release of two payloads [11,10], as well as micro-patterned reservoirs of multiple payloads [12] in ARSs, little is known about their vaporization dynamics and release kinetics.

Insight into the physics of vaporization will help formulation and selection of PFC emulsions for specific applications. The complex dynamics of ADV have been studied theoretically and experimentally using high-speed microscopy for PFP emulsions dispersed in water [13,14,15]. Based on earlier high-speed studies, ADV was initiated by homogeneous nucleation within the emulsion along the axis of ultrasound propagation [16]. Stability of the generated PFP bubbles post-ADV have also been studied optically [17,18]. Due to their low bulk boiling point, micron-size PFP emulsions are superheated (i.e., above their bulk boiling point) under physiological conditions. Therefore, once triggered, the ADV process is irreversible, thus leading to stable bubble formation. The stability of a bubble after ADV, which is dependent on the bulk boiling point of the PFC and the size of the generated bubble, significantly impacts payload release kinetics from ARSs [2]. For example, slower rates of payload release were observed in ARSs where ADV generated stable bubbles versus transient bubbles [19]. These differences in payload release kinetics ultimately elicited different biological responses. For example, blood vessel formation was dependent on the kinetics of angiogenic growth factor release from an ARS [20,21]. In addition to drug release, ADV and resulting bubble stability can also alter the hydrogel component of the ARS [22], which can impact cell migration [23] and lead to changes in cell phenotype [24]. Thus, there is clear motivation to better understand the ADV mechanism within ARSs.

In this study, we used ultra-high-speed brightfield microscopy to investigate ADV dynamics in ARSs containing three different formulations of PFC emulsions (i.e., PF2, PFH, and PFO) in microsecond and millisecond timescales. Stability and growth dynamics of the ADV-generated bubbles were compared. The effects of fibrin concentration and pulse duration on the maximum radial expansion of the ADV-generated bubbles were quantified. In addition, fluorescent markers were used to label the PFC phase and payload in phase-shift emulsions to visualize their response to ultrasound via ultra-high-speed fluorescence microscopy. Release distance and velocity, during and post-ADV, were characterized in microsecond and millisecond timescales. To our knowledge, this is the first time that ultra-fast ADV dynamics of higher molecular weight PSDEs as well as ADV-triggered release kinetics have been investigated. The results provide physical insight enabling the modulation of bubble dynamics with ADV and hence release kinetics, which can be used for theranostic applications of ultrasound.

### 2. Material and methods

#### 2.1. Microfluidic production of PSDEs

Monodisperse PSDEs with a W1-in-PFC-in-W2 morphology were produced using a microfluidic chip (Cat# 3200146, junction: 14 µm × 17 µm, Dolomite, Royston, United Kingdom), as described previously [2]. PFP (CAS# 355-42-0, Strem Chemicals, Newburyport, MA, USA), PFH (CAS# 355-42-0, Strem Chemicals) or PFO (CAS# 307–34-6, Sigma-Aldrich, St. Louis, MO, USA) were used as the PFC phase. In this work, each PSDE is designated by its respective PFC (e.g., PFP-PSDE). The monodisperse PSDEs were fabricated in two steps: the formation of a primary emulsion (i.e., W1/PFC in a 1: 2 volumetric ratio) via sonication in aniced water bath, and its encapsulation by the W2 phase via the microfluidic chip. The W1 phase consisted of fluorescent markers in phosphate buffered saline (PBS, Life Technologies), stabilized by a fluorinated surfactant. Alexa Fluor 647-labeled dextran (AF647, 10 kDa, Life Technologies, Grand Island, NY, USA) was used as a fluorescent marker to visualize payload release dynamics. The PFC phase was fluorescently labeled with a fluoruous cyanine dye (F80,Cy5), which was generously provided by Dr. Ellen Sletten [25]. The W2 phase was 50 mg/mL Pluronic F68 (CAS# 9003–11-6, Sigma-Aldrich) in PBS.

For comparing ADV dynamics, single emulsions of PFH were prepared similarly. The emulsions were characterized using a Coulter Counter (Multisizer 4, Beckman Coulter, Brea, CA, USA) with a 30 µm aperture tube. Sizing characteristics of the prepared PSDEs are summarized in Table 1. The thermophysical properties of the three different PFC liquids used in our study are summarized in Table 2 [2,26].

