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Laser-activated perfluorocarbon nanodroplets: a new tool for blood brain barrier opening

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Abstract: A major obstacle in the monitoring and treatment of neurological diseases is the blood brain barrier (BBB), a semipermeable barrier that prevents the delivery of many therapeutics and imaging contrast agents to the brain. In this work, we explored the possibility of laser-activated perfluorocarbon nanodroplets (PFCnDs) to open the BBB and deliver agents to the brain tissue. Specifically, near infrared (NIR) dye-loaded PFCnDs comprised of a perfluorocarbon (PFC) core with a boiling point above physiological temperature were repeatedly vaporized and recondensed from liquid droplet to gas bubble under pulsed laser excitation. As a result, this pulse-to-pulse repeated behavior enabled the recurring interaction of PFCnDs with the endothelial lining of the BBB, allowing for a BBB opening and extravasation of dye into the brain tissue. The blood brain barrier opening and delivery of agents to tissue was confirmed on the macro and the molecular level by evaluating Evans Blue staining, ultrasound-guided photoacoustic (USPA) imaging, and histological tissue analysis. The demonstrated PFCnD-assisted pulsed laser method for BBB opening, therefore, represents a tool that has the potential to enable non-invasive, cost-effective, and efficient image-guided delivery of contrast and therapeutic agents to the brain.

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References and links
1. Introduction

Noninvasive treatment and monitoring of neurological disease is greatly hindered by the blood brain barrier (BBB), which often blocks delivery of therapeutics and contrast agents to the brain tissue [1,2]. To overcome this barrier, many methods of delivering contrast and therapeutics to the brain for disease diagnosis and treatment have been developed and are currently used in the clinic [2–4]. However, the methods developed, such as surgical resection, device implantation, or chemical manipulation of tissue via pharmacological substances, have challenges and pitfalls. These methods can be invasive, and their effects can result in widespread opening of the BBB, which is not always desirable, as this greatly increases the risk of complications such as infection [2,3]. Furthermore, these interventions often do little to prevent the recurrence rate of high mortality diseases, like glioblastoma [4]. This main challenge of overcoming the BBB is difficult, as it prevents the passage of large molecules (>500 Da) from entering into the brain tissue [1,2]. In recent years, focused ultrasound (FUS) has been developed as an alternative method to transiently open BBB by using the interaction between the FUS field and microbubbles [1,3,5–7]. Although the biological mechanisms of this method are not fully understood, it is widely believed that the oscillation or cavitation of microbubbles, which interact with the endothelial lining of the brain vasculature, stretch tight junctions and increase the uptake through mechanotransduction pathways, allowing for larger molecules to pass through the BBB [1,6,8]. Because of its localized effect, BBB opening occurs only where the FUS field and microbubbles are present, and delivery of contrast and therapeutic agents can be targeted to precise locations. FUS mediated delivery of therapeutics to the brain has shown promise, resulting in initial clinical trials [9,10].

Another possible, noninvasive means of opening the BBB is through the use of perfluorocarbon nanodroplets (PFCnDs). PFCnDs are unique particles, that when activated by electromagnetic or acoustic energy, can phase change form liquid to gas [11–13]. They are composed of a liquid core, a shell, and when activated via laser light, an optical trigger [11,13]. PFCnDs, activated by an FUS field and converted to gas microbubble in situ have been investigated to examine their ability to open the blood brain barrier [14,15]. Acoustically activated PFCnDs phase change to microbubbles when exposed to sufficient rarefractional pressure provided by FUS and are capable of providing BBB opening through the phase change and stable cavitation of the produced microbubbles [14]. Because of their similarity to microbubbles in their gaseous state, it was demonstrated that PFCnDs are capable of producing similar BBB opening effects [14,15]. Although acoustic droplet vaporization (ADV) enables BBB opening, it involves the use of below body temperature perfluorocarbon such as perfluorobutane (boiling point −1.7°C) or octafluoropropane (boiling point −36.7°C) which can result in premature, spontaneous droplet vaporization in vivo [15]. These lower boiling point PFCs are used for acoustic droplet vaporization because PFCs with boiling points above body temperature would require potentially unsafe, higher pressure acoustic waves to cause phase change [12]. Thus, there are limitations to the use of ADV as a means to open the BBB.

In general, PFCnDs are multifaceted as they are small in size (i.e. hundreds of nanometers) in their liquid state and are capable of carrying therapeutics or other particles, which are either encapsulated or attached to the droplet shell [16]. More importantly, PFCnDs act as a versatile agent when activated to their gaseous phase via multiple forms of energy including electromagnetic waves such as pulsed laser light [12,13,17]. Specifically, the absorption of laser energy by the droplet’s optical trigger causes localized heating and expansion and produces a photoacoustic pressure wave, with these phenomena together phase changing a PFCnD from liquid to gas [13]. Vaporization under a laser pulse of low and,
therefore, safe fluence produces ultrasound (US) and photoacoustic (PA) contrast, resulting in the development of laser-activated PFCnDs as dynamic US and PA imaging contrast agents [12,13,17–19]. Laser activation of PFCnDs due to the optical trigger located within the droplet allows for implementation of higher boiling point PFCnDs, such as perfluoropentane (boiling point 29°C) or perfluorohexane (boiling point 57°C) [20]. Thus, laser-activated PFCnDs can be synthesized with many different components, using varying combinations of photoabsorbers, perfluorocarbon (PFC) species, as well as PFCnD sizes, creating application-specific PFCnDs [13,17,18,21].

Laser-activated PFCnDs have the potential to aid in the diagnosis and treatment of neurological diseases. Indeed, optically activated PFCnDs are an attractive tool for BBB opening, as they can be selectively and repeatedly vaporized at the particular wavelength at which their optical trigger absorbs the most laser light energy [13,17,18]. To enable successful BBB opening, PFCnD parameters (size, PFC species, and concentration) and laser parameters (fluence and lasing duration) must be selected appropriately in order to achieve BBB opening. In this work, we harness the characteristics of NIR dye-loaded perfluorohexane nanodroplets (PFHnDs) to open the BBB and deliver substances to the tissue.

2. Materials and methods

2.1 Synthesis of laser-activated perfluorohexane nanodroplets (PFHnDs)

Due to its boiling point being above body temperature (37°C), perfluorohexane (FluoroMed, L.P.) was used as the core of the synthesized PFCnDs [20]. In addition to 0.3 mL of perfluorohexane (PFH), 3 mL 1X PBS, 1 mL Zonyl FSO fluorosurfactant (1% v/v, Sigma Aldrich), and 2 mg Epolight 3072 dye (Epolin, Inc.) – a near infrared (NIR) dye with a peak absorption around 1064 nm – were used. All materials were added to a 7 mL scintillation vial and vortexed for 10 seconds (Vortex Mixer, Fisher Scientific). The vial was placed in an ice bath and sonicated using a probe sonicator (Q500, QSonica LLC) for a total of 60 seconds at the lowest sonicator amplitude of 1. The emulsion was then transferred to two, 2 mL tubes and spun in a mini centrifuge (Mini-Spin, Eppendorf) at 400 rcf for 2 min to remove excess dye and size separate PFHnDs. The supernatant containing smaller sized PFHnDs was transferred to another 2 mL tube and spun again at 400 rcf for 4 min. To concentrate the droplets, the supernatant resulting from this second centrifugation step was removed and the pellet was resuspended in 0.5 mL 1X PBS using a water bath sonicator (VWR, 180 W). When preparing larger sized PFHnDs for BBB opening that would enable larger (i.e. micrometer) sized constructs to extravasate, droplets were sonicated and size separated as described above, but the pellet of the first centrifugation step was kept, resuspended in 1 mL 1X PBS, and the supernatant discarded. For in vivo experiments, the droplets were left under UV light for 30 minutes for sterilization. A spectrophotometer (Evolution 220, Thermo Scientific) was used to measure the absorbance of PFHnDs both with and without NIR dye, and the blank droplet spectra was subtracted from the spectra of the PFHnDs containing NIR dye. Droplet zeta potential and size were measured in PBS using a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instruments Ltd.).

2.2 Preparation of the imaging phantom containing laser-activated PFHnDs

To evaluate droplet vaporization and recondensation dynamics, a polyacrylamide phantom containing solely PFHnDs was fabricated. The polyacrylamide phantom was prepared by first adding 127 mL nanopure water (Thermo Scientific), 42.5 mL 40% polyacrylamide solution (VWR), and 1.7 mL 10% aqueous ammonium persulfate (Sigma Aldrich) to a Büchner flask and mixing with a magnetic stir bar and stir plate. The solution was degassed by sealing the top of the flask with a rubber stopper, attaching the flask to a vacuum, and sonicating using the water bath sonicator for 10 minutes. The solution was returned to the stir plate and 170 µL PFHnDs were added to 212.5 µL of tetramethylethlenediamine...
The phantom was poured into a plastic container and allowed to solidify before removal from the mold.

2.3 Imaging and processing of laser-induced PFHnD vaporization and recondensation dynamics

To visualize the phase change dynamics of PFHnDs, the prepared polyacrylamide phantom embedded with PFHnDs was imaged using a Verasonics Vantage 256 ultrasound system (Verasonics Inc.) and an Nd:YAG Phocus laser (10 Hz, 5-7 ns pulse length, Opotek Inc.) operating at 1064 nm and a fluence of 46 mJ/cm². An array transducer operating at 5 MHz (L11-4v, Verasonics Inc.) was used to capture ultrasound at a frame rate of 500 frames per second. Photoacoustic signal was captured at the rate of 10 Hz (laser PRF). Ultrasound data was collected for six frames after each laser pulse (12 milliseconds total), and pixels containing droplets were identified using compounded harmonic ultrasound difference images, similar to a previously described method [22]. To visualize PFHnD recondensation dynamics over time, linear ultrasound intensity of the identified droplet pixