The Effects of Droplet Composition on Nanodroplet-Mediated Histotripsy

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Abstract

Nanodroplet-mediated histotripsy (NMH) is a targeted ablation technique combining histotripsy with nanodroplets that can be selectively delivered to tumor cells. In two previous studies, polymer encapsulated perfluoropentane nanodroplets were used to generate well-defined ablation similar to histotripsy but at significantly lower pressure when NMH therapy was applied at a pulse repetition frequency (PRF) of 10 Hz. However, cavitation was not maintained over multiple pulses when ultrasound was applied at a lower PRF (i.e. 1-5 Hz). In this study, we hypothesize that nanodroplets with a higher boiling point perfluorocarbon core will provide sustainable cavitation nuclei allowing cavitation to be maintained over multiple pulses even at low PRF, which is needed for efficient and complete tissue fractionation via histotripsy. To test this hypothesis, we investigate the effects of droplet composition on NMH therapy by applying histotripsy at various frequencies (345 kHz, 500 kHz, 1.5 MHz, 3 MHz) to tissue phantoms containing perfluoropentane (PFP, boiling point~29°C, surface tension~9.5 mN/m) and perfluorohexane (PFH, boiling point ~56°C, surface tension~11.9 mN/m) nanodroplets. First, the effects of droplet composition on the NMH cavitation threshold were investigated, with results demonstrating a significant decrease (>10 MPa) in the peak negative pressure (p_-) cavitation threshold for both types of nanodroplets compared to controls. A slight decrease (~1-3 MPa) in threshold was observed for PFP phantoms compared to PFH phantoms. Next, the ability of nanodroplets to function as sustainable cavitation nuclei over multiple pulses was investigated, with results demonstrating that PFH nanodroplets were sustainable cavitation nuclei over 1,000 pulses, while PFP nanodroplets were destroyed during the first few pulses (<50 pulses), likely due to the lower boiling point. Finally, tissue phantoms containing a layer of embedded red blood cells were used to compare the damage generated for NMH treatments using PFP and PFH droplets, with results showing PFH nanodroplets significantly improved NMH ablation, allowing for well-defined lesions to be generated at all frequencies and

- 1 **PRFs tested.** Overall, the results of this study provide significant insight in the role of droplet
- 2 composition in NMH therapy and provide a rational basis to tailor droplet parameters in order to
- 3 improve NMH tissue fractionation.
- 4 **Keywords:** Nanodroplet, histotripsy, perfluoropentane, perfluorohexane, cavitation

1 **Introduction**

2 Histotripsy is a noninvasive tissue ablation method that controllably fractionates soft tissue
3 through cavitation generated by high pressure, short duration ultrasound pulses (Xu et al. 2005b;
4 Parsons et al. 2006a; Roberts et al. 2006). Histotripsy depends on the initiation and maintenance of
5 a dense cavitation bubble cloud to produce mechanical tissue fractionation (Xu et al. 2005a; Parsons
6 et al. 2007). Previous work has shown that, using a 1-2 cycle pulse with a single dominant negative
7 pressure phase, cavitation bubbles can be reproducibly generated in tissue when the peak negative
8 pressure (p_-) is raised above the histotripsy intrinsic threshold of ~25-30 MPa (Maxwell et al. 2013;
9 Vlaisavljevich et al. 2015b). In order to effectively fractionate tissue into acellular debris,
10 histotripsy requires a dense cavitation bubble cloud to be initiated and maintained over multiple
11 pulses (often >100) until the tissue is completely fractionated into a liquid-appearing homogenate
12 with no cellular structures remaining (Xu et al. 2005b; Roberts et al. 2006; Hall et al. 2007).
13 Histotripsy is currently being studied for many clinical applications where non-invasive tissue
14 removal is desired including benign prostatic hyperplasia (Hempel et al. 2011), deep vein
15 thrombosis (Maxwell et al. 2011), congenital heart disease (Xu et al. 2010; Owens et al. 2011), and
16 cancer (Styn et al. 2010; Vlaisavljevich et al. 2013b).

17 Although histotripsy has shown promise for many clinical applications including tumor
18 ablation, this approach is limited to applications in which the target tissue can be identified and
19 imaged prior to treatment, which is often not feasible in cancer patients with many small tumor
20 nodules and micro-metastases. As a result, our group has developed a targeted ablation approach
21 combining polymer encapsulated nanodroplets with histotripsy (Vlaisavljevich et al. 2013a; Yuksel
22 Durmaz et al. 2014; Vlaisavljevich et al. 2015a). This nanodroplet-mediated histotripsy (NMH)
23 approach takes advantage of the significantly reduced cavitation threshold of the nanodroplets,
24 allowing for cavitation to be selectively generated only in regions where nanodroplets localize

(Vlaisavljevich et al. 2013a). NMH has the potential for selective ablation of tumors given the small size (~100-400 nm) of the synthesized nanodroplets, which enables their diffusion across the leaky tumor vasculature and preferential accumulation in the tumor tissue (Vlaisavljevich et al. 2013a; Yuksel Durmaz et al. 2014). Previous work has demonstrated that NMH can be used to create well-defined ablation similar to histotripsy but at significantly lower pressure and has demonstrated the potential to use NMH for simultaneous multi-focal ablation (Vlaisavljevich et al. 2013a). Furthermore, a previous study by Yuksel Durmaz *et al* investigated the optimal characteristics of polymer encapsulated perfluoropentane (PFP) nanodroplets, with results showing optimal NMH ablation for nanodroplets with a shell-cross-linked triblock amphiphilic copolymer composed of a poly(ethylene glycol) (PEG) block that forms a biocompatible corona, a poly(acrylic acid) (PAA) middle block reacting with the cross-linker to form a flexible shell, and a poly(heptadecafluorodecyl methacrylate-*co*-methyl methacrylate) (P(HDFMA-*co*-MMA)) fluorinated hydrophobic block encapsulating 2% v/v PFP (Yuksel Durmaz et al. 2014). However, although this previous study determined the optimal conditions for PFP nanodroplets, the effects of perfluorocarbon boiling temperature on NMH therapy have not been previously investigated.