#### 2.2. Preparation of ARSs

ARSs were prepared according to the previously published methods [2,27]. Briefly, fibrinogen solution was prepared by reconstituting bovine fibrinogen (Sigma-Aldrich) in FluoroBrite Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) at 40 mg/mL clottable protein. Fibrinogen solution was gently vortex-mixed for 30 s and then degassed in a vacuum chamber (at ≈ 6 kPa for 60 min, Isotemp vacuum oven, Model 282A, Fisher Scientific, Dubuque, IA, USA) at room temperature to minimize dissolved gases. ARSs (height: 1.3 ± 0.3 mm) were polymerized in a custom-made PVC cylinder (inner diameter: 33 mm, height: 3 mm) with an acoustically and optically transparent bottom membrane (Tegaderm, 3 M Healthcare, St. Paul, MN, USA) with an attenuation coefficient of 0.12 ± 0.07 dB/cm/MHz [27]. The final concentrations of fibrin, bovine lung aprotinin (Sigma-Aldrich), PSDE, and recombinant human thrombin (Recomthrom, Baxter, Deerfield, IL, USA) were 10 mg/mL, 0.05 U/mL, 0.01% v/v, and 2 U/mL, respectively. To study the effect of fibrinogen concentration on the ADV dynamics of PFP-PSDEs, ARSs were made with final fibrinogen concentrations of 5 mg/mL to 20 mg/mL.

#### Table 1

Size characterization of perfluorocarbon (PFC) phase-shift emulsions used in ultra-high-speed brightfield and fluorescence microscopies. Dynamics of acoustic droplet vaporization (ADV), payload release, and PFC phase were compared for perfluoropentane (PFP), perfluorohexane (PFH), and perfluorooctane (PFO) phase-shift emulsions as shown schematically in Fig. 1A. The resulting concentrations were (0.1–0.4) × 109 particles/mL.

| PFC liquid | ADV dynamics | Payload release dynamics | PFC dynamics |
|------------|--------------|--------------------------|--------------|
|            | Average diameter (µm) | Average diameter (µm) | Average diameter (µm) |
| PFP        | 11.6 ± 0.1    | 15.9 ± 0.5               | 15.3 ± 0.7   |
| PFH        | 10.2 ± 0.1    | 16.3 ± 0.4               | 15.0 ± 0.9   |
| PFO        | 12.7 ± 0.09   | 15.2 ± 0.6               | --           |
The PVC cylinder containing the ARS, which was mounted on a 3-axis translation stage, was placed in a water tank (30 cm × 15 cm × 13 cm) filled with degassed, deionized water (≈ 37 °C). An upright microscope (Eclipse, Nikon LV100ND, Melville, NY, USA) was customized by removing the base of the main body to accommodate the water tank as shown in Fig. 1B. The upright microscope, which was equipped with water-immersion objectives at 100x (NA: 1.1, WD: 2 mm, Plan, Nikon) or 40x (NA: 0.8, WD: 2 mm, Fluor, Nikon), was paired with an ultra-high-speed framing camera (SM802 model, Specialised Imaging Ltd, Pitstone, UK) providing a frame rate up to 200 million frames per second (Mfps). The camera had eight discrete CCD channels and was able to record sixteen 12-bit images (i.e., each image was 1,280 × 960 pixels) per trigger. For brightfield microscopy, transmission illumination was provided by a high intensity flash lamp (750 J, total illumination duration: 750 μs in a continuous mode) (Fig. 1B) through a liquid light guide (core diameter: 3 mm, Newport, Irvine, CA, USA) aligned with the objective. A high intensity pulsed laser (400 W, 640 nm, total illumination time: 30 μs, Cavitar Ltd, Tampere, Finland) was used in reflectance mode for ultra-high-speed fluorescence microscopy. The laser was focused through the imaging objective, using the same liquid light guide, through the back port of the upright microscope. An appropriate fluorescence filter kit (Cy5 filter cube, Nikon) was used for high-speed fluorescence microscopy to detect fluorescence from PSDEs. Images were acquired at frame rates up to 30 Mfps and 0.5 Mfps for brightfield and fluorescence microscopies, respectively. Based on the speed of sound in degassed water at 37 °C and the focal length of the transducers, the camera recording was delayed appropriately to start a few microseconds before ultrasound arrival. In all images, 0 μs or 0 ms denotes the reference frame prior to the arrival of ultrasound. Due to the limited numbers of frames (i.e., 16), dynamics in microsecond and millisecond timescales were done in separate experiments.

