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Lipid Shell Composition Plays a Critical Role in the Stable Size Reduction of Perfluorocarbon Nanodroplets

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Abstract

Perfluorocarbon nanodroplets (PFCnDs) are phase-change contrast agents that have the potential to enable extravascular contrast-enhanced ultrasound and photoacoustic (US/PA) imaging. Producing consistently small, monodisperse PFCnDs remains a challenge without resorting to technically challenging methods. We investigated the impact of variable shell composition on PFCnD size and US/PA image properties. Our results suggest that increasing the molar percentage of PEGylated lipid reduces the size and size variance of PFCnDs. Furthermore, our imaging studies demonstrate that nanodroplets with more PEGylated lipids produce increased US/PA signal compared to those with the standard formulation. Finally, we highlight the ability of this approach to facilitate US/PA imaging in a murine model of breast cancer. These data indicate that, through a facile synthesis process, it is possible to produce monodisperse, small-sized PFCnDs. Novel in their simplicity, these methods may promote the use of PFCnDs among a broader user base to study a variety of extravascular phenomena.

Keywords

perfluorocarbon nanodroplets; nanoemulsions; ultrasound; photoacoustics; lipid; cancer

Introduction

Ultrasound is a portable, cost-effective, non-ionizing imaging modality that is increasingly gaining clinical traction in point-of-care clinical settings. Ultrasound image contrast is determined by variations in underlying tissue density and the speed of sound, which define the acoustic impedance to incident sound waves (Szabo 2004). Unfortunately, soft tissues have similar echogenicity (Chivers 1977, Senapati, et al. 1972, Stakutis, et al. 1955), and so it is difficult to distinguish some organs or to identify small lesions indicative of disease

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using standard “b-mode” ultrasound alone. To address this limitation, gas microbubbles were developed for use as exogenous ultrasound contrast agents (Goldberg, et al. 1994). The density and compressibility of these microbubbles allows them to oscillate within an applied acoustic field, leading to enhanced backscatter of incident waves to increase contrast in ultrasound imaging (Kogan, et al. 2010, Stride and Saffari 2003).

Although a measureable portion of particles from standard microbubble formulations are submicrometer in size (Goertz, et al. 2007, Gorce, et al. 2000), the fraction of these agents that produce perceptible echogenicity on ultrasound have diameters on the micrometer scale (Gorce, et al. 2000, Helfield and Goertz 2013, Wilson and Burns 2010). Therefore, these agents are practically restricted to use within the systemic vasculature. Their minutes-long stability within systemic vasculature notwithstanding (Kabalnov, et al. 1998, Kwan and Borden 2012, Wilson and Burns 2010), microbubbles within the useable fraction are still too large to extravasate through “leaky” cancer neovasculature or to traffic within extravascular locations in times permissive of clinical imaging. In order for contrast agents to interact with tumor cells, metastases, and other sources of pathology, they must first reach and travel through extravascular spaces. Extravascular trafficking necessitates small contrast agent size. Gaps between endothelial cells in leaky tumor neovasculature have been reported to be between 380 to 780 nm (Barua and Mitragotri 2014, Hashizume, et al. 2000, Hobbs, et al. 1998), and the xenograft models used to produce these estimates were likely biased towards the larger side of what is observed clinically in human solid tumors (Prabhakar, et al. 2013). Similarly, though particles larger than 100 nm will preferentially drain to lymphatics versus blood vessels, particles larger than this will also exhibit size-dependent reduction in lymphatic trafficking speed and uptake efficiency (Strand and Bergqvist 1989, Swartz 2001, Swartz, et al. 1996).

To achieve the nanoscale particle size necessary for successful extravascular imaging, perfluorocarbon nanodroplets (PFCnDs) were created as a new class of ultrasound contrast agent (Figure 1A, inset) (Sheeran, et al. 2012, Wilson, et al. 2012). Nanodroplets are structurally similar to microbubbles; however, they contain a core composed of liquid perfluorocarbon instead of gas. This change allows PFCnDs to remain stable at the nanoscale. Because of their size relative to ultrasound wavelength and smaller difference in acoustic impedance from tissue, PFCnDs produce minimal contrast in their nascent, liquid-core state. However, when exposed to a burst of energy, typically acoustic, the perfluorocarbon core undergoes a phase change. This phase-change event effectively transforms the PFCnDs into microbubbles *in situ* (Rapoport 2012, Sheeran and Dayton 2012), which allows for traditional contrast-enhanced ultrasound imaging.

The phase-change event can also be optically-triggered (Figure 1B, i) by incorporating a photoabsorber, such as a near-infrared dye, within the nanodroplet construct (Figure 1A). In this case, the initial pressure waves created from PFCnD vaporization events can be received ultrasonically to reconstruct a high-contrast photoacoustic image (Figure 1B, ii) (Santiesteban, et al. 2017, Wilson, et al. 2012). When using a “low-boiling point” perfluorocarbon (e.g., perfluoropentane; Boiling Point [BP]: 28°C), the PFCnD phase change is permanent (Kripfgans, et al. 2000, Santiesteban, et al. 2017). Conversely, PFCnDs constructed with “high-boiling point” perfluorocarbons (e.g., perfluorohexane; BP: 56°C)

will recondense into their original liquid state after exposure to an energy pulse (Figure 1B, iii) (Hannah, et al. 2016). The ability to repeatedly induce a phase change in “high-boiling point” nanodroplets allows for more robust and longitudinal imaging studies with a single PFCnD population (Yoon, et al. 2017).

Extravascular ultrasound imaging applications with PFCnDs are predicated on the ability to produce a small and monodisperse nanodroplet population. In solid tumors, for instance, only nanodroplets that are smaller than a tumor-specific pore cutoff size will be able to extravasate through gaps between neovascular endothelial cells into the tumor stroma (Figure 1C) (Mei, et al. 2016). Using a monodisperse population of PFCnDs would allow for a higher percentage of the injected dose to reach the extravascular space, assuming the median size is below the pore cutoff. Additionally, the acoustic response from monodisperse PFCnDs would be relatively uniform, allowing for more accurate, image-based estimates of *in vivo* nanodroplet concentration. Unfortunately, many facile synthesis methods (e.g., sonication, extrusion) produce nanodroplets closer to 500 nm than to 100 nm in size (Hannah, et al. 2013, Rapoport 2012, Rapoport, et al. 2011, Wilson, et al. 2012), and these larger, polydisperse droplets may be ill-suited for extravascular use. Several methods have been employed to achieve a stable reduction in PFCnD size and particle dispersity: condensation synthesis (Sheeran, et al. 2011, Sheeran, et al. 2012), size-separation (Arnal, et al. 2015), and microfluidics (Martz, et al. 2012, Seo and Matsuura 2012). Still, each of these methods is technically challenging and has its own weaknesses.

Condensation synthesis, which requires the formation of stable microbubbles, cannot be applied practically to “high-boiling point” perfluorocarbon formulations. Size-separation techniques have high variability in size reduction, low particle yields, and often require differential centrifugation, which adds time to the manufacturing process and is challenging to execute at scale. Microfluidic devices also have small sample yields and can be prohibitively expensive without local expertise in their fabrication. The ability to achieve PFCnD size reduction and monodispersity without these methods would facilitate their use amongst a broader cadre of researchers and would have the potential to place more emphasis on applications-based research with nanodroplets.

In the context of these lingering challenges, we investigated the impact of alterations in the nanodroplet shell composition on the size of PFCnDs. We restricted this analysis to lipid-shelled formulations because of their strong translational benefits: chiefly, their demonstrable biocompatibility and well-studied linker strategies for downstream functionalization. Previous research notes that the yield of and ultrasound signal produced by nanodroplets is increased by using a higher molar percentage of unsaturated phospholipids (Chattaraj, et al. 2016). These same studies also describe that nanodroplets formed with 10% and 20% molar DSPE-mPEG2000 demonstrate a solid-liquid disordered phase existence, which is structurally similar to the formulations displaying the most ideal yields and imaging properties (Chattaraj, et al. 2016). Standard formulations for nanodroplets with acoustically-triggered vaporization have historically utilized no more than 10% molar PEGylated phospholipid (Dayton, et al. 2006, Luke, et al. 2016, Sheeran, et al. 2011, Sheeran, et al. 2012). However, literature from mixed-micelles, which have similar structure to the lipid shells of nanodroplets, suggests that increasing the molar concentration

of PEGylated lipids up to 90% would confer stable size reduction (Ashok, et al. 2004, Weissig, et al. 1998), potentially due to increased steric stabilization.

The objectives of this work were to assess the effect of varying the percentage of PEGylated phospholipids on the size and size distribution of lipid-shelled PFCnDs and to assess the impact of these changes on the ultrasound and photoacoustic contrast produced by the PFCnDs. Changes in the shell composition may have both direct and indirect, via size modulation, effects on the PFCnDs' acoustic response. Then, we highlighted one *in vivo* application of an optimized formulation of PFCnDs by performing contrast-enhanced imaging in a murine model of primary breast cancer.

Materials and Methods

Nanodroplet Synthesis

Perfluorocarbon nanodroplets (PFCnDs) were synthesized using a sonication-based method, modified from one described by our group previously (Hannah, et al. 2016, Luke, et al. 2016). In this protocol, 2 μ mol of phospholipids, consisting of a mixture of DSPC and DSPE-mPEG2000 (Avanti Polar Lipids, Inc., Alabaster, AL, USA), were isolated from a chloroform suspension using a rotary evaporator (Rotavapor R-215, Buchi AG, Flawil, CHE) with water bath, adhering to the "20/40/60 Rule." For PFCnDs used in imaging experiments, the evaporated chloroform suspension also contained 2 mg of near-infrared absorbing dye (Epolight™ 3072, Epolin, Inc., Newark, NJ, USA), which was obtained from a 2 mg/mL chloroform suspension. After a minimum of 30 minutes in the Rotavapor, the resulting lipid cake was allowed to dry under vacuum overnight.

After drying, the lipids were resuspended in 1 mL of phosphate buffered saline. This solution was vortexed for 2 minutes and sonicated with 20 kHz ultrasound in a water bath sonicator (1510 Ultrasonic Cleaner, Branson Ultrasonics Corp., Danbury, CT, USA) at room temperature (25°C) in 5-minute increments until homogeneous. To this mixed micelle solution, we added 50 μ L of perfluorohexane (FluoroMed, L.P., Round Rock, TX, USA). The mixture was placed into an ice bath and positioned within a microtipped probe sonicator (QSonica, Newtown, CT, USA). The ice-cold mixture was allowed to equilibrate for 5 minutes and then exposed to two rounds of probe sonication, one low intensity (1% power, 1 s on, 5 s off, 20 total pulses) and one high intensity (50% power, 1 s on, 10 s off, 5 total pulses), with 5 minutes in between sequences. Prior to size measurements or use in imaging, these PFCnDs were diluted 1:20 in phosphate buffered saline and exposed to 5 minutes of 20 kHz water bath sonication at room temperature to produce the final samples.

Size Characterization

Size characterization studies were performed on 1 mL samples of 1:1 phosphate buffered saline dilutions of the 1:20 dilution of PFCnDs described above. Size measurements were taken by a standard dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., Worcestershire, UK) at various time points spanning from immediately post-synthesis to 6 hours post-synthesis. The quartz cuvette containing the 1 mL sample was inverted immediately prior to each measurement, and the cuvette remained within the 25°C

temperature-buffered instrument holder between measurements. After a 60-second sample equilibration period, the DLS instrument obtained three measurements at each time point, each measurement an average of 10 individual measurements. The measurement sequence for each time point was executed over approximately eight minutes, including the equilibration step. Measurement data were exported as comma-separated values and displayed in R (R Core Team) using the ggplot2 data visualization package.

Absorption Characterization

The absorption of the near-infrared dye encapsulated within the PFCnDs was characterized by UV-Vis spectroscopy. A UV-Vis spectrophotometer (Evolution 220, Thermo Scientific, Waltham, MA, USA) was used to measure the absorbance of 1:1000 PBS dilutions of both unloaded PFCnDs and PFCnDs containing the near-infrared dye. The spectrum of the unloaded PFCnDs was subtracted from that of the dye-loaded PFCnDs, and the resulting differential absorption spectrum was normalized for display purposes.

Ultrasound/Photoacoustic Imaging of Phantom

Ultrasound and photoacoustic (US/PA) imaging was performed using an integrated setup described elsewhere (Yoon and Emelianov 2018). This system combines a custom ultrasound system (Vantage 256, Verasonics, Inc., Kirkland, WA, USA) with a tunable nanosecond pulsed Nd:YAG laser (Phocus, Opotek, Inc., Carlsbad, CA, USA).

The Nd:YAG laser was used to produce pulses at the fundamental 1064 nm wavelength at a 10 Hz pulse repetition frequency (PRF). Within this imaging study, each laser pulse deposited 28 mJ of energy, resulting in a local fluence of approximately 13 mJ/cm². The laser pulses were delivered via optical fibers coupled to the imaging transducer. These fibers were oriented such that the incident light intersected the imaging plane at 20±3 mm in the axial dimension.

The coupled Vantage 256 ultrasound system utilized an L11-4v (Verasonics, Inc., Kirkland, WA, USA) linear array transducer, which has a -6 dB bandwidth ranging from 11 to 4 MHz. These imaging studies utilized a transmit center frequency of 5 MHz and a transmit voltage of 40V. Each ultrasound frame is the result of three angularly compounded plane waves. These plane waves span 6 degrees (-3°, 0°, 3°) and were acquired at 3 kHz for a resulting ultrasound imaging frame rate of 1 kHz.

Each imaging acquisition consisted of six packets of data. The last five packets were preceded by a single laser pulse. Each packet contains one photoacoustic image (receive only) followed by 50 ultrafast ultrasound frames, as described above. The resulting imaging sequence occurs over the course of 600 ms, as dictated by the 10 Hz laser PRF, with the resulting US/PA data acquired over the first 50 ms in each 100 ms packet.

Imaging data were processed offline in MATLAB (Mathworks, Natick, MA, USA). All images were linearly interpolated in two-dimensions by a factor of three for display purposes. Quantitative values from images were obtained from the raw (i.e., non-interpolated) data.

Imaging Phantom Setup

The base of the imaging phantoms were created from polyacrylamide in a process detailed by our lab previously.(Hannah, et al. 2016, Yoon and Emelianov 2018) In brief, 40% (w/v) acrylamide (OmniPur® Acrylamide Bis-acrylamide 29:1, MilliporeSigma, St. Louis, MO, USA) and 10% (w/v) ammonium persulfate (ACS Grade, VWR International, Radnor, PA, USA) solutions were diluted to 10% and 0.002%, respectively, in degassed, deionized water. To this solution, we added silica gel particles (40-63 μm particle size, MilliporeSigma, St. Louis, MO, USA) to achieve a final 1% (w/v) concentration of silica. A catalytic amount of TEMED (Proteomics Grade, AMRESCO Inc., Solon, OH, USA) was added to initiate the crosslinking of aqueous acrylamide to solid polyacrylamide gel.

Two cylindrical inclusions were made in the polyacrylamide phantom. Viewed from the orientation of the imaging plane, these inclusions were 5 mm in diameter, separated by 20 mm, and located 20 mm at depth from the top surface of the polyacrylamide. Then, 1 mL of the 1:20 dilution of PFCnDs described previously was mixed into 10 mL of ultrasound gel (Sonigel, 3B Scientific, Hamburg, DEU) with a 1% (w/v) concentration of silica. After removing air bubbles from the gel mixture via 2 minutes of centrifugation at 1000 ref, the 1:200 PFCnD gel mixture was added via syringe to fill one of the empty inclusions in the polyacrylamide gel. Each inclusion ultimately contained gel with one of the batches of PFCnDs to be analyzed by US/PA imaging, as detailed above.

Murine Model of Primary Breast Cancer

All animal procedures were conducted under the oversight of the Institutional Animal Care and Use (IACUC) of the Georgia Institute of Technology (Protocol A16018). A six-week-old female athymic nude mouse (Charles River Laboratories, Wilmington, MA, USA) was anesthetized with isoflurane (5% induction, 2.5% maintenance) and inoculated with 1×10^6 4T1 cells (CRL-2539, ATCC, Manassas, VA, USA) in a 200 μL of mixture of 1:1 media and Matrigel membrane matrix (Corning Inc., Corning, NY, USA) in the right-lower mammary fat pad. The mouse was examined daily for signs of distress and infection, and the primary tumor size was assessed via inspection and manual palpation. After one week, the primary tumor grew to sufficient size to allow for downstream US/PA imaging studies.

Ultrasound/Photoacoustic Imaging of Mice

The murine imaging studies utilized the integrated setup detailed for the phantom studies. However, instead of the L11-4v linear array transducer, we instead utilized a commercial US/PA imaging transducer (LZ400, FUJIFILM VisualSonics, Toronto, ON, CAN) with an integrated optical fiber bundle. This linear array transducer has an imaging bandwidth ranging from 38 to 18 MHz. We utilized a transmit center frequency of 21 MHz and a transmit voltage of 20V. Each ultrasound frame is the result of 15 angularly compounded plane waves. These plane waves span 12 degrees and were acquired at 15 kHz for a resulting ultrasound imaging frame rate of 1 kHz.

As before, each imaging acquisition consisted of six packets of data. The last five packets were preceded by a single laser pulse. Each laser pulse deposited 60 mJ of energy, resulting in an incident fluence of approximately 24 mJ/cm² Each packet contains one photoacoustic

image (receive only) followed by 5 ultrafast ultrasound frames, as described above. The resulting imaging sequence occurs over the course of 600 ms, as dictated by the 10 Hz laser PRF, with the resulting US/PA data acquired over the first 5 ms in each 100 ms packet.

The mouse was anesthetized with isoflurane (5% induction, 2.5% maintenance) and placed in the supine position for US/PA imaging. The imaging transducer was oriented above the primary tumor and coupled with ultrasound gel (Sonigel, 3B Scientific, Hamburg, DEU). A series of imaging sequences were then acquired in the axial dimension across the length of the tumor.

After this preliminary US/PA imaging session, the mouse was injected intravenously with a 50 μ L bolus of 10^{10} droplet/mL solution via the right jugular vein. This concentrated bolus of “10:90” PFCnDs was prepared by centrifuging a 1 mL 1:20 diluted and sonicated stock, as detailed under the synthesis section, at 1000 rcf for 20 minutes and then resuspending the pelleted PFCnDs in 50 μ L of the PBS supernatant. The order-of-magnitude estimate of PFCnD concentration was calculated based on measurements obtained with the Archimedes Particle Metrology System (Malvern Panalytical, Worcestershire, UK) on a 1:40 diluted and sonicated stock solution using the Nano-D4868E sensor with ParticleLab version 1.20.17004.1. Following the injection, the mouse was allowed to wake up and monitored for signs of distress for 30 minutes before being returned in isolation to the animal housing facility.

Twenty-four hours post-injection, the same mouse was again placed under general anesthesia and reimaged in the tumor region, as described above. After this second round of US/PA imaging was completed, the mouse was euthanized per our IACUC protocol in a manner consistent with the AMVA Guidelines for the Euthanasia of Animals. In brief, while still under general anesthesia, the mouse was injected with a lethal dose of Euthasol (390 mg/kg, Henry Schein, Inc., Melville, NY, USA). Cervical dislocation was performed as secondary euthanasia after respiration ceased.

Imaging data were processed offline in MATLAB (Mathworks, Natick, MA, USA). All images were linearly interpolated in two-dimensions by a factor of three for display purposes.

Results and Discussion

Nanodroplet Characterization

We synthesized batches of PFCnDs in one of two cohorts: nanodroplets with a 10:90 molar ratio of DSPC:DSPE-mPEG2000 and nanodroplets with a more standard 90:10 molar ratio, hereafter referenced by these defining numeric ratios. We then obtained average size measurements via dynamic light scattering (DLS) over the first six hours post-synthesis (Figure 2). Representative DLS size distributions can be found in the supplemental (Figure S1). Initially, the standard formulation produced nanodroplets that were relatively large (median: 647 nm) and variable (Interquartile Range [IQR]: 484 nm) in size. Nonetheless, the minimum size observed (366 nm) was consistent with reported values within seminal PFCnD literature using sonication-based synthesis methods (Reznik, et al. 2011). Over the

first three hours post-synthesis, the average size of the 90:10 nanodroplets trended upward, potentially due to Ostwald ripening (Kabalnov and Shchukin 1992). This hypothesis is further supported by a reduction in the inter-quartile range over time (137 nm by hour six). After the third hour post-synthesis, however, the median droplet size measurement began to decrease (624 nm by hour six). This observed phenomenon is likely due to the spontaneous vaporization of larger (i.e., $1 + \mu\text{m}$) PFCnDs within the solution (Kripfgans, et al. 2000).

By comparison, the initial size of 10:90 nanodroplets was smaller (median: 159 nm) and more consistent between batches (IQR: 5 nm). The low variance in size persisted for six hours post-synthesis, though the average size measurements did trend upward (median: 218 nm at 1 hour, 284 at 6 hours) similar to the 90:10 nanodroplets. Again, this phenomenon may potentially be due to Ostwald ripening of PFCnDs. Still, nanodroplets with an inverted molar ratio of PEGylated phospholipids remain small for an appreciable amount of time post-synthesis (median < 250 nm at 2 hours). This is a significant finding because downstream PFCnD modification protocols (e.g., bioconjugation) are time-consuming, and the nanodroplets will grow in size over this time. Many researchers only report initial size measurements from their nanodroplets, and these measurements may not accurately reflect the ultimate size of PFCnDs used in their *in vitro* and *in vivo* experiments.

We also investigated batches of PFCnDs with an “intermediate” molar ratio of DSPC:DSPE-mPEG2000 of 50:50. One might expect that the size data to fall halfway between the previous 90:10 and 10:90 batch data. Surprisingly, the intermediate nanodroplets behaved far more similarly to the 10:90 nanodroplets than to the 90:10 nanodroplets. These nanodroplets were initially slightly larger (median: 178 nm) and more variable (IQR: 25 nm) than 10:90 nanodroplets. However, such differences in the data distributions were small, and the size distributions were effectively the same after the first 2 hours post-synthesis. These data imply that the relationship between increased lipid shell PEGylation and reduced droplet size is nonlinear. It is possible that there is a critical concentration of PEG necessary to achieve sufficient steric stabilization such that increasing PEGylation further will not have a demonstrable impact on reducing nanodroplet coalescence. Further study is necessary to fully characterize this relationship.

Much of the differences in size variance at each time point, detailed through the interquartile ranges described previously, can be explained by differences in the batch-to-batch variability of the different formulations tested. Both the 10:90 and 50:50 PFCnDs produced nearly identically sized particles batch-to-batch. However, the more traditional 90:10 PFCnDs were highly variable batch-to-batch, from 366 to 1085 nm immediately post-synthesis. More detailed descriptive statistics can be found in the supplemental tables: Table S1, Table S2, and Table S3. It is clear that increasing the molar percentage of PEGylated lipid reduces the size of resulting PFCnDs as well as variance between synthesized batches.