In this study, we compare perfluoropentane (PFP, boiling point ~29°C) and perfluorohexane (PFH, boiling point ~56°C) nanodroplets for NMH therapy. Based on previous work comparing PFP and PFH droplets for acoustic droplet vaporization (Giesecke and Hynynen 2003; Fabiilli et al. 2009), we hypothesize that PFH nanodroplets will have a slightly higher cavitation threshold than PFP droplets but the cavitation threshold of both droplets will be significantly lower than the histotripsy intrinsic threshold. To test this hypothesis, tissue phantoms containing PFP nanodroplets, PFH nanodroplets, and no nanodroplets were exposed to histotripsy pulses produced by 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz custom-built histotripsy transducers. The probability of generating inertial cavitation from a single 1-2 cycle histotripsy pulse was measured, with the cavitation

threshold defined as the peak negative pressure at which the probability of generating cavitation, p_{cav} , from a single histotripsy pulse was $p_{cav}=0.5$. In addition to the effects of droplet composition on the cavitation threshold, we also investigate the effects of droplet composition on cavitation sustainability over multiple histotripsy pulses. In a previous studies, PFP nanodroplets were used to create consistent, well-defined fractionation at pressure levels (11-20 MPa) significantly below the histotripsy intrinsic threshold (~26-30 MPa) in tissue phantoms at a pulse repetition frequency (PRF) of 10 Hz by maintaining cavitation over multiple pulses (Vlaisavljevich et al. 2013a; Yuksel Durmaz et al. 2014). However, it was also observed that cavitation was not maintained over multiple pulses when ultrasound was applied at a lower PRF (1 Hz) (Vlaisavljevich et al. 2013a; Yuksel Durmaz et al. 2014; Vlaisavljevich et al. 2015a). A low PRF (1 Hz) has been shown to produce more efficient tissue fractionation and is not affected by the cavitation memory effect, in which residual gas bubbles from previous cavitation events function as nuclei for generating cavitation on a subsequent pulse (Wang et al. 2012). This result suggests that PFP nanodroplets are destroyed during the first few pulses, requiring cavitation on subsequent pulses to be generated from residual nuclei remaining from previous pulses. We hypothesize that, due to their higher boiling point, PFH nanodroplets will re-condense into a liquid after cavitation and remain as sustainable nuclei over multiple (>100) pulses, allowing cavitation to be maintained over multiple pulses even at low PRF. To test this hypothesis, 1000 histotripsy pulses were applied to a single focal zone tissue in phantoms containing PFP and PFH nanodroplets, and the number of NMH bubbles generated was compared after each pulse. In addition, tissue phantoms containing a layer of embedded red blood cells were used to compare the damage generated for NMH treatments using PFP and PFH droplets. Overall, these results will improve our understanding of the NMH process and help to determine the optimal nanodroplet characteristics for NMH therapy.

1 **Materials**

2 Methyl methacrylate (MMA, Sigma-Aldrich, 99 %), 3,3,4,4,5,5,6,6,7,7,8,8,9,9,10,10,10
3 heptadecafluorodecyl methacrylate (HDFMA, Sigma-Aldrich, 97 %), *tert*-butyl acrylate (*t*BA,
4 Sigma-Aldrich, 98 %) and *N, N, N', N'', N''*-pentamethyldiethylenetriamine (PMDETA, Sigma-
5 Aldrich, 99 %) were passed through a basic alumina column to remove the inhibitor. Copper (I)
6 bromide (CuBr, Sigma-Aldrich, 99.9 %), 2-bromoisobutyryl bromide (Fluka, >97 %),
7 tetrahydrofuran anhydrous (THF, Sigma-Aldrich, >99.9 %), *N,N'*-Dicyclohexylcarbodiimide (DCC,
8 Sigma-Aldrich, 99 %), dimethylaminopyridine (DMAP, Acros, 99 %), 4-pentynoic acid (Sigma-
9 Aldrich, 99 %), furan (Sigma-Aldrich, ≥99 %), maleic anhydride (Fluka, ≥99 %), 9-anthracene
10 methanol (Aldrich, ≥ 99%), perfluoropentane (PFP, Alfa Aesar, 97% ca. 85% *n*-isomer),
11 perfluorohexane (PFH, SynQuest Lab, > 98%) *N*-hydroxy succinimide (NHS, Fluka, 97 %), *N*-(3-
12 Dimethylaminopropyl)-*N* ethylcarbodiimide hydrochloride (EDC, Fluka >98 %), poly(ethylene
13 glycol) monomethylether (Me-PEG, M_n : 2000 g/mol, Sigma-Aldrich), sodium azide (NaN₃, Acros,
14 99 %), 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES, Acros, 99 %), triethylamine
15 (TEA, Sigma-Aldrich, ≥ 99%), trifluoroacetic acid (TFA, Acros, 99 %), ethylene carbonate (Sigma-
16 Aldrich, 98 %), 2,2`-(ethylenedioxy)-bis(ethylamine) (Sigma-Aldrich, 98%) agarose powder (Type
17 VII, Sigma-Aldrich), citratephosphate-dextrose (CPD, Sigma-Aldrich), heptane fraction (Sigma-
18 Aldrich, > 99%), dichloromethane (DCM or CH₂Cl₂, Sigma-Aldrich, >99.5%) were used as
19 received.

20

21 **Methods**

22 **Nanodroplet Formulation and Characterization**

23 A well-defined, triblock amphiphilic copolymer containing a hydrophilic poly(ethylene
24 glycol) (PEG) block, a middle block poly(acrylic acid) (PAA) block, and a hydrophobic random

1 copolymer of heptadecafluorodecyl methacrylate (HDFMA) and methyl methacrylate (MMA) was
2 synthesized using a combination of atom transfer radical polymerization (ATRP) and “click”
3 coupling techniques as previously described (**Fig.1**) (Yuksel Durmaz et al. 2014). The synthesized
4 P(HDFMA_{8-co}-MMA₂₀)-*b*-PAA₁₂-*b*-PEG₄₅ triblock amphiphilic copolymer was used to prepare
5 PFP- and PFH-loaded nanodroplets. Briefly, the copolymers were dissolved in tetrahydrofuran
6 (THF) (0.2% w/v) and cooled down to 0 °C before the addition of PFP (2% v/v) or PFH (2% v/v)
7 while vigorously stirring the copolymer-perfluorocarbon mixture. An equal amount of deionized
8 water was added drop-wise to this solution mixture to initiate micelle formation and the mixture
9 was stirred for 1h in an ice bath. The micelles solution was transferred into a dialysis bag (MWCO
10 of 1 KDa, Spectrum, Rancho Dominguez, CA) and dialyzed overnight against ice-cold MES
11 solution (pH 5.5) to remove the THF and get a milky solution of non-cross-linked PFP-loaded
12 nanodroplets and non-cross-linked PFH-loaded nanodroplets. The milky nanodroplets solutions
13 were transferred to round bottom flasks and mixed with the 2,2'-(ethylenedioxy)-bis(ethylamine)
14 cross-linker, which reacts with the carboxyl groups of the central PAA block in the copolymer via
15 NHS/EDC coupling chemistry forming cross-linked nanodroplets with a flexible polymer shell.
16 Shell cross-linked nanodroplets were dialyzed against ice-cold water for 12 hours to remove
17 unreacted cross-linker and reaction byproducts.

18 Concentration and size distribution of the nanodroplets were measured using Nanoparticle
19 Tracking Analysis (NTA). Briefly, the NanoSight™ LM10 (Malvern Instruments, Amesbury, UK),
20 equipped with a temperature-controlled 405 nm laser module, high sensitivity scientific
21 Complementary Metal–Oxide–Semiconductor (sCMOS) camera (Hamamatsu, Orca, Hamamatsu
22 City, Japan), and a syringe pump was used for the collection of NTA data. Upon diluting the
23 nanodroplets solution to the appropriate particle concentration with deionized water, image capture
24 and analysis was carried out using the NTA software (Version 3.0, Build 0066) at 37 °C.

Concentration and size distribution of the nanodroplets in each sample were analyzed using 5 videos per sample each lasting 60s. Based on these videos, nanodroplet concentration was plotted as 6 a function of droplet size, with the error bars representing the standard deviation of the repeat 7 measurements of each sample. The mean size and standard deviation values obtained by the NTA 8 software correspond to arithmetic values calculated with the sizes of all particles analyzed for each 9 sample (n=5).

Preparation of Tissue Phantoms

Agarose phantoms were used to provide a well-controlled viscoelastic medium for this study. Tissue phantoms containing 1% agarose w/v were prepared by slowly mixing agarose powder (Agarose Type VII; Sigma-Aldrich, St. Louis, MO, USA) into saline solution (0.9% sodium chloride; Hospira, Lake Forest, Illinois, USA) heated to boiling temperature. The solution was stirred on a hot plate until the gel turned completely transparent and then allowed to boil for ten minutes. After boiling, solutions were allowed to cool and were degassed under a partial vacuum (~20 kPa, absolute) for 30 minutes. After degassing, phantoms containing nanodroplets were prepared by slowly adding the nanodroplets (2.0×10^8 particles/ml) into the agarose solution while stirring. The agarose mixtures were poured into rectangular polycarbonate holders with acoustic windows and placed in a refrigerator at 4°C to allow the solution to solidify, forming tissue phantoms with embedded PFP nanodroplets, PFH nanodroplets, or without nanodroplets (control).

The temperature of the agarose was ~40°C when the nanodroplets were added. As this temperature is close to the temperature at which the NTA analysis was performed (37°C), it is expected that the size and concentration of nanodroplets was not be significantly affected by the casting process. This hypothesis is further supported by previous studies showing that PFP and PFH nanodroplets have vaporization temperatures much higher than their reported boiling points. For example, the

vaporization temperatures of PFP and PFH nanodroplets between 250-350 nm have been reported to be higher than 80°C and 140°C, respectively, suggesting that the droplets will remain stable during the casting process (40°C) and experimental temperatures (37°C) (Rapoport et al. 2009; Sheeran et al. 2011).

For cell fractionation experiments, tissue phantoms with a red blood cell (RBC) layer were prepared using porcine RBCs in 0.9% isotonic saline and 1% agarose solutions with PFP or PFH nanodroplets. Fresh porcine blood was obtained from research subjects in an unrelated study and added to an anticoagulant solution of citratephosphate-dextrose (CPD) (Sigma-Aldrich Co., St. Louis, MO) with a CPD-to-blood ratio of 1:9 mL. Whole blood was separated in a centrifuge at 3000 rpm for 10 min. The plasma and white buffy coat were removed, and the RBCs were saved for addition to the phantom. To prepare the RBC phantom, an initial layer of 1% agarose mixture (with PFP or PFH nanodroplets) was poured into a rectangular polycarbonate housing to fill half of it at 40°C. The housing was placed in a refrigerator at 4°C to allow the agarose to cool and solidify. The remaining solution was kept at 40°C. A small amount of agarose solution was mixed with the RBCs (5% RBCs v/v). The frame with solidified agarose was removed from refrigeration, and a thin layer of the RBC-agarose solution was poured onto the gel surface to allow the entire surface to coat in a thin layer. After 5 min, the RBC-agarose layer was solidified, and the remaining agarose solution without RBCs was poured to completely fill the frame. This procedure created a thin layer of RBCs suspended in the center of the agarose phantom.

Histotripsy Pulse Generation

Histotripsy pulses were generated at four ultrasound frequencies (345 kHz, 500 kHz, 1.5 MHz, and 3 MHz) using three custom-built histotripsy transducers. The 345 kHz pulses were generated by a twenty-element array transducer with a geometric focus of 150 mm, an aperture size

of 272 mm, and an effective f-number of 0.55. The 1.5 MHz pulses were generated by a six-element array transducer with a geometric focus of 55 mm, an aperture of 79 mm in the elevational direction and 69 mm in the lateral direction, and effective f-numbers of 0.7 and 0.8 in the elevational and lateral directions, respectively. The 500 kHz and 3 MHz pulses were generated by a dual frequency array transducer that consisted of twelve 500-kHz elements and seven 3-MHz elements. For the 500 kHz elements, the geometric focus was 40 mm, the aperture size was 71 mm, and the effective f-number was 0.56. For the 3 MHz elements, the geometric focus was 40 mm, the aperture size was 80 mm, and the effective f-number was 0.5. The design of the dual frequency transducer has been described in detail in a previous study (Lin et al. 2014a).

To compare the NMH cavitation threshold with the histotripsy intrinsic threshold, short pulses with a single dominant negative pressure half-cycle were applied to the tissue phantoms with and without nanodroplets inside a water bath heated to at 37 °C. To generate a short therapy pulse, a custom high-voltage pulser developed in-house was used to drive the transducers. The pulser was connected to a field-programmable gate array (FPGA) board (Altera DE0-Nano Terasic Technology, Dover, DE, USA) specifically programmed for histotripsy therapy pulsing. This setup allowed the transducers to output short pulses of less than two cycles. A fiber-optic probe hydrophone built in-house (Parsons et al. 2006b) was used to measure the acoustic output pressure of the transducers. At higher pressure levels ($p > 23$ MPa), the acoustic output could not be directly measured due to cavitation at the fiber tip. These pressures were estimated by a summation of the output focal p - values from individual transducer elements. This approximation assumes minimal nonlinear distortion of the waveform occurs within the focal region. In a previous study (Maxwell et al. 2013), this estimated p - was found to be accurate within 15% compared to direct focal pressure measurements in water and in a medium (1,3 butanediol) with a higher cavitation threshold. Sample acoustic waveforms produced by the four frequency transducers are shown in **Figure 2A**.

Optical Imaging and Image Processing

High speed optical imaging was used to capture images of the focal zone after the propagation of each pulse through the focus using two high speed cameras (**Fig. 2B**). For experiments with the 345 kHz and 1.5 MHz transducers, a high-speed, 1 megapixel CCD camera (Phantom V210, Vision Research, Wayne, NJ, USA) was aligned with the transducer and backlit by a continuous white-light source. The camera was focused using a macro-bellows lens (Tominon, Kyocera), giving the captured images a resolution of approximately 5.9 μm per pixel and 3.4 μm per pixel for 345 kHz and 1.5 MHz, respectively. For experiments with the 500 kHz and 3 MHz dual frequency transducer, a digital, 1.3-megapixel CCD camera (PN: FL3-U3-13Y3M-C, Flea® 3, PointGrey, Richmond, BC, Canada) was positioned perpendicularly to the dual-frequency array transducer facing one of the transducer's optical windows. A Nikon 4X objective was attached to the camera with extension tubes to magnify the image plane, giving the captured images a resolution of approximately 2.5 μm per pixel. A pulsed white-light LED was placed on the diametrically-opposed optical window of the dual-frequency array transducer, which provided backlit illumination. The cameras were triggered to record one image for each applied pulse. After acquisition, shadowgraph images were converted from grayscale to binary by an intensity threshold determined by the background intensity using image processing software (MATLAB, The Mathworks, Natick, MA, USA), as described in a previous study (Maxwell et al. 2013). Bubbles were indicated as any black regions >5 pixels. By this criterion, the minimum resolvable bubble radius was 14.75 μm , 6.25 μm , 8.5 μm , and 6.25 μm for the 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz transducers, respectively.

Passive Cavitation Detection (PCD)

In addition to high speed imaging, an acoustic method was used to identify cavitation in the focal zone for cavitation threshold experiments. For each experiment, one of the transducer's therapy elements was also used for PCD to detect the presence of cavitation in the focal region (**Fig. 2B**). The PCD signal was connected to an oscilloscope (LT372; Lecroy, Chestnut Ridge, NY, USA) with the time window selected to record the backscattering of the therapy pulse from cavitation bubbles (Maxwell et al. 2013; Vlasisavljevich et al. 2014; Vlasisavljevich et al. 2015b). To determine whether cavitation occurred during a pulse, the signal generated by backscattering of the incident pulse from the focus was analyzed following the method used in previous studies (Maxwell et al. 2013; Vlasisavljevich et al. 2015b). A significant fraction of the incident wave energy is scattered when a cavitation bubble expands, greatly increasing the backscattered pressure amplitude received by the PCD. This signal appeared on the PCD at the time point corresponding to two times the time of flight for the focal length of the respective transducers. The integrated frequency power spectrum (S_{PCD}) of the backscatter signal was used as a measure of whether cavitation occurred according to the method previously described by Maxwell *et al* (Maxwell et al. 2013).

NMH Cavitation Threshold

For cavitation threshold experiments, 100 pulses were applied to each sample **inside a water bath heated to 37 °C** at each pressure level at a pulse repetition frequency (PRF) of 0.5 Hz. The PRF was kept low to minimize the possibility that cavitation from one pulse would change the probability of cavitation on a subsequent pulse. In a previous study, it was demonstrated that cavitation during a pulse increased the likelihood of cavitation on a following pulse for PRFs > 1 Hz, , but this effect was not observed for PRFs < 1 Hz (Maxwell et al. 2013). In addition to this low PRF, the focus was translated for each pulse by 1 mm transverse to the acoustic propagation direction in a 10 × 10 grid in order to minimize the effects of cavitation damage to the nanodroplets

or tissue phantoms from altering the probability of cavitation. For each pulse, cavitation was monitored using both high speed imaging and PCD, and the fraction of total pulses (out of 100) for which cavitation was detected was determined as the cavitation probability.

The probability of observing cavitation followed a sigmoid function, given by

$$P(p_-) = \frac{1}{2} + \operatorname{erf}\left(\frac{p_- - p_t}{\sqrt{2}\sigma}\right) \quad (\text{E1})$$

where erf is the error function, p_t is the negative pressure at which the probability $p_{cav}=0.5$, σ is a variable related to the width of the transition between $p_{cav}=0$ and $p_{cav}=1$, with $\pm \sigma$ giving the difference in pressure from about $p_{cav}=0.15$ to $p_{cav}=0.85$ for the fit (Maxwell et al. 2013). The cavitation threshold for each sample, p_t , is defined as the p - corresponding to $p_{cav}=0.5$ as calculated by the curve fit. Curve fitting for all data sets was performed using an OriginLab curve fitting program (OriginPro 9.1; OriginLab Corporation, Northampton, MA, USA). The fit curves for all samples were analyzed statistically to determine whether the differences in the values of p_t were significantly different from each other. The standard errors for p_t were estimated by a covariance matrix using the delta method (Hosmer and Lemeshow 1992). The curves were compared using a two-sample t-test with statistic $t\left(p_{int1} - p_{int2}, \sqrt{SE_1^2 + SE_2^2}\right)$ at a 95% confidence interval. Results were considered statistically significant for $p < 0.05$. Note that the standard error does not include the uncertainty in absolute pressure from the hydrophone measurement, only the uncertainty in the fit. A sample size of 3 tissue phantoms was used for each experimental condition (i.e. PFP nanodroplets, PFH nanodroplets, or no nanodroplets).

NMH Multi-pulse Sustainability

To test the hypothesis that PFP nanodroplets will remain sustainable cavitation nuclei over multiple pulses while PFP nanodroplets will be destroyed during the first few pulses, 1000

ultrasound pulses were applied to a single focal region in tissue phantoms containing PFP and PFH nanodroplets inside a water bath heated to 37 °C. Pulses were applied at a PRF of 1 Hz and peak negative pressures of 11.8 MPa (345 kHz), 12.6 MPa (500 kHz), 14.3 MPa (1.5 MHz), and 15.6 MPa (3 MHz). The PRF in this study was kept low (1 Hz) to minimize the contributions of residual nuclei from a previous pulse from effecting cavitation generation on a subsequent pulse in order to determine if PFP and PFH nanodroplets continue to function as viable cavitation nuclei after the first few pulses or if the nanodroplets are destroyed in the cavitation process. Furthermore, a PRF of 1 Hz was also chosen as previous work has shown an increase in ablation efficiency for 1 Hz treatments in comparison to higher PRF treatments that rely on residual nuclei from previous pulses to maintain the cavitation bubble cloud (Wang et al. 2012; Lin et al. 2014b). Cavitation was monitored using high speed optical imaging, and the number of bubbles produced by each pulse was compared for 1000 histotripsy pulses in each sample.

NMH RBC Phantom Ablation

Agarose gel phantoms with an embedded RBC layer were used to characterize cell fractionation induced by NMH. Fractionation of the RBCs turns the color of the embedded cell layer from opaque red to translucent as the RBCs are lysed, which allows direct visualization of the histotripsy-induced fractionation process (Maxwell et al. 2010). Previous studies have also shown that the lesion visualized in RBC phantoms is similar to the lesion generated in tissue identified by histology (Maxwell et al. 2010). For RBC experiments, 2000 histotripsy pulses were applied to the center of the red blood cell phantom layer at PRFs of 1 Hz and 10 Hz (n=4). The bubble cloud and resulting cell fractionation were recorded by high-speed optical imaging after each pulse. Cell fraction was compared between RBC phantoms with PFP and PFH nanodroplets.

Results

Nanodroplets Characterization

In this study, we aimed to encapsulate PFH into the triblock amphiphilic P(HDFMA₈-*co*-MMA₂₀)-*b*-PAA₁₂-*b*-PEG₄₅ copolymer in order to compare the cavitation ability of PFH-loaded nanodroplets with PFP-loaded nanodroplets. In our previous study, we demonstrated that PFP can be encapsulated in the fluorinated copolymer, forming nanodroplets containing an elastic shell with an average size of ~100-400 nm (Yuksel Durmaz et al. 2014). In contrast to PFP, the solubility of PFH is limited in most common polar solvents as well as water. Moreover, PFH is not miscible with any of these solvents and exhibits a clear phase separation from organic solvents. In this study, we hypothesized that PFH could be encapsulated by the P(HDFMA₈-*co*-MMA₂₀)-*b*-PAA₁₂-*b*-PEG₄₅ copolymer forming nanodroplets with similar size and characteristics to the PFP droplets described in our previous study (Yuksel Durmaz et al. 2014). Results demonstrate that both PFP and PFH encapsulated into stable nanodroplets in the desired size range (100-600 nm). NTA size analysis showed similar characteristics for the PFP and PFH droplets (**Fig.3**). PFP-loaded nanodroplets size ranged from 100-450 nm with the major peak <300 nm. The size distribution of PFH-loaded nanodroplets was slightly larger than the PFP-loaded ones, ranging from 100-600 nm. Three additional high-intense peaks in the range of 200-300 nm, 300-450 nm, and 450-600 nm were observed in the large size portion of the PFH nanodroplet size plot (**Fig.3B**). The larger size of the PFH-loaded nanodroplets is possibly due to the difference in PFH miscibility with the THF/copolymer mixture compared to PFP. For example, PFP was observed to homogeneously disperse in the THF/copolymer mixture in the beginning of droplets formulation, whereas PFH remained separate from the mixture on the bottom of the round-bottom flask. This energetically-favored phase separation was broken up by drop-wise addition of water to trigger the micellization process of the amphiphilic copolymer under vigorous stirring. This strategy allowed for uniform

1 mixing of the PFH and efficient encapsulation in the droplets core. Both types of droplets were
2 dispersed throughout the aqueous solution without aggregation or settling down in solution due to
3 the hydrophilic PEG corona. PFP- and PFH-loaded nanodroplets exhibited similar concentrations
4 and size distribution (**Fig.3**). The error bars on the size distribution plots represent the standard
5 deviation of the repeat measurements of each sample. The mean size and standard deviation values
6 obtained by the NTA software correspond to arithmetic values calculated with the sizes of all
7 particles analyzed for each sample (n=5). Results from all samples demonstrated an average size of
8 177.9 ± 1.9 nm and 233.9 ± 3.9 nm for PFP and PFH nanodroplets, respectively.

10 **NMH Cavitation Threshold**

11 To investigate the effects of nanodroplet composition on the NMH threshold, histotripsy
12 pulses were applied to tissue-mimicking agarose phantoms with PFP nanodroplets, PFH
13 nanodroplets, and no nanodroplets using the 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz histotripsy
14 transducers. For all experimental conditions, cavitation bubbles were observed on the high-speed
15 camera in an increasingly larger area with increasing pressure once a certain negative pressure was
16 exceeded, with complete agreement between optical imaging and PCD detection methods (**Fig.4**),
17 as seen in previous studies (Maxwell et al. 2013; Vlaisavljevich et al. 2015b). Plotting the
18 probability of cavitation as a function of peak negative pressure demonstrated a significant decrease
19 in the cavitation threshold with both PFP and PFH nanodroplets compared to controls at all
20 frequencies (**Fig.5**). Additionally, results showed a slight increase in the nanodroplet cavitation
21 threshold for PFH nanodroplets compared to PFP nanodroplets (**Fig.5**). Cavitation threshold results
22 for all experimental conditions are listed in **Table 1**. Comparing the threshold results for phantoms
23 containing PFH nanodroplets with control phantoms demonstrated that the cavitation threshold was
24 decreased by 14.4 MPa, 15 MPa, 13.7 MPa, and 11.9 MPa at 345 kHz, 500 kHz, 1.5 MHz, and 3

MHz, respectively (**Fig.6**). When comparing phantoms containing PFP and PFH nanodroplets (**Fig.6**), a decrease of 3 MPa, 1.3 MPa, 2.5 MPa, and 1.7 MPa was observed for PFP phantoms at 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz, respectively (**Fig.6**). For all experimental conditions, the cavitation threshold was observed to decrease at lower frequency, as has been observed in previous studies (**Fig.6**) (Vlaisavljevich et al. 2013a; Vlaisavljevich et al. 2015b). A two-way ANOVA was performed on the data shown in **Figure 6**, demonstrating all differences between the cavitation threshold in PFP, PFH, and control phantoms were significant ($p<0.05$).