Calibrated, focused single-element transducers (H-108 (##: 0.83), H-101 (##: 1), Sonic Concepts Inc., Bothell, WA, USA) induced ADV within the ARSs at different excitation frequencies. Each transducer was mounted on a 3-axis micro-positioner to enable precise alignment of acoustic and optical foci. The transducers were driven by a single, sine-wave ultrasound burst generated by a function generator (33500B, Agilent Technologies, Santa Clara, CA, USA), and amplified by a gated radiofrequency amplifier (GA-2500A Ritec Inc., Warwick, RI, USA). For ADV dynamic studies, three single bursts of 2 μs, 6 μs, and 12 μs at three excitation frequencies of 1 MHz, 2.5 MHz, and 8.6 MHz were investigated in separate studies. All studies on payload release dynamics were conducted at 2.5 MHz, and a single burst of 6 μs. Signals were monitored in real-time on an oscilloscope (HDO4034, Teledyne LeCroy, Chestnut Ridge, NY, USA). The transducers were calibrated in free field at the focus using an in-house fiber optic hydrophone (sensitivity: 16.6 mV/MPa) with a fiber diameter of 105 μm [28]. Experiments were conducted at three peak rarefractional pressures: 5 MPa (at 1 MHz), 6.5 MPa (at 2.5 MHz), and 7 MPa (at 8.6 MHz). These pressures, which are close to the highest output of the transducers, were suprathreshold for ADV in ARSs containing PFP, PFH, and PFO phase-shift emulsions [11,19]. Unless otherwise noted, all acoustic pressures are listed as peak rarefractional pressures. In our setup, all triggers were synchronized with the camera. The relatively longer working distance of the objectives as well as tightly focused transducers, placed at an angle with respect to the samples, minimized the formation of a complex standing wave field [11].

### Table 2
Thermophysical properties of perfluorocarbon (PFC) liquids (at 37 °C) used in ultra-high-speed microscopy taken from [2,26]. Phase-shift emulsions with three different PFC liquids were studied: perfluoropentane (PFP), perfluoroctane (PFO), and perfluorohexane (PFH).

| PFC liquid | Surface tension (mN m⁻¹) | Vapor pressure (kPa) | Bulk boiling point (°C) | Mass density (kg m⁻³) | Molecular mass (kg mol⁻¹) |
|------------|--------------------------|----------------------|------------------------|-----------------------|--------------------------|
| PFP        | 9.5                      | 135                  | 29                     | 1590                  | 0.288                    |
| PFH        | 11.2                     | 46                   | 56                     | 1690                  | 0.338                    |
| PFO        | 12.8                     | 0.8                  | 100                    | 1770                  | 0.438                    |

2.3. Ultra-high-speed microscopy setup

The PVC cylinder containing the ARS, which was mounted on a 3-axis translation stage, was placed in a water tank (30 cm × 15 cm × 13 cm) filled with degassed, deionized water (≈ 37 °C). An upright microscope (Eclipse, Nikon LV100ND, Melville, NY, USA) was customized by removing the base of the main body to accommodate the water tank as shown in Fig. 1B. The upright microscope, which was equipped with water-immersion objectives at 100x (NA: 1.1, WD: 2 mm, Plan, Nikon) or 40x (NA: 0.8, WD: 2 mm, Fluor, Nikon), was paired with an ultra-high-speed framing camera (SM802 model, Specialised Imaging Ltd, Pitstone, UK) providing a frame rate up to 200 million frames per second (Mfps). The camera had eight discrete CCD channels and was able to record sixteen 12-bit images (i.e., each image was 1,280 × 960 pixels) per trigger. For brightfield microscopy, transmission illumination was provided by a high intensity flash lamp (750 J, total illumination duration: 750 μs in a continuous mode) (Fig. 1B) through a liquid light guide (core diameter: 3 mm, Newport, Irvine, CA, USA) aligned with the objective. A high intensity pulsed laser (400 W, 640 nm, total illumination time: 30 μs, Cavitar Ltd, Tampere, Finland) was used in reflectance mode for ultra-high-speed fluorescence microscopy. The laser was focused through the imaging objective, using the same liquid light guide, through the back port of the upright microscope. An appropriate fluorescence filter kit (Cy5 filter cube, Nikon) was used for high-speed fluorescence microscopy to detect fluorescence from PSDEs. Images were acquired at frame rates up to 30 Mfps and 0.5 Mfps for brightfield and fluorescence microscopies, respectively. Based on the speed of sound in degassed water at 37 °C and the focal length of the transducers, the camera recording was delayed appropriately to start a few microseconds before ultrasound arrival. In all images, 0 μs or 0 ms denotes the reference frame prior to the arrival of ultrasound. Due to the limited numbers of frames (i.e., 16), dynamics in microsecond and millisecond timescales were done in separate experiments.

