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TITLE:

Synthesis and Characterization of Multi-Modal Phase-Change Porphyrin Droplets

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SUMMARY:

In this protocol, methods for synthesizing and characterizing multi-modal phase-change porphyrin droplets are outlined.

ABSTRACT:

Phase-change droplets are a class of ultrasound contrast agents that can convert into echogenic microbubbles *in situ* with the application of sufficient acoustic energy. Droplets are smaller and more stable than their microbubble counterparts. However, traditional ultrasound contrast agents are not trackable beyond acoustic feedback measurements, which makes quantifying contrast agent bio-distribution or accumulation *ex vivo* difficult. Researchers may have to rely on fluorescent or optically absorbent companion diagnostic particles to infer bio-distribution. The purpose of this protocol is to detail steps for creating multi-modal phase-change porphyrin droplets using a condensation method. Porphyrins are fluorescent molecules with distinct absorbance bands that can be conjugated onto lipids and incorporated into droplets to extend droplet versatility, enabling more robust bio-distribution while retaining acoustic properties. Seven formulations with varying porphyrin-lipid and base lipid contents were made to investigate microbubble and droplet size distributions. Characterizations suited to porphyrin-containing

structures are also described in the protocol to demonstrate their analytic versatility in-solution. Sizing showed that the post-condensed mean diameters were 1.72 to 2.38 times smaller than precursor populations. Absorbance characterization showed intact assemblies had a Q-band peak of 700 nm while disrupted samples had an absorbance peak at 671 nm. Fluorescence characterization showed intact 30% porphyrin-lipid assemblies were fluorescently quenched (>97%), with fluorescence recovery achieved upon disruption. Acoustic vaporization showed that porphyrin droplets were non-echogenic at lower pressures and could be converted into echogenic microbubbles with sufficient pressures. These characterizations show the potential for porphyrin droplets to eliminate the need for absorbance or fluorescence-based companion diagnostic strategies to quantify ultrasound contrast agent bio-distribution for delivery or therapeutic applications *in vivo* or *ex vivo*.

INTRODUCTION:

Ultrasound imaging is a non-invasive, non-ionizing form of medical imaging that utilizes acoustic waves. While ultrasound scanners are more portable and can provide real-time images, ultrasound imaging can suffer from low contrast, making it difficult for sonographers to reliably distinguish similarly echogenic pathological features. To counteract this limitation, microbubbles can be injected into the host to improve vascular contrast. Microbubbles are micron-sized gas filled contrast agents that are highly echogenic to acoustic waves and can provide enhanced vessel contrast[1,2]. The shells and gas cores of microbubbles can be tailored for different applications, such as imaging, thrombolysis, cell membrane permeabilization, or transient vascular opening[2].

A drawback of microbubbles is their short circulation half-lives. For example, clinically available perflutren lipid microspheres only have a half-life of 1.3 minutes[3]. For long imaging sessions, multiple injections of microbubbles are needed. Another drawback of microbubbles is their large diameters. While perflutren lipid microspheres are around 1 to 3 µm in diameter, small enough to circulate in vasculature, they are too large to extravasate and passively accumulate into tissues of interest, such as tumors[4]. One strategy to overcome these limitations is to condense the gas-core microbubbles into smaller, liquid-core droplets[5,6]. While droplets are not echogenic in their liquid state, they can be vaporized into microbubbles upon exposure to ultrasound with sufficiently high peak negative pressure, regaining their ability to provide contrast. This allows for the droplet to take advantage of the more favorable pharmacokinetics of a small liquid-core, while retaining the ability to provide contrast when insonated and without changing the chemical composition[4,7].

Decafluorobutane is an ideal perfluorocarbon compound for phase-shifting between gaseous and liquid states[5,6,7]. Decafluorobutane allows for condensation of microbubbles into droplets with temperature reduction alone, whereas less dense perfluorocarbons require additional pressurization[5]. This gentle method minimizes destruction of bubbles during condensation[7,8,9]. As their cores are liquid, droplets are non-echogenic and invisible to ultrasound. However, with the application of sufficient acoustic or thermal energy, the liquid cores can vaporize back into a gaseous state, generating echogenic microbubbles[8]. This vaporization allows for control of when and where to generate microbubbles.

While droplets are useful for passive accumulation, *in situ* vaporization, or improving cell permeability[4], droplets (and their fragments) cannot be imaged or quantified *ex vivo*. Therefore, quantifiable companion diagnostic agent, such as fluorescent[4,10,11], magnetic particles[12], optically absorbent agents[13], are utilized as an analogue to gauge droplet delivery to tissues of interest. For example, Helfield et al. used a co-injection of fluorescent nano-beads for histology image quantification of mouse organs as droplets could not be detected fluorescently[4]. The disadvantage of companion diagnostic agents is the trackable component may act independently from the droplet depending on its individual pharmacokinetic profile.

Fortunately, the shell of microbubbles and droplets can be customized. For example, Huynh et al. demonstrated ultrasound contrast agents with porphyrin-lipid shells, creating multi-modal microbubbles[14]. Porphyrins are a class of organic compounds with an aromatic macrocylic structure[14,15]. They are optically absorbent, fluorescent, and can be chelated to a wide variety of metals for radiotherapy, radionuclide-based imaging, or trace metal-based quantification[14]. One example of porphyrin is pyropheophorbide (Pyro). By conjugating Pyro onto lipids, incorporating Pyro-lipids in microbubbles or droplets allow them to be imaged and tracked through multiple modalities: acoustically, fluorescently, and through absorbance[14]. This multi-modal contrast agent could be used to track and quantify accumulation. This could eliminate the need for companion diagnostic agents as the quantifiable component is now conjugated onto the shell, enabling more accurate delivery quantification[16].

Herein, a protocol for creating multi-modal phase-change porphyrin droplets is outlined. As ultrasound contrasts agents can be used as a platform for drug delivery to tissues of interest, such as tumors[2,4], extending their detectability beyond ultrasound could prove useful for delivery efficacy quantification. The purpose of these droplets is to provide trackable ultrasound contrast agents capable of passive accumulation *in vivo*, *in situ* vaporization and acoustics, and with the potential to quantify bio-distribution or accumulation from *ex vivo* organs without the reliance on secondary sensors. Characterization methods are also outlined to showcase porphyrin droplets' potential as bio-distribution sensors. The effects of Pyro-lipid loading in the shell (0% to 50% by molar ratio) are also discussed.

PROTOCOL:

1. Dehydrated lipid films

1.1. Calculate the masses of each of the shell components needed (see Supplementary Information "Lipid Formula Sheet").

NOTE: For this protocol, the shell composition will be: 10 molar % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] ammonium salt (DSPE-PEG5K), *x* molar % pyropheophorbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (Pyro-SPC), and (90 - *x*) molar % 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). The amount of Pyro-SPC will be varied across 7 shell compositions (*x* = 0, 1, 10, 20, 30, 40, 50). Check the

molecular weight of DSPE-PEG5K on the stock bottle.

1.1.1. Scale the protocol to any lipid volume with a minimum volume of 1 mL. For this protocol, a total lipid volume of 10 mL with a total lipid concentration of 1 mg per mL was used for all formulations. The excipient solution will be: 10% propylene glycol, 10% glycerol, and 80% phosphate buffer saline (PBS, 1X, 7.4 pH) (% v/v/v) (see Step 2.3 and "Lipid Formula Sheet").

NOTE: Synthesis protocol of Pyro-lipids is outlined in the Supplementary Information "Other Protocols and Data" Steps S1 to S1.19, which was modified from the work done by Zheng et al.[15].

1.2. Based on the calculated masses (see Step 1.1 and "Lipid Formula Sheet"), weigh out each of the non-Pyro-lipids and transfer to a sufficiently sized borosilicate glass vial with a screw-on cap.

1.3. Cap the vial, label the cap and vial, and cover the bottom and walls of the lipid vial with aluminum foil. This vial will be referred to as the "Lipid Vial" for the rest of the protocol. Store the Lipid Vial in a cool, dry, dark area.

1.4. If the formulation contains Pyro-SPC, dissolve 10 mg of Pyro-SPC dry film (see "Other Protocols and Data") into 1 mL of chloroform. Vortex for 5 s, measure the absorbance, and calculate the appropriate volume to add to the Lipid Vial.

CAUTION: Chloroform is a health hazard, irritant, and toxic. Wear a protective lab coat, eye protection, gloves, and avoid breathing fumes.

NOTE: As Pyro-SPC is light sensitive, reduce the lights in the working area if possible when handling Pyro-SPC. Keep Pyro-SPC sealed and covered when not in use.

1.4.1. On the ultraviolet-visible spectrophotometer, set it to measure absorbance from a wavelength range of 800 nm to 300 nm with 0.5 nm increments, and measure a baseline with 2000 µL of pure methanol in a compatible 1 cm path length cuvette.

CAUTION: Methanol is a health hazard, irritant, toxic, and flammable. Wear a protective lab coat, eye protection, gloves, and avoid breathing fumes. Keep away from sparks and heat.

1.4.2. Add 2 µL of the Pyro-SPC in chloroform into 2000 µL of methanol, and vortex for 30 s. Transfer it into a clean, compatible 1 cm cuvette, and measure the absorbance. Adjust this dilution factor if the absorbance peak at 667 nm out of the ultraviolet-visible spectrophotometer's absorbance range.

NOTE: Whenever transferring chloroform or methanol, use a clean glass syringe or a positive-displacement pipette rather than a mechanical pipette for better accuracy.

1.4.3. Repeat Step 1.4.2 two more times to get triplicate absorbance values.

1.4.4. Average the absorbance peak values at 667 nm and use the following equation to calculate the volume of Pyro-SPC in chloroform needed for the Lipid Vial[14,15]:

$$V = \frac{m \div M}{\left(\frac{A \cdot d}{L \cdot \varepsilon}\right)}$$

where *V* is the volume of Pyro-SPC in chloroform needed, *m* is the required mass of Pyro-SPC (see Step 1.1 and "Lipid Formula Sheet"), *M* is the molecular weight of Pyro-SPC at 1040.317 $g \cdot mol^{-1}$, *A* is the averaged absorbance at 667 nm, *d* is the dilution factor based on the methanol and Pyro-SPC in chloroform volumes (Step 1.4.2), *L* is the cuvette path length at 1 cm, and ε is 667 nm molar attenuation coefficient of Pyro-SPC at 45000 $L \cdot mol^{-1} \cdot cm^{-1}$.

NOTE: The denominator of the equation is the Beer-Lambert Law, which relates the concentration of an analyte in solution to the measured optical absorbance over a distance.

1.4.5. Add the calculated volume of Pyro-SPC in chloroform from the previous step to the Lipid Vial using a clean glass syringe (**Figure 1A**) and then cap and cover the vial.

NOTE: **Figure 1** only shows the 30% Pyro-lipid formulation only.

1.4.6. If there is any Pyro-SPC in chloroform remaining: In a fume hood, uncap the Pyro-SPC + chloroform vial from Step 1.4, partially tilt the vial to its side and continuously flow nitrogen gas as gently as possible into the Pyro-SPC/chloroform vial. Rotate the vial to dry out the chloroform using the nitrogen gas flow and to evenly coat the Pyro-SPC onto the interior wall of the vial as it dries. Ensure no splashes are made and none of the solution falls out.

1.4.7. Once the Pyro-SPC lipid film appears dry and coated onto the wall of the vial, turn off the nitrogen gas flow. Cap the vial, seal the vial neck with wax film, and store the vial at -20 °C and in the dark.

1.5. Prepare a solution of 90% chloroform and 10% methanol (% v/v), and add 5 mL of it to the Lipid Vial. Cap the Lipid Vial, and gently swirl to homogenize the contents (**Figure 1B**).

NOTE: If the formulation contains phosphatidic acid lipids (such as 1,2-distearoyl-sn-glycero-3-phosphate sodium salt (DSPA)), add: 60% chloroform, 32% methanol, and 8% double-deionized water (% v/v/v) to the Lipid Vial instead. More intense swirling may be necessary to fully dissolve the lipid contents.

1.6. In a fume hood, uncap the Lipid Vial, partially tilt the Lipid Vial to its side and continuously flow nitrogen gas as gently as possible into the Lipid Vial. Rotate the Lipid Vial to dry out the solution using the nitrogen gas flow and evenly coat the lipid film onto the interior wall of the Lipid Vial as it dries. Ensure no splashes are made and none of the solution falls out.

1.7. Once the lipids appear dry and coated onto the interior walls of the lipid vial (**Figure 1C**),

turn off the nitrogen gas flow, cover the bottom and wall of the Lipid Vial with aluminum foil, and cover the top opening with aluminum foil poked with a few holes with a needle for venting (**Figure 1D**).

1.8. Label and place the covered Lipid Vial inside a vacuum desiccator and allow the lipids to dry further for at least 24 h but no more than 72 h.

NOTE: The protocol can be resumed later, after 24 to 72 h.

2. Lipid Hydration

2.1. Fill a bath sonicator with water and heat it to 70 °C. Turn on the sonication to help mix the water.

CAUTION: The water and sonicator are at high temperatures. Avoid touching the water and the sonicator. Wear eye protection, a protective lab coat, and protective gloves.

2.2. Once the bath sonicator has reached 70 °C, remove the Lipid Vial from the vacuum desiccator. Reduce the lights in the working area if possible.

2.3. Prepare an excipient solution of 10% propylene glycol, 10% glycerol, 80% PBS (% v/v/v) and add 10 mL of it to the Lipid Vial (see Step 1.1.1 and "Lipid Formula Sheet").

NOTE: If using a standard air-displacement pipette, use care when handling viscous solvents like propylene glycol and glycerol. Aspirate and plunge the volume slowly and wait for the residual volume to reach the bottom of the pipette tip. Ensure liquid does not cling to the outside of the pipette tip when transferring volumes by moving slowly.

2.4. Cap the Lipid Vial, remove the aluminum cover, and gently swirl the vial in the bath sonicator for 15 min while the sonication is on to dissolve the lipids. Make sure the Lipid Vial's neck is above the water. Occasionally check if the vial cap is securely closed.

2.4.1. Occasionally, remove the Lipid Vial from the water bath. Briefly hold it to the light to check if the contents are fully dissolved (**Figure 1E**).

2.4.2. If the Lipid Vial contents are not homogenizing, remove the Lipid Vial from the bath sonicator. Secure the cap, swirl more aggressively, and return it to the bath sonicator.

2.5. Remove the Lipid Vial from the bath sonicator, and turn off the bath sonicator. Dry the Lipid Vial exterior with paper towels, and re-label the Lipid Vial.

2.6. Cover the Lipid Vial with aluminum foil, and cool the Lipid Vial at room temperature in a cool, dark, dry area for 10 minutes.

2.7. Aliquot the Lipid Vial contents: about 2 mL of the lipid solution in 3 mL borosilicate glass clear serum vials (7 mm inner mouth diameter, 13 mm outer mouth diameter).

NOTE: Some protocols may use 1.5 mL of lipid solution in the 3 mL vial[7].

2.8. Cap the serum vials with lyophilization-style gray chlorobutyl rubber stoppers (7 mm inner mouth diameter, 13 mm outer mouth diameter) and secure the rubber stopper with tear-off aluminum seals (13 mm outer mouth diameter) and a crimper (**Figure 1F**).

2.9. Vacuum, degas, and re-pressurize the lipid solution in each of the serum vials[4,5,7] (**Figure 2**).

NOTE: Refer to "Other Protocols and Data" for instructions on how to assemble the Gas Exchanger and more details.

2.9.1. Close every valve between and including Pressure Valve A and Gas Cylinder Valve (**Figure 2**). Connect the serum vials to the manifold needles, then open the corresponding Manifold Valves, then open Vacuum Valve A and Vacuum Valve B, and vacuum at -90 kPa (-13 psi, -900 mbar) for 5 min to remove atmospheric air. DO NOT vacuum out any of the liquid (see "Other Protocols and Data" Steps S3 to S3.1.5).

CAUTION: The vacuum pump can burst if handled incorrectly. Do not use the vacuum pump with organic, acidic, or basic chemicals.

2.9.2. With the vacuum still on, hold a serum vial (to prevent it from swinging) and tap it rapidly with a pen or marker to degas. Keep tapping until no bubbles form and there are no bubbles in the vial. DO NOT let any liquid be vacuumed out. Pause tapping if necessary. Repeat for all connected serum vials. After degassing all serum vials, **CLOSE** Vacuum Valve A and Vacuum Valve B and **TURN OFF** the vacuum pump (see "Other Protocols and Data" Steps S3.2 to S3.2.3).

2.9.3. With the serum vial still connected to the needle and with the vacuum pump turned off, slowly turn the Gas Cylinder Valve 1/16 to 1/8 (about 22.5 to 45 ° of full revolution) counterclockwise to partially open, then open the T-Handle Valve, and **SLOWLY** turn the Air Regulator Valve clockwise to 3 psi (20.7 kPa) gauge. Then, open Pressure Valve A and Pressure Valve B (see "Other Protocols and Data" Steps S3.3 to S3.3.21).

CAUTION: The decafluorobutane gas cylinder is under pressure and can explode if heated. Keep way from heat and impact. Decafluorobutane gas may cause oxygen displacement and suffocation. Wear proper eye protection and handle in a fume hood. If handled incorrectly, it is possible to vacuum the gas cylinder, which can cause rapid de-pressurization and implosion. Opening the Gas Cylinder Valve more than 1/8 turn can damage the Air Regulator.

2.9.4. After 30 s of pressurization (gauge should still read 3 psi (20.7 kPa)), close all Manifold Valves with serum vials, disconnect the serum vials, sheath the needles, and **CLOSE** the Gas

Cylinder Valve.

2.9.5. Relieve the built-up pressure by partially opening a single Manifold Valve until Air Regulator gauge needle goes to its resting position. Then close everything between and including the Manifold Valves and the T-Handle.

2.10. Label serum vials and store them at 4 °C and in the dark. Ensure all Gas Exchanger valves are closed and the vacuum pump is turned off afterwards.

NOTE: The serum should be stable for up to 4 months in this state. At this step, the protocol can be resumed later, after 4 months at most.

3. Decafluorobutane vials

3.1. With clean, empty 3 mL borosilicate glass clear serum vials (7 mm inner mouth diameter, 13 mm outer mouth diameter), cap them with lyophilization-style gray chlorobutyl rubber stoppers (7 mm inner mouth diameter, 13 mm outer mouth diameter) and secure the rubber stopper with tear-off aluminum seals (13 mm outer mouth diameter) and a crimper[4,7,8].

3.2. Follow Step 2.9.1 to vacuum the atmospheric air (see "Other Protocols and Data" Steps S3.1 to S3.1.5).

3.3. Skip the degassing and follow Steps 2.9.3 to 2.9.5 to re-pressurize the vial (see "Other Protocols and Data" Steps S3.3 to S3.3.21).

3.4. Label the decafluorobutane vials and store them at 4 °C and in the dark. Ensure all Gas Exchanger valves are closed and the vacuum pump is turned off afterwards.

NOTE: Serum vials filled with decafluorobutane gas will be needed for the droplet condensation. They should be stable for up to 4 months in this state. At this step, the protocol can be resumed later, after 4 months at most.

4. Droplet formation

4.1. Remove a hydrated lipid solution in the serum vial (from Step 2.10) from the fridge.

4.2. Using a decapper, remove the aluminum seal on the serum vial and transfer 1 mL of the lipid solution to a 1.85 mL borosilicate glass sample vial (with a phenolic screw cap) by letting the lipid solution flow down the interior wall. Do not create bubbles.

4.2.1. If there is any remaining lipid solution in the serum vial, follow Steps 2.9 to 2.10 to degas and re-pressurize the serum vial for storage (see "Other Protocols and Data" Steps S3 to S3.3.21).

4.3. With the 1.85 mL sample vial, gently flow in decafluorobutane gas into the sample vial

headspace using the Gas Exchanger (see **Figure 2** for the specific valve names).

4.3.1. Ensure that all the valves on the Gas Exchanger are properly closed and the pump is turned off.

CAUTION: If done incorrectly, it is possible to vacuum out the gas cylinder causing rapid decompression and implosion.

4.3.2. Open one manifold valve and carefully unsheathe the corresponding needle from the manifold.

CAUTION: Sharp object, avoid contact/piercing.

4.3.3. Open Pressure Valve A and Pressure Valve B and turn the Gas Cylinder Valve 1/16 to 1/8 (about 22.5 to 45 ° of full revolution) counterclockwise to partially open.

CAUTION: Do not open the Gas Cylinder Valve more than this as it could cause damage to the Air Regulator.

4.3.4. Open the T-Handle Valve and ***SLOWLY*** turn the Air Regulator Valve clockwise until the Air Regulator gauge needle moves slightly from its resting position and perfluorocarbon gas is gently flowing out of the manifold needle.

NOTE: As the system is now open, the Air Regulator gauge cannot properly read pressure.

4.3.5. Uncap the sample vial with the lipid solution, place the needle above the liquid-air interface, and let the perfluorocarbon gas gently flow into the headspace for 30 s. Do not create bubbles. Adjust the Air Regulator valve if necessary.

NOTE: The liquid-air interface should be slightly perturbed by the decafluorobutane gas flow.

4.3.6. After 30 s, carefully and quickly cap the sample vial without moving the vial too much.

4.3.7. Close the Gas Cylinder Valve (clockwise), the T-Handle Valve, Air Regulator Valve (counterclockwise), Pressure Valve A, Pressure Valve B, and Manifold Valve.

4.3.8. Carefully sheath the needle.

4.3.9. Label the sample vial and seal the neck with wax film going clockwise (**Figure 3A** and **3B**).

NOTE: **Figures 3B** to **3F** only show the 30% Pyro-lipid formulation.

4.4. Store the sample vial in the dark and at 4 °C for at least 10 min or up to 24 h.

NOTE: At this step, the protocol can be resumed later, 24 h at most.

4.5. Place about 100 g of dry ice (carbon dioxide) in an insulated container and place regular ice in another insulated container. Retrieve prepared decafluorobutane serum vials mentioned in Step 3, two 3.81 cm (1.5 inch) 20-gauge needles, a 1 mL plastic syringe, a 200 mL container, metal tongs, and a thermometer (-20 to 100 °C).

NOTE: If only making microbubbles, then there is no need for isopropanol, dry ice, and ice.

4.6. Place the sample vial with the lipid solution in the mechanical agitator and agitate for 45 s (**Figure 3C**).

4.7. After the mechanical agitation, stand the sample vial right side-up, shielded from light, and start a 15 min countdown to cool down the vial and size-select the microbubbles[8,17].

4.7.1. When the 15 min countdown has reached 10 minutes (5 minutes left on the countdown), fill a container with about 200 mL of isopropanol and cool it to -20 °C with dry ice using metal tongs.

NOTE: The target temperature is -15 to -17 °C but the isopropanol will warm up while handling the microbubbles.

CAUTION: Isopropanol is flammable. Keep away from heat and sparks. Dry ice can cause skin damage. Handle with tongs. Wear gloves, eye protection, and a protective lab coat.