NMH Multi-pulse Sustainability

To compare the ability of nanodroplets to act as sustainable cavitation nuclei over multiple pulses, 1000 histotripsy pulses were applied to a single focal region in phantoms containing PFP and PFH nanodroplets at a PRF of 1 Hz. Results demonstrated that a bubble cloud consisting of many bubbles was observed after the first pulse for both types of droplets (**Fig.7**). However, the bubble cloud generated in PFH phantoms was a more well-defined bubble cloud, similar to those previously observed for histotripsy above the intrinsic threshold (Vlaisavljevich et al. 2015b; Vlaisavljevich et al. 2015c). At all frequencies, over all 1,000 pulses, a dense bubble cloud was produced in PFH phantoms, with the bubbles more tightly confined inside the focal region and no significant change in the number of bubbles inside the cloud (**Fig. 7-8**). In comparison, after the first pulse, the bubble cloud generated in the PFP phantom is sparsely populated (**Fig.7-8**). At all frequencies, the number of bubbles observed inside PFP phantoms significantly decreased with increasing number of pulses, and bubbles distinguished after ~5-50 pulses. At a higher frequency, the bubble cloud distinguished with fewer pulses. For example, no bubbles were observed after ~50 pulses in PFP phantoms exposed to 500 kHz ($p=12.6$ MPa) pulses and after ~5 pulses exposed to 3 MHz ($p=15.6$ MPa) pulses.

NMH RBC Phantom Ablation

Agarose tissue phantoms embedded with RBC layers were used to compare NMH ablation for phantoms containing PFP and PFH nanodroplets. Results demonstrated that NMH generated consistent, well-defined lesions for RBC phantoms containing PFH nanodroplets, with the results being consistent for NMH treatments applied at 1 Hz and 10 Hz PRF. For example, **Figures 9-10** show images of NMH lesions generated inside RBC phantoms containing PFH droplets using the 345 kHz (**Fig.9**) and 1.5 MHz (**Fig.10**) transducers. For all cases, dense cavitation bubble clouds were generated inside the PFH phantom on every pulse, resulting in sharp lesions with well-defined boundaries between the fractionated lesion and intact RBCs (**Fig.9-10**). In contrast, only sparse lesions were formed inside the PFP phantoms. For example, for the 10 Hz PRF treatments using the 345 kHz transducer, sparse cavitation bubble clouds were observed over the course of the treatment, with the location of the bubbles remaining consistent from pulse to pulse inside the PFP phantoms, resulting in sparse lesions (**Fig.9**). For the 1 Hz treatments inside the PFP phantoms using the 345 kHz transducer, cavitation was only observed on the initial pulses (<10 pulses) and was not maintained for the duration of the treatment, resulting in very small lesions after treatment (**Fig.9**). At higher frequency (i.e. 1.5 MHz), no visible lesions were observed in the PFP phantoms for either 1 Hz or 10 Hz treatments, with bubbles only visible on the first pulse (**Fig. 10**). Quantifying the lesions areas for RBC phantoms (n=4) demonstrated a significant decrease in lesion size for phantoms containing PFP droplets in comparison to PFH phantoms. For example, at 345 kHz the lesion areas inside the PFH phantoms were $14.35 \pm 1.13 \text{ mm}^2$ (1 Hz) and $15.01 \pm 1.61 \text{ mm}^2$ (10 Hz), which were significantly larger than the lesion areas inside the PFP phantoms of $0.05 \pm 0.04 \text{ mm}^2$ (1 Hz) and $3.24 \pm 0.29 \text{ mm}^2$ (10 Hz). At 1.5 MHz, the lesion areas inside the PFH phantoms were

1 $1.31 \pm 0.09 \text{ mm}^2$ (1 Hz) and $1.17 \pm 0.13 \text{ mm}^2$ (10 Hz), which were significantly larger than the lesion
2 areas inside the PFP phantoms of $0.00 \pm 0.00 \text{ mm}^2$ (1 Hz, 10 Hz).

4 Discussion

5 In this study, the effects of droplet composition on NMH therapy were investigated using
6 perfluoropentane (boiling point $\sim 29^\circ\text{C}$, surface tension $\sim 9.5 \text{ mN/m}$) and perfluorohexane (boiling
7 point $\sim 56^\circ$, surface tension $\sim 11.9 \text{ mN/m}$) nanodroplets. In the first part of this study, PFP and PFH
8 droplets were synthesized using a previously developed method (Yuksel Durmaz et al. 2014). The
9 results from the nanodroplet characterization suggest that the droplet preparation method described
10 by Yuksel Durmaz *et al* can be used to create nanodroplets with similar surface characteristics
11 within the media while the composition of the encapsulated perfluorocarbon can be modulated as
12 desired (Yuksel Durmaz et al. 2014). The design of the triblock amphiphilic copolymer acts to
13 increase the particle's solubility in aqueous environments due to the hydrophilic character of the outer
14 PEG block while also improving hemocompatibility (i.e. no protein adsorption on the particles, no
15 macrophage recognition, and minimum interaction with blood cells during blood circulation) (Kim
16 et al. 2005; Dobrovolskaia et al. 2008; Yuksel Durmaz et al. 2014). The results of this study suggest
17 that the PFC inside the nanodroplets can be easily modified to take advantage of perfluorocarbons
18 with different boiling points without losing the benefits of the functionalized polymer shell.

19 In the second part of this study, the effects of droplet composition on the histotripsy cavitation
20 threshold were investigated, with results showing a significant decrease in the cavitation threshold
21 for both types of nanodroplets compared to controls, with a slightly lower threshold observed for
22 PFP phantoms, likely due to the decrease in surface tension for PFP. These results support our
23 hypothesis that both PFP and PFH nanodroplets can be used to significantly reduce the pressure
24 required to generate histotripsy bubbles for NMH therapy. At all frequencies tested, the NMH

1 threshold for both types of droplets was significantly lower (>10 MPa decrease) than the histotripsy
2 intrinsic threshold, while maintaining a steep threshold behavior. In fact, the σ_{mean} values calculated
3 by the curve fit decreased (i.e. sharper threshold curve) for phantoms containing nanodroplets, with
4 the lowest σ_{mean} values observed for phantoms containing PFH droplets (**Table 1**). This distinct
5 threshold behavior is promising for the development of NMH therapy in which the applied pressure
6 must be chosen in the region above the NMH threshold but below the histotripsy intrinsic threshold
7 to ensure cavitation is only generated in regions containing nanodroplets. The results of these
8 threshold experiments suggest that both PFP and PFH nanodroplets could be used for NMH, with
9 PFP droplets offering a slightly lower threshold (~ 1 -3 MPa decrease). With everything else equal,
10 one might expect a lower cavitation threshold for the larger PFH droplets (233.9 ± 3.9 nm) compared
11 to PFP droplets (177.9 ± 1.9 nm). It is possible that the slight decrease in threshold for PFP droplets
12 is due to the lower surface tension of PFP (9.5 mN/m) compared to PFH (11.9 mN/m), as previous
13 work has demonstrated that the cavitation threshold is highly dependent upon the surface tension of
14 the media (i.e. bulk fluid inside the droplet) when using the 1-2 cycle pulses used in this study
15 (Maxwell et al. 2013; Vlasisavljevich et al. 2015b). These results suggest that the nanodroplets may
16 actually decrease the cavitation threshold by carrying a lower threshold medium, rather than each
17 droplet acting as single cavitation nuclei as would be the case for an air contrast agent. Based on
18 this finding, the results of this work suggest that the NMH threshold can be selectively modulated
19 by changing the droplet composition, with lower surface tension/boiling point droplets resulting in a
20 decreased cavitation threshold. The finding that the NMH threshold can be finely tuned by changing
21 droplet composition while maintaining the distinct threshold behavior is a significant benefit for the
22 development of NMH therapy as well as for other nanodroplet applications such as selective drug
23 delivery (Fabiilli et al. 2013).

1 In the final part of this study, the effects of droplet composition on cavitation sustainability
2 and tissue fractionation were investigated, with results showing that PFH nanodroplets were
3 sustainable cavitation nuclei over multiple pulses, while PFP nanodroplets were destroyed during
4 the initial pulses. This effect resulted in well-defined lesions being generated inside the red blood
5 cell phantom containing PFH droplets under all treatment conditions. In contrast, only sparse
6 lesions were formed inside the PFP phantoms for the 345 kHz treatments applied at 10 Hz PRF,
7 with no visible lesions observed at higher frequency (i.e. 1.5 MHz) or lower PRF (i.e. 1 Hz). The
8 increased cavitation sustainability of the PFH droplets is most likely due to the re-condensing of
9 PFH droplets into a liquid after the cavitation event occurs, due to the higher boiling point of the
10 PFH droplets. The finding that PFH nanodroplets act as sustainable cavitation nuclei over multiple
11 pulses, while PFP droplets are destroyed during the initial pulses, supports our hypothesis that using
12 droplets with a higher boiling point is advantageous for NMH therapy. Results suggest that PFH
13 droplets may re-condense into a liquid after the collapse of the cavitation bubble while the PFP
14 bubbles are destroyed by the cavitation process (i.e. do not return to liquid form). These results
15 suggest that higher boiling point droplets can be used to significantly reduce the nucleation
16 threshold for generating cavitation bubbles over multiple pulses, allowing NMH therapy to be
17 applied for multiple pulses until the targeted tissue is completely fractionated, even at low PRF. In
18 contrast, due to the lower boiling point, PFP nanodroplets are only capable of reducing the
19 cavitation threshold on the initial pulses, requiring cavitation on subsequent pulses to be generated
20 from residual nuclei remaining in the focal region from previous pulses. The finding that higher
21 boiling point droplets can serve as functional cavitation nuclei over multiple pulses is therefore a
22 major benefit for NMH therapy, as previous work has shown a decrease in ablation efficiency for
23 higher PRF treatments that rely on residual nuclei from previous pulses to maintain the cavitation
24 bubble cloud (Wang et al. 2012).

In addition to the finding that PFH droplets acted as sustainable cavitation nuclei over multiple pulses, it was also observed that the NMH bubble clouds produced from PFH droplets were more well-defined and densely populated compared to the sparse bubble clouds produced by the PFP droplets, even when comparing bubble clouds produced on the first pulse. The well confined, dense bubble clouds produced inside the PFH phantoms closely match the behavior of bubble clouds previously observed for histotripsy treatments above the intrinsic threshold, which have been shown to be efficient and precise at fractionating the target tissue (Maxwell et al. 2013; Lin et al. 2014b; Vlaisavljevich et al. 2015b; Vlaisavljevich et al. 2015c). In contrast, the more sparse bubble clouds produced inside the PFP phantoms resemble bubble clouds generated at higher PRF or bubble clouds that rely upon residual nuclei in order to maintain cavitation, which have been shown to be less efficient at fractionating tissue while increasing collateral damage to surrounding tissue (Wang et al. 2012). This observation suggested that NMH bubble clouds produced using PFH droplets will be more efficient at fractionating tissue and provide less collateral damage to surrounding tissue compared to NMH therapy using PFP droplets, which was validated by the final set of experiments in this study comparing NMH ablation in red blood cell phantoms.

Conclusion

In this work, the effects of droplet composition on NMH therapy were investigated using perfluoropentane (boiling point~29°C, surface tension~9.5 mN/m) and perfluorohexane (boiling point~56°, surface tension~11.9 mN/m) droplets. The results demonstrated a significant decrease in the cavitation threshold for both types of nanodroplets compared to controls, with a slightly lower threshold observed for PFP phantoms, likely due to the decrease in surface tension for PFP. Results further demonstrated that PFH nanodroplets were sustainable cavitation nuclei over multiple pulses, while PFP nanodroplets were destroyed during the initial pulses. This effect is most likely due to the

re-condensing of PFH droplets into a liquid after the cavitation event occurs, due to the higher boiling point of the PFH droplets. In the final part of this study, tissue phantoms containing a layer of embedded red blood cells were used to compare the damage generated for NMH treatments using PFP and PFH droplets, with results showing PFH nanodroplets significantly improved NMH ablation, allowing for well-defined lesions to be generated at all frequencies and PRFs tested. The results of this study suggest that NMH therapy can be significantly enhanced by modulating droplet composition in order to optimize the cavitation threshold (decrease droplet surface tension) and increase the multi-pulse sustainability (increase droplet boiling point). Overall, the results of this study provide significant insight in the role of droplet composition in NMH therapy and will provide a rational basis to specifically tailor droplet parameters in order to improve NMH tissue fractionation.

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38

1 Tables.

2 **Table 1. Threshold results.** Table shows the values for the peak negative pressure, p_t , at which the
 3 fit curve set $P_{cav} = 0.5$ for each sample as well as the mean values for p_t and σ . A two-way ANOVA
 4 showed all differences in p_{t_mean} between samples were statistically significant ($p < 0.05$).

Frequency	Material	P_t (1)	P_t (2)	P_t (3)	P_t (mean)	σ (mean)
345 kHz	No Droplets	25.9	23.7	24.7	24.8	2.0
	PFP Droplets	7.3	7.4	7.4	7.4	1.4
	PFH Droplets	10.5	10.1	10.6	10.4	0.9
500 kHz	No Droplets	23.5	26.3	26.7	25.5	1.8
	PFP Droplets	9.4	10.0	8.2	9.2	0.8
	PFH Droplets	10.4	10.8	10.3	10.5	0.8
1.5 kHz	No Droplets	26.8	27.0	26.3	26.7	1.0
	PFP Droplets	10.3	10.7	10.5	10.5	0.4
	PFH Droplets	12.8	13.0	13.1	13.0	0.3
3 MHz	No Droplets	26.9	27.2	26.3	26.8	0.9
	PFP Droplets	13.1	12.9	13.7	13.2	0.6
	PFH Droplets	14.6	14.6	15.4	14.9	0.4

Figures.

Figure 1. Synthesis of the P(HDFMA-*co*-MMA)-*b*-PtBA-*b*-PEG triblock copolymer and formulation of PFP- and PFH-loaded nanodroplets **(A)** The chemical structure of each block and **(B)** the one-pot “click” reaction to obtain P(HDFMA-*co*-MMA)-*b*-PtBA-*b*-PEG copolymer. **(C)** Hydrolysis of the *t*BA group to obtain the P(HDFMA-*co*-MMA)-*b*-PAA-*b*-PEG polymer. **(D)** Formulation of the P(HDFMA-*co*-MMA)-*b*-PtBA-*b*-PEG polymer into PFP- and PFH-loaded nanodroplets.

Figure 2. **(A)** Example waveforms for 2-cycle histotripsy pulses generated by custom-built 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz transducers. **(B)** Experimental set-up showing the focus of the histotripsy transducers aligned inside tissue phantoms containing PFP, PFH, or no nanodroplets. Cavitation was monitored using high speed optical imaging and passive cavitation detection using one of the therapy elements.

Figure 3. Nanoparticle Tracking Analysis (left) demonstrated similar characteristics for **(A)** PFP- and **(B)** PFH-loaded nanodroplets. Size distribution plots (right) show the average size of the droplets was 177.9 ± 1.9 nm and 233.9 ± 3.9 nm for PFP and PFH nanodroplets, respectively.

Figure 4. Cavitation detection. Sample PCD signals (top) and high speed optical imaging (bottom) were used for cavitation detection for cavitation threshold experiments. Results showed good agreement between the two methods. Representative images shown above are from 1.5 MHz histotripsy pulses applied to tissue phantoms containing PFH nanodroplets.

Figure 5. Plots show sample cavitation probability curves for tissue phantoms containing PFP nanodroplets, PFH nanodroplets, and no nanodroplets at 345 kHz, 500 kHz, 1.5 MHz, and 3 MHz. At all frequencies, results showed a significant decrease in the cavitation threshold with both PFP and PFH nanodroplets compared to controls. Results also showed a slight increase in the nanodroplet cavitation threshold for PFH nanodroplets compared to PFP nanodroplets.

Figure 6. Bar plots show the complete cavitation threshold results for tissue phantoms containing PFP nanodroplets, PFH nanodroplets, and no nanodroplets. All differences in the cavitation threshold between samples were statistically significant ($p < 0.05$).

Figure 7. Optical images show NMH bubbles produced by 500 kHz ($p = 12.6$ MPa) pulses at a single focal point in tissue phantoms containing PFP and PFH nanodroplets at a PRF of 1 Hz. Results for PFP phantoms showed a decrease in the number of bubbles observed at the focus, with increasing pulse number, with no bubble observed after ~50 pulses. PFH phantom results demonstrated no significant decrease in the number of bubbles observed at the focus with increasing pulse number, with well-defined dense bubble clouds still observed after 1,000 pulses.

Figure 8. Optical images show NMH bubbles produced by 3 MHz ($p = 15.6$ MPa) pulses at a single focal point in tissue phantoms containing PFP and PFH nanodroplets at a PRF of 1 Hz. Results for PFP phantoms showed a decrease in the number of bubbles observed at the focus, with increasing pulse number, with no bubble observed after ~5 pulses. PFH phantom results demonstrated well-defined NMH bubble clouds were still observed after 1,000 pulses.

Figure 9. Optical images show NMH fractionation produced by 345 kHz ($p=12.6$ MPa) pulses in RBC phantoms containing PFP and PFH nanodroplets at PRFs of 1 Hz and 10 Hz. Results showed significantly larger and more well-defined lesions generated inside the PFH tissue phantoms, compared to the sparse lesions inside the PFP tissue phantoms.

Figure 10. Optical images show NMH fractionation produced by 1.5 MHz ($p=15.7$ MPa) pulses in RBC phantoms containing PFP and PFH nanodroplets at PRFs of 1 Hz and 10 Hz. Results showed well-defined lesions generated inside the PFH tissue phantoms. No visible lesions were observed for treatments applied to tissue phantoms containing PFP droplets.

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