Images were processed offline using ImageJ (National Institutes of Health, Bethesda, MD, USA) and MATLAB (The MathWorks, Natick, MA, USA). Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard deviation. The number of independent replicates is given in the caption for each figure. Significant differences between groups were determined using one-way ANOVA followed by Tukey’s multiple comparisons test. A significance level of 0.05 was used.

3. Results

3.1. ADV dynamics

Ultra-high-speed brightfield images of a PFP-PSDE in ARSs of...

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**Fig. 1.** Ultra-high-speed microscopy was used to capture the dynamics of acoustic droplet vaporization (ADV), payload release, as well as the perfluorocarbon (PFC) phase in phase-shift double emulsions (PSDEs) at varying acoustic parameters (A). PSDEs with three different PFC liquid cores were studied: perfluoropentane, perfluoroctane, and perfluorohexane. A schematic representation of the experimental setup is shown in B. Ultra-high-speed brightfield microscopy was performed in a back illumination configuration using a high intensity flash lamp at frame rates up to 30 million frames per second. Ultra-high-speed fluorescence microscopy was performed in a front illumination configuration using a high intensity pulsed laser at frame rates up to 0.5 million frames per second. Scale bar: 15 μm.
varying fibrin concentrations exposed to a single burst of 6 µs at 2.5 MHz (6.5 MPa) are presented in Fig. 2A-C. Based on the change in the refractive index of a liquid emulsion and a gas bubble, the images indicate that phase change was completed within microseconds following the arrival of ultrasound. During ADV, both monotonic expansion, with a typical velocities around 1 ms\(^{-1}\) to 2.5 ms\(^{-1}\), and sinusoidal perturbations due to successive ultrasound cycles were seen for a PFP-PSDE. The ADV-generated PFP bubble continued to grow after ultrasound was turned off (frames with timestamp of 46.0 µs and greater). The maximum expansion ratio, defined as the maximum bubble diameter during ADV normalized by the corresponding initial PSDE diameter, was 3.9 ± 0.6, 4.0 ± 0.7, and 3.1 ± 0.4 in ARSs with fibrin concentrations of 5 mg/mL, 10 mg/mL, and 20 mg/mL, respectively (Fig. 2D, Videos S1, S2, and S3). The diameter of the resulting ADV-generated bubbles 30 s post-ADV correlated inversely with fibrin concentration (Fig. 2E). At the ultrasound pulse durations interrogated in ARSs with 10 mg/mL fibrin, the maximum diameter of the generated PFP bubbles during and after ADV was not significantly different (Fig. 2F & G).

High-speed fluorescence images of a PFP-PSDE, which contained fluorescently-labeled PFP liquid, showed that prior to ADV, the fluorescence signal was homogeneously localized within the PFP emulsion (Fig. 3). However, following ADV, the fluorescence signal diminished within the vaporized bubble core, due to the drastic change in density between the liquid and gas phases, and remained at the interface between the bubble and the ARS after ultrasound was turned off.

PFH-PSDE, with a higher bulk boiling point (see Table 2), underwent ADV at similar acoustic parameters (Fig. 4A, Video S4). However, once the ultrasound pulse was turned off, a significantly smaller bubble persisted (= 5 µm at 466 µs).

To further study the fate of the induced bubble in a PFH-PSDE milliseconds post-ADV, the camera was trigger-delayed to the maximum time allowed (i.e., 200 ms). With an illumination window of 750 µs, we recorded the growth dynamics of the generated bubble post ADV. Fig. 4B shows that the generated bubble in PFH-PSDE persisted 200 ms post-ADV and grew on a millisecond timescale to a large bubble due, in part, to inward diffusion of air from the surrounding ARS (i.e., passive diffusion). Due to the limited illumination window and the time it required to upload and save the images, it was not possible to record growth dynamics from 200.7 ms to 30 s. There were no significant differences in the average diameter of the ADV-induced bubbles in PFH-PSDE at any timepoint during and post-ADV when two different ultrasound pulse durations of 6 µs and 12 µs were applied (Fig. 4C). To further understand the ADV dynamics in PFH emulsions, experiments were repeated on PFH single emulsions with fluorescently-labeled PFH liquid. Brightfield images of PFH single emulsions displayed similar ADV dynamics to PFH-PSDEs in microsecond as well as millisecond timescales (Fig. 5A & B). Similar to Fig. 3, Fig. 5C shows that prior to ADV, the fluorescence signal was localized within the PFH emulsion. However, within microseconds after ultrasound was turned off, the fluorescence signal recovered, suggesting partial or complete recondensation of the PFH phase, leaving a small region without detectable fluorescence signal. The region without fluorescence signal (i.e., the ADV-generated bubble) continued to grow (Fig. 5D) on a millisecond timescale. A dim fluorescent rim was retained at the interface of the fully-grown bubble and the ARS after 30 s.

Fig. 6 demonstrates that a PFO-PSDE underwent repeated vaporization and recondensation after consecutive ultrasound bursts until it was fragmented by the 8th ultrasound burst. A modified ideal gas law [29], including the effect of Laplace pressure, was used to predict the relationship between the expanded bubble and the corresponding initial emulsion for the three different PFC emulsions as follows:

\[
\tau = \sqrt{\frac{M_r^2 (P + \frac{\gamma}{\rho})}{\rho R T}}
\]  

(Fig. 6)
where $r_i$ is the radius of the initial PSDE, $r_g$ is the radius of the ADV-induced bubble, $M$ is the molar mass, $P$ is the ambient pressure, $R$ is the ideal gas constant, $T$ is the ambient temperature, $\rho_l$ is the liquid density (see Table 2), and $\sigma$ is the interfacial tension between the PSDE and the surrounding medium (Pluronic copolymers: $42 \text{ mN m}^{-1}$ [30]). The modified ideal gas law predicts and the experimental data confirmed that PFO-PSDEs underwent a lower expansion during ADV compared to PFP- and PFH-PSDEs at similar acoustic parameters (Fig. 7). The ultimate fate of the ADV-generated bubbles from three different PSDEs at varying acoustic parameters are summarized in Fig. 8.
Depending on the PFC liquid core and the applied acoustic parameters, four distinct ADV-generated responses were observed. ADV resulted in stable bubble formation in PFP-PSDE (without recondensation) at all acoustic parameters studied here. Stable bubble formation with recondensation was observed for a PFH-PSDE except at 8.6 MHz and 2 μs pulse duration. ADV resulted in transient bubble formation and recondensation in PFO-PSDE (see Video S5) except for the highest frequency studied here. No ADV was observed at 8.6 MHz and any pulse duration for PFO.

As shown in Fig. 8, ADV was highly stochastic at the lowest frequency studied here, which highlights the important interplay between the size of the emulsion and the wavelength of the incident wave.

### 3.2. Payload release dynamics

ADV-triggered payload release was visualized in microsecond as well as millisecond timescales in ARSs containing three different PSDEs. The homogeneous distribution of the payload in a PFP-PSDE was disrupted following a single ultrasound burst (2.5 MHz, 6.5 MPa, and 6 μs), resulting in a radial distribution of the released payload around the newly-generated bubble (Fig. 9A). Both homogeneous and heterogeneous fluorescence patterns were observed in the distribution of the released payload around the generated bubbles. The ADV-induced payload release, in the form of cloud release, remained at the bubble-ARS interface and transported further as the bubble grew in size (Fig. 9B, Video S6). The measured release distance, defined as the distance of the front of the released payload with respect to the initial position of the corresponding emulsion wall, reached 7.0 ± 3.5 μm during the 6 μs ultrasound burst.

The transport of released payload continued after ultrasound was turned off, due to passive diffusion, reaching 39.4 ± 2.6 μm at 100 ms which was five times the initial PFP-PSDE size. The release velocity was the highest during ADV (1.4 ± 0.3 ms⁻¹) and slowed down significantly post-ADV (Fig. 9C & D). The fluorescence signal was undetectable at a longer timescale recorded here (i.e., 2 min), possibly due to diffusion of released payloads out of the focal plane (Fig. S1). A significantly different payload release dynamic, illustrated in Fig. 10A, was recorded for a PFH-PSDE. During ADV, the fluorescence signal was localized between the newly formed bubble and the surrounding medium, followed by the inward transport of the payload after the ultrasound pulse was turned off (Video S7). The newly generated bubble, indicated by the region with no fluorescence signal, persisted and grew on millisecond timescales and transported the payload further (Fig. 10B).

For a PFH-PSDE, the maximum release distance and velocity during ADV were 6.0 ± 3.2 μm and 2.2 ± 1.2 ms⁻¹ (Fig. 10C), respectively. Although there was no significant difference in the release velocity during ADV between PFP- and PFH-PSDEs, the release velocity in millisecond timescales was three orders of magnitude slower for PFO.
Fig. 8. The ultimate fate of a bubble generated via acoustic droplet vaporization is graphically represented as a function of pulse duration and frequency for three perfluorocarbon phase-shift double emulsions: perfluoropentane (PFP), perfluorohexane (PFH), and perfluorooctane (PFO).

Fig. 9. Payload release in acoustically-responsive scaffolds containing perfluoropentane phase shift double emulsion was studied using ultra-high-speed fluorescence microscopy. Alexa Fluor 647-labeled dextran was used as a fluorescent marker to visualize payload release dynamics. A single ultrasound burst of 6 µs at 2.5 MHz, and 6.5 MPa induced acoustic droplet vaporization (ADV). ADV-triggered payload release from perfluoropentane phase-shift double emulsions were monitored in microsecond (A) as well as millisecond (B) timescales, in separate experiments. Frames containing ultrasound are denoted with an asterisk. Scale bar: 15 µm. The corresponding release distance and release velocity were quantified in microsecond (n = 22) (C) and millisecond (n = 15) (D) timescales.
Fig. 10. Ultra-high-speed fluorescence microscopy was used to visualize payload release in acoustically-responsive scaffolds containing perfluorohexane phase-shift double emulsions. Alexa Fluor 647-labeled dextran was used, as a fluorescent marker, to visualize payload release dynamics. A single ultrasound burst of 6 µs at 2.5 MHz, and 6.5 MPa induced acoustic droplet vaporization (ADV). ADV-triggered payload release from perfluorohexane phase-shift double emulsion was monitored in microsecond (A) as well as millisecond (B) timescales in two separate studies. Frames containing ultrasound are denoted with an asterisk. Scale bar: 15 µm. The corresponding release distance and release velocity were quantified in microsecond ($n=14$) (C) and millisecond ($n=10$) (D) timescales.

Fig. 11. Ultra-high-speed fluorescence microscopy was used to study payload release triggered by acoustic droplet vaporization (ADV) in acoustically-responsive scaffolds. Alexa Fluor 647-labeled dextran was used as a fluorescent marker to visualize payload release dynamics. A single ultrasound burst of 6 µs at 2.5 MHz, and 6.5 MPa induced ADV. ADV-triggered payload release from perfluorooctane (PFO) phase-shift double emulsions were monitored in microsecond (A) as well as millisecond timescales (B). Frames containing ultrasound are denoted with an asterisk. Scale bar: 15 µm. The corresponding release distance and release velocity were quantified ($n=6$) (B). The intensity of four different PFO-PSDE is plotted after each consecutive ultrasound burst before fragmentation and compared with the control (i.e., without ultrasound (-US)) (C). Repeated pulsed laser exposures without application of ultrasound did not impact the intensity of the fluorescently-labeled payloads (i.e., no photobleaching).
PSDEs compared to PFP-PSDEs (Fig. 10D). In ARSs containing the highest bulk boiling point PFC emulsion (i.e., PFO-PSDE), ADV did not result in a cloud release after a single burst (Fig. 11A). Instead, after each ultrasound burst (i.e., vaporization and recondensation) the fluorescence intensity of the payload decreased (Fig. 11C), suggesting partial payload release. Once the final ultrasound burst fragmented the PFO emulsion, the fluorescence signal was too low to detect, suggesting complete payload release. Control studies showed that thermal effects of laser illumination as well as photobleaching were insignificant with the short pulses used here and with the highly photostable AF647 (Fig. 11C).

4. Discussion

We used ultra-high-speed microscopy to understand the underlying physics of ADV and ADV-triggered payload release at different timescales in ARSs containing three different PSDEs. Prior studies have explored the ADV dynamics of submicron [31] and micron-size [32] PFP emulsions. Earlier high-speed imaging studies resolved the inception of nucleation within the PFP emulsion following ADV [33,16], and other studies investigated the stability of the generated PFP bubbles post-ADV [18 17]. PFP has been very commonly studied for theranostic applications of ultrasound since its bulk boiling point is below physiological temperature. However, higher molecular weight PFCs like PFH or PFO offer much enhanced thermal stability, which is required for ARSs that remain implanted for days to weeks. Therefore, understanding the underlying physics of ADV in higher bulk boiling point PFC emulsions is critical for the development of emulsions for specific applications. To our knowledge, this is the first time ultra-fast ADV dynamics as well as release kinetics of higher bulk boiling point PSDEs have been studied. Additionally, the prior high-speed studies were conducted on PFP emulsions primarily dispersed in water, and not within a hydrogel as studied here.