This design approach is compatible with other shell modifications that could be implemented to further reduce the size and size variability of PFCnDs. Previous studies in the literature using “low-boiling point” perfluorocarbons have demonstrated an inverse relationship between the acyl chain length of phospholipids in the shell and the median sizes of resulting PFCnDs (Yoo, et al. 2018). How these design choices are impacted by other

factors, such as the addition of cholesterol or unsaturated lipids into the shell, remains an active area of research.

Phantom Imaging

In addition to these sizing studies, combined ultrasound and photoacoustic (US/PA) imaging was performed on PFCnDs with varying shell PEGylation. A tissue-mimicking phantom was constructed such that two circular inclusions containing diluted batches of 90:10 and 10:90 PFCnDs in scattering media were visible within a single US/PA imaging plane (Figure 3A). Optical fibers adjacent to the imaging transducer were oriented such that both inclusions could simultaneously be evenly illuminated, allowing a more fair comparison of the two droplet populations. Because PFCnDs were loaded with a near-infrared absorbing dye to trigger phase-change events, it was possible to analyze both ultrasound and photoacoustic contrast within the same imaging sequence.

The resulting imaging data were divided into six distinct packets: one without a pre-acquisition laser pulse and five with a pre-acquisition near-infrared laser pulse. Examining the differential ultrasound signal (Figure 3B) as a function of time reveals spikes in intensity within the inclusions immediately after the laser pulse (Figure 4A), which are characteristic of the repeatable vaporization-recondensation or “blinking” of perfluorohexane nanodroplets (Hannah, et al. 2016). This differential signal returns to baseline for both inclusions after the first millisecond of ultrasound acquisitions.

Analyzing the integrated differential ultrasound signal within each inclusion from the post-laser-pulse frames, the total signal from 10:90 PFCnDs is 1.5 times the total signal from 90:10 PFCnDs (Figure 4B). In plane-wave b-mode ultrasound, the backscattered signal (P_{US}) received from a region of interest containing PFCnDs is proportional to the total *en face* surface area of nanodroplets ($n_{PFCnDs} \cdot SA_{PFCnD}$) encountered by plane waves travelling through the region. That is to say,

$$P_{US} = P_{PFCnDs} \propto n_{PFCnDs} \cdot SA_{PFCnD} \quad (1)$$

Batches of both the 10:90 and 90:10 PFCnDs were synthesized with equivalent molar concentrations of perfluorocarbon and surfactant phospholipids. Assuming that the amount of surfactant is non-limiting, the number of droplets within each batch should be inversely proportional to the cube of the diameter of the PFCnDs because the total volume of perfluorocarbon (V_{PFC}) is constant between batches and,

$$V_{PFC} = n_{PFCnDs} \cdot V_{PFCnD} = n_{PFCnDs} \cdot (4/3)\pi(d/2)^3 \quad (2)$$

Because the surface area of a droplet is proportional to the square of its diameter, combining Equations 1 and 2 would imply that the signal from PFCnDs in these experiments should be inversely proportional to the corresponding droplet diameter (d). Theoretically, this suggests that there should be approximately 2.8 times the signal from the inverted PFCnD inclusion than the standard PFCnD inclusion.

The 1.5-fold enhancement observed is much less than the theoretical one. Because the synthesis is sonication-based, it is likely that the total volume of perfluorocarbon is not conserved, as some nanodroplets will be vaporized by the 20 kHz ultrasound during sonication, which would lead to a decrease in the observed contrast enhancement. Additionally, the quoted droplet diameters were for immediately post-synthesis PFCnDs, prior to incorporation into gel mixtures for imaging. It is possible that the nanodroplets continued to grow in size, as observed in Figure 2, after synthesis but prior to imaging. Since the size of 10:90 nanodroplets trends much larger relative to their initial size, this could reduce the size ratio used to predict theoretical signal enhancement.

As aforementioned, the laser-activated PFCnDs used for imaging also produce a photoacoustic signal, which can be visualized independently for contrast enhancement (Figure 3C). The photoacoustic signal produced from nanodroplets can be attributed to two distinct components (Wilson, et al. 2012). The first component is due to the pressure wave created by the optical droplet vaporization (ODV) and the resulting volumetric expansion. Because the partial derivative of spherical volume in the radial direction is equivalent to surface area, the resulting pressure of this component follows the logic of the ultrasound signal enhancement detailed previously.

If volumetric expansion were the only component of the photoacoustic signal emitted by PFCnDs, then the signal enhancement for 10:90 nanodroplets should be equivalent across ultrasound and photoacoustic imaging. However, experimentally the photoacoustic signal enhancement is higher: 2.2 for photoacoustic signal (Figure 4C) versus 1.5 for differential ultrasound (Figure 4B). One potential explanation for this observation is the photoacoustic signal emitted from the encapsulated photoabsorber, which is the second component of laser-activated PFCnD photoacoustic signal (Wilson, et al. 2012). A study in mixed micelles demonstrated that water-insoluble drugs have increased solubility in DSPE compared to DSPC (Krishnadas, et al. 2003). Similar logic would suggest that inverted nanodroplets, with an appreciably higher DSPE shell content, should solubilize more of the water-insoluble, near-infrared photoabsorber and may be preferable for encapsulating non-polar therapeutics when using PFCnDs for applications in controlled delivery and release.

In Vivo Imaging

The enhanced potential for therapeutic delivery and increased contrast enhancement from inverted nanodroplets can only be fully exploited if these nanodroplets' stable size reduction truly allows for extravascular US/PA imaging. To assess these prospects, we performed an *in vivo* imaging experiment with 10:90 nanodroplets in an orthotopic murine model of primary breast cancer. In brief, we inoculated an athymic mouse with 4T1 triple-negative breast carcinoma cells in the right-lower mammary fat pad. We allowed the primary tumor to grow for one week prior to US/PA imaging.

Initial US/PA imaging revealed only weak background photoacoustic signal at the tumor site (Figure 5A). Following this preliminary imaging, we injected the mouse intravenously with a solution of 10:90 nanodroplets and allowed these to circulate and clear from systemic circulation for 24 hours. After this washout period, we again performed US/PA imaging on

the same mouse. This time, the PA images revealed strong photoacoustic contrast enhancement within the primary tumor region (Figure 5B).

Studies in liposomal literature have hypothesized that the “pore cutoff size” for particles to extravasate from tumor neovasculature in the orthotopic 4T1 model is on the order of 250 nm (Charrois and Allen 2003). Additionally, biodistribution studies of an MRI contrast agent similar in hydrodynamic size to our 10:90 PFCnDs show complete clearance from systemic circulation by the reticuloendothelial system within 24 hours postinjection (Mei, et al. 2016). These factors suggest that it is possible that PFCnDs may have extravasated in our proof-of-concept study; however, a constellation of fully powered *in vivo* studies would be necessary to conclusively prove this phenomenon. In any case, our representative *in vivo* data support the ability of 10:90 PFCnDs to produce contrast enhancement in a model of primary breast cancer, which could be used to study nanodroplet extravasation.

For PFCnDs, as with other nanoparticle agents, successful extravascular imaging is dependent upon effective delivery. Canonically, the enhanced permeability of cancer vasculature, coupled with decreased lymphatic filtration, results in passive accumulation of particles within solid tumors (Matsumura and Maeda 1986). This phenomenon, known as the enhanced permeability and retention (EPR) effect, has historically justified decades of passively targeted nanoparticle research. However, significant inter- and intratumoral heterogeneity with respect to the EPR effect call its basal impact on particle delivery into question (Prabhakar, et al. 2013). Tumor vasculature is spatially and temporally variable, both in terms of flow and porosity (Jain 1999). Furthermore, the high interstitial pressure of solid tumors serves as a barrier to particle convection (Jain 1999). These factors inhibit the passive transport of larger particles, such as PFCnDs, more than they do small molecules (Sharma, et al. 2006).

Various approaches exist to improve the retention of particles within solid tumors, which could enhance the ability to use PFCnDs for imaging of primary cancer. Modulation of the tumor microenvironment provides a means of enhancing the EPR effect. Vasoactive drugs can be administered concomitantly to increase tumoral blood flow and neovascular pore size (Maeda, et al. 2013). Additional strategies to “normalize” the tumor microenvironment have also been proposed to enhance vascular delivery (Chauhan, et al. 2013, Jain 2013). Complementary to modulation of the underlying biology, modulation of particle composition provides another avenue for enhancing delivery in cancer. In terms of PFCnDs, various methods of bioconjugation have been explored to enhance the molecular specificity and retention of these particles in tumors for extravascular imaging (Luke, et al. 2016, Marshalek, et al. 2016, Mitcham, et al. 2018, Santiesteban, et al. 2019). Although the PFCnD size modulation described in these studies represents an important advancement, consistently successful extravascular imaging of solid tumors with PFCnDs will necessitate a multifaceted strategy in order to ensure maximal particle accumulation.

Conclusion

In conclusion, we have investigated the effects of variable lipid shell composition on the particle and imaging characteristics of lipid-shelled, laser-activated PFCnDs. Our results

suggest that increasing the molar concentration of PEGylated phospholipid compared to standard formulations reduces the size and size variability of nanodroplets. Additionally, our imaging studies demonstrate that nanodroplets from batches with an inverted molar ratio of lipids produce increased ultrasound and photoacoustic signal compared to batches of 90:10 nanodroplets, likely because of increased total surface area and dye encapsulation in 10:90 nanodroplets.

These data confirm that it is possible to produce small-sized, monodisperse PFCnDs without the need for more involved synthesis strategies. Further, we have demonstrated the ability of this design approach to create nanodroplets that can provide contrast enhancement within a model of primary murine breast cancer 24 hours after systemic delivery. We also note that the strategy of increased shell PEGylation is not mutually exclusive with alternative approaches: it could potentially be combined with others (e.g., size separation, microfluidics, acyl chain elongation) to further tune PFCnD size or percent yield. Although these studies utilized nanodroplets for US/PA imaging, the dynamic contrast of these agents could potentially be used with optical coherence tomography (Barton, et al. 2002), magnetic resonance imaging (Alexander, et al. 1996, Díaz-López, et al. 2010), and other techniques that can use microbubbles or perfluorocarbon for contrast generation. Moving forward, we anticipate that the shell-focused strategy for PFCnD synthesis will facilitate the use of PFCnDs amongst more investigators in a wide array of applications-based research endeavors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A) Absorption spectrum of the near-infrared photoabsorber encapsulated within PFCnDs; **B)** A schematic of optical droplet vaporization (ODV). First, a laser pulse is absorbed by encapsulated photoabsorbers within PFCnDs (i) to induce a phase change (ii). This mechanism can produce contrast through both US and photoacoustic (PA) imaging (ii, insets). “High-boiling point” perfluorocarbons, such as perfluorohexane (BP: 56°C), may recondense into the droplet state after the laser pulse ends (iii), allowing for repeatable contrast generation from the same PFCnD population (i-iii); **C)** Cartoon depicting extravascular imaging of a primary tumor with PFCnDs. Only PFCnDs smaller than the gaps between endothelial gaps in cancer neovasculature (blue) will be able to extravasate into the tumor stroma. Larger PFCnDs (red-orange) will be restricted to the vasculature.

Figure 2.

Average size data for PFCnDs as a function of time post-synthesis. Each box represents size data from four batches of lipid-shelled PFCnDs with shells containing a molar ratio of 90:10 (red-orange), 50:50 (green), or 10:90 (blue) DSPC:DSPE-mPEG2000. For each batch at each time point, the dynamic light scattering instrument yielded three Z-Average values for average particle size, each Z-Average an average of 10 successive measurements. Samples were kept at room temperature (25°C) during and between measurements.

Figure 3.

Tissue-mimicking polyacrylamide phantom doped with 1% (w/v) silica gel and containing two inclusions with 1:200 stock dilutions of “10:90” (left, blue circle; average size: 170 nm at 0 hours post-synthesis) and “90:10” (right, red-orange circle; average size: 470 nm at 0 hours post-synthesis) PFCnDs. **A)** Plane-wave b-mode ultrasound image prior to PFCnD laser activation. Three successive plane waves (at -3° , 0° , and 3° , respectively) were compounded to create each ultrasound image. This image is normalized, interpolated, and displayed using a 40 dB dynamic range. Scale bar is 5 mm; **B)** Absolute differential ultrasound image immediately post-laser pulse. Each differential ultrasound image was generated by taking the absolute value of the forward-looking difference of the linear ultrasound signal after compounding each unique ultrasound frame. This image is interpolated and displayed over a linear scale; **C)** Photoacoustic image immediately post-

laser pulse. This image is normalized, interpolated, and displayed using a 22 dB dynamic range.

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Figure 4.

A) Absolute linear differential ultrasound signal as a function of time for the phantom shown in Figure 3. The differential ultrasound signal was integrated across an area-equivalent region of interest containing the “10:90” PFCnDs (red-orange), the “90:10” PFCnDs (blue), and a control region centered within the polyacrylamide gel background in between the two inclusions (gray). Discontinuities within the graph correspond to times when no data are acquired by the US/PA imaging sequence; **B)** Absolute linear differential ultrasound signal post-laser pulse integrated across the “10:90” (blue) and “90:10” (red-orange) PFCnD inclusions; **C)** Linear photoacoustic signal integrated across the “10:90” (blue) and “90:10” (red-orange) PFCnD inclusions.

Figure 5.

US/PA imaging in a murine model of primary breast cancer. Ultrasound and photoacoustic data are normalized, interpolated, and displayed using 50 dB and 20 dB dynamic ranges, respectively. Scale bar is 2 mm. **A)** A representative pre-injection US/PA image showing only background PA signal within the tumor (yellow dashed line). After baseline imaging, the mouse was injected intravenously with a bolus of 10:90 PFCnDs (average size: 203 nm at 0 hours pre-injection); **B)** A representative US/PA image taken 24 hours post-injection showing significant PA contrast enhancement within the tumor (yellow dashed line).