4.8. After the microbubbles have been size-selected for 15 min, look for the size-selected partition inside the sample vial (**Figure 3D**).

4.9. Keeping the sample vial right side-up, carefully uncap the sample vial, and withdraw about 0.7 mL of the bottom partition with a 1.5 inch 20-gauge needle attached to a 1 mL plastic syringe. Ensure none of the top partition is withdrawn. Do not flick the syringe to remove air pockets.

4.10. Insert a different 20-gauge needle into a decafluorobutane serum vial (keeping the needle near the top of the serum vial) to vent and then insert the needle/syringe with the size-selected microbubbles.

4.11. Slowly transfer the size-selected microbubbles. Tilt the vial and angle the syringe to let the liquid slide down the interior wall of the decafluorobutane serum vial.

4.12. Once all the size-selected microbubble solution has been transferred, remove the needle with the syringe but keep the venting needle in to relieve negative pressure (**Figure 3E**).

4.12.1. If making only size-selected microbubbles, stop here. Keep the venting needle in and near the top. Keep the vial in the dark and at room temperature.

4.13. Add small amounts of dry ice or room temperature isopropanol to the isopropanol bath to ensure the bath temperature is between -15 to -17 °C.

4.14. With the 20-gauge venting needle inserted near the top of the serum vial, place the serum vial in the isopropanol bath, keeping the microbubble level below the level of the isopropanol but the vial neck above it, and intermittently swirl the serum vial for 2 min to condense the microbubbles.

NOTE: This step was modified from the work done by Sheeran et al.[6]

4.14.1. Do no swirl the serum vial continuously in the isopropanol and do not let the solution freeze. Swirl for about 5 s, and lift the serum vial out of the isopropanol. Check for ice nucleation, and resume swirling in isopropanol. If there is ice formation, swirl the serum vial in the air until it dissipates.

4.15. After the 2-minute condensation, remove the serum vial from the isopropanol bath and remove the venting needle.

NOTE: The microbubbles should have been condensed into droplets, as indicated by the change in translucency (**Figure 3E** versus **Figure 3F** for the 30% Pyro-lipid formulation).

4.16. Wipe the serum vial, label it, and place it on regular ice in a dark, insulated container until ready for use. Unopened (intact aluminum seal) droplets should be stable in this state for up to 6 h as long as the melted ice gets replaced as needed.

4.17. When ready to use, remove the aluminum seal with the decapper. Keep droplets (even open vials) on ice and in dark while not in use. Keep microbubbles in dark and at room temperature.

5. Morphological and Optical Characterization

5.1. Prepare 1% Triton by: adding 5 mL of Triton X-100 into 500 mL of PBS (1x, pH 7.4) and stir with a magnetic stir bar until homogenous[14].

NOTE: Triton X-100 very viscous. If using a standard air-displacement pipette, use care when handling. Aspirate and plunge the volume slowly and wait for the residual volume to reach the bottom of the pipette. Ensure liquid does not cling to the outside of the pipette tip when transferring volumes by moving slowly.

5.2. Prepare droplets (Step 4). If microbubbles are also being characterized, collect a small volume of the size-selected microbubbles prior to condensation (Step 4.9).

5.3. Size the microbubbles or droplets on a Coulter Counter (CC) from 0.2 to 6 µm to obtain

size-distributions and concentrations (**Figure 4**).

5.3.1. Fill a clean 20 mL cuvette with 10 mL of CC electrolyte that was filtered through 0.2 µm pore polyethersulfone membrane filter. Measure it on the CC with three runs to get a baseline.

5.3.2. In the same CC electrolyte, add 5 µL of microbubble or droplet sample and gently mix.

NOTE: 2 to 20 µL of sample can be added depending on how concentrated the sample is.

5.3.3. Run the sample on the CC (3 runs), subtract the averaged baseline, and calculate the size distribution and concentration (number per mL).

5.4. Measure the droplet absorbance with UV-Vis spectroscopy (**Figure 5**).

NOTE: **Figure 5** only shows the 30% Pyro-lipid formulation only.

5.4.1. On the UV-Vis spectrophotometer, set the absorbance measurement as 800 nm to 300 nm wavelengths with 0.5 nm increments and enable baseline correction.

5.4.2. Using a clean 1 cm path length cuvette filled with PBS, perform a baseline measurement. Ensure the volume is high enough to intersect the spectroscope beam path.

5.4.3. Dilute the Pyro-lipid droplets into PBS (recommend 2 µL to 500 µL of droplets into 2000 µL of diluent) and mix by pipetting.

NOTE: **DO NOT vortex** or else the assemblies will be destroyed.

5.4.4. Transfer the diluted droplets into a cleaned cuvette and measure the absorbance. Alter the dilutions if necessary.

5.4.5. Repeat Steps 5.4.1 to 5.4.4 but use 1% Triton instead of PBS. After dilution, transfer sample to a sealable/capped vial and vortex for 30 s before measuring.

5.5. Measure the fluorescence of microbubbles or droplets (**Figure 6**).

NOTE: **Figure 6** only shows the 30% Pyro-lipid formulation only.

5.5.1. On the fluorescence spectrophotometer, set the excitation wavelength as 410 nm and the emission wavelength range from 600 to 750 nm with 1 nm increments.

5.5.2. Measure the fluorescence of the PBS diluent to get a baseline using a cuvette that is compatible with the fluorescence spectrophotometer.

5.5.3. Dilute the Pyro-lipid microbubbles or droplets into PBS (recommend 0.5 µL to 10 µL of

droplets into 2000 µL of diluent) and mix by pipetting.

NOTE: **DO NOT vortex** or else the assemblies will be destroyed.

5.5.4. Transfer the diluted sample to the cleaned, baselined cuvette and measure the fluorescence. Alter the dilutions if necessary and avoid signal saturation.

5.5.5. Repeat Steps 5.5.1 to 5.5.4 but use 1% Triton instead of PBS. After diluting in 1% Triton, transfer the diluted sample to a sealable/capped vial and vortex for 30 s before measuring. Add enough volume to ensure bubbles generated from vortexing are above the laser path.

NOTE: The fluorescence signal of the sample in Triton will be much higher than in PBS due to fluorescence unquenching (**Figure 6**).

6. Vaporization imaging

6.1. Fill an appropriately sized water tank with deionized water and let it rest for 24 hours to equilibrate the gases in the water with the atmosphere.

6.2. Prepare droplets and keep on ice and in dark until use.

6.3. Make a flow phantom tube from 2% agar as described by Pellow et al.[18] Submerge the phantom into a water tank heated to 37 °C.

6.4. Warm up PBS to 37 °C and flow through the phantom.

6.5. With a pre-clinical ultrasound system and 21 MHz linear array transducer (see **Table of Materials**), align the view to the flow phantom, set it to B-mode imaging, set the output pressures, and capture videos or images to acquire baselines at each pressure.

6.6. Dilute 20 µL of the droplet into 50 mL of 37 °C PBS and mix gently. Transfer the solution into a 30 mL plastic syringe and push the solution through the agar phantom.

6.7. Keeping the same alignment as Step 6.5, increase the output pressures until vaporization is observed (bright speckles in the phantom, see **Figure 7**).

NOTE: **Figure 7** shows the 30% Pyro-lipid droplet sample. This commercially available 21 MHz linear array transducer is capable of both imaging and vaporizing droplets.

RESULTS:

Pre-condensed, size-selected microbubble samples (n = 3) and post-condensed droplet samples (n = 3) were sized on a Coulter Counter (CC) with a 10 µm aperture. A limitation of the 10 µm aperture is it cannot measure particles smaller than 200 nm, which can bias the mean size and concentration. **Figure 4** shows the sizing data for each of the Pyro-lipid content formulations.

Table 1 shows statistics based on the sizing data. Using a ratio of pre- and post-condensed mean diameters, the results showed that the 0% Pyro-lipid formulation had the smallest mean diameter shift at 1.72 ± 0.02. The 50% Pyro-lipid formulation had the greatest mean diameter at 2.38 ± 0.08. The 1% Pyro-lipid droplet sample had the highest observed concentration at (2.71 ± 0.13) × 10^{10} /mL while the 40% Pyro-lipid droplet sample had the lowest observed concentration at (7.36 ± 0.81) × 10^{9} /mL. Sizing data showed the 10% Pyro-lipid droplet sample had the smallest peak dimeter at 261 ± 13nm while the 50% Pyro-lipid droplet sample had the largest at 390 ± 55 nm. Generally, as the Pyro-lipid content increased, the concentration decreased and the mean diameter increased. As the post-condensed samples are based on the precursor microbubble sample, the trend occurred for both types of ultrasound contrast agents. As the Pyro-lipid content increased, a microbubble subpopulation (with a peak size at approximately 2000 µm) started to form. This secondary peak was not present in the 0% Pyro-lipid microbubble sample and most apparent in the 40% and 50% Pyro-lipid populations.

Figure 5 shows representative absorbance measurements of the 30% Pyro-lipid droplet sample. The peak of the intact sample in PBS was 700 nm while the disrupted sample in Triton shifted the peak to 671 nm. This showed that the intact assembles have different optical properties compared to the individual, unassembled lipid components.

Figure 6A shows representative fluorescence measurements of the pre-condensed microbubble sample while **Figure 6B** shows post-condensed droplet sample with 30% Pyro-lipid. The intact sample in PBS had a fluorescence peak at 704 nm while the disrupted form had a peak at 674 nm. Subtracting the disrupted area under the curve with the intact area under the curve, and dividing the difference by the disrupted area under the curve gives the quenching efficiency, which works out to be 98.61% and 98.07% for the 30% Pyro-lipid microbubble sample and droplet sample, respectively.

To demonstrate droplets converting to microbubbles, diluted droplets were imaged and vaporized in a 37 °C flow phantom with an ultrasound system. **Figure 7** shows representative ultrasound images of the 30% Pyro-lipid droplet sample imaged at different pressures. At low pressures (**Figure 7A**), there was very little signal, only background signal from air bubbles stuck from the agar synthesis. This is because droplets are non-echogenic and do not scatter ultrasound. At a slightly high power, a few microbubbles were generated (**Figure 7B**) as shown by the appearance of bright speckles. As the pressure increased, more microbubbles were generated (**Figure 7C** and **7D**). This also demonstrated that the droplets will not spontaneously vaporize at 37 °C.

FIGURE AND TABLE LEGENDS:

Figure 1: **Images of the steps to form the 30% Pyro-lipid solution. A**) Lipid powder plus Pyro-SPC in chloroform. **B**) Dissolving solution added. **C**) Lipid film dried and coated onto interior wall of vial. **D**) Lipid vial wrapped in aluminum foil (exterior foil taped for reuse). **E**) Hydrated lipid solution. **F**) Lipid solution in serum vial.

Figure 2: **The 10-manifold gas exchanger.** The valves referenced in the protocol are labelled. See

Supplementary Information "Other Protocols and Data" for instructions on how to assemble the Gas Exchanger.

Figure 3: **A**) The lipid solutions of the 7 formulations (0% to 50% Pyro-SPC) in sample vials. **Figures B** to **D** show images of the steps taken to make 30% Pyro-lipid droplets. **B**) 30% Pyro-lipid solution in a sample vial. **C**) Post-agitation. **D**) 15 min size-selected. **E**) Bottom partition transferred to decafluorobutane vial. **D**) Post-condensation.

Figure 4: **Coulter Counter (CC) sizing data of the size-selected microbubble and droplet samples with different Pyro-lipid shell content (*n* = 3).** The solid green lines represent microbubbles and the dotted cyan lines represent droplets. **A**) 0% Pyro-SPC. **B**) 1% Pyro-SPC. **C**) 10% Pyro-SPC. **D**) 20% Pyro-SPC. **E**) 30% Pyro-SPC. **F**) 40% Pyro-SPC. **G**) 50% Pyro-SPC. **H**) The total observed concentrations of microbubble and droplet samples from the CC based on Pyro-SPC content in the shell. All error bars indicate standard deviation. All measurements were performed using a 10 µm aperture which has a size range of 200 nm to 6000 nm.

Figure 5: **Representative ultraviolet–visible (UV-Vis) spectroscopy absorbance measurements from 300 to 800 nm of the post-condensed 30% Pyro-lipid droplet sample diluted in PBS and in 1% Triton.**

Figure 6: **Representative fluorescence emission from 600 to 750 nm excited at 410 nm. A**) Size-selected, pre-condensed 30% Pyro-lipid microbubble sample in PBS and in 1% Triton. **B**) Post-condensed 30% Pyro-lipid droplet sample in PBS and in 1% Triton.

Figure 7: **Representative ultrasound images of a 37 °C agar flow phantom taken with a pre-clinical 21 MHz linear array transducer in B-mode (see Table of Materials).** The left column (**Figures A**, **C**, **E**, & **G**) shows PBS controls. The right column (**Figures B**, **D**, **F**, & **H**) shows 20 µL of post-condensed 30% Pyro-lipid droplet sample diluted into 50 mL of 37 °C PBS. Each row represents free-field peak negative pressures, which were estimated from the work done by Sheeran et al.[8] The yellow triangles indicate focus depth.

Table 1: **Sizing data statistics of the microbubble and droplet samples with different Pyro-SPC content from Coulter Counter (CC) (*n* = 3). All errors indicate standard deviation.**

DISCUSSION:

After adding all the lipid components together (Steps 1.2 and 1.4.5, **Figure 1A**), a solution of chloroform and methanol (and water if phosphatidic acid lipids like DSPA are present) was added to ensure the Pyro-lipid and non-Pyro lipid components were fully homogenized (Step 1.5, **Figure 1B**). To prevent the formation of lipid vesicles with heterogeneous lipid composition, the dissolved lipids were dried and coated onto the interior of the wall of the vial as a thin film (**Figure 1C**). The coating (Step 1.6) also makes the hydration (Step 2.1 to 2.4) easier as it increases the surface area of the dried film. The drying (Step 1.6, **Figure 1C**) and vacuuming (Step 1.8, **Figure 1D**) were done to ensure the chloroform and methanol were fully evaporated as these chemicals can disrupt the formation of microbubbles. While the protocol can be scaled down to make lipid

solution volumes as low as 1 mL, larger volumes can reduce vial-to-vial variation. While this may run the risk of degrading the Pyro-SPC while not in use, the storage condition of the lipid solution (Step 2.9 to 2.10) was meant to reduce that risk. The degassing step with the gas exchanger (Step 2.9.2, **Figure 1F** and **Figure 2**) serves to eliminate as much oxygen as possible to prevent oxidization. It is not recommended storing lipid solutions containing porphyrin-lipids while atmospheric gases are still dissolved in the solution (**Figure 1E**).

In step 2.10, the lipid solution is in a serum vial with a pressurized headspace, similar to how the clinically approved ultrasound contrast agent perflutren lipid microspheres are sold (similar to **Figure 1F**). Internal work has shown stable microbubbles could not be generated via mechanical agitation with the presence of Pyro-lipids if the cap was a soft material like the rubber stopper. Therefore, the lipid solution was transferred to a sample vial with a non-rubber phenolic cap (Steps 4.1 to 4.4, **Figure 3A** and **3B**). When the decafluorobutane gas was flowed into the sample vial (Steps 4.1 to 4.4), the denser decafluorobutane should displace the atmospheric air in the sample vial headspace. Currently, it is unknown why Pyro-lipids are unable to form microbubbles with rubber stoppers. With no Pyro-lipids, stable microbubbles can be made directly in the serum vials with rubber stoppers[4,7]. Thus, it is recommended using the Gas Exchanger to degas and re-pressurize the serum vial then agitate the serum vial itself for non-Pyro-lipid formulations[4,5,6,7] (see "Other Protocols and Data"). The advantage of being able to mechanically agitate in serum vial is the headspace can be pressurized and size-selection can be done by inverting the serum vial upside-down[8]. In this protocol, the 0% Pyro-lipid formulation was transferred to a sample vial (Steps 4.1 to 4.4) to be consistent with the formulations that did contain Pyro-lipids. Additionally, longer acyl lipid chain lengths result in more stable droplets due to better van der Waals interactions[19]. The lipid shell composition was chosen based on what was commercially available, 18-acyl chain length for all lipid types. DSPE-PEG5K was incorporated in all the formulation (Step 1.1) as the presence of the polyethylene glycol chains prevents coalescence of structures via repulsive steric forces[19]. During lipid hydration, the bath sonicator bath was set to 70 °C (Step 2.1) as high enough to fully disperse the 18-acyl chain length lipid film[18]. For longer acyl chain lengths, higher temperatures will be required.

Higher Pyro-lipid loading would increase the concentration of optically absorbing and fluorescing components, which may be desired for certain applications that benefit from maximized porphyrin loading. However, as the Pyro-lipid content increased, the observable droplet concentration decreased and the diameters increased (**Figure 4** and **Table 1**). This illustrates a trade-off between optical fluorescence and absorbance properties versus droplet concentration and diameter. To researchers that must prioritize small diameters for *in vivo* accumulation through small leaky vessels or if a high concentration of droplets needs to be injected, increasing Pyro-lipid loading may not be worth the increase in droplet dimeter or decrease in droplet concentration. If high droplet concentrations and/or small droplet diameters are paramount, similarly sized companion diagnostic agents should be considered instead of Pyro-lipids. While 1% Pyro-lipid droplets did not result in a decrease in concentration or increase in size, 1% Pyro-lipid loading may be too low to be reasonably detectable from tissue background fluorescently. However, the flexibility of porphyrin moiety provides multiple options for functionalization which will impart alternative means of quantification more suitable for low-concentration applications.

For example, Pyro-lipids can be chelated with copper-64 for positon emission tomography imaging and gamma counting[20], or with palladium for trace-metal quantification using mass spectrometry, or with manganese for magnetic resonance imaging[14].

While some experiments may only require a small volume of the droplet solution, 1 mL of the lipid solution is needed to fill the 1.85 mL sample vial. Goertz et al. demonstrated that changes to handling, headspace pressure, liquid-to-gas ratio, and even the vial shape can all affect microbubble populations[17]. Vial temperature during agitation and size-selection can also influence the size distribution. Therefore, for the methods optimized by the end-user, it is critical to be as consistent as possible when making droplets. Unopened droplets may be frozen (-20 °C) and thawed later for future use but this will affect size populations.

The agitation procedure that activates a lipid solution into microbubbles does not produce a morphologically homogeneously population (Step 4.6); rather, the sample is filled with microbubbles, multilamellar vesicles, liposomes, and micelles[18,21,22]. While microbubble sizes span the micron and nanometer range, the other structures are largely below 800 nm [23]. The sizing techniques used do not distinguish between these various structures, and thus the post-agitated microbubble samples (Step 4.6, **Figure 3C**) and the post-condensed droplet samples (Step 4.14, **Figure 3F**) must be assumed as mixtures. The ultrasound-insensitive assemblies (multilamellar vesicles, liposomes, and micelles) are likely conserved post-condensation and will not change size as they do not have phase-changeable cores. Since the Coulter Counter cannot distinguish between these different supramolecular assemblies, the shift in population size following condensation should be interpreted with the assumption that some proportion of the nanoscale structures are inconvertible and contribute to the observed population in that size region. Additionally, these structures contribute to the spectroscopic and fluorescent signatures of these samples[14]. The fluorescence and absorbance signatures of micelles, liposome/vesicles, and droplets are all similar, including their degree of fluorescence quenching[14]. Thus, it is important to consider that there is a mixture of assemblies in **Figures 3C** to **3F**, **Figure 4**, the PBS diluted sample in **Figure 5**, and the PBS diluted sample in **Figure 6**.

After size-selecting and prior to condensation (Step 4.9), it is possible to eliminate the non-bubble assemblies by centrifuging the microbubble sample to separate the buoyant bubbles from the non-buoyant assemblies as described by Feshitan et al.[21] The degree of separation can be controlled by adjusting the spin force and duration. However, experiments of microbubble condensation of such size-isolated samples revealed that using the larger precursor microbubble populations that are selected using size isolation procedures yielded larger droplets (see "Other Protocols and Data" Step S5 for post-spun bubble and droplet sizing). Since an intended application of droplets produced with this protocol is a platform for passive extravasation and accumulation due to their small size compared to microbubbles[4,8], droplet populations that are as small as possible were desired. Thus, this protocol used post-agitated microbubble samples that were not size-isolated via centrifugation, even if that meant ultrasound-insensitive micelles, liposomes, and vesicles were present in the final solution. This does imply that quantification procedures for bio-distribution will derive signal for all of the injected structures and are not limited to just the droplets. However, since these similarly-sized structures most likely

accumulate via a passive mechanism that is primarily dictated by size, it is not suspected that this should change the main inferences that can be made if this platform is to be utilized *in vivo*, although all these aspects should be individually considered depending on the context in which the platform may be used. Tests using experimental arms with and without ultrasound can be performed to ensure that it is the ultrasound-sensitive droplets that are responsible for any changes in bio-distribution, as only the perfluorocarbon core assemblies in the solution will respond to ultrasound.

After agitation, the vial was rested for 15 minutes and a partition was observed in the vial (**Figure 3C** versus **3D**). Size-selection via buoyancy is a simple method of eliminating the larger structures/bubbles from an activated microbubble solution[8,17]. In this case, particles with diameters greater than 5 µm were mostly removed after size-selection (**Figure 4**). The extent of size-selection can be tuned by controlling the duration of the size-selection[17]. Sheeran et al. has shown that not size-selecting can result in generated microbubbles that occlude vasculature[8].

Pefluorocarbons have the advantage of being biologically inert[7]. While decafluorobutane's boiling point is -1.7 °C, above body temperature, the droplets do not immediately vaporize when exposed to 37 °C (**Figure 7B**). As the droplets are meta-stable at 37 °C, additional acoustic energy is needed to vaporize the droplets to microbubbles[7,9]. Poprosky et al. has demonstrated porphyrin droplets condensed via pressurization[22]. This is a viable and even essential method when using less dense perfluorocarbons but high pressures may destroy some bubbles in the process. Octafluoropropane (C_3F_8) has a boiling point of -36.7 °C, so both cooling and pressurization is needed for droplet condensation. However, the lighter perfluorocarbon leads to less stable droplets. Dodecafluoropentane (C_5F_{12}) can lead to more stable droplets with a boiling point of 28 °C. However, it is a liquid at room temperature and will need stronger acoustic energies to vaporize. Thus, choice of the containing gas of the ultrasound contrast agent should consider the conditions of its intended biological application in addition to the parameters of its fabrication. In this protocol, the isopropanol bath for condensation was set to -15 to -17 °C (Step 4.7.1 and Step 4.13) while other protocols used -10 °C [5,6]. Even with a common decafluorobutane core, the condensation temperature may vary depending on excipient composition, total lipid concentration, and lipid shell composition. If using other formulations, optimization may be required to ensure proper droplet condensation without causing the solution to freeze.

As the droplets are smaller and more stable than their microbubble precursor[7], they can take better advantage of passive accumulation mechanisms to extravasate into certain tissues of interest, such as the enhanced permeability and retention effect of certain tumor types[4,24]. With fluorescent, optically absorbent, and acoustic methods of detection[14], it is possible to use a single formulation to quantify uptake. Additionally, this platform can be used to investigate whether the acoustic vaporization of droplets can improve delivered agent fraction beyond passive levels[16]. To quantify agent bio-distribution in tissues and organs of interest after injection, a known amount of Pyro-lipid droplets should be injected into the animal, ultrasound may or may not be applied depending on the control set, the animal should be sacrificed a pre-specified time-point, and the organs should be removed and weighed. The organs should be homogenized, filtered, diluted in surfactant (detergent) to decellularize the tissue, and quantified with

fluorescence or UV-Vis spectroscopy to obtain injected dose percentages per organ mass based on the Pyro signals. For Step 5.4.5 (**Figure 5**) and Step 5.5.5 (**Figure 6**), Triton X-100 surfactant (detergent) was used to disrupt the samples as it is non-fluorescent at 410 nm and its absorbance wavelengths do not overlap with Pyro's.

Microbubbles were not characterized with UV-Vis absorbance. As the UV-Vis spectroscope's laser source is parallel with the detector, any large bubbles could scatter light away from the detector, making them appear more optically absorbent[14]. Unlike the UV-Vis spectrophotometer, the fluorescence spectrophotometer's detector is/should be perpendicular to the laser source to prevent the source from interfering with the detector. UV-Vis was used to quantify the absorbance of the intact and disrupted droplet samples (Step 5.4, **Figure 5**). 300 to 800 nm was chosen as the absorbance wavelengths as the two main absorbance bands of pyro-lipid, the Soret band (340 to 500 nm) and the Q-band (640 to 730 nm), fall within this range[14]. When assembled into a droplet (or other supramolecular structures), the Q-band peak of Pyro-lipid is red-shifted from 671 nm to 700 nm (**Figure 5**). When this supramolecular structure is disrupted by a surfactant like Triton, the peak shifts back to 671 nm[14,15]. Based on this shift, it is possible to tell whether the Pyro-lipids are in an assembled state or in a disrupted state. The ratio of the two peaks can be used to estimate the decay of the assemblies over time.

For the fluorescence measurements (Step 5.5, **Figure 6**), an excitation wavelength of 410 nm was chosen as it corresponds to the Soret band peak for unassembled Pyro-lipid[14]. An emission wavelength range from 600 to 800 nm was selected as the peaks of the intact assemblies in PBS and disrupted Pyro-lipids in Triton are contained within this range. The shift and increase in fluorescence (**Figure 6**) between the intact (704 nm in PBS) and disrupted (674 nm in Triton) samples occurred because of structure-induced quenching. In the assembled form, the Pyro-lipid molecules were packed closely together so generated photons were absorbed by nearby Pyro-lipid molecules. This is evident in the intact versus disrupted quenching efficiency. Thus, it is necessary to dilute samples in surfactant (detergent) like 1% Triton X-100 to relieve quenching and maximize signal for bio-distribution quantification[14].

For simplicity, the same linear array ultrasound transducer was used to both vaporize and image (Steps 6.5 and 6.7, **Figure 7**). This ultrasound transducer (Table of Materials) was capable of reaching the necessary peak negative pressures needed to vaporize droplets[8]. Filling a tank with deionized water from a tap generates gases that become dissolved in the water (Step 6.1). To minimize interference from the dissolved gases in the water with vaporization and imaging, the water was rested for 24 h in the tank to allow the gases in the water to equilibrate with the atmosphere (Step 6.1). Alternatively, the deionized water can be degassed with a sufficiently sized, sealable container connected to a sufficiently powerful vacuum. The ultrasound images demonstrated the microbubbles were successfully condensed as the droplets were unobservable/non-echogenic at low pressures (**Figure 7B**). It was only at higher output pressures that the droplets vaporized into observable, echogenic microbubbles (**Figure 7D, 7F, 7H**). While the post-condensed droplet sample contains micelles and liposomes/vesicles, these assemblies are non-echogenic and only droplets can vaporize into echogenic microbubbles. A PBS control was flowed through the phantom to establish baseline images (**Figures 7A**, **7C**, **7E**, **7G**). As the

pressure increased in the PBS, no contrast was generated. This indicated that the high pressures from the transducer could not produce spontaneous cavitation in a water-based medium alone, and thus all other generated contrast could be attributed to the ultrasound contrast agent employed. If the output pressure is too high, generated microbubbles can be destroyed. By incrementally increasing the pressure and observing the generated contrast, the optimal pressure can be found[8]. The circulation half-life of the droplets can be determined in a similar way by vaporizing the droplets are certain time intervals and observing the contrast generated over time[7].

In summary, multi-modal phase-change droplets with varying Pyro-lipid content were created with the condensation method. Sizing showed that there was a trade-off between Pyro-lipid loading and microbubble/droplet concentration. Characterizations showed that there were differences in intact and disrupted forms in both absorbance and fluorescence. Ultrasound imaging showed droplets were non-echogenic at 37 °C and were vaporizable into echogenic microbubbles at sufficient pressures. Characterizations also showed the potential for Pyro-lipid droplets to replace companion diagnostic agents for droplet bio-distribution or accumulation tests. Future work will investigate in-solution vaporization thresholds, in-solution stability, and *in vivo* circulation durations in nude mice.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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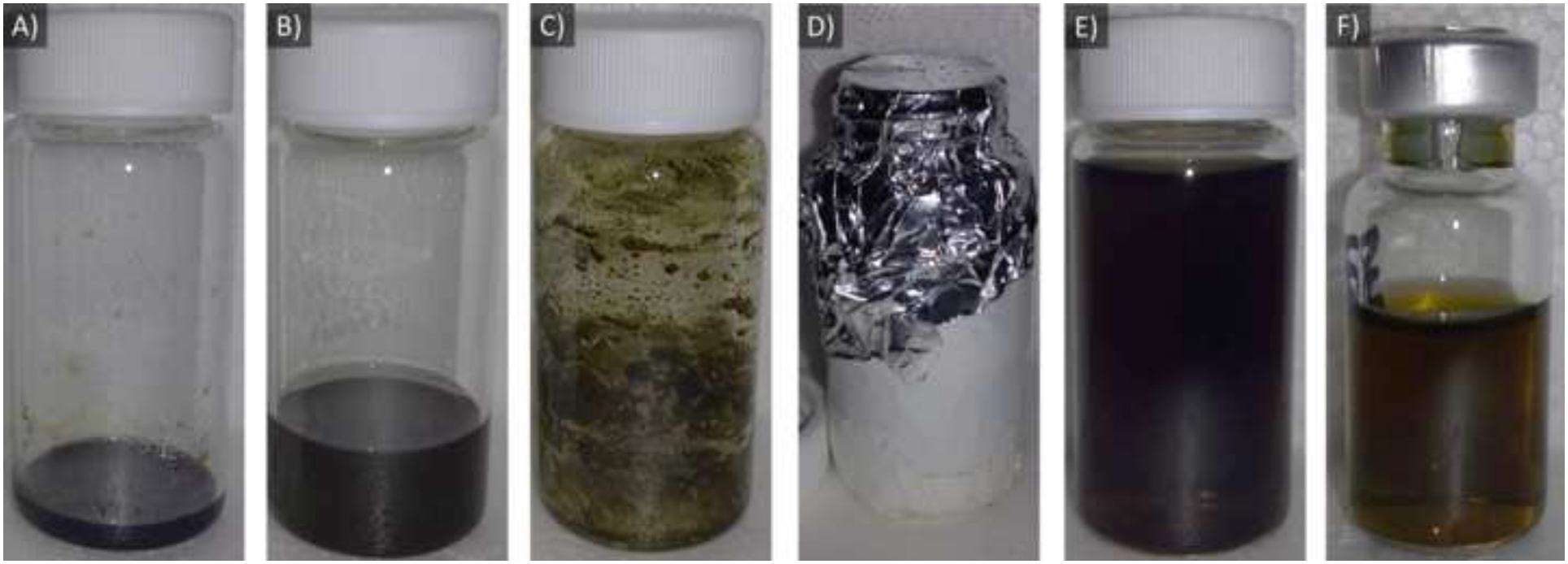

Figure 2

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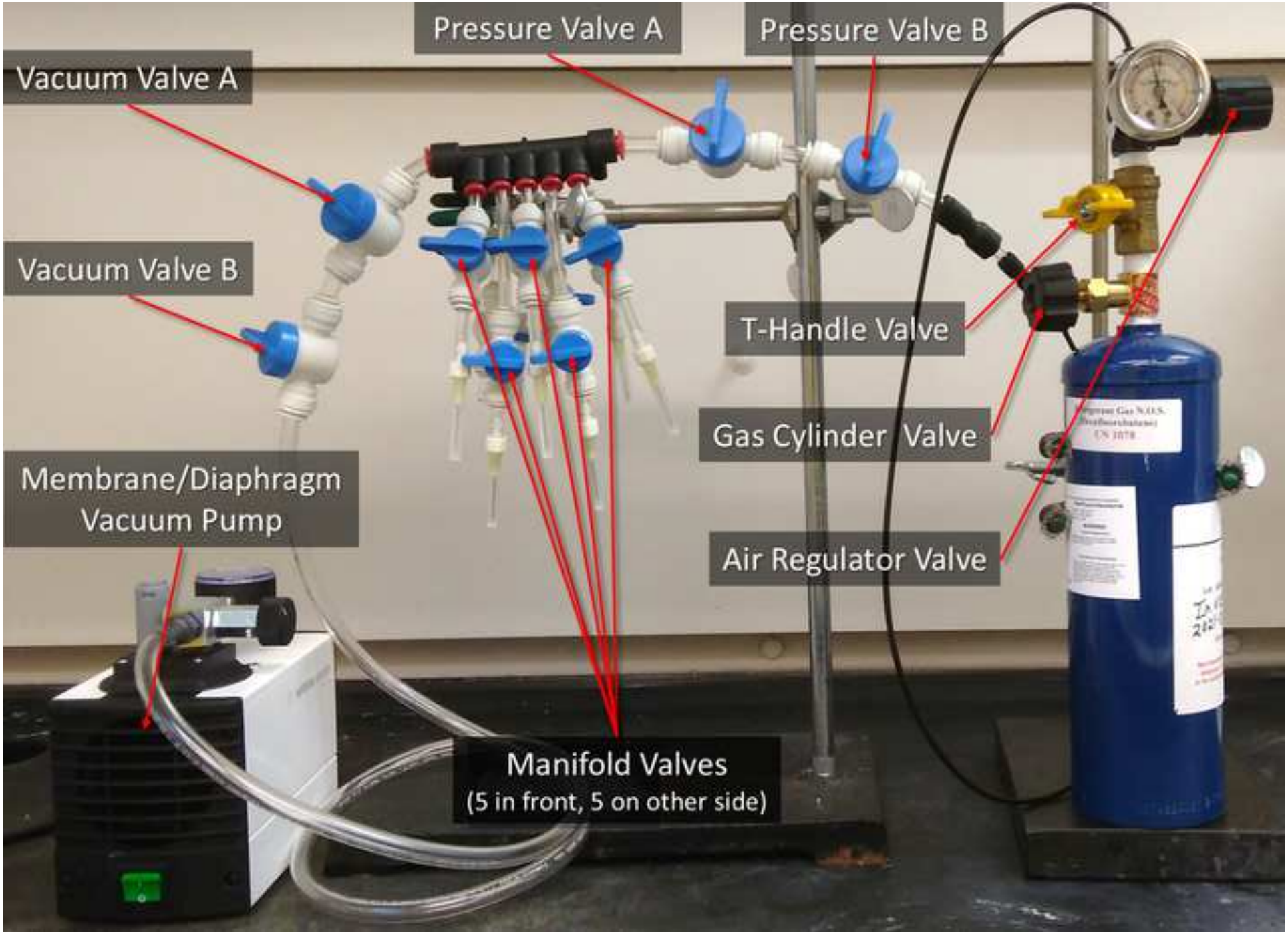

Figure 3

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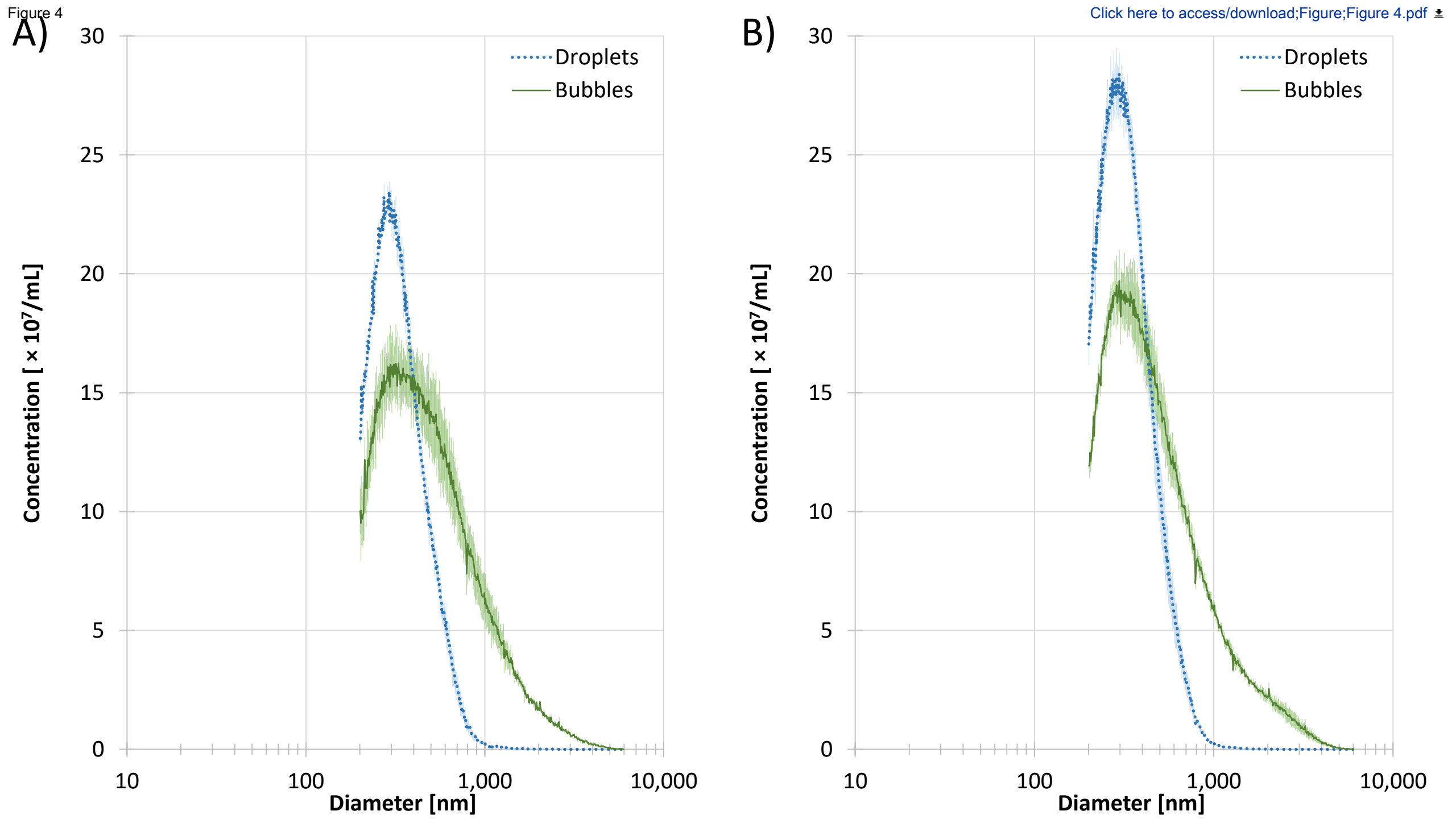
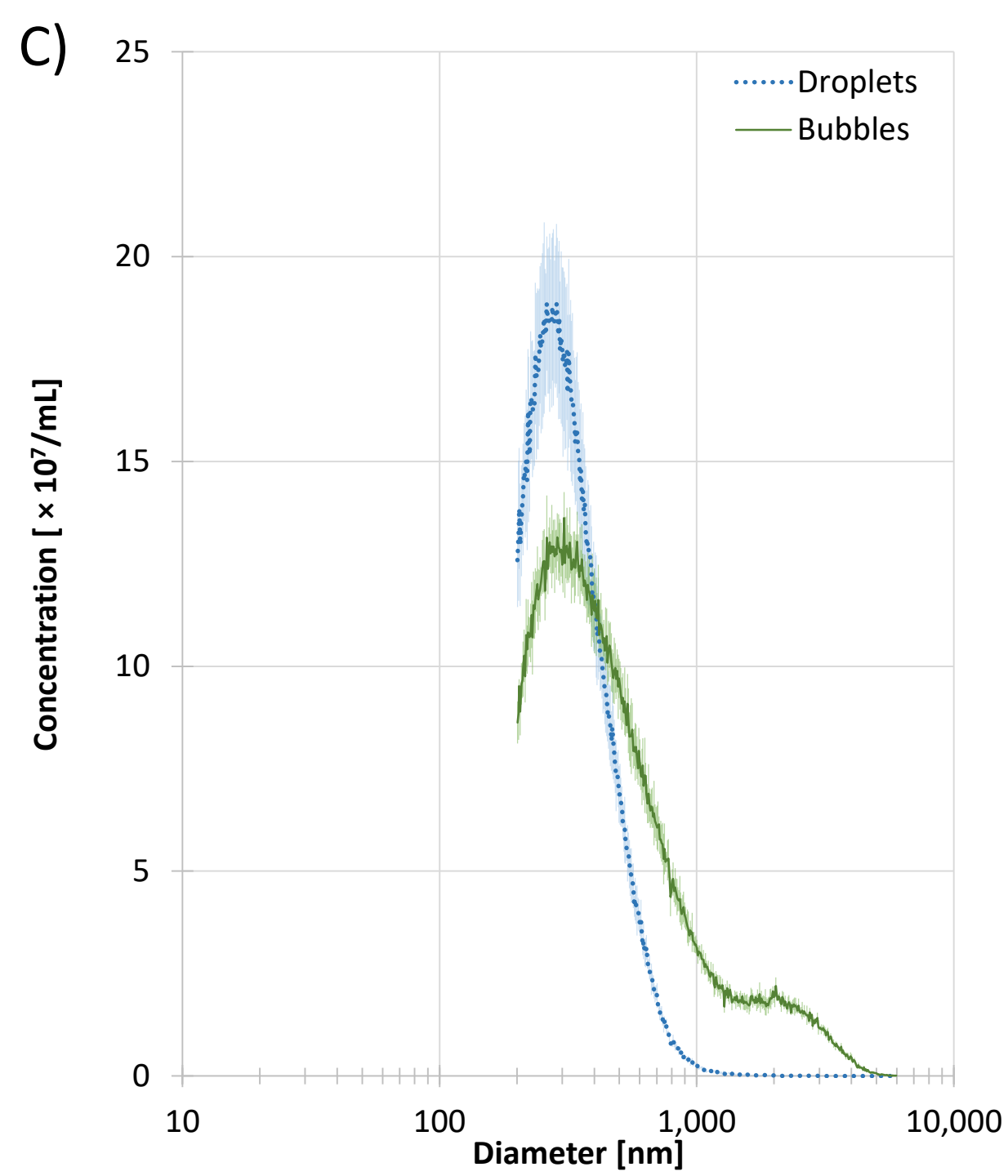
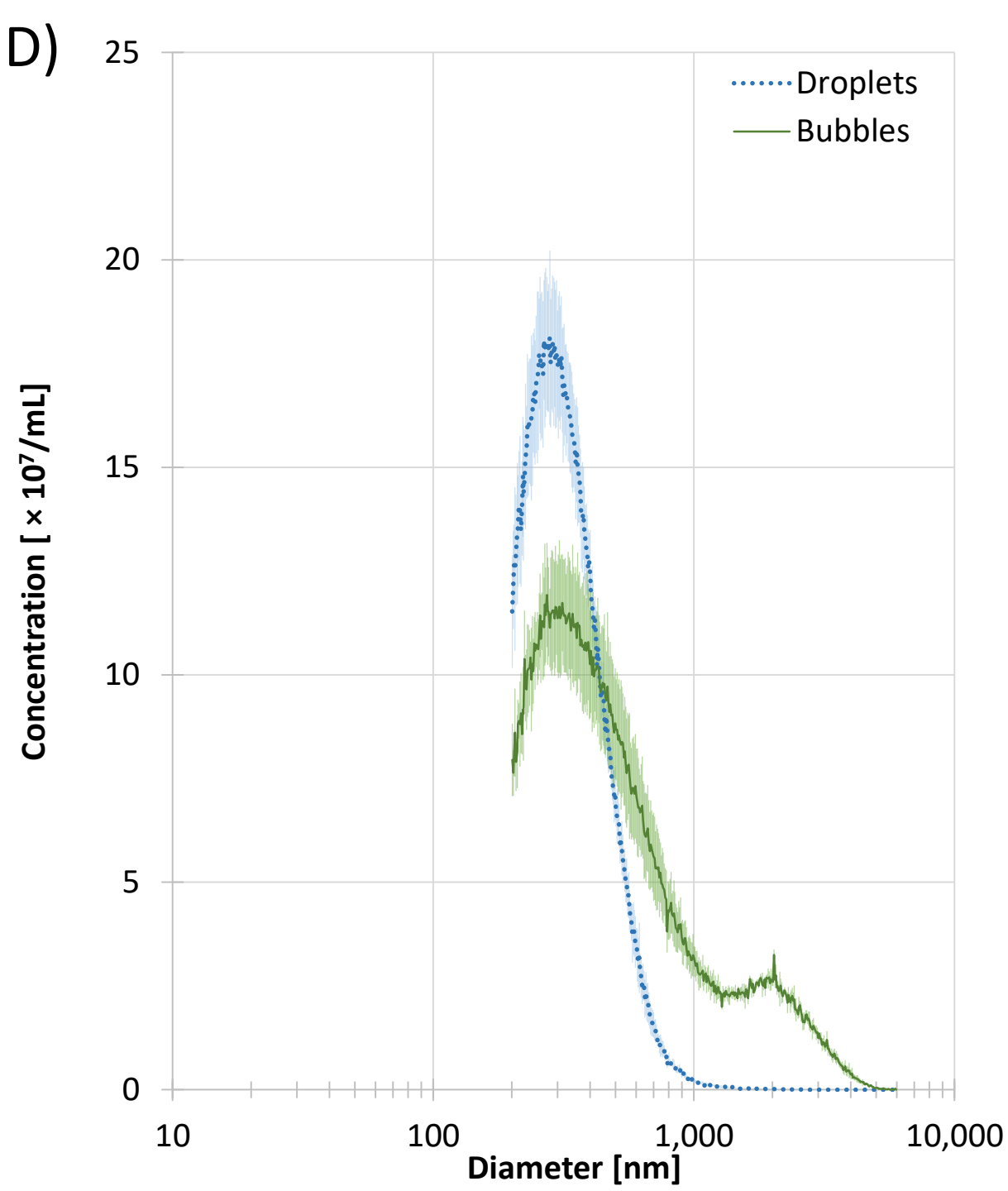
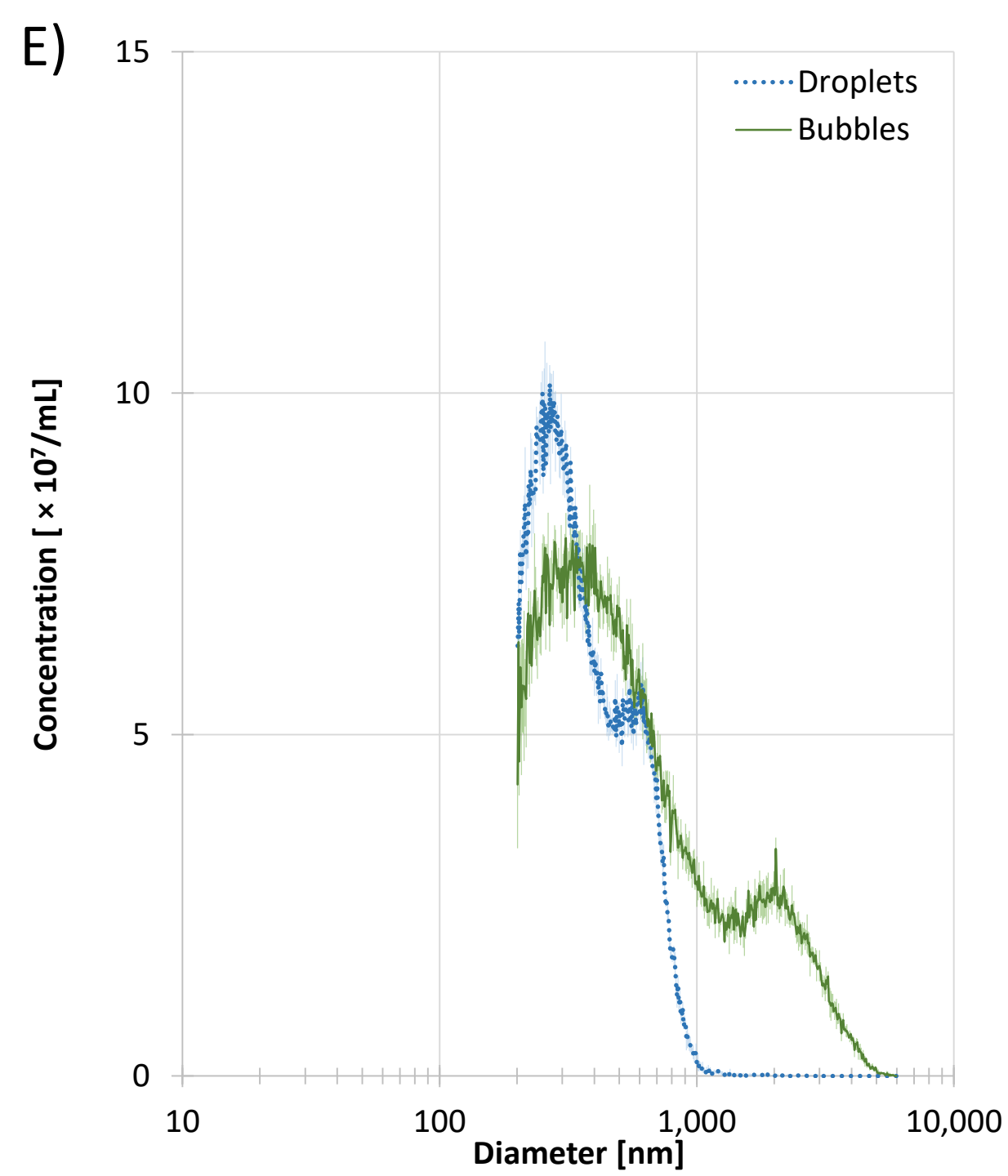
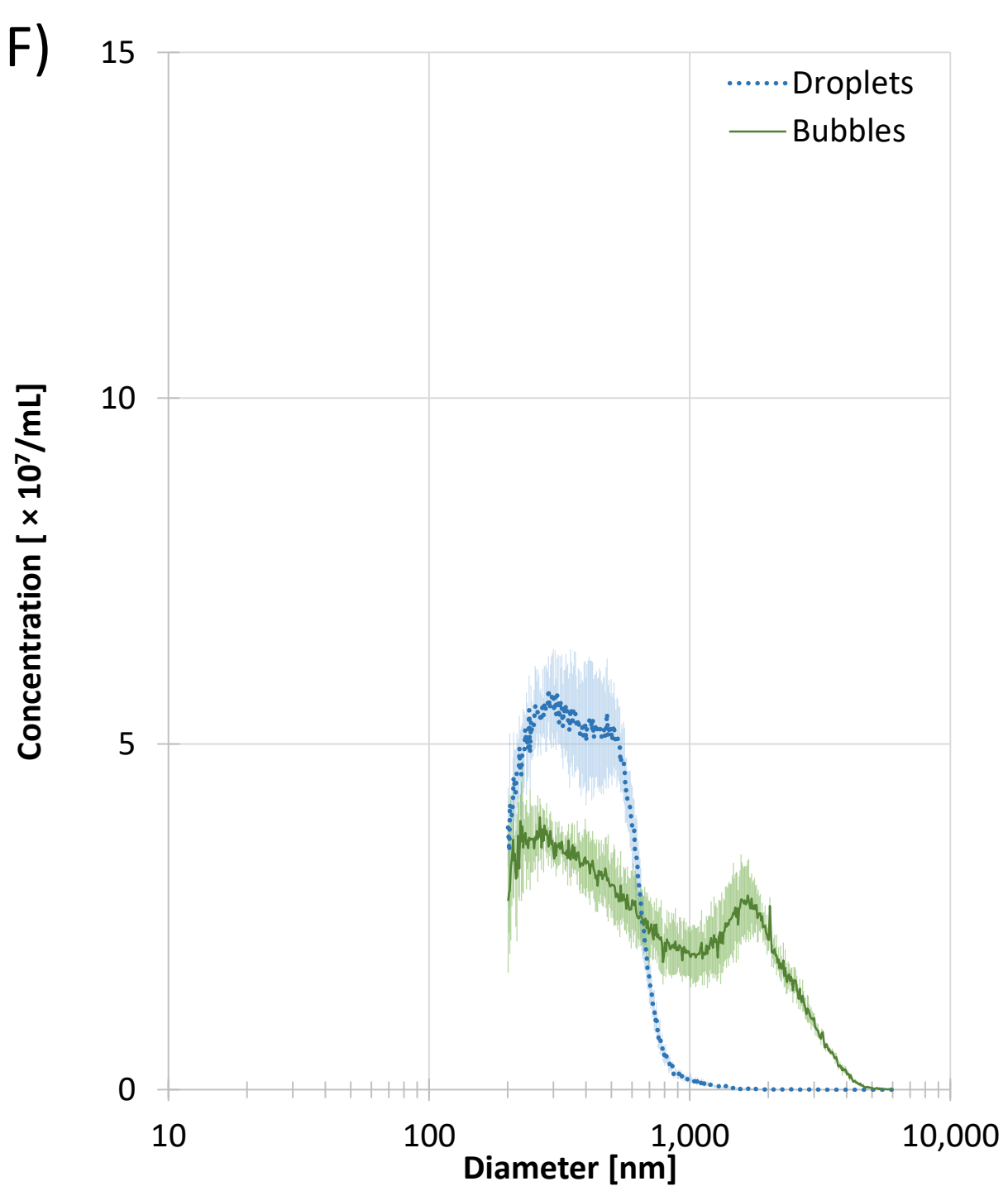
Figure 4