Our brightfield, high-speed images captured the conversion of the liquid PFC emulsions to PFC vapor within microseconds upon arrival of ultrasound (Figs. 2, 4 & 6). Due to the stochastic nature of vapor nucleation, not all PSDEs nucleated in the first rarefractional half cycle. Transient behavior of the transducer might also affect the nucleation within the initial cycles. A radial expansion ratio of ≈5 was estimated for sufficiently sized PFP emulsions in water [1]. Here, we included the effect of Laplace pressure to predict the expansion ratio for three different PSDEs. Both experimental data and theoretical predictions indicated a lower expansion ratio for a PFO emulsion (Fig. 7). The experimentally measured, average radial expansion ratios were lower than those predicted likely due to the presence of a viscoelastic gel, which was not accounted for in the theoretical predictions, as well as the limited sampling rate. Although stable bubble formation was observed for both PFP- and PFH-PSDEs at the acoustic parameters shown in Fig. 8, the growth dynamics of ADV-generated bubbles were substantially different (Figs. 2 & 4). Note that at the experimental conditions, only PFP-PSDEs were in a superheated state (degree of superheat: 8 °C). Furthermore, the contribution of Laplace pressure was minimal for the generated PFP bubbles here. Therefore, once the PFP bubble was triggered, it continued to grow even after ultrasound was turned off. While no recondensation was observed for PFP bubbles (Fig. 3), a PFP bubble underwent partial or complete recondensation with a smaller bubble remaining stable within the emulsion post-ultrasound. By fluorescently labeling the PFH liquid, recondensation of PFH single emulsions was captured within microseconds after ultrasound was turned off (Fig. 5C). The slower growth of the bubble remaining in PFH emulsion was due to both interfacial tension and the surrounding liquid PFH density [34]. A previous numerical study showed that in the absence of inward gas diffusion during ADV, the generated bubble would not survive the first collapse in response to the compressional wave, thus highlighting the critical role of gas diffusion during ADV [13]. Depending on the extent of inward gas diffusion, the ADV-induced bubble in PFH emulsions may recondense or continue to grow. Therefore, it is likely that the observed trapped bubble in PFH emulsions in our studies was the result of sufficient in-gassing during ADV, enhanced by the higher amplitude and long pulse durations used here (i.e., rectified diffusion). The fact that no stable bubble formation was observed for PFH emulsion at 8.6 MHz and a pulse duration of 2 µs points to insufficient in-gassing at higher frequencies and shorter pulse durations. In studies using a laser to thermally induce vaporization of PFH phase-shift emulsions, bubble formation was reversible (i.e., recondensation without stable bubble formation) at low laser powers and irreversible when a higher power was applied [35]. Stable bubble formation at higher laser powers was associated with the longer time it took for the generated bubble to cool down to ambient temperature and condense back to liquid, allowing more air influx from the surrounding to occur.

Unlike PFP and PFH, stable bubble formation was not observed for PFO-PSDEs at any acoustic parameters studied here due to the significantly higher supersaturation ratio (i.e., pressure inside the ADV-generated bubble to the equilibrium vapor pressure), which is in agreement with our predictions using classical nucleation theory [2]. A lower expansion ratio during ADV, as well as lower solubility and mass diffusivity of oxygen in PFO liquid [36] decrease the survival of an air bubble inside the emulsion. Other parameters studied here were the effects of fibrin concentration and pulse duration on the ADV dynamics of PFP-PSDEs. A 4-fold increase in fibrin density resulted in a 35% decrease in the diameter of ADV-generated bubbles 30 s post-ADV. Although fibrin density significantly affected the generated ADV bubbles in PFP-PSDEs, bubble growth rate did not depend on the pulse durations interrogated here.

Based on both the brightfield and fluorescence images, stable bubble formation yielded complete payload release for PFP- and PFH-PSDEs, although the release dynamics were significantly different. High-speed fluorescence microscopy was performed at frame rates lower than the frequency of ultrasound excitation to permit optical visualization of the selected fluorophores. Therefore, release dynamics on a nanosecond timescale could not be captured. Furthermore, the release of the W1 phase (i.e., the fluorescently-labeled payload) could have been too small to resolve individually or could have expelled outside of the field of view. Consequently, we observed a mist or cloud release at the surface of the newly-generated bubbles. Both homogeneous and heterogeneous distribution patterns of the released payload were recorded for PFP- and PFH-PSDEs. The higher driving pressures used here (i.e., 6.5 MPa) could lead to non-spherical oscillations of the generated bubbles, thus generating different boundary conditions. The brighter regions of fluorescence signal observed in the rim of the generated bubbles following ADV suggest spatial redistribution of the fluorophore (Figs. 9 & 10). It is worth noting that the observed phenomena were limited by the optical, focal plane. For a complete 3D observation of the ADV-triggered release dynamics, a side view camera would be necessary.

To evaluate the length scale that the released payload was transported, the maximum distance from the edge of the fluorescently labeled payload was determined in each frame. Both release distance and velocity were significantly different post-ADV for PFP- and PFH-PSDEs. Similar release velocities on the order of 0.2 mms⁻¹ to 1 mms⁻¹ have been reported for the release of drug-loaded liposomes from microbubbles in water at driving pressures of 40 kPa to 100 kPa and pulse lengths of 10–1,000 cycles [37]. At the highest pressure and longest pulse duration, the released liposomes propagated over a distance of twenty times the initial bubble radius. This suggests that displacement of the released payload in ARSs can also be modulated for specific applications by applying higher pressures and longer pulses.

Recondensation and fragmentation were observed in our high-speed studies as two main release dynamics mechanisms in ARSs containing PFO-PSDEs. Transient bubble formation via ADV resulted in partial release of the payload in these ARSs through repeated vaporization and recondensation evident by the gradual decrease in the fluorescence intensity of the emulsion after each ultrasound burst (Fig. 11C). Complete release of the payload was achieved once the emulsion was fragmented,
which was dependent on the number of applied ultrasound bursts. This is consistent with our previous in vitro release studies where we showed that ARSs containing PFO-PSDEs required multiple rasters of ultrasound to reach similar amounts of release generated by a single raster of ultrasound in ARSs with the same volume fraction of PFH-PSDEs [19].

In microbubble-assisted drug delivery, shell damage, either through acoustic dissolution during compression or static dissolution, has been reported as a key mechanism for payload release. Although in our studies the shell was not fluorescently labeled, the volumetric expansion of the PFC phase during ADV (Fig. 7) and successive oscillations could have considerably decreased the surface density of surfactant molecules for those with fluid shells (such as lipids) or cause irreversible shell fragmentation for those with stiffer shells (such as polymers).

ADV-triggered stable or transient bubble formation enables a unique platform to optimize release kinetics of therapeutics to achieve a desired response. Release kinetics of growth factors have been shown to play a key role in regulating cellular responses. For example, sustained release of basic fibroblast growth factor (bFGF) was up to 3-fold more effective than bolus release in increasing vascular endothelial and smooth muscle cell proliferation, while the reverse was true for transforming growth factor-β1 (TGF-β1) [38]. A bolus of TGF-β1 inhibited vascular cells up to 3.8-fold more efficiently than the same amount of TGF-β1 if control-released. The findings here can be useful in applications beyond drug delivery. For example, repeated vaporization and recondensation of phase-shift emulsions can be leveraged for an on-and-off blinking contrast signal between consecutive ultrasound pulses, offering better localization of bubble signal from tissue [39]. The ADV-induced stable or transient bubble formation can also be utilized to tune micro-mechanical properties of strain stiffening biomaterials in an on-demand, non-invasive manner [22, 24]. Future studies will focus on how the distinct release kinetics observed here can impact cellular responses. Additionally, although laser-induced bubble dynamics in viscoelastic biomaterials have been modeled using cavitation bubble dynamics [40], similar studies should be performed using ADV-bubble dynamics.

5. Conclusions

The combination of ultra-high-speed brightfield and fluorescence microscopy provided insight into ADV-dynamics and payload release kinetics for three different PSDEs in ARSs. Tuning the acoustic parameters and PFC liquid properties enabled distinct ADV bubble dynamics. Although stable bubble formation was observed in ARSs with PFP- and PFH-PSDEs, the growth rates post-ADV were substantially different. Once a vapor nucleus was initiated in a PFP-PSDE, the bubble continued to grow. For a PFH-PSDE, fluorescence microscopy revealed partial/complete recondensation of the PFH phase. However, stable bubble formation was likely associated with the influx of air during ADV. Transient bubble formation followed by recondensation was observed for PFH-PSDE, the highest bulk boiling point PFC liquid emulsion studied here. The distinct ADV bubble dynamics resulted in distinct release kinetics. Repeated vaporization and recondensation was associated with partial release while stable bubble formation yielded complete release. The ability to generate stable and transient bubble formation, in an on-demand and spatiotemporally-controlled manner, could be desirable for both diagnostic and therapeutic applications of ultrasound.

CRediT authorship contribution statement

Mitra Aliabouzar: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Oliver D. Kripfgans: Conceptualization, Resources. Jonathan B. Estrada: Resources. J. Brian Fowlkes: Conceptualization. Mario L. Fabiilli: Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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