Video Article

Synthesis of Phase-shift Nanoemulsions with Narrow Size Distributions for Acoustic Droplet Vaporization and Bubble-enhanced Ultrasound-mediated Ablation

Jonathan A. Kopechek, Peng Zhang, Mark T. Burgess, Tyrone M. Porter

Department of Mechanical Engineering, Boston University

Correspondence to: Tyrone M. Porter at tmp@bu.edu

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Abstract

High-intensity focused ultrasound (HIFU) is used clinically to thermally ablate tumors. To enhance localized heating and improve thermal ablation in tumors, lipid-coated perfluorocarbon droplets have been developed which can be vaporized by HIFU. The vasculature in many tumors is abnormally leaky due to their rapid growth, and nanoparticles are able to penetrate the fenestrations and passively accumulate within tumors. Thus, controlling the size of the droplets can result in better accumulation within tumors. In this report, the preparation of stable droplets in a phase-shift nanoemulsion (PSNE) with a narrow size distribution is described. PSNE were synthesized by sonicating a lipid solution in the presence of liquid perfluorocarbon. A narrow size distribution was obtained by extruding the PSNE multiple times using filters with pore sizes of 100 or 200 nm. The size distribution was measured over a 7-day period using dynamic light scattering. Polyacrylamide hydrogels containing PSNE were prepared for *in vitro* experiments. PSNE droplets in the hydrogels were vaporized with ultrasound and the resulting bubbles enhanced localized heating. Vaporized PSNE enables more rapid heating and also reduces the ultrasound intensity needed for thermal ablation. Thus, PSNE is expected to enhance thermal ablation in tumors, potentially improving therapeutic outcomes of HIFU-mediated thermal ablation treatments.

Video Link

The video component of this article can be found at http://www.jove.com/video/4308/

Protocol

1. Preparation of Phase-shift Nanoemulsion (PSNE)

1. Dissolve 11 mg DPPC and 1.68 mg DSPE-PEG2000 in chloroform
2. Evaporate the organic solvent to form a dry lipid film in a glass round-bottom flask
3. Dessicate the lipid film overnight
4. Rehydrate the lipid film with 5.5 ml of phosphate-buffered saline (PBS)
5. Heat solution in a 45 °C water bath until lipid film dissolves, vortexing periodically
6. Transfer lipid solution into 7 ml vial
7. Sonicate lipid solution for 2 min at 20% amplitude
8. Divide solution into two vials of 2.5 ml each (discard remaining 0.5 ml)
9. Add 2.5 ml PBS to each vial
10. Place each vial in a 0 °C ice-water bath
11. Add 50 µl DDFP to each vial
12. Sonicate each vial in the ice-water bath using the following settings: 25% amplitude, pulsed mode (10 sec on, 50 sec off), 60 sec total on time
13. Transfer PSNE solutions to 20 ml scintillation vials
14. Add 5 ml PBS to each vial, resulting in 10 ml final volume
15. Assemble extruder following directions provided by manufacturer
    1. Rinse each part with deionized water
    2. Place the stainless steel support disc in the center of the filter support base
    3. Place the stainless steel mesh on top of the stainless steel support disc
    4. Using tweezers, place an extruder drain disc membrane (shiny side up) on the stainless steel mesh
    5. Using tweezers, place the extruder filter (shiny side up) on the drain disc membrane
    6. Carefully place the small O-ring on the filter and place the thermobarrel and extruder top above the support base
    7. Partially tighten each wing-nut first, then completely tighten the wing-nuts by hand in an alternating fashion
    8. Connect the extruder to a nitrogen gas line

9. To prime the extruder, pipette 10 ml deionized water into the top sample port, cap the opening, and tighten the vent valve
10. Slowly open the nitrogen gas line to increase the pressure, forcing the sample through the membranes, and collect the sample from the outlet tubing
11. After use, disassemble in reverse order, rinse the extruder parts with deionized water, and discard the membrane filter and membrane drain disc

16. For 100 nm droplets only, pre-condition PSNE by extruding 10 times through 200 nm filter
17. Extrude PSNE 16 times through 100 nm or 200 nm filter to obtain narrow size distribution

2. Preparation of Polyacrylamide Hydrogel Containing PSNE

1. Prepare 24% BSA solution by diluting 1.2 g BSA powder in 5 ml deionized water
2. Prepare 10% APS solution by diluted 0.1 g APS powder in 1 ml deionized water
3. In the following order, mix 2.1 ml acrylamide solution, 1.2 ml Tris buffer, 0.1 ml 10% APS, 4.5 ml 24% BSA solution, and 3.6 ml deionized water in plastic chamber
4. Heat to 40 °C and place under vacuum for 1 hr
5. Add 480 µl of PSNE and thoroughly mix by gently swirling the plastic chamber.
6. Add 12 µl TEMED and place the chamber in a 12 °C water bath for 2 hr

3. Representative Results

A schematic of the setup for ultrasound experiments with tissue-mimicking hydrogel phantoms is shown in **Figure 1**. This protocol results in lipid-coated perfluorocarbon droplets with a narrow size distribution that are stable in solution for at least a week. The size distribution measured with dynamic light scattering (90Plus Particle Size Analyzer, Brookhaven Instruments, Holtsville, NY) is shown in **Figure 2** for PSNE extruded using 100 and 200 nm filters. The PSNE effective diameter over time, measured using dynamic light scattering, is listed in Table 1, demonstrating that PSNE are stable for at least a week. B-mode images of PSNE before and after vaporization in a polyacrylamide hydrogel are shown in **Figure 3**. Also, a lesion formed by 15 sec of HIFU-mediated heating in a polyacrylamide hydrogel containing albumin and PSNE is shown in **Figure 4**. The asymmetric shape of the lesion is a result of prefocal heating that occurs due to the presence of the bubble cloud in the ultrasound path. It is important to note that prefocal heating and lesion formation due to scatter from bubbles can be minimized by reducing the transmitted acoustic power.

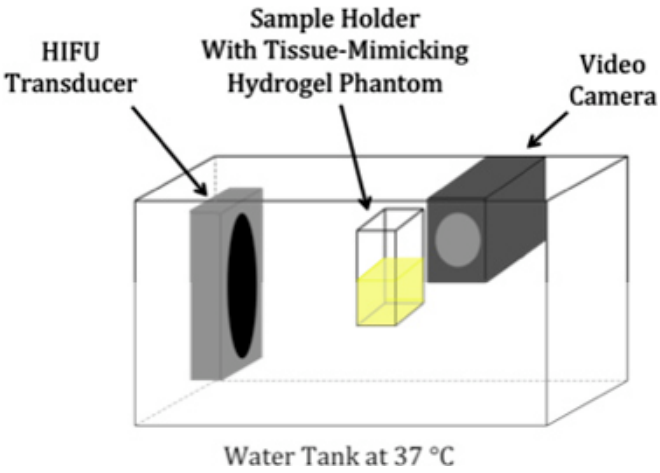

Figure 1. Schematic diagram of experimental setup for ultrasound experiments with tissue-mimicking hydrogels.

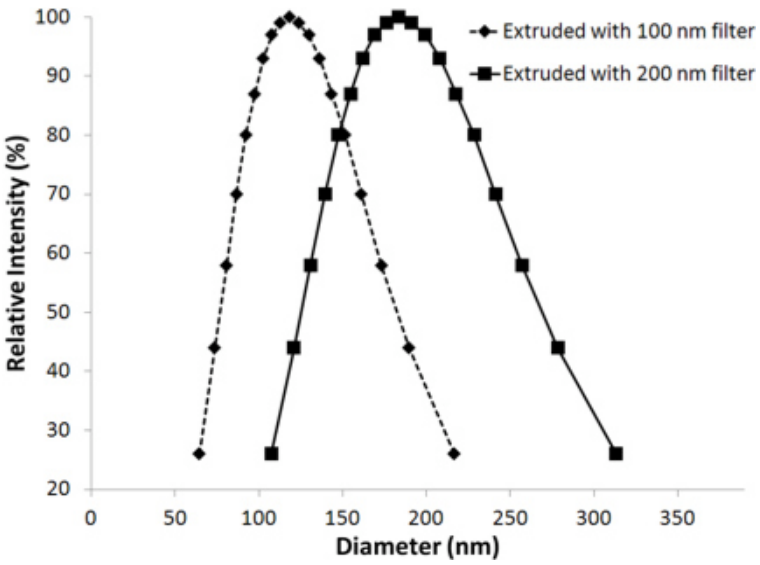

Figure 2. Size distribution of PSNE extruded through 100 nm or 200 nm filters, measured using dynamic light scattering. The units of the ordinate axes are based on the intensity of scattered light from particles of a certain size relative to the total scattered light intensity from the sample.

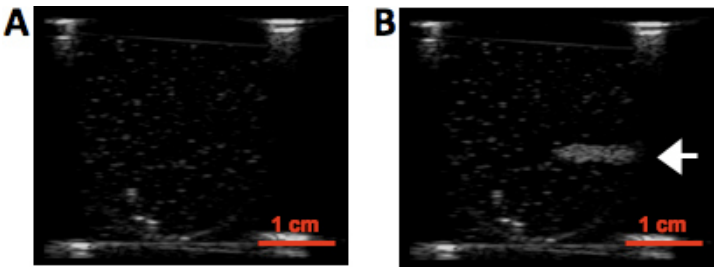

Figure 3. B-mode images (a) before and (b) after PSNE vaporization in a polyacrylamide hydrogel. The arrow indicates the focal region where a bubble cloud was formed by PSNE vaporization.

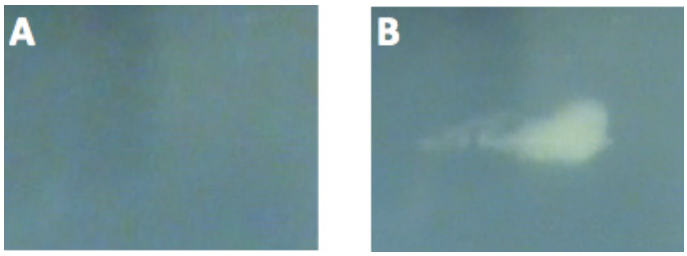

Figure 4. Images of polyacrylamide hydrogel containing albumin and PSNE (a) before and (b) after vaporization and sonication with HIFU, demonstrating lesion formation as a result of ultrasound-induced heating. The ultrasound center frequency was 3.3 MHz. The ultrasound signal consisted of an initial 30-cycle, 6.4 W pulse to vaporize PSNE, immediately followed by 15 sec of continuous ultrasound at 0.77 W.

Days after extrusion	***Extruded with 200 nm filter***		***Extruded with 100 nm filter***	
	Mean Dia. (nm)	**Std. Dev. (nm)**	**Mean Dia. (nm)**	**Std. Dev. (nm)**
1	182.9	4.9	118.0	0.9
7	177.7	2.5	124.8	3.1

Table 1. Mean diameter and standard deviation of PSNE at one and seven days after extrusion with 100 nm and 200 nm filters.

Discussion

High-intensity focused ultrasound (HIFU) is used clinically to thermally ablate tumors.[1] To enhance localized heating and improve thermal ablation in tumors, lipid-coated perfluorocarbon droplets have been developed which can be vaporized by HIFU. The vasculature in many tumors

is abnormally leaky due to their rapid growth.[2] Thus, nanoparticles are able to penetrate the fenestrations and passively accumulate within tumors, a process known as the enhanced permeability and retention (EPR) effect.[3] It has been shown that nanoparticles between 70 and 200 nm accumulate most efficiently in tumors.[4] The procedure described in this report produces a stable phase-shift nanoemulsion (PSNE) of lipid-coated perfluorocarbon droplets with a narrow size distribution. In the past, most studies used polydisperse size distributions of PSNE, but recent studies have focused on producing PSNE with narrow size distributions.[5, 6] The extrusion method described in this protocol allows one to control the size in order to increase the percentage of droplets administered systemically that will accumulate within tumors.

The dodecafluoropentane core of the nanodroplets has a boiling temperature of 29 °C.[7] Thus, it is important to maintain a low temperature during each step of the PSNE preparation. Sonication increases the temperature of the solution, but using a pulsed sonication sequence and placing the sample in an ice-water bath during sonication can reduce evaporation. Once the lipid-coated droplets have formed, the boiling temperature increases above 60 °C due to surface tension.[8] PSNE vaporization is temperature- and pressure-dependent and also depends on the size and composition of the liquid perfluorocarbon droplets.[9] For example, it was found that peak rarefactional pressures above 3.8 MPa were needed to vaporize 200 nm DDFP droplets at 37 °C.[10] Coating the droplets with lipids conjugated with poly(ethylene glycol) (PEG) inhibits fusion, thus increasing the size stability of PSNE over multiple days. Additionally, it has been documented that PEG can increase the circulation time of lipid-based vesicles,[11-13] which may increase the fraction of systemically administered PSNE that accumulate in localized malignancies.[14, 15]

The perfluorocarbon droplets can be suspended in a tissue-mimicking polyacrylamide hydrogel phantom containing albumin for *in vitro* thermal ablation studies.[16] The PSNE-loaded hydrogels are useful for assessing the vaporization thresholds as well as studying lesion formation from bubble-enhanced HIFU-mediated heating. The hydrogels absorb and convert acoustic energy into heat, and once the temperature in the hydrogel exceeds 58°C, albumin in the hydrogel denatures and becomes opaque.[17] Because the hydrogels are optically transparent, it is possible to observe protein denaturation in real time. Vaporization of PSNE within the hydrogels creates bubbles, which are used to increase the efficiency of ultrasound-mediated heating. Using a focused transducer, PSNE vaporization and bubble-enhanced heating can be localized, thus avoiding unwanted heating in intervening biological media (*i.e.* tissue). In the phantoms, the vaporized bubble cloud can affect the ultrasound beam propagation and cause prefocal heating, provided the acoustic power exceeds a threshold. Below this threshold, the scattered power is too low to ablate tissue in the prefocal region; consequently, the ablated volume is confined to the location of the bubble cloud. The use of PSNE to enhance localized heating *in vivo* could potentially improve outcomes of HIFU tumor ablation therapies. As a first step, an extrusion-based protocol has been developed to control the size of narrowly distributed PSNE. Using PSNE dispersed within optically-transparent tissue-mimicking hydrogels, it is possible to investigate the impact of vaporized PSNE on ultrasound-mediated heating and thermal ablation. Delivery of therapeutic agents and nanoparticles to the tumor core *in vivo* remains a challenge due to increased interstitial pressures that are found there. It is likely that PSNE would preferentially accumulate within the tumor periphery and may not easily penetrate the tumor core. Studies in hydrogels have shown that bubbles can redirect acoustic energy toward the transducer resulting in ablated volumes in the prefocal region. This phenomenon occurs when the transmitted acoustic power exceeds a specific threshold. Thus, it is possible to localize bubble-enhanced tumor ablation to the tumor periphery using one power setting as well as ablate the inner core by reflecting acoustic energy off bubbles created in distal margin at a higher power setting. Furthermore, precise ablation of the tumor periphery that avoids damaging the surrounding healthy tissue would still represent a significant breakthrough and could potentially allow previously non-resectable tumors to be removed surgically. Although there are differences between *in vivo* conditions and the tissue-mimicking hydrogels, the phantoms are useful for understanding the physical mechanisms of ultrasound-enhanced heating with PSNE in order to optimize the ultrasound parameters for thermal ablation. These are critical steps for translating the use of PSNE for enhancing ultrasound-mediated ablation from the laboratory to the clinic.

Disclosures

No conflicts of interest declared.

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