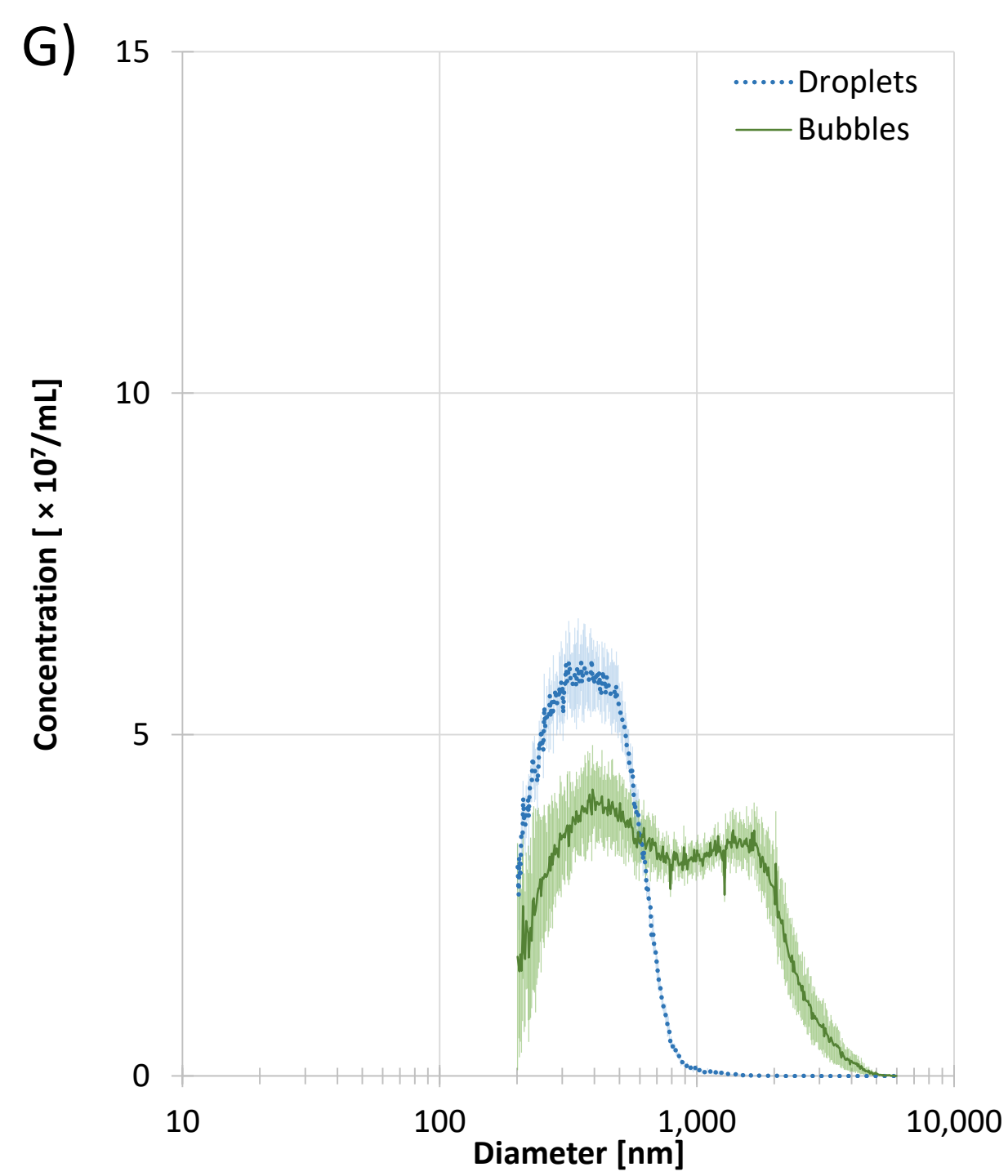



C)
Droplets
Bubbles
Concentration [× 10⁷/mL]
25
20
15
10
5
0
10
100
1,000
10,000
Diameter [nm]


D)
Droplets
Bubbles
Concentration [× 10⁷/mL]
25
20
15
10
5
0
10
100
1,000
10,000
Diameter [nm]


E)
15
10
5
0
Concentration [× 10⁷/mL]
Droplets
Bubbles
10
100
1,000
10,000
Diameter [nm]


F)
15
10
5
0
Concentration [× 10⁷/mL]
Droplets
Bubbles
10
100
1,000
10,000
Diameter [nm]


G)
Droplets
Bubbles
Concentration [× 10^7/mL]
15
10
5
0
10
100
1,000
10,000
Diameter [nm]

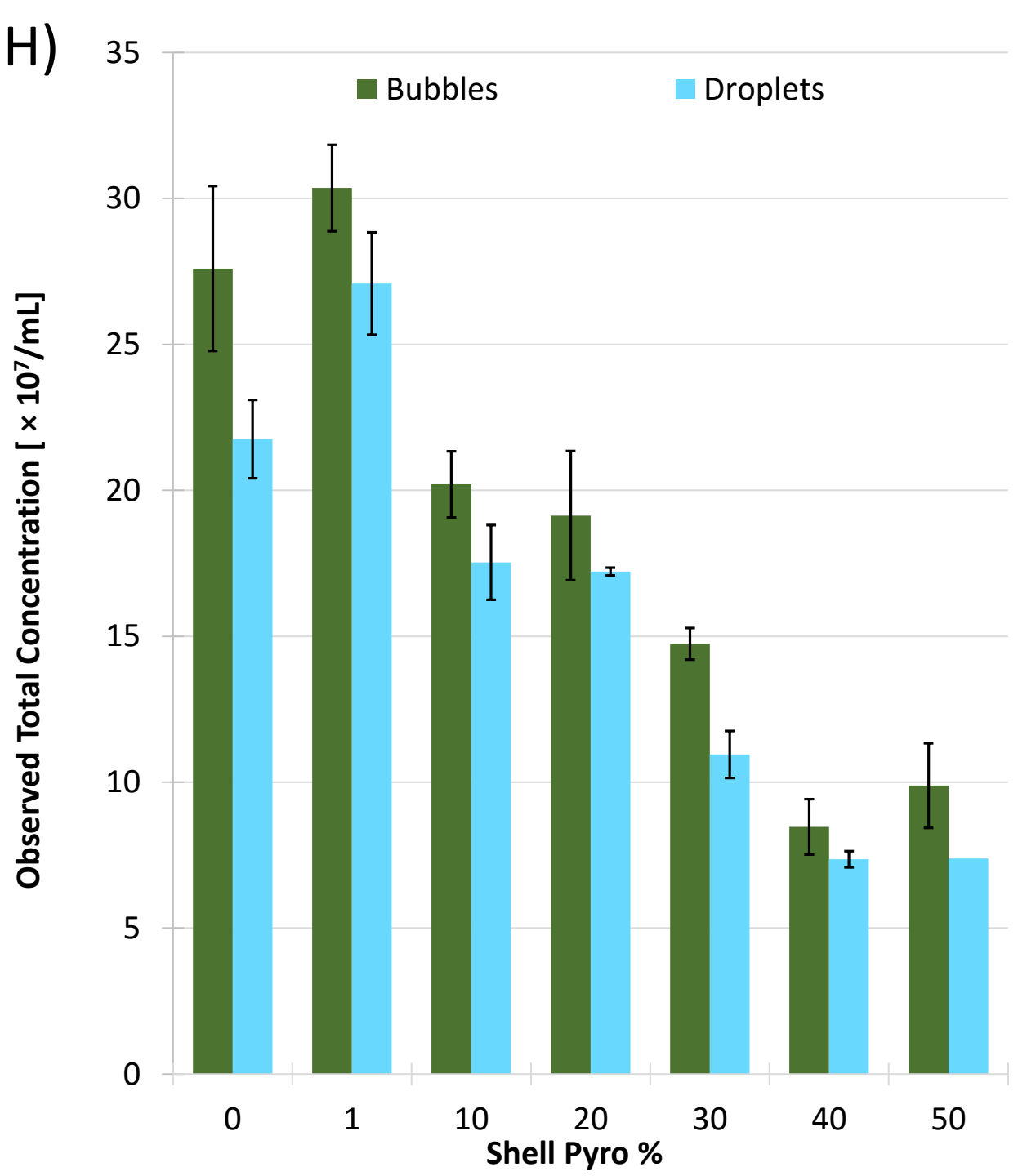

H)
Bubbles
Droplets
Observed Total Concentration [× 10^7/mL]
35
30
25
20
15
10
5
0
0
1
10
20
30
40
50
Shell Pyro %

Figure 5

Click here to access/download;Figure;Figure 5.pdf

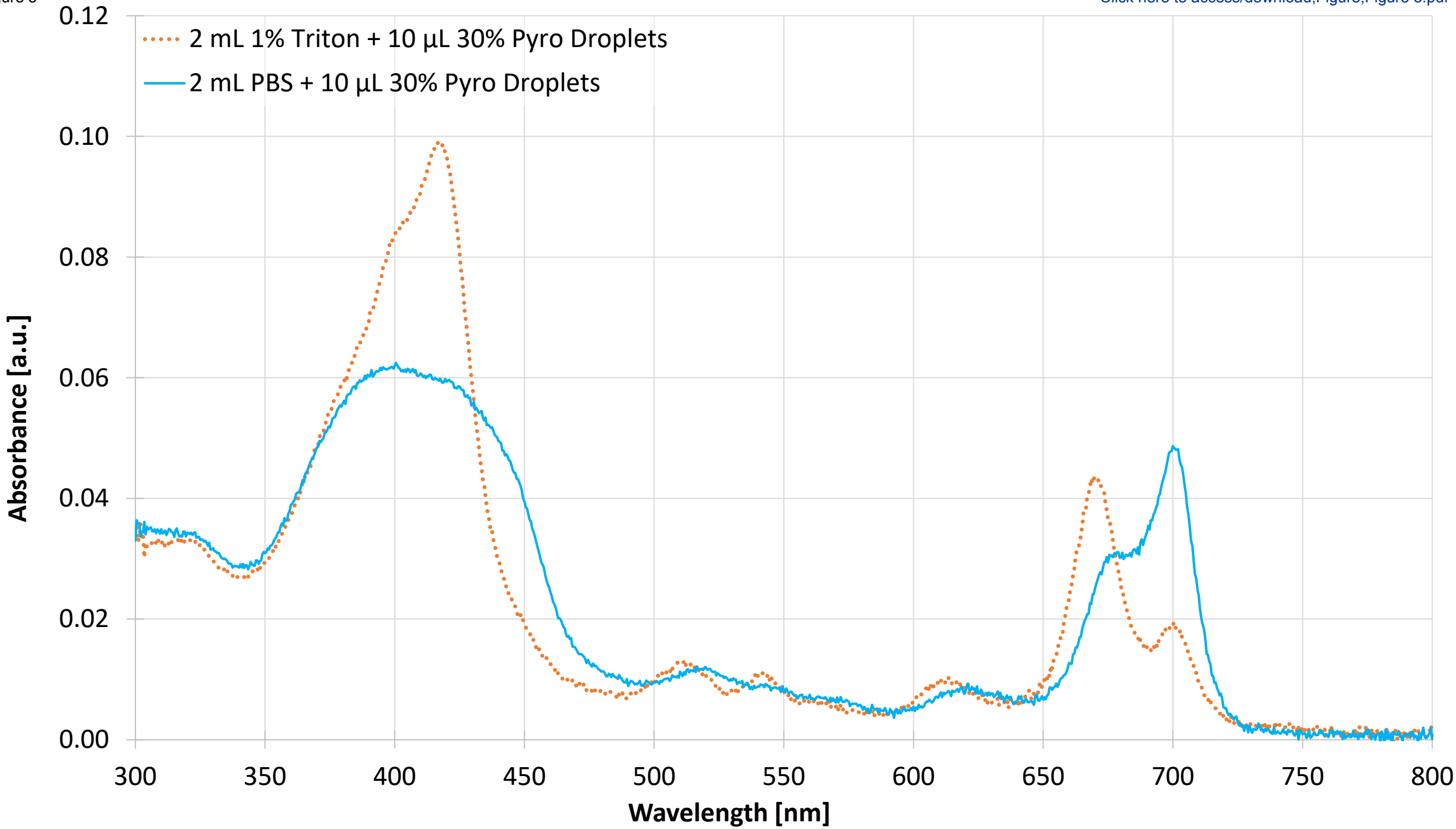


Figure 6

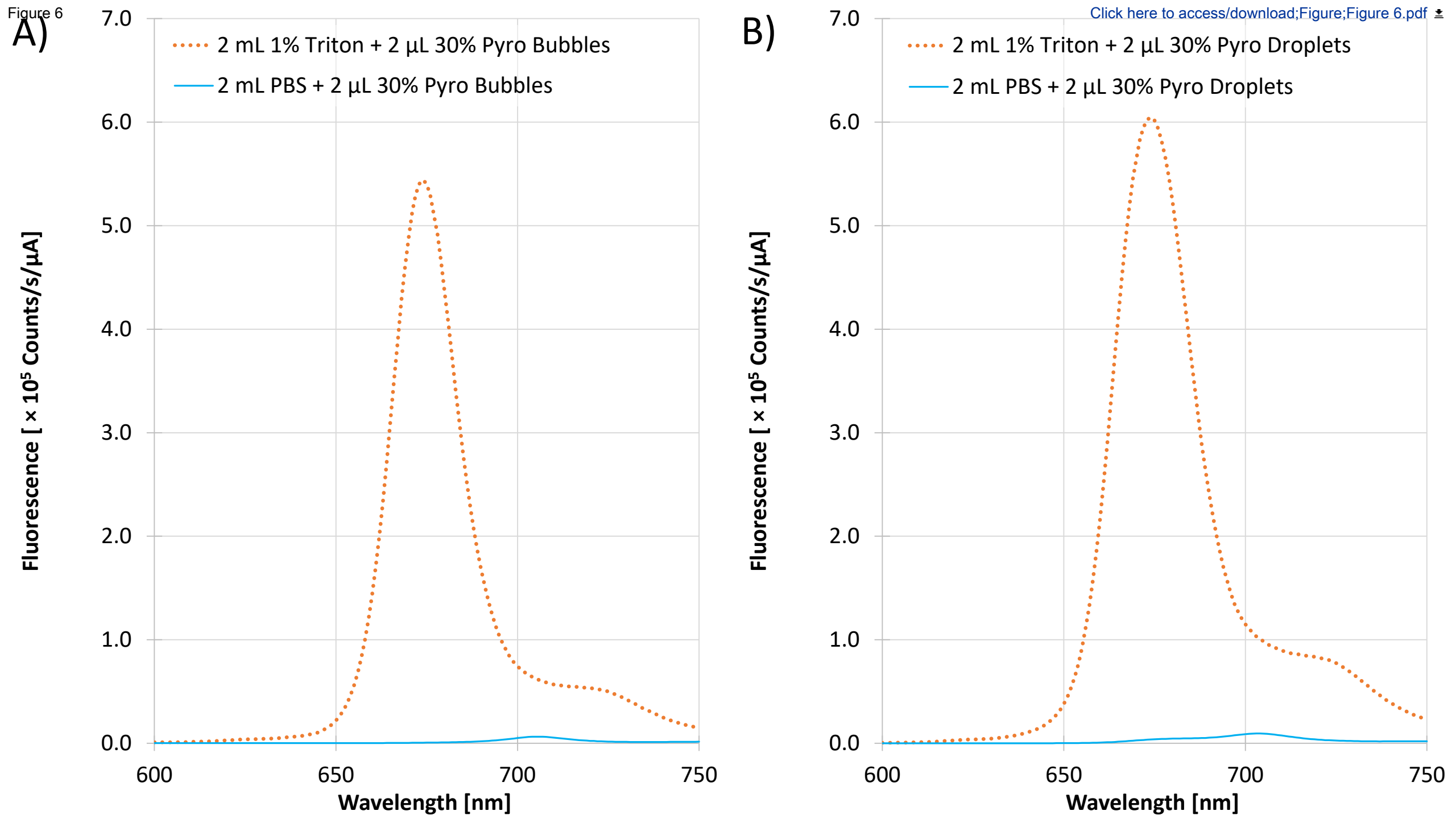


Figure 7

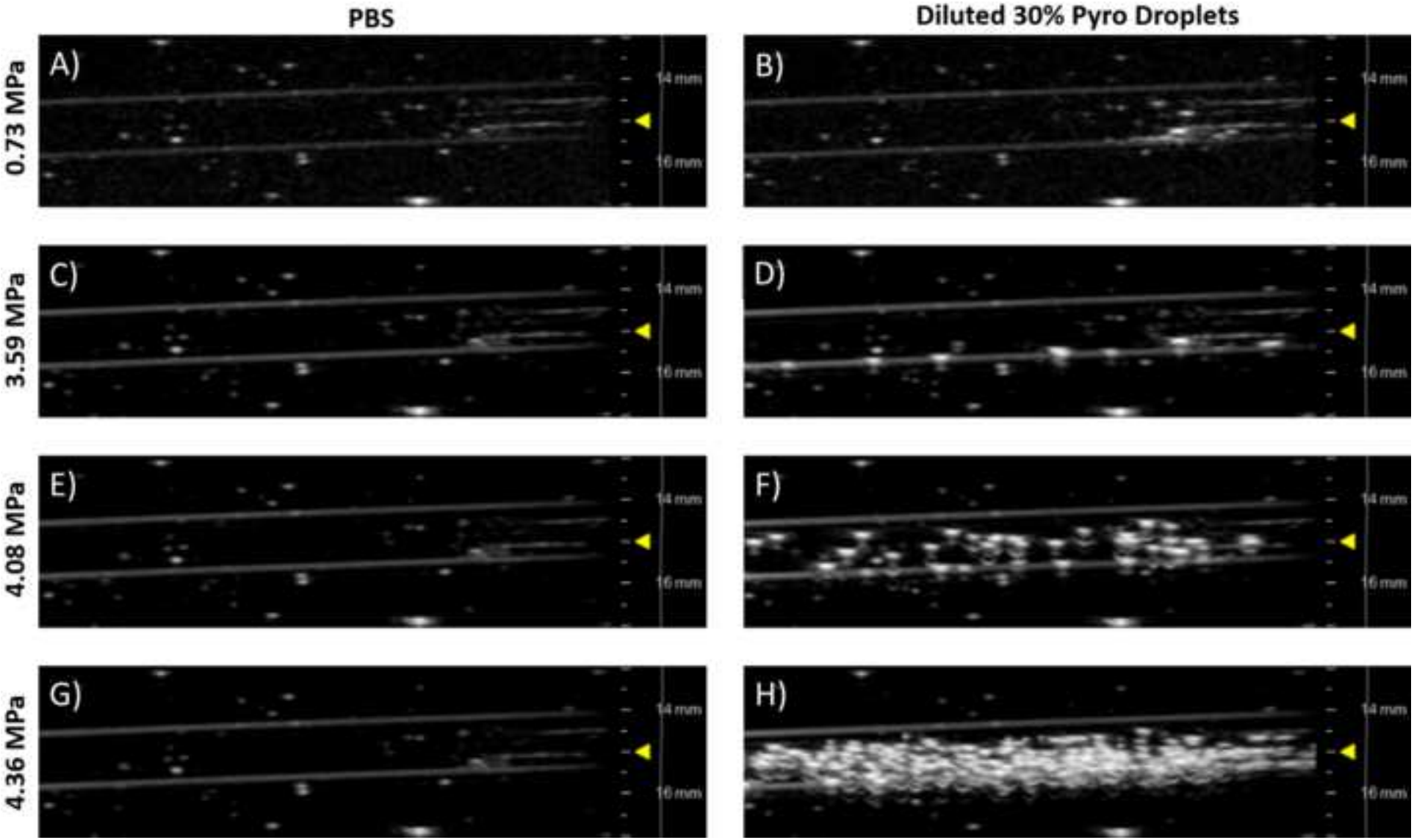


Table 1

Method	Agent	Pyro Shell %	0	1
CC	Bubbles	Conc. [/mL]	(2.76 ± 0.28) × 10^10	(3.04 ± 0.15) × 10^10
CC	Bubbles	Peak [nm]	329 ± 6	297 ± 15
CC	Bubbles	Mean [nm]	609 ± 2	603 ± 15
CC	Bubbles	Median [nm]	450 ± 6	421 ± 6
CC	Droplets	Conc. [/mL]	(2.18 ± 0.07) × 10^10	(2.71 ± 0.13) × 10^10
CC	Droplets	Peak [nm]	292 ± 0	297 ± 17
CC	Droplets	Mean [nm]	353 ± 5	350 ± 5
CC	Droplets	Median [nm]	318 ± 4	318 ± 4

10	20	30	40
(2.02 ± 0.11) × 10^10	(1.91 ± 0.22) × 10^10	(1.47 ± 0.05) × 10^10	(8.47 ± 0.95) × 10^9
305 ± 21	273 ± 14	310 ± 40	266 ± 33
635 ± 6	690 ± 8	812 ± 1	935 ± 22
414 ± 6	432 ± 5	490 ± 2	596 ± 37
(1.75 ± 0.18) × 10^10	(1.72 ± 0.13) × 10^10	(1.09 ± 0.01) × 10^10	(7.36 ± 0.81) × 10^9
261 ± 13	280 ± 9	268 ± 17	287 ± 38
347 ± 1	347 ± 4	397 ± 1	399 ± 6
310 ± 1	315 ± 2	340 ± 0	367 ± 5

50

(9.89 ± 0.15) × 10^9
393 ± 89
950 ± 55
695 ± 41

(7.38 ± 0.28) × 10^9
390 ± 55
400 ± 7
370 ± 9

Name of Material/ Equipment

1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt)
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
Aluminum Foil
Aluminum Seals, Tear-Off
Bath Sonicator
Bio-Stor Screw Cap Vials
Borosilicate glass clear serum vials
Borosilicate Glass Sample Vial with Phenolic Screw Cap
Borosilicate Glass Vial with Screw-On cap
Chloroform
MultiSizer 4e
Coulter Counter Elctrolyte Diluent
Decafluorobutane (C4F10)
Double-Deionized Water
Dry Ice (Carbon Dioxide)
Dynamic Light Scattering (DLS) Particle Analyzer
E-Z Crimper, 13 mm
E-Z Decapper, 13 mm
Fluorescent Spectrophotometer
Fluorescent Spectrophotometer Compatible Cuvette
Gas Exchanger
Glass syringes
GLWR Custom Aperture Tube 10 um
Glycerol
Insulated Styrofaom containers with lids
Isopropanol
Lyophilization-Style Rubber Stoppers
Membrane Diaphram Vacuum Pump
Metal Tongs
Methanol
MS250 21 MHz Linear Array Ultrasound Transducer

Nalgene Rapid-Flow Sterile Single Use Vacuum Filter Units
Needles, Conventional
Nitrogen Gas
Parafilm
Phosphate Buffered Saline (PBS)
Pipette Tips
Pipette
Plastic Syringes
Polyethersulfone (PES) Membrane Filter
Propylene Glycol
Pyropheophorbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
Thermometer
Triton X-100
Ultraviolet–Visible (UV-Vis) Spectrophotometer
Ultraviolet–Visible (UV-Vis) Spectrophotometer Compatible Cuvette, 1 cm Path Length
Vacuum Desiccator
Vevo 2100 Ultrasound Imaging Platform
Vialmix
Vortex Mixer

Company	Catalog Number
Avanti Polar Lipids	880220
Avanti Polar Lipids	855775
Any brand	
VWR	16171-840
Any brand	
National Scientific	BS20NABP
VWR	16171-285
VWR	66011-020
Any brand	
Any brand	
Beckman Coulter	
Any brand	
FluoroMed	355-25-9
Any brand	
Any brand	
Any brand	
Wheaton	W225302
Wheaton	W225352
Any brand	
Any brand	
Made in-house	
Any brand	
Beckman Coulter	B42812
Any brand	
Any brand	
Any brand	
VWR	71000-060
Sartorius Stedim	16694-1-60-06
Any brand	
Any brand	
VisualSonics	

Thermo Scientific	567-0010
BD	305176
Any brand	
Any brand	
Any brand	
Any brand	
Any brand	
Any brand	
Any brand	
Any brand	
Made in-house	
Any brand	
Any brand	
Any brand	
Any brand	
Any brand	
VisualSonics	
Bristol-Myers-Squibb	
Any brand	

Comments/Description

Sometimes referred as "DSPE-PEG5K"
Sometimes referred as "DSPC"

Standard Aluminum, 13 mm outer diameter
Capable of sonicating and heating up to 70 °C,
Plastic, 2 mL Skirted, with O-ring
3 mL, 7 mm inner mouth diameter, 13 mm outer mouth diameter
1.85 mL, Short Form Style, 12 mm outer diameter, 35 mm height, 8-425 cap size
Sizes will depend on desired volumes

Capable of sizing from 0.2µm to 6 µm
Compatible with Coulter Counter

Capable of temperature control
13 mm Standard Aluminum Seals
13 mm Standard Aluminum Seals
Capable of 400 to 600 excitation and 300 to 800 nm emission detection, detector perpendicular to laser source
Can hold at least 2 mL, capable of 300 to 800 nm, all four sides are optical windows
Refer to Supplementary Information - "Other Protocols and Data" for assembly instructions.
Sizes will depend on desired volumes
10 µm aperture, compatible with Beckman Coulter MultiSizer 4e

7 mm inner mouth diameter, 13 mm outer mouth diameter, 2-leg, Chlorobutyl
Adjustable pressure

21 MHz, Capable of B-mode and non-linear imaging.

Polyethersulfone (PES) membrane, 0.1µm pore size, 1000 mL volume. As Isoton II is non-sterile, can use Filter units multiple times
20 gauge, 1.5 inch length
Make sure there are regulator valves and tubes to direct the flow. Setup will be dependend on brand and source.
Referred to as "wax film" in the protocol.
1X, 7.4 pH
Sizes will depend on desired volumes
Sizes will depend on desired volumes
1 mL, 3 mL, and 30 mL. With Luer Lock connections
0.2 µm pore size

Also known as "Pyro-SPC". Refer to "Supplementary Information - Other Protocols and Data" for synthesis.
(-20 to 100 °C)
Also known as "2-[4-(2,4,4-trimethylpentan-2-yl)phenoxy]ethanol"
Capable of absorbance from 300 to 800 nm, at least 0.5 nm resolution
Can hold at least 2 mL, capable of 300 to 800 nm

Pre-clinical ultrasound imaging system
Referred to as "mechanical agitator" in the protocol. Agitates for 45 s.

Editorial Comment 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
• Updated. • The authors would like to thank the editor for the feedback and comments.

Editorial Comment 2: Please provide an email address for each author.
• Alexander Dhaliwal, Juan Chen, and Paul S. Sheeran were added as a co-authors. • The email addresses for each author are on Lines 5 to 7.

Editorial Comment 3: Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.
• The abstract has been rewritten to state the goals of the protocol and it still between 150 to 300 words.

Editorial Comment 4: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Definity, etc.
• Commercial names were updated: "Parafilm" to "wax film", "Definity" to "perflutren lipid microspheres", "VialMix" to "mechanical agitator". • Definity (perflutren lipid microspheres) is not a material or reagent needed for the protocols. The entire protocol can be completed without it. It is only mentioned to make comparisons to the microbubbles and droplets made in the protocol. That's why it was not listed in the Table of Materials and Reagents.

Editorial Comment 5: Please define all abbreviation during the first-time use.
• Updated.

Editorial Comment 6: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
• Updated.

Editorial Comment 7: Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?
• The authors would like to thank the editor for the suggestions. The protocol steps have been elaborated upon in order to more clearly address how each step is performed.

Editorial Comment 8: 2.1 How do you synthesize pyro-SPC?
• The complete synthesis of Pyro-SPC has now been added in the Supplementary Information "Other Protocols and Data".

Editorial Comment 9: 4.1 what excipient is used in your case?
• Now on Step 1.1 (Lines 124 to 125) and Step 2.3 (Lines 224 to 225). The steps were rewritten to illustrate this more clearly, including how the excipient was prepared from standard materials.

Editorial Comment 10 & 11: There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Parts of the supplemental protocol which shows how to perform a particular step should be included in the main text. Some of the highlighted steps have reference to the supplemental protocol. We can only film the main text.
• Parts of the Protocol have been reshuffled and different parts have been highlighted so the essential steps for the video are now in the main manuscript.

Editorial Comment 12: Please ensure the results are described in the context of the presented technique e.g., e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
• Figures 1, 2, and 3 are more visuals aid to help readers follow along the protocol rather than just highlight results. However, we expanded the first paragraph of the Discussion to put Figures 1, 2, and 3 into context with the techniques. • For Figures 4 to 7, the context regarding the data was expanded upon in the Discussion.

Editorial Comment 13: Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a

.doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
• The authors made all the figures in the manuscript and Supplementary Information. None of the figures, including the ones in the Supplementary Information, have been previously published.

Editorial Comment 14: As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol b) Any modifications and troubleshooting of the technique c) Any limitations of the technique d) The significance with respect to existing methods e) Any future applications of the technique
• The Discussion was expanded and parts were rewritten. Disadvantages and limitations of the method regarding increased size and decreased concentration were added (now Lines 656 to 666). • An explanation of a potential bio-distribution tests was also added in the Discussion (now Lines 745 to 755). • The potential applications were expanded (now Lines 671 to 674, Lines 773 to 782)

Reviewer 1 Summary: This manuscript documents the protocol of producing pyropheoprobide loaded phase-change lipid stabilized decafluorobutane nanodroplets through condensation of mechanically agitated microbubbles. The authors also document microbubble/nanodroplet characterization using DLC, Coulter Counter, UV-Vis spectroscopy, Fluorescence spectroscopy, and acoustic vaporization using a 21 MHz linear array transducer. DLS and Coulter Counter results indicate that the authors produced nanometer scale droplets that may be distinct from microbubble precursors. The authors demonstrated that pyropheoprobide was incorporated in the nanodroplet shell through UV-vis and fluorescence spectroscopy when analyzed under a Triton-X100 solution. Comparison of B-mode ultrasound images at increasing pressures may indicate acoustic droplet vaporization. STRENGTHS: Reporting of acoustically activated phase-change droplets with pyropheoprobide loading and without pressurization during the condensation step as well as activating the droplets using an imaging transducer has not been documented. The described protocol is novel. The author took care in describing safety considerations. The authors demonstrated their experience in working with pyropheoprobide.
• The authors would like to thank the reviewer for the feedback and comments.

Reviewer 1, Major Weakness 1a: The authors do not explain the motivation in performing their characterization experiments in sufficient detail. Since the host journal is intended for knowledge transfer it is key that the reader understand why they are performing these experiments. Specific excerpts are listed:

Lines 471 and 472: What do the authors mean by "checking agent assembly"? Is this to confirm that Pyro-SPC is incorporated into the lipid shell? The function of TritonX-100 may need to be described in more detail.

Line 495: What is the purpose of this experiment? Should the reader expect the fluorescent spectrum to change if TritonX-100 was added to the solution? Was this step also taken to confirm that Pyro-SPC was incorporated into the lipid shell? Was Triton used to simulate sonoporation to show that the droplets were multimodal? If so, why not use ultrasound for popping the droplets.

• Yes, by checking the UV-Vis and fluorescence signatures in PBS (intact assemblies) and in Triton (disrupted), we can check if the Pyro-SPC formed assemblies by comparing the signals between the two diluents (now Lines 673 to 771). The statement "check the agent assembly" was removed.

• Additionally, Triton's UV-Vis optical absorbance and fluorescence bands do not overlap with Pyro's. Additionally, for potential bio-distribution, a detergent/surfactant like Triton may also be used to decelluarize tissue before optical measurements. Thus, Triton was chosen. Parts of the Discussion were rewritten to provide details for the purpose of Triton (now Lines 755 to 757, Lines 766 to 771).

• The 390 nm excitation fluorescence data was removed as we felt it was redundant to the 410 nm excitation data (now Figure 6).

• Triton was not used to simulate sonoporation.

Reviewer 1, Major Weakness 1b: Line 531 to 532: Why was an imaging transducer chosen for this application?

• To demonstrate that the droplets made in the protocols can be converted into microbubbles, an imaging transducer was chosen to visually confirm that non-echogenic droplet could indeed be converted echogenic microbubbles. For simplicity, this transducer chosen to both vaporize and image. Step 6 and the Discussion (now Lines 784 to 805) was updated to reflect this.

Reviewer 1, Major Weakness 1c: Line 550: Does DLS have any inherent advantages to Coulter Counting? If DLS cannot report concentration, why include the data at all? What is

the motivation behind using intensity or number weighted DLS results? What conclusions can be reached given the differences between DLSi and DLSn peaks?
• As the 10 μm aperture on the Coulter counter could not detect particles below 200 nm, we used the DLS to sort of make up for the Coulter counter's limitation. • DLS data was removed from the main text and added to the Supplementary Information "Other protocols and data" instead.

Reviewer 1, Major Weakness 2a: The experiment design that was presented leave large room for unanswered questions if repeated by the reader for separate experiment. The present concern is that readers may encounter questions from reviewers regarding due diligence if the present protocol design is replicated for future publications. Specific sections are listed: Line 667: What does the author mean by large diameter microbubbles? Is this referring to the foam or general bubbles above a specific size range?
• The "large diameter" statement was rewritten, now stating upper bound cut-off (now Lines 725 to 727).

Reviewer 1, Major Weakness 2b: Lines 668 to 669: What is the method of destruction associated with centrifugation and is this mechanism specific to pyro-incorporated microbubbles? Mechanical methods of bubble creation do not have 100% yield in terms of lipid input. Excipient filled multilamellar vesicles or liposomes can be formed as waste which do not participate in echo generation or condensation. This waste may not be distinguishable from nanometer scale bubbles using the characterization methods presented. Size selection via centrifugation also serves to remove lipid stabilized particles that are not bubbles and it should be noted that "washing" out non-bubbles components by centrifugation is often repeated within studies involving conjugation/loading in literature. If the intent of plotting bubbles with droplet results on Figure 3 was to demonstrate that condensation did occur, then it is suggested that a centrifuge size selected population of larger size (e.g. 2 μm lower bound, 4 μm upper bound) be prepared for condensation. It is evident that acoustic activation may have occurred when referencing Figure 6, but Figure 3 can be made to be more convincing. It is not known how non-droplet/bubble sub-micrometer lipid stabilized particles contribute to the presented population distribution in the Figure 3 in the presented work. Lines 561 to 584: Given that the waste material has not been removed, it is difficult to attribute these results directly to the droplets. Line 685 to 695: Cannot make conclusions directly attributing Pyro-SPC effects to bilayer disruption of droplets given lack of lower bound in bubble size selection for waste.

• These are good points. Post-agitation, non-bubble structures such as micelles, liposomes and, multilamellar vesicles are present.

• However, a major priority for these droplets was to have a small diameter to provide better passive accumulation *in-vivo* compared to microbubbles. We wanted to conserve as many microbubbles as possible even if it meant it included micelles, liposomes, and multilamellar vesicles, so we did not spin and wash the bubbles.

• We performed a spinning down and washing[17,19] test on 30% Pyro bubbles to get a peak bubble diameter of about 2 μm. These bubbles resulted in droplets with a peak diameter of about 700 nm post-condensation. For the applications we were aiming for (multi-modal droplets capable of passive accumulation), 700 nm is too large[4]. We have included this test in the Supplementary Information "Other Protocols and Data".

• We expanded the Discussion to address these issues (now Lines 684 to 722).

• "Destruction" via centrifugation statement was removed.

• We also changed some of the wording to indicate the products are not homogenous populations (e.g.: calling "microbubbles" to "microbubble samples" and calling "droplets" to "droplet samples").

Reviewer 1, Major Weakness 2c: Lines 586 to 593: Authors may need to include a blank control at each pressure point to confirm that the bubble signals were not caused by high PNP ultrasound.

• Good idea. Blank control images were added (Step 6.5, Figure 7) and discussed (Lines 791 to 801).

Reviewer 1, Major Weakness 2d: Line 618 to 621: Much more information regarding the ultrasound parameters is needed for repeatability. All specifications of the transducer should be reported. Need to report the scanner system being used. It would be reasonable for authors to account for transducers made specifically for therapy. The authors should refer to tutorial papers in the ultrasound community for best practices in reporting. It is not clear why "any transducer with center frequency greater than 10 MHz" is cited in the materials list. Was the water used in the vaporization test degassed?

• A specific, commercially available ultrasound imaging system and transducer were added in the materials list and the "any transducer with center frequency greater than 10 MHz" was removed.

• The free-field peak negative pressures and other parameters for the specified commercially available ultrasound transducer (see updated Table of Materials) were estimated based on the work done by Sheeran et al.[8] (now Figure 7).

• The deionized water used in the vaporization test was not degassed but it was air-equilibrated by letting it rest in the water tank 24 hour before the test. The manuscript was updated with this information (now Step 6.1, Lines 508 to 509, Lines 786 to 791).

Reviewer 1, Major Weakness 2e: Lines 700 to 702: Figure 6 demonstrates qualitative comparison but not quantitative results. It is recommended that the authors quantify droplet activation to show this optimization to future readers.

• Now Figure 7: Internally, we have done linearized pixel analysis in the phantom's region-of-interest. By incrementally increasing the peak negative pressure and observing the increase in contrast in the cine-loops, we did find a vaporization threshold[7,8]. However, we think this image analysis is outside the scope of the protocol. We believe it would be more appropriate if we included quantitative results along with in-solution acoustics and *in-vivo* performance in mice as a separate paper.
• Here, we wanted to describe a way by which droplets activation could be validated using a commercially available system.

Reviewer 1, Comment 1: Abstract: The abstract needs to be more concise. The type and quantity of information that is presented makes the abstract read like a second introduction.

• Abstract has been rewritten.

Review 1, Comment 2: Introduction: The authors may need to elaborate on what type of application these droplets are intended for.

• Added, (e.g.: replacement for companion diagnostic agents for ultrasound contrast agents), (now Lines 80 to 87 and Lines 100 to 108).

Reviewer 1, Comment 3: Methods: Line 154: Safety notices should be included at the first mention of a potentially dangerous chemical.

• Updated, now on Step 1.4 and Lines 142 to 143.

Reviewer 1, Comment 4: Author needs to go into more detail about the function of TritonX-100.

• Details regarding Triton and its use in disruption, decellularization (now Lines 749 to 757), and optical unquenching (now Lines 766 to 769, Lines 776 to 782) were added in the Discussion

Reviewer 1, Comment 5: Instances of "at user discretion" (Line 189, 480) also need to be more detailed to increase repeatability.
• Removed.

Reviewer 1, Comment 4: Results: The authors need to walk through all sub figures in the writing. Lines 561 to 569: The Figure 3 results text did not have any guidance. It is unclear why the authors chose to include error bars on only their Coulter Counter results in Figure 3.
• Updated. • Now Figure 4, DLS data was removed from main manuscript and moved to the supplementary as we felt it was redundant and to declutter the Coulter counter data.

Reviewer 1, Comment 5: Results: The authors have an inconsistent representation of data on graphs. In Figure 3, why are some lines dotted or dashed? In Figure 5, why is the Triton result dashed if the PBS line is solid, why does this not mirror Figure 4 which used almost the same colors but were both solid?
• In the old Figure 3 (now Figure S7), the orange line was solid and the yellow line was dashed to give more differentiation between DLSi and DLSn. DLS data has been removed from the main manuscript. • For the absorbance (now Figure 5), I forgot to make the Triton line dotted. This has been updated and now the absorbance graph (now Figure 5) matches the fluorescence graphs (Figure 6).

Reviewer 1, Comment 6: Tick marker text can be cleaned up by placing exponents in the units.
• Updated, now Figures 4 and 6.

Reviewer 1, Comment 7: Discussion: Some of the information here belongs in the introduction, Lines 685 to 695's paragraph has information that the reader needs to understand why the emission and excitation wavelengths were chosen in the methods. Lines 667 to 671 is justification for the buoyancy sorting method.
• Information about the application of porphyrin droplets were added to the introduction (Lines 80 to 87 and Lines 100 to 108). • The wavelengths chosen based on the Soret and Q-bands of Pyro[14]. This information has now been added in the Discussion (Lines 764 to 769, Lines 773 to 774)

Reviewer 1, Comment 8: Discussion: The authors need to make direct reference to figure and sub-figure in their results analysis for their discussion.
• Updated, all figures and subfigures are now referenced in the Discussion.

Reviewer 1, Comment 9: Discussion: Lines 711 to 712, The future works section needs more examples of experiment planning and justifications for those experiments. There is a leap in logic between the presented work and what is proposed in future work resulting in unanswered questions. For example, the 21 MHz transducer may experience attenuation for deeper tumors what steps will be taken to address this and will the cancer model in the proposed study affect translation of the findings presented here?
• Future works section (Lines 813 to 815) has been rewritten. The mention of tumors has been removed from the future works. • Yes, higher frequencies attenuate faster with distance. However, for our applications on *in-vivo*/*ex-vivo* organ bio-distributions and accumulation in small animals (such as mice), attenuation is less of a concern (see updated future work, now Lines 813 to 815). • Additionally, 21 MHz was used Figure 7 and Step 6 as that was what was available at the time. However, this does not mean all future tests are locked to 21 MHz.

Reviewer 2, Summary: The protocol is about synthesizing and characterizing porphyrin droplets. How different concentration of pyro on the shell changes the size distribution and concentration of bubbles. Overall: This protocol will be very helpful with the microbubble and ultrasound community to have a well-documented protocol to making droplets in the lab. There are many articles for applications with droplets but not detailed information on the creation of the droplets. The authors did a great job at detailing the steps throughout the protocol. It is very detailed but sometimes hard to follow steps. I would encourage to go through the document one more time and make it a bit clearer in certain steps. If more sentences are needed to make it detailed, please add them. There is a part of the protocol that just references another paper on how to create specific portions for the steps, for instance the Pyro-lipids right at the beginning of the protocol. The protocol should be added into the protocol for a well-documented protocol on pyro loading droplets. This manuscript would serve as a good reference for people in the droplet community.
• The authors would like to thank the reviewer for the feedback and comments.

Reviewer 2, Major Concern 1: Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." There are many sections throughout the document that just make statements. Please elaborate these areas.
• Updated.

Reviewer 2, Major Concern 2: Title is a misleading to what the article is about. Please consider revisiting the title name to emphasize the sub-micron bubbles and specifically the creation of Pyro-loaded coated bubbles. As stated in the authors summary, "synthesizing and characterizing porphyrin droplets" seems more of an adequate title for the protocol manuscript.
• The title has been changed to "Synthesis and characterization of multi-modal phase-change porphyrin droplets".

Reviewer 2, Major Concern 3: Lines 125- 128: Authors tells reader to look up how to synthesize Pyro-SPC by citing an article. I looked up the article, will anyone be able to just stop reading this procedure and do the other steps in Zheng [13]? I see a major problem with this as the protocol is not detailed. This means someone would need to read two different protocols to do this current submitted protocol. For example, how long will it take a reader to do the other steps from a different article?
• The complete synthesis of Pyro-SPC has now been added but in the Supplementary Information "Other Protocols and Data" instead of the main text due to the 10-page limitation for the protocol. • The main protocol's wording for this has also been updated (now Step 1.1, Lines 111 to 128).

Reviewer 2, Major Concern 4: Lines 191 - 195: Explain what Beer-Lambert Law is, show equation and results for molar concentration reader should be expecting based on the protocol described.
• The equation and description were added, now Step 1.4.4 (now Lines 164 to 173).

Reviewer 2, Major Concern 5: Line 487: Explain why Triton is being used.

• Triton was used to disrupt the assemblies and look at how the optical signatures change when Pyro-SPC is in intact assemblies (like droplets in PBS) and when unassembled (in Triton) (now Lines 767 to 770, Lines 776 to 780).
• Additionally, as intact assemblies are fluorescently quenched, Triton is used to disrupt the assemblies, unquench the fluorescence, and maximize signal for quantification (now Lines 778 to 782).
• Detergents/surfactants like Triton are used to decellularize tissue before optical analysis and Triton was chosen as it's optical absorbance and fluorescence bands do not overlap with Pyro's (now Lines 749 to 757).

Reviewer 2, Major Concern 6: Line 206: How are they flowing Nitrogen into the vial?

• A continuous gas flow to into the vial was used to evaporate the chloroform/methanol. The wording has been updated, (now Step 1.4.6 and Lines 181 to 186, now Step 1.6 and Lines 199 to 203).

Reviewer 3, Major Concern 7: Line 260: Here and throughout protocol, authors states to see Lipid Formula Sheet, if there is a volume to be added to a step, add amount in the step instead of making readers go see the Formula sheet.

• The step can now be followed without the referring to the Lipid Formula Sheet (now in Step 2.3 and Lines 224 to 225).

Reviewer 2, Major Concern 8: Line 548: how could have the microbubble affected the laser?

• Microbubbles are large enough to scatter the UV-Vis laser away from the detector, making microbubbles appear more optically absorbent. Comments were added to addressing the UV-Vis laser in the Discussion (now Lines 759 to 763).
• The fluorescence at 390 nm excitation was removed as we felt like it was redundant given that 410 nm excitations gave similar data (now Figure 6).
• Fluorescence data for 30% Pyro microbubble samples were added to demonstrate that pre-condensed microbubble samples still showed fluorescence quenching (now Figure 6A).

Reviewer 2, Major Concern 9: Lines 138: By how much will the DSPE-PEF5k vary? Let the reader know how much they should expect the vary to be and at what point is not proper.

• Good point. Unfortunately, we don't have that information and the manufacturer does not report the extreme ranges of DSPE-PEG5K's molecular weight. From our experience, it never found it deviate more than 0.1% from the target molecular weight but this is anecdotal. The manufacturer states the batch's molecular weight on the bottle itself. The wording has been changed to reflect this (now Step 1.1, Lines 120 to 121).

Reviewer 2, Minor Concern 1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. There are certain portions of the document that has common acronyms without spelling it out. Please look throughout the document to catch these.
• Updated.

Reviewer 2, Minor Concern 2: The word agents are used interchangeably but this can be confusing for the reader, if not familiar with the area, please keep it common with term usage. Don't just say agent, say ultrasound contrast agent.
• Updated.

Reviewer 2, Minor Concern 3: In certain steps, it mentions notes.
• Updated.

Reviewer 2, Minor Concern 3: Please make sure to remove the notes or commentary into note sections. Avoid the use of commercial language, including ™/®/© symbols or company brand names before/after an instrument or reagent. Cite these in the Table of Materials instead.
• Commercial names were updated: "Parafilm" to "wax film", "Definity" to "perflutren lipid microspheres", "VialMix" to "mechanical agitator".

Reviewer 2, Minor Concern 4: Make sure protocol steps are in the proper format. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
• Updated.

Reviewer 2, Minor Concern 5: Lines 103-105, Lines 107 - 112: These are not steps, should be a note. Anything not as a step should be placed as a NOTE. In addition to these lines being vague, there should be more background explanation to this statement.
• Updated.

Reviewer 2, Minor Concern 6: The authors states to consider the application, listing some examples and with information that would help push the use of these type of bubbles to be created.

• Statement was removed. Now in the Discussion (now Lines 670 to 674), it states other applications with porphyrin droplets, such as magnetic resonance, gamma counting, and mass spectrometry.

Reviewer 2, Minor Concern 7: Also, Lines 145 - 148 & Lines 150, 158 are also not steps.
• Updated.

Reviewer 2, Minor Concern 8: Lines 114 - 116: list out volume concentration in %V/V, %W/W, %W/V. In addition, we need to tell the reader how to create these chemical calculations, not just list them. Or did you purchase them already at those concentrations? Use appropriate symbols and units throughout protocol.
• Updated, now Step 1.1 (Lines 124 to 125) and now Step 2.3 (Lines 224 to 225).

Reviewer 2, Minor Concern 9: Line 136: Spell out DSPE-PEF5k before using the acronym.
• Now in Step 1.1, Lines 115 to 117.

Reviewer 2, Minor Concern 10: Line 367: Remove company product names and use standard name of instrument used.
• Updated, now called "mechanical agitator" in Step 4.6 (Line 364).

Reviewer 2, Minor Concern 11: Line 164: List compatible cuvette in Materials List.
• Added.

Reviewer 2, Minor Concern 12: Line 197: State that "1040.317" is the MW of Pyro-SPC. It is not clear what MW you are referring to.
• Added, now Step 1.4.4 (Lines 167 to 168).

Reviewer 2, Minor Concern 13: Line 201: Transfer to what to what? Please be clear on the steps the user should be following.
• The step (now Step 1.4.5, Lines 176 to 178) has been rewritten.

Reviewer 2, Minor Concern 14: Lines 221: It is hard to understand what is meant by spilling of lipids and referencing Figure 1B does not look like any spilling is occurring. Was a different Figure supposed to be referenced?
• No, it's the same Figure. The step (now Step 1.6, Lines 199 to 203) has been rewritten.

Reviewer 2, Minor Concern 15: Line 236: Where there is a stop for the authors put it as a "Note", so it does not look as a step. Do the same throughout the protocol.
• Updated.

Reviewer 2, Minor Concern 16: Lines 380 & 389: The amount of time for microbubble sizing is different. One states 10 mins and the other 15 mins. How much time is it?
• The 10 minutes is referring to a time-point of when to start preparing the isopropanol bath. The step (now Step 4.7.2, Lines 375 to 377) has been rewritten.

Reviewer 2, Minor Concern 17: Line 428: Increased transparency should be more of a change to opaque color. From the transition of the vials from one color to the next is quite different. Also moving forward through the article, the authors need to state that a range of pyro-loaded bubbles will be shown but that 30% will be the main pyro-loaded shell discussed more often, especially starting with Figure 1.
• The step (now Step 4.15, Lines 424 to 425) was modified. • The references to the 30% Pyro-SPC shell has been added (Step 1.4.5, Step 4.3.9, Step 4.15, Step 5.4, Step 5.5, Step 6.7).

Reviewer 2, Minor Concern 18: Lines 91-93: States there are disadvantages but lists none. Please add the disadvantages and cite them. It is left as a teaser. If there is more discussion later, state at least one example of why it is disadvantage.
• The line was removed from the introduction but disadvantages (such as trade-offs with small diameters that may be needed for passive accumulation or decrease in particle concentration for limited injection volume) are now addressed in the Discussion (Lines 655 to 668).

Supplementary Information for "Synthesis and characterization of multi-modal phase-change porphyrin droplets": Other Protocols and Data

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1. Synthesis of Pyro-Lipids

The following steps outline how to synthesize approximately 80 mg of pyropheophorbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (Pyro-SPC) with a purity greater than 95%. This synthesis protocol was modified from the work done by Zheng et al.[S1] Refer to **Table S1** for List of Materials.

1.1. Prepare 15 mL of chloroform as the main diluent for the reaction.
CAUTION: Chloroform is a health hazard, irritant, and toxic. Wear a protective lab coat, eye protection, protective gloves, and avoid breathing fumes.

1.2. Add 100 mg of spirulina pacifica algae derived pyropheophorbide *a* (Pyro)[S1] to the 15 mL of chloroform.

1.3. Add 93 mg of 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (SPC) to the chloroform.

1.4. Add 71.5 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl) to the chloroform.
CAUTION: EDC-HCl is toxic and may cause organ damage. Wear a protective lab coat, eye protection, and protected gloves.

1.5. Add 11.8 mg of 4-(dimethylamino)pyridine (DMAP) to the chloroform.
CAUTION: DMAP is toxic and can be absorbed through the skin. Wear eye protection, protected gloves, and a protective lab coat.

1.6. Add 19 µL of N,N-diisopropylethylamine (DIPEA) to the chloroform.
CAUTION: DIPEA is flammable, corrosive, and toxic. Keep away from heat, flames, and sparks and avoid breathing fumes. Wear a protective lab coat, protective gloves, eye protection, and face protection. Handle in fume hood.

1.7. Shield the reaction mixture from light and stir under argon gas at room temperature for 36 h.
NOTE: The conjugation efficiency should be about 80% [S1].

1.8. After the 36 h, place the reaction mixture in a rotary evaporator and evaporate the reaction solvent by setting the vacuum to -30 kPa (room temperature) for 10 min.

1.9. Set the rotary evaporator vacuum to -10 kPa gauge (room temperature) for 10 min to obtain crude Pyro-SPC.

1.10. Prepare a silica gel column by loading it with 12 g of silica gel (high purity grade, 6 nm diameter pores, 70-230 mesh) and pre-elute the column with dichloromethane (DCM) in a fume hood.
CAUTION: DCM is an irritant and may cause cancer. Wear a protective lab coat, protective gloves, eye protection, and avoid breathing fumes. Handle in fume hood.

1.11. Dissolve the crude Pyro-SPC in 1 mL of DCM and load it into the silica gel column.

1.12. Elute the crude Pyro-SPC in DCM through a silica gel column with DCM.

1.13. Prepare a solution of 95% DCM and 5% methanol %V/V and further elute the mixture in the silica gel column with it.
CAUTION: Methanol is a health hazard, irritant, toxic, and flammable. Wear a protective lab coat, eye protection, gloves, and avoid breathing fumes. Keep away from sparks and heat.

1.14. Prepare a solution of 84% DCM, 15% methanol, and 1% double-deionized water %V/V/V and further elute the mixture in the silica gel column with it.

1.15. Prepare a solution of 70% DCM, 28% methanol, and 2% double-deionized water %V/V/V and further elute and collect the fraction in the silica gel column.

1.16. After collecting the Pyro-SPC fraction, dehydrate it in the rotary evaporator at 37 °C with a vacuum at -5 kPa gauge for 30 min (creating 80 mg of Pyro-SPC with greater

than 95% purity).

1.17. Collect and dissolve the purified Pyro-SPC into 8 mL of chloroform, then aliquot 1 mL into 2 mL plastic vials with caps. Label the vials.
OPTIONAL: Measure the Pyro-SPC concentration as described in Step 1.4 to 1.4.4 in the main protocol before aliquoting.

1.18. Vacuum spin the vials for 24 hours in a vacuum concentrator system (room temperature, -110 °C cool trap, centrifuge speed and pressure may depend on model) to remove moisture and solvents and create dried Pyro-SPC films.

1.19. Cap and label the vials and store them in the dark and at -20 °C.

2. **Gas Exchanger Assembly**
The following protocol outlines how to assemble the gas exchanger. The gas exchanger serves the dual purpose to both eliminate atmospheric air dissolved in the aqueous solution and re-pressurize the vial headspaces with inert gases. The gas exchanger is based on the work by Sheeran et al.[S1] Refer to **Table S2** for List of Materials.
NOTE: "OD" stands for "Outer Diameter", "ID" stands for "Inner Diameter", and "NPT" stands for "(American) National Pipe Thread". Many of the units in this document are in Imperial units as the supplier also reports in Imperial.

2.1. Build the Gas Cylinder Assembly as shown in **Figure S1**.
CAUTION: The perfluorocarbon gas cylinder is under pressure and can explode if heated. Keep way from heat and impact. Perfluorocarbon gas is may cause oxygen displacement and suffocation. Wear proper eye protection and handle in a fume hood.

2.1.1. Ensure all valves are securely closed. The valves with the handles are closed when the handles are perpendicular to the flow path.

2.1.2. Applying thread seal tape to the (Push-to-Connect Tube Fitting for Air Straight Adapter, for 1/8" Tube OD x 1/4 NPT Male) threads clockwise with no more than 3 layers. Keep the vent/port clear of tape.

2.1.3. Ensure that the Air Regulator (Compact Compressed Air Regulator, Nonrelieving, Acetal Housing, 1/4 NPT Female, 0-25 psi) valve is closed (pull and turn counter-clockwise).

2.1.4. Look for the flow direction indicator arrows on the back of the Air Regulator (**Figure S1A**). On the 1/4 NPT port with the arrow pointing out of the Air Regulator, screw in the component from the previous step with an adjustable wrench while holding the Air Regulator by hand.
NOTE: Avoid overtightening any screw-on components as it can damage the

threads.

2.1.5. Apply thread seal tap to a sealing screw (packaged with Air Regulator) and screw it on to one of the 1/8 NPT ports on the Air Regulator with a flat screwdriver (**Figure S1A**). This Air Regulator Assembly will be needed later (Step S2.1.10).

2.1.6. Ensure that the perfluorocarbon gas cylinder valve is securely closed (turn clockwise). Then remove the brass cap on the gas cylinder with adjustable wrenches.
CAUTION: Not closing the gas cylinder valve properly can cause the gas cylinder to vent or decompress.

2.1.7. Apply Thread Seal tape to the gas cylinder's threads clockwise with no more than 3 layers. Keep the vent/port hole clear of tape.

2.1.8. Screw on the (Brass Body On/Off Valve with T-Handle, 1/4 NPT Female x 1/4 NPT Male) onto the gas cylinder with adjustable wrenches (**Figure S1B**). Ensure the valve is closed.
NOTE: A way of checking for leaks is to apply dish soap to the connection points after assembly and watch if bubbles appear.

2.1.9. Apply Thread Seal tape to the (Brass Body On/Off Valve with T-Handle, 1/4 NPT Female x 1/4 NPT Male) threads clockwise with no more than 3 layers. Keep the vent/port hole clear of tape.

2.1.10. Screw on the Air Regulator Assembly (Step S2.1.5, **Figure S1A**) onto the T-Handle Valve by hand while holding the (Brass Body On/Off Valve with T-Handle, 1/4 NPT Female x 1/4 NPT Male) with an adjustable wrench (**Figure S1B**).

2.1.11. Apply thread seal tape to the (Vibration-&Corrosion-Resistant Gauge with Dual Scale, Compound, 1-1/2" Dial, 1/8 NPT Male Center Back Connection, 0-15 psi) threads with no more than 3 layers. Keep the vent/port clear of tape.

2.1.12. Screw component from the previous step on to an open 1/8 NPT port on the (Compact Compressed Air Regulator Nonrelieving, Acetal Housing, 1/4 NPT Female, 0-25 psi) by hand (**Figure S1B**).

2.2. Assemble the Manifold Inlets as shown in **Figure S2**.

2.2.1. Cut a piece of the (Tygon PVC Soft Plastic Tubing for Air and Water, Clear, 1/8" ID, 1/4" OD) (recommended length of 3.5 cm, see **Figure S2A**).

2.2.2. Cut five pieces of the (Tygon PVC Soft Plastic Tubing for Air and Water, Clear, 1/4" ID, 3/8" OD), (recommended length of 5 cm each, see **Figure S2A** and **S2B**).

2.2.3. On one of the 3/8" outlet port of the (Push-to-Connect Tube Fitting for Air and Water, 10 Outlet Manifold Reducer, 3/8" Inlet Tube OD), push the pieces that are shown in **Figure S3A** together to connect. The tubing should click-in when they are locked in place. This is the Gas Cylinder to Manifold connection.

2.2.4. On the other 3/8" outlet port of the (Push-to-Connect Tube Fitting for Air and Water, 10 Outlet Manifold Reducer, 3/8" Inlet Tube OD), push the pieces that are shown in **Figure S3B** together to connect. The tubing should click-in when they are locked in place. This is the Manifold to Vacuum connection.

2.3. Assemble the Manifold Outlets as shown in **Figure S3**.

2.3.1. Cut four pieces of (Tygon PVC Soft Plastic Tubing for Air and Water, Clear, 1/8" ID, 1/4" OD) (recommended lengths of 9.5 cm, 4.5 cm, 4.5 cm, & 3 cm, see **Figure S3A**).

2.3.2. Push or twist the pieces shown in **Figure S3A** together to connect. The tubes should click-in when they are locked in place.

2.3.3. Repeat Steps 2.3.1 to 2.3.2 five more times (alternating the lengths so the Polypropylene On/Off Valves aren't pushing against each other and the needles are at different heights, see **Figure S3B**) until all 10 Manifold Outlet ports are filled.

2.4. Connect the assembled pieces together as shown in **Figure S4**.

2.4.1. Link the Gas Cylinder Assembly (Step S2.1, **Figure S1**) to the Gas Cylinder side of the Manifold Inlet (Step S2.2.3, **Figure S2A**) with (Firm Polyurethane Tubing for Air and Water 1/16" ID, 1/8" OD). The tubing length will depend on the working area, equipment placement, and user's discretion.

2.4.2. Link the Vacuum side of the Manifold Outlet (Step S2.2.4, **Figure S2B**) to the membrane/diaphragm vacuum pump with (Tygon PVC Soft Plastic Tubing for Air and Water Clear, 1/8" ID, 1/4" OD). The tubing length will depend on the working area, equipment placement, and user's discretion.
CAUTION: The vacuum pump can burst if handled incorrectly. Do not use the vacuum pump with organic, acidic, or basic chemicals. Handle in fume hood and wear proper eye protection.
NOTE: The membrane/diaphragm vacuum pump should be capable of

reaching -90 kPa (-13 psi, -900 mbar) gauge.

2.4.3. Place the Gas Exchanger in a fume hood, secure the gas cylinder with retort clamps, and place manifold on a different retort clamp to keep the needles off of the floor (see **Figure S4**).

3. **Gas Exchanging**

The following steps outline how to use the Gas Exchanger for vacuuming, degassing, and re-pressurizing vials. Refer to **Figure S4** for the names of specific valves.

NOTE: The gas exchanger requires serum vial(s) capped with a lyophilization-style gray chlorobutyl rubber stoppers and crimped with a tear-off aluminum seals (see Steps 2.9 and 3 in the main protocol).

CAUTION: Do not use the vacuum pump with organic, acidic, or basic chemicals. Wear proper eye protection and handle in fume hood.

3.1. Vacuum the headspace of the serum vial.

3.1.1. Ensure that **ALL** the valves are properly closed (turn Gas Cylinder Valve clockwise and turn Air Regulator Valve counter-clockwise) and the pump is turned off.

CAUTION: If done incorrectly, it is possible to vacuum out the gas cylinder causing rapid decompression and implosion.

3.1.2. Insert the manifold's needle through the center of the lyophilization-style gray chlorobutyl rubber stoppers on the sealed and crimped serum vial(s). Ensure the needle tip stays near the top of the serum vial to prevent any liquids from getting evacuated.

3.1.3. Turn on the vacuum and slowly dial the vacuum pump to -90 kPa (-13 psi, -900 mbar).

3.1.4. Open the appropriate manifold valves (ones with serum vials connected), then open Vacuum Valve A and Vacuum Valve B.

3.1.5. Wait at least 5 min to vacuum the serum vial headspace.

3.2. Degas the liquid in the serum vial.

3.2.1. After vacuuming the headspaces, leave the vacuum on, hold one of the serum vials to prevent from swinging, and rapidly tap the serum vial with a heavy pen or marker until gas bubbles and/or foam start to appear in the vial. Do not let any liquid or bubbles get vacuumed out.

NOTE: The vacuum alone (without boiling) doesn't have enough energy to

nucleate the gases out of the liquid so the tapping is required.

3.2.2. Pause tapping (or tap another vial) if the bubble level rises too closely to the needle and resume tapping once the bubble level goes down. Occasionally check that the needle tip is well above the liquid as not to vacuum the liquid. Once there is no bubbles/foam in a vial and no new bubbles form when tapped (approximately 5 to 10 minutes per vial), the vial is degassed.
NOTE: This process is very tedious. Get an undergrad to do it.

3.2.3. Repeat Steps S3.2.1 to S3.2.2 for each serum vial. Rotate the retort stand to get better access to the other side of the manifold.

3.3. Re-pressurize the serum vial.
NOTE: The method in the main protocol (Steps 2.92 to 2.93) is a single-cycle gas exchange. The method in the supplementary protocol here is a three-cycle gas exchange, which is more secure.

3.3.1. Check that the needle tips are well above the liquid. Keep the serum vials connected in the same manifold needles as the manifold is connected to both the vacuum and gas cylinder. Keep the pump turned on and the appropriate Manifold Valves open.

3.3.2. Close Vacuum Valve A and Vacuum Valve B. Ensure everything between and including Pressure Valve A to the Gas Cylinder Valve are closed.
Double check to ensure the needles aren't too close to the liquid in the serum vial.
CAUTION: If done incorrectly, it is possible to vacuum out the gas cylinder causing rapid decompression and implosion.

3.3.3. Turn the Gas Cylinder Valve 1/16 to 1/8 counter-clockwise to partially open.
CAUTION: Do not open the Gas Cylinder Valve more than this as it could cause damage to the Air Regulator.

3.3.4. Open the T-Handle Valve.

3.3.5. ***SLOWLY*** turn the Air Regulator Valve clockwise until the gauge on the Air Regulator reads 3 psi (20.7 kPa).

3.3.6. (Double check and) close both Vacuum Valve A and Vacuum Valve B.

3.3.7. Open Pressure Valve A and Pressure Valve B. If there is liquid in the serum vial, you should see the gas flow perturb the liquid. Wait 30 s and keep your hands on the Pressure Valves in case of leaks (the gauge should still read 3 psi (20.7 kPa)).

CAUTION: If done incorrectly, it is possible to vacuum out the gas cylinder causing rapid decompression and implosion.

3.3.8. Close Pressure Valve A and Pressure Valve B.

3.3.9. Open Vacuum Valve A and Vacuum Valve B. Wait 30 s while keeping your hands on the Vacuum Valves in case of leaks.
CAUTION: If done incorrectly, it is possible to vacuum out the gas cylinder causing rapid decompression and implosion.

3.3.10. Repeat Step 3.3.6 to 3.3.9 two more times to ensure the gas has been properly cycled.

3.3.11. Close both Vacuum Valve A and Vacuum Valve B.

3.3.12. Turn off the vacuum pump.

3.3.13. Open Pressure Valve A and Pressure Valve B. Wait 30 s and keep your hands on the Pressure Valves in case of leaks (the gauge should still read 3 psi (20.7 kPa)).

3.3.14. One manifold valve & serum vial at a time: close a Manifold Valve, remove the corresponding serum vial from the needle and carefully sheath the needle. The gauge should still read 3 psi (20.7 kPa).

3.3.15. Repeat Step 3.3.14 for each inserted serum vial.

3.3.16. Ensure all the Manifold Valves are closed.

3.3.17. Securely close the Gas Cylinder Valve by turning it clockwise.

3.3.18. Relieve positive pressure in the Air Regulator by: slightly open one of the Manifold Valves until the gauge reads 0 psi (or comes to a resting position) and close the Manifold Valve.

3.3.19. Close the T-Handle Valve, close the Air Regulator Valve (turn counter-clockwise), close Pressure Valve B and Pressure Valve A.

3.3.20. Ensure **ALL** valves are closed, all needles are sheathed, and the pump is turned off.

3.3.21. Label the serum vials to note they have been gas exchanged.

4. **Dynamic Light Scattering**

Droplets were also sized with dynamic light scattering (DLS) to obtain both the intensity-weighted (DLSi) and number-weighted (DLSn) size distributions to supplement the Counter counting data. Refer to **Table S3A** for List of Materials.

4.1. Turn on the DLS machine, wait for the laser to equilibrate, and set the temperature to 10 °C and set the scan for at least 3 replicates.

4.2. Once the DLS machine is ready, dilute the droplets 20-fold by volume into filtered (0.2 µm pore polyethersulfone) phosphate buffered saline (PBS, 7.4 pH, 1X), mix without creating bubbles.

NOTE: Since DLS operates on the principle that the same is homogeneously distributed across the scan time, it is not ideal for buoyant samples such as microbubbles which float/dissolve out of solution in PBS across a sizing measurement, affecting the results over time.

4.3. Fill a 1 cm path length cuvette (that's compatible with the DLS machine) with the diluted droplets to a sufficient height (depends on machine and cuvette model), and measure. See Figures S9 for DLS graphs and Table S3 for sizing statistics.

NOTE: **Table S3B** and **S3C** reports the DLS sizing statistics. Errors indicate standard deviation. **Figure S5** shows representative graphs of the different Pyro loaded droplets.

5. **Spinning and washing bubbles**

The post-agitation and size-selected microbubble sample will inevitably contain assemblies that are not bubbles, such as multilamellar vesicles, liposome, and micelles. A possible method of eliminating the smaller, non-bubble assemblies is to centrifuge the post-agitated, size-selected sample to the larger, more buoyant bubbles. However, this process both reduces the total population of bubbles capable of participating in the condensation procedure and is not able to isolate smaller micro- and nanobubbles, resulting in larger droplet populations with lower overall yield. Because such populations do not suit an intended droplet application that takes advantage of passive accumulation mechanisms unique to nanomaterials below a certain size threshold, they were not utilized in the main body of this work. Nonetheless, the process is described here, as it may be useful for other groups with different intended applications, especially those that prioritize a product that is purely droplets. This may hinder passive accumulation *in-vivo*. The additional materials needed are listed in **Table S4**. The methods were based on the work done by Feshitan et al.[S3]

5.1. Mix 1 mL of propylene glycol, 1 mL of glycerol, 8 mL of phosphate buffer saline (PBS, 1X, 7.4 pH) until homeneous.

5.2. Aliquot 2 mL of the solution from Step S5.1 into 3 mL borosilicate glass clear serum vials (7 mm inner mouth diameter, 13 mm outer mouth diameter).

NOTE: This will be referred to as the Excipient Diluent.

5.3. Cap the serum vials with lyophilization-style gray chlorobutyl rubber stoppers (7 mm inner mouth diameter, 13 mm outer mouth diameter) and secure the rubber stopper with tear-off aluminum seals (13 mm outer mouth diameter) and a crimper.

5.4. Vacuum, degas, and re-pressurize the Excipient Diluent as described in Steps S3 to S3.3.21.

5.5. Store the Excipient Diluent at 4 °C until ready to use.

5.6. Prepare size-selected bubbles as described in the main protocol Steps 4 to 4.12.1, but withdraw 0.7 mL of the size-selected microbubble sample into a 3 mL plastic syringe instead of a 1 mL syringe and a 20-gauge needle.

5.7. Equilibrate the Excipient Diluent from Step S5.5 to room temperature while size-selecting the microbubbles.

5.8. Uncap the Excipient Diluent with a decapper. With a fresh 20-gauge needle, withdraw the Excipient Diluent into the 3 mL syringe containing the size-selected microbubble sample to a total volume of 1 mL. Then carefully remove the needle.

5.9. Keep excess air in the syringe then carefully close the syringe nozzle with a syringe twist-on syringe cap. With excess air in the syringe, gently invert and revert the capped syringe until uniformly mixed. Holding the syringe upwards, uncap, carefully expel any remaining air, and then recap the syringe.

5.10. Place the syringe nozzle down/plunger up in a balanced centrifuge, and spin the syringe at 50 relative centrifugal force (rcf) for 8 minutes [S3,S4].

5.11. Immediately after the centrifugation step finishes, carefully retrieve/move the syringe while keeping the nozzle down/plunger up orientation, carefully uncap the syringe, and push out the infranatant (the more translucent, bottom partition) into a fresh vial. Reserve the supernatant (the bubble/foaming "cake"-like partition, approximately 50 µL).

5.12. Dilute the supernatant "cake" with Excipient Diluent up to a total volume of 1 mL into the same 3 mL syringe. Repeat Step 5.9 to homogenize syringe contents.

5.13. Repeat Steps S5.10 to S5.12.

5.14. Transfer a small volume in the syringe to size on the Coulter counter (CC) as described in Step 5.3 to 5.3.3 in the main protocol to verify if the precursor microbubble

population falls within the intended size range for the chosen application.

5.15. Transfer the remaining volume in the 3 mL syringe into a vented decafluorobutane vial and condense as described in Steps 4.10 to 4.16 in the main protocol.

5.16. Size the condensed product from the previous step as described in Steps 5.2 to 5.2.4 in the main protocol.
NOTE: **Figure S6** shows the size distribution of spun 30% Pyro bubbles from Step S5.14 and corresponding condensed droplets from Step S5.16 sized on the Coulter counter with the 10 µm aperture.

Table S1: Materials needed to synthesize Pyro-SPC

Name	Company	Catalog #
Spirulina Pacifica algae derived pyropheophorbide *a*	Cyanotech	
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine	Avanti	855775
N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl)	Any brand	
4-(dimethylamino)pyridine (DMAP)	Any brand	
N,N-diisopropylethylamine (DIPEA)	Any brand	
Chloroform	Any brand	
Dichloromethane (DCM)	Any brand	
Methanol	Any brand	
Double-deionized water	Any brand	
Rotary Evaporator, capable of up to -30 kPa vacuum	Any brand	
Silica Gel, high purity grade, 6 nm diameter pores, 70-230 mesh	Any brand	
Gel Column with bottom valve	Any brand	
Vacuum Concentrator	Any brand	

Table S2: Gas Exchanger Components

Name	Quantity	Company	Catalog #
Brass Body On/Off Valve with T-Handle, 1/4 NPT Female x 1/4 NPT Male	1	McMaster Carr	4082T42
Compact Compressed Air Regulator, Nonrelieving, Acetal Housing, 1/4 NPT Female (0-25 psi)	1	McMaster Carr	6746K42-6746K27
Vibration-&Corrosion-Resistant Gauge with Dual Scale, Compound, 1-1/2" Dial, 1/8 NPT Male Center Back Connection (0-15 psi)	1	McMaster Carr	38545K29-38545K52
Push-to-Connect Tube Fitting for Air, Straight Adapter, for 1/8" Tube OD x 1/4 NPT Male	1	McMaster Carr	5779K243
Firm Polyurethane Tubing for Air and Water, Black, 1/16" ID, 1/8" OD	5 ft 1.52 m	McMaster Carr	5648K22-5648K221
Push-to-Connect Tube Fitting for Air, Straight Reducer, for 1/4" x 1/8" Tube OD	1	McMaster Carr	5779K352
Push-to-Connect Tube Fitting for Air, Straight Reducer, for 3/8" x 1/4" Tube OD	1	McMaster Carr	5779K355
Tygon PVC Soft Plastic Tubing for Air and Water, Clear, 1/4" ID, 3/8" OD	5 ft 1.52 m	McMaster Carr	6516T21
Tygon PVC Soft Plastic Tubing for Air and Water, Clear, 1/8" ID, 1/4" OD	5 ft 1.52 m	McMaster Carr	6516T14
Push-to-Connect Tube Fitting for Air and Water, 10 Outlet Manifold Reducer, 3/8" Inlet Tube OD	1	McMaster Carr	52045K206
Polypropylene On/Off Valve for Drinking Water, Push-to-Connect Female for 1/4" Tube OD	10	McMaster Carr	4503K23
Polypropylene On/Off Valve for Drinking Water, Push-to-Connect Female for 3/8" Tube OD	4	McMaster Carr	4503K25
Plastic Quick-Turn Tube Coupling, Plugs, for 1/8" Barbed Tube ID, Nylon	1 pack = 10	McMaster Carr	51525K123
Conventional Needles, Luer Lock Connection, Regular Bevel Tip, 20 gauge, 1" length	1 box = 100	BD	305175
Conventional Needles, Luer Lock Connection, Regular Bevel Tip, 23 gauge, 3/4" length	1 box = 100	BD	305143
Conventional Needles, Luer Lock Connection, Regular Bevel Tip, 25 gauge, 5/8" length	1 box = 100	BD	305122
Polytetrafluoroethylene Thread Seal Tape, 0.5 to 1 inch width	1	Any brand	
Membrane Vacuum Pump, Adjustable Setting	1	Sartorius Stedim	16694-1-60-06
Decafluorobutane (C_4F_{10}) Gas Cylinder	1	FluoroMed	APF-N2M

Table S3A: Materials needed for Dynamic Light Scattering (DLS) Sizing

Name	Company	Catalog #
Dynamic Light Scattering (DLS) Machine, Capable of temperature control	Any brand	
Dynamic Light Scattering (DLS) Machine compatible Cuvette	Any brand	
Phosphate Buffer Saline (PBS, 1X, 7.4 pH)	Any brand	
Plastic Syringe	Any brand	
Polyethersulfone (PES) Membrane Filter, 0.2 µm pore size	Any brand	

Table S3B: Intensity-Weighted DLS Statistics for Droplets with different Pyro loadings.

Pyro %	0	1	10	20	30	40	50
Peak [nm]	295.3 ± 19.0	295.3 ± 59.3	342.0 ± 58.1	255.0 ± 0.0	342.0 ± 70.6	342.0 ± 27.1	295.3 ± 70.6
Mean [nm]	319.0 ± 25.2	342.3 ± 82.3	359.6 ± 65.9	291.0 ± 2.5	370.8 ± 88.8	386.6 ± 54.9	363.0 ± 74.6
Median [nm]	295.3 ± 19.0	295.3 ± 59.3	342.0 ± 66.5	255.0 ± 0.0	342.0 ± 70.6	342.0 ± 27.1	295.3 ± 50.4

Errors indicate standard deviation.

Table S3C: Number-Weighted DLS Statistics for Droplets with different Pyro loadings

Pyro %	0	1	10	20	30	40	50
Peak [nm]	190.1 ± 37.4	255.0 ± 53.7	255.0 ± 43.3	164.2 ± 26.4	255.0 ± 52.6	255.0 ± 52.6	255.0 ± 0.0
Mean [nm]	220.1 ± 22.7	238.2 ± 67.2	275.0 ± 47.1	199.0 ± 18.9	258.4 ± 50.0	296.4 ± 9.3	276.3 ± 12.8
Median [nm]	190.1 ± 26.4	220.2 ± 62.7	255.0 ± 49.6	190.1 ± 12.2	255.0 ± 52.6	255.0 ± 20.2	255.0 ± 0.0

Errors indicate standard deviation.

Table S4: Additional materials needed to eliminate lower populations

Name	Company	Catalog #
Plastic Syringe, 3 mL	Any brand	
Twist-on (Luer Lock) syringe cap that compatible with the 3mL plastic syringe	Any brand	
Centrifuge capable of holding at least two 3 mL syringes, and capable of reaching 50 relative centrifugal force	Any brand	

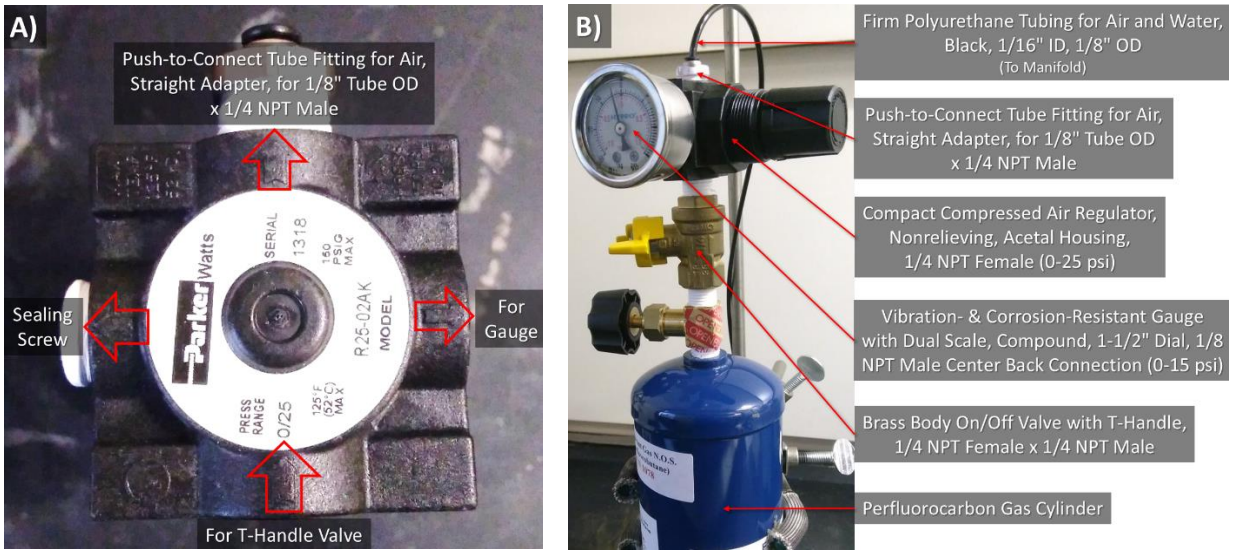
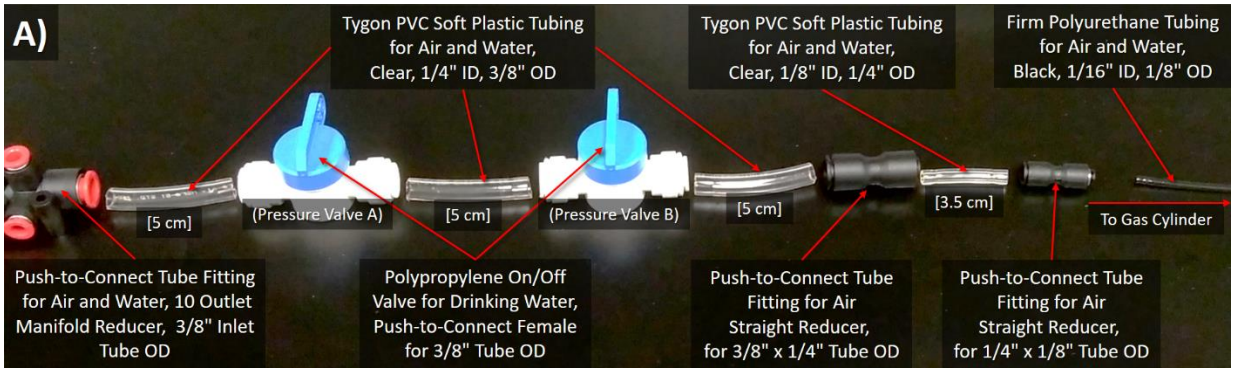


Figure S1: Gas Cylinder Assembly. **A**) Air Regulator Assembly. The gas flow direction arrow indicators have been highlighted with red arrows for clarity. **B**) Components attached on top of the perfluorocarbon gas cylinder.

497
498
499



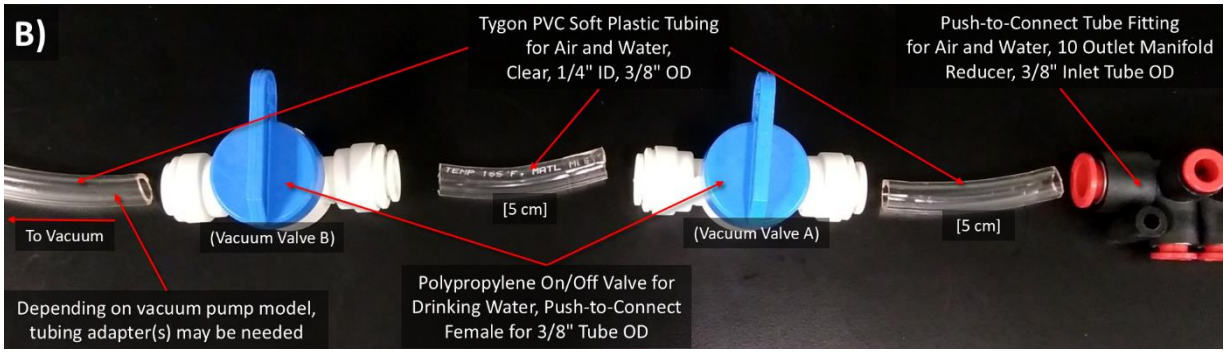


Figure S2: Manifold Inlet connections. Simply push the shown pieces in to connect. Square brackets indicate recommended tubing lengths. **A**) Gas Cylinder to Manifold connections. **B**) Manifold to Vacuum connections.

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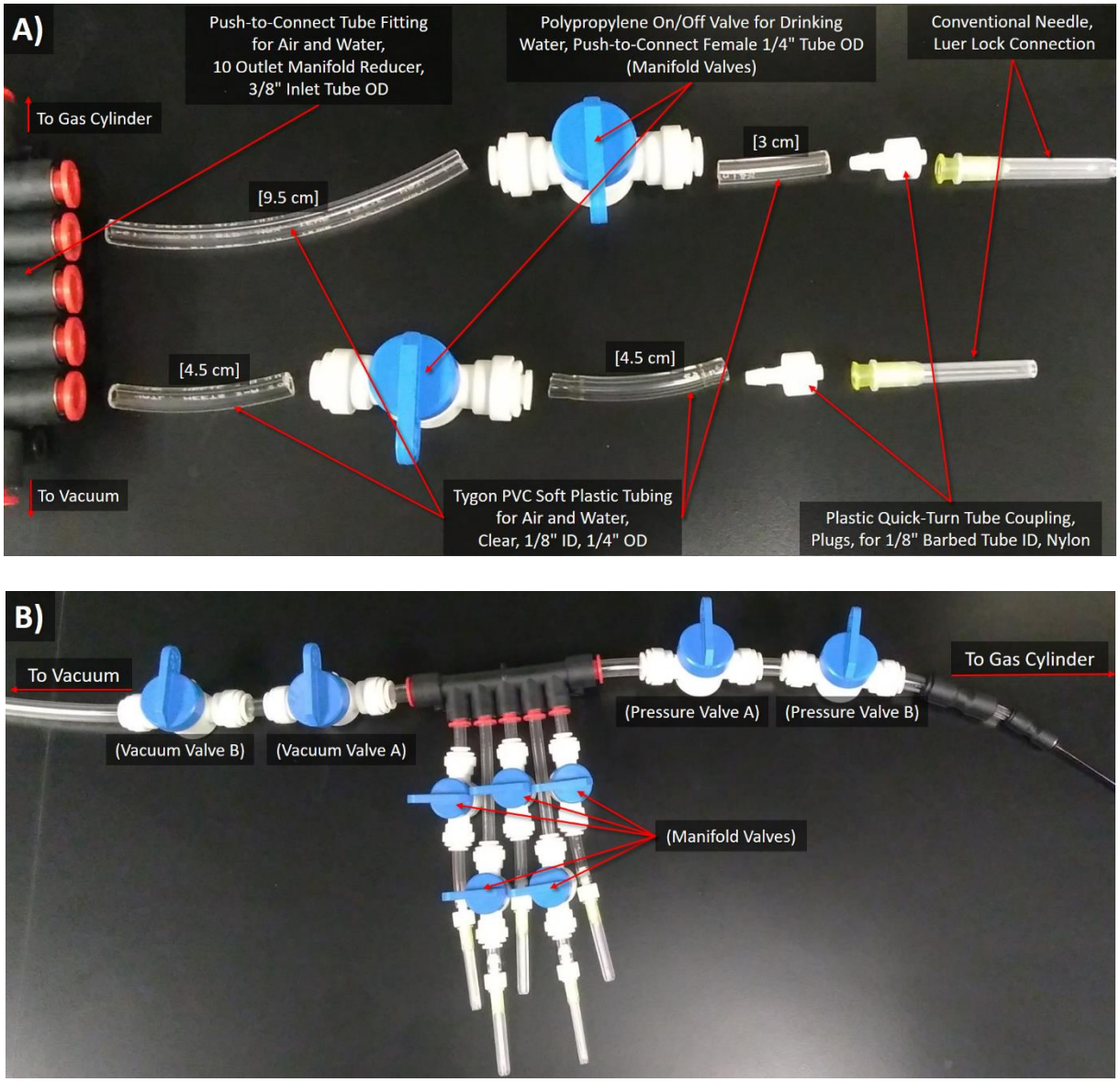

Figure S3: Manifold Outlet connections. **A**) Pieces needed for the Manifold Outlets. Simply push or twist the shown pieces to connect. Square brackets indicate recommended tubing lengths. **B**) One completed side of the Manifold Outlets. Different tubing lengths are used so the valves won't push against each other.

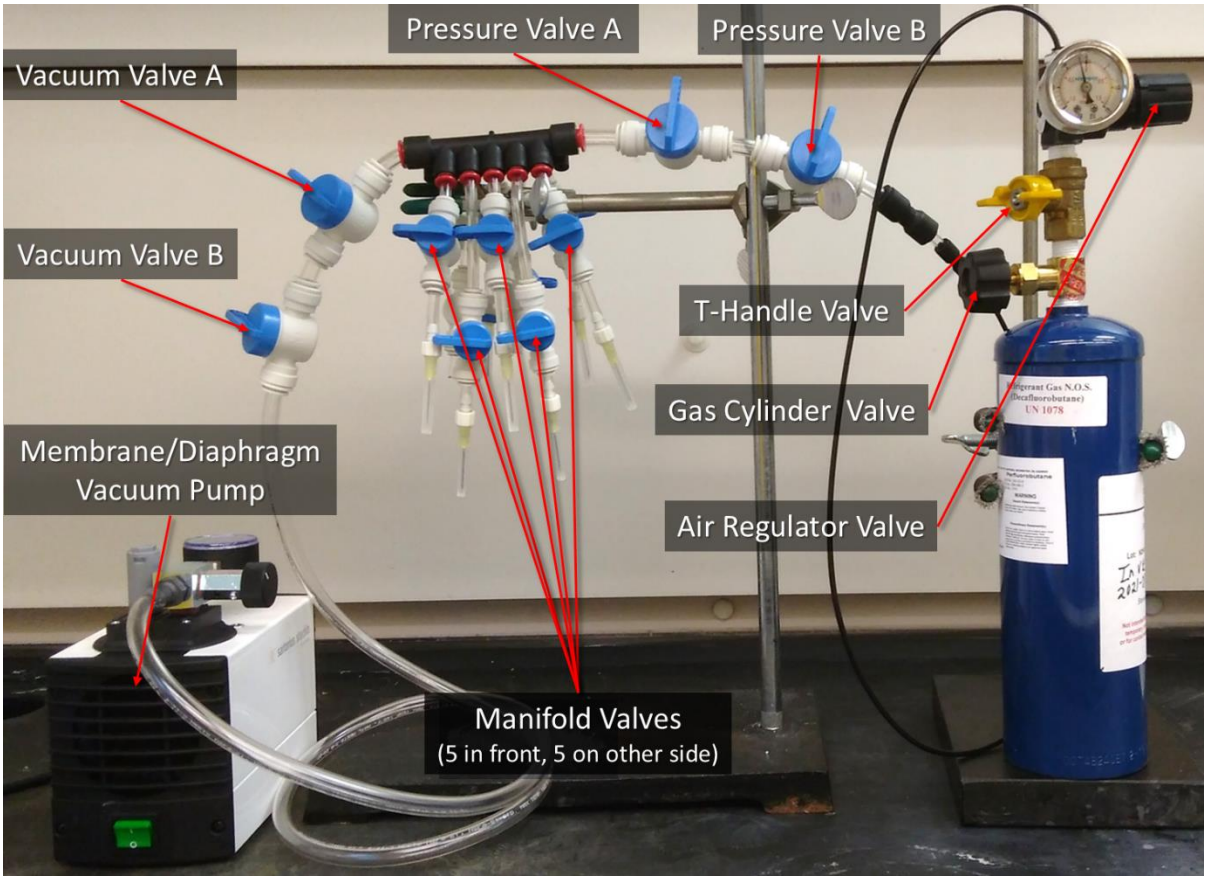

Figure S4: The completed Gas Exchanger. Valves have been labelled for reference. Retort bars are used to hold the manifold off of the floor and secure the gas cylinder.

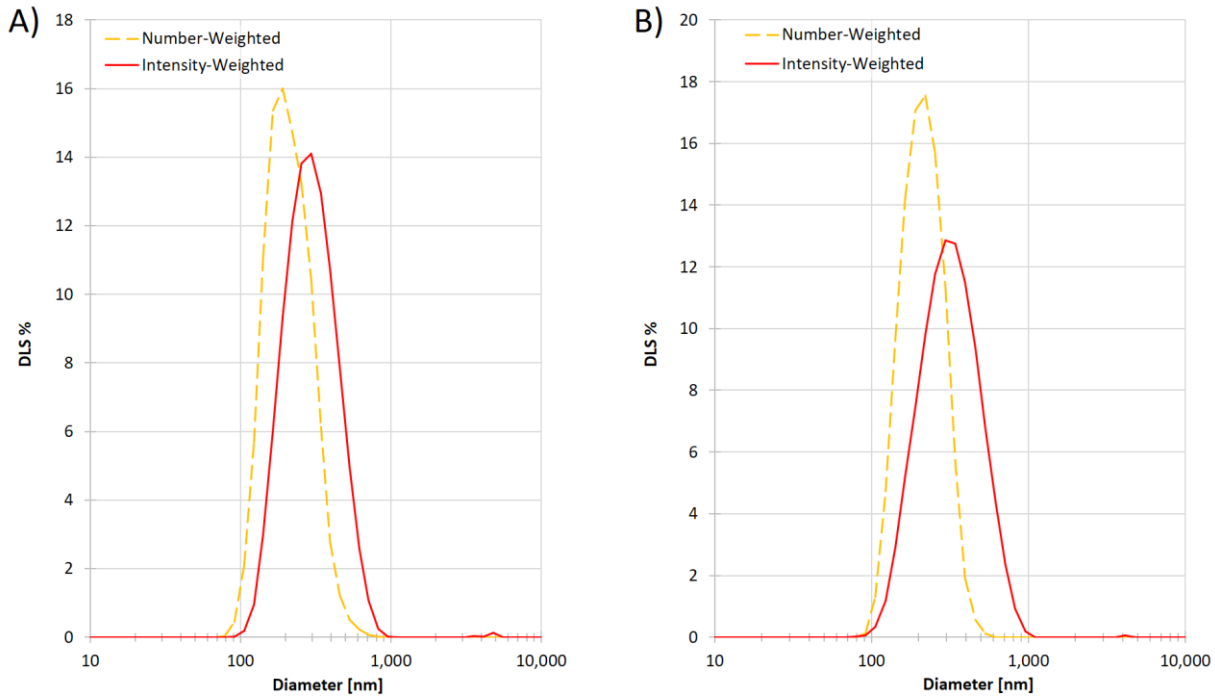

Figure S5: Representative DLS sizing for **A**) 0% Pyro Droplets. **B**) 1% Pyro Droplets

508
509
510

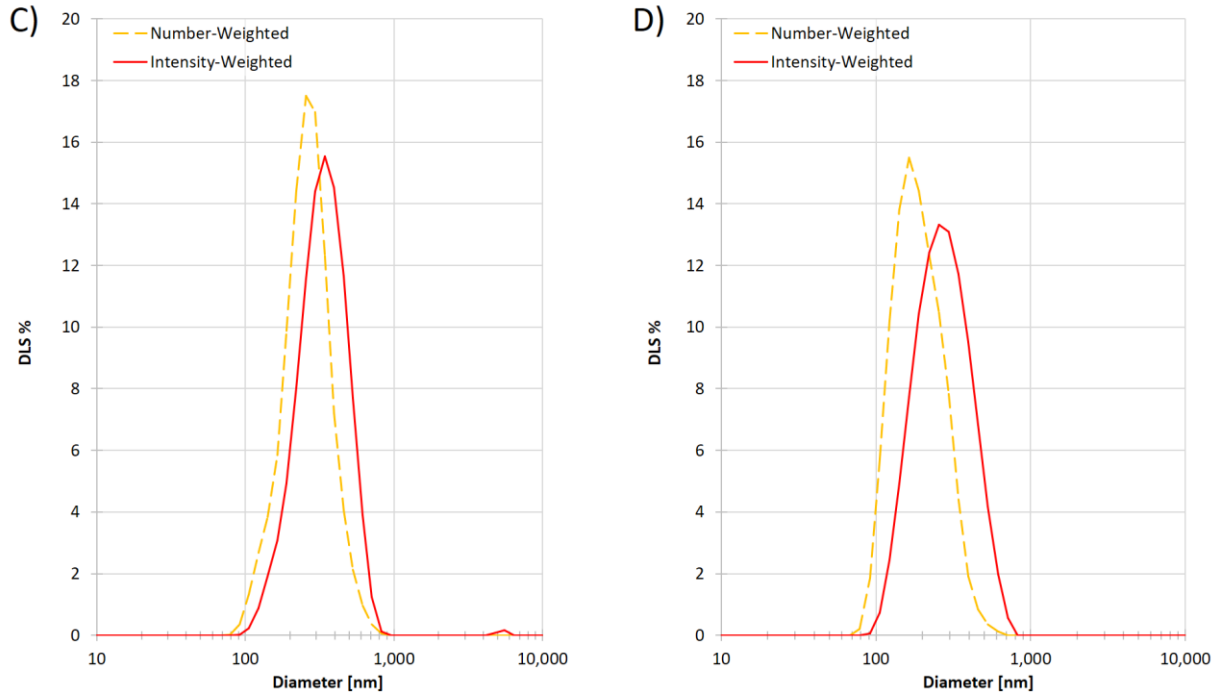

Figure S5: Representative DLS sizing for **C**) 10% Pyro Droplets. **D**) 20% Pyro Droplets

511
512
513

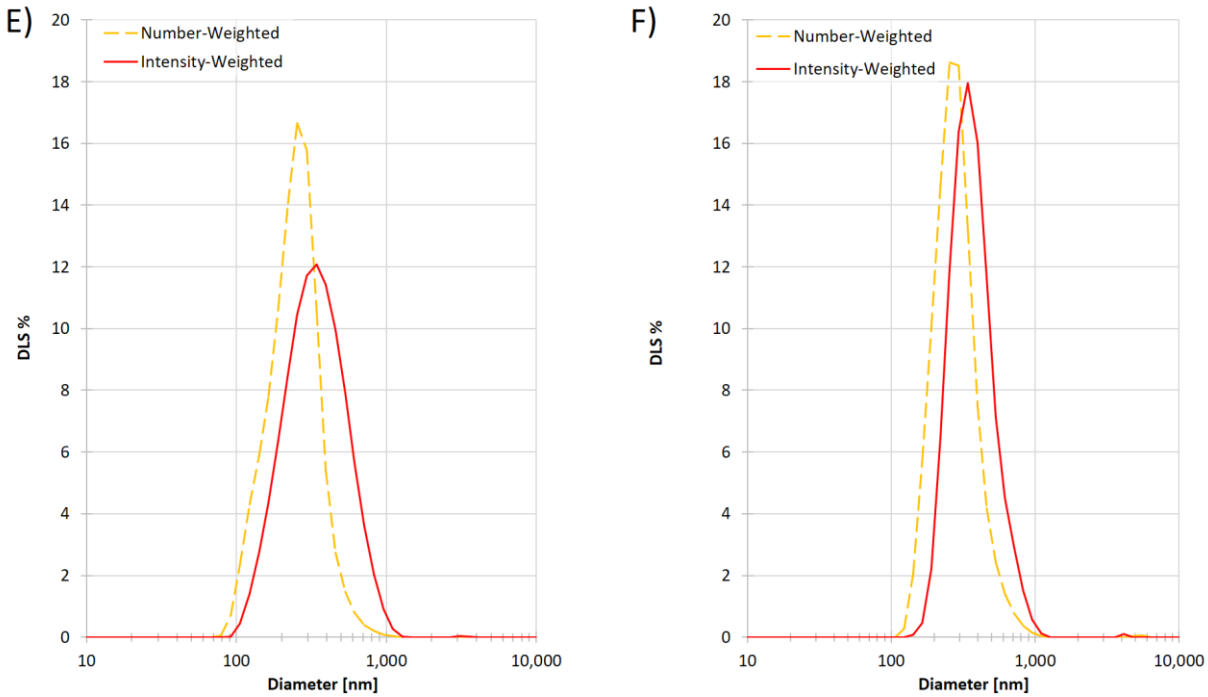

Figure S5: Representative DLS sizing for **E**) 30% Pyro Droplets. **F**) 40% Pyro Droplets

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515
516

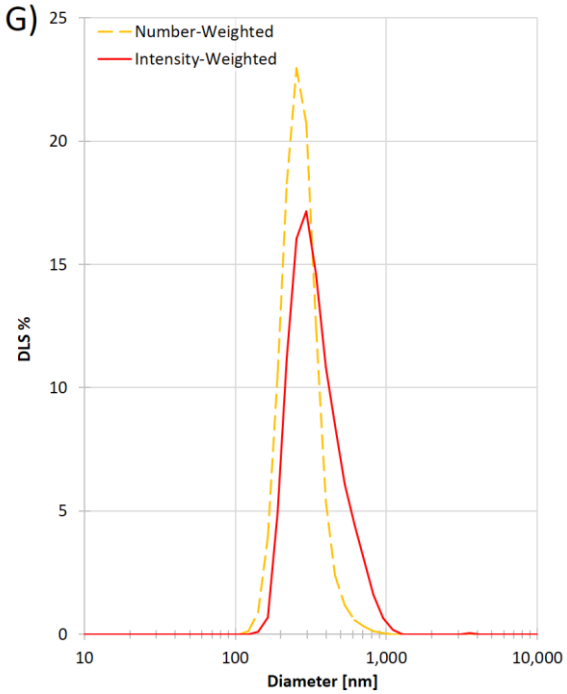

Figure S5: Representative DLS sizing for **G**) 50% Pyro Droplets.

517
518

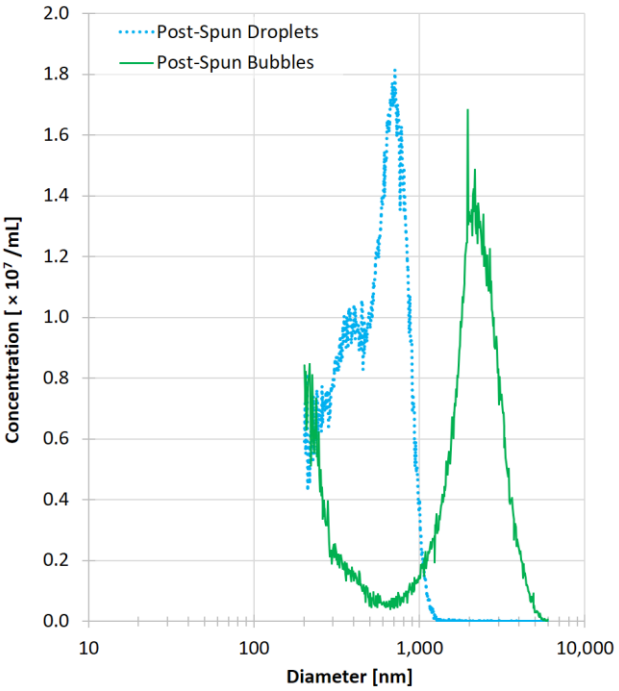

Figure S6: Centrifuged and washed 30% Pyro bubbles and corresponding droplets sized on the Coulter counter with a 10 µm aperture (*n* = 1). The post-spun bubble population had a peak diameter or 1961 nm while the post-spun droplets had a peak diameter of 713 nm.

ACKNOWLEDGMENTS:

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S3. Feshitan, J, Chen, C. C., Kwan, J. J., Borden, M. A. Microbubble size isolation by differential centrifugation. *Journal of Colloid and Interface Science*. **329** (2), 316-324.

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Lipid Concentration and Total Lipid Volume		
Total Lipid Conc. [mg/mL]	**1.00**	mg/mL
Total Volume [mL]	**10**	mL
Total Lipid Mass [mg]	10.00	mg

Group	Species	MW [g/mol]
mPEG 5000 †	DSPE-PEG5K (18:0 PEG5K PE) †	**5801.071**
Phosphate	DSPA (18:0 PA)	726.979
Phosphocholine	DSPC (18:0 PC)	790.145
Pyro-Lipid	Pyro-SPC (18:0 L PC) ‡	1040.317
	Pd-Pyro-SPC (18:0 L PC) ‡	1146.738
	Cu-Pyro-SPC (18:0 L PC) ‡	1103.863

† Due to polydispersity, check molecular weight
‡ DO NOT mix pyro-lipid types or else the docum

UV-Vis Absorbance Measurement		
Absorbance (600 to 800nm Peak)	**0.481452467**	abs [a.u.]
Chloroform Added to Pyro-Lipid [uL]	**1000**	uL
Cuvette: Methanol Volume [uL]	**2000**	uL
Cuvette: (Pyro-Lipid + Chloroform) Vol [uL]	**2**	uL
Cuvette Path Length [cm]	**1.00**	cm
Dilution Factor	1001.00	
Molar attenuation coef. [L/mol/cm] ‡	45,000	L/mol/cm
Conc Pyro-Lipid [mol/L]	1.071E-02	mol/L
Molecular Weight [g/mol]	1040.317	g/mol
Check Sample Mass [mg]	**11.141**	mg

Add (Pyro-Lipid + Chloroform) to Lipid Vial	Volume [uL]	
Pyro-SPC (18:0 PC)	**205.02**	uL
Pd-Pyro-SPC (18:0 PC)	**0.00**	uL
Cu-Pyro-Lipid (18:0 PC)	**0.00**	uL

Dissolving Solution	Vol Fraction	Volume [uL]

Chloroform	**0.90**	**4,500**
Methanol	**0.10**	**500**
Double-Distilled Water (ddWater)	**0.00**	**0**
Total	**1.00**	**5,000**

Rehydrate Excipient for Lipid Solution	**Vol Fraction**	**Volume [uL]**
Propylene Glycol	0.10	**1,000**
Glycerol	0.10	**1,000**
Phosphate Buffer Saline (PBS)	0.80	**8,000**
Total	**1.00**	**10,000**

Cell Legend

INPUT
OUTPUT
Background Calculation
Matched Values
Mismatched Values

Mole Fraction	MW*Fraction [g/mol]	Molar atten. coef. [L/mol/cm]	Mass [mg]	Moles
0.1	580.107	na	**4.25**	**7.319E-07**
0	0.000	na	**0.00**	**0.000E+00**
0.6	474.087	na	**3.47**	**4.391E-06**
0.3	312.095	45,000	**2.28**	**2.196E-06**
0	0.000	52,500	**0.00**	**0.000E+00**
0	0.000	66,000	**0.00**	**0.000E+00**
1.000	**1366.289**		10.00	7.319E-06
fraction	[g/mol]		[mg]	[mol]

on stock bottle.
ıent will get confused regarding the molar atten. coef.

DO NOT MIX PYRO-LIPIDS TYPES
DO NOT MIX PYRO-LIPIDS TYPES
DO NOT MIX PYRO-LIPIDS TYPES
DO NOT MIX PYRO-LIPIDS TYPES

uL	If no DSPA, use 0.9:0.1 = chloroform:methanol.
uL	If no DSPA, use 0.9:0.1 = chloroform:methanol.
uL	If DSPA, use: 0.6:0.32:0.08 = chloroform:methanol:ddWater.
uL	TOTAL

uL	Solvent and anti-freeze
uL	Anti-freeze
uL	Solvent and buffer
uL	TOTAL

Check Mole Fraction	Transition [°C]	Link 1	Link 2	Full Name
0.10		Avanti		1,2-distearoyl-sn-glycero-3-ph
0.00		Avanti		1,2-distearoyl-sn-glycero-3-ph
0.60	55	Avanti		1,2-distearoyl-*sn*-glycero-3-ph
0.30	55			Spirulina pacifica-derived pyro
0.00	55			Palladium-chelated pyropheop
0.00	55			Copper-chelated pyropheopho
1.00				
fraction	[deg C]			

osphoethanolamine-N-[methoxy(polyethylene glycol)-5000] ammonium salt
osphate sodium salt
nosphocholine
pheophorbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
horbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine
rbide conjugated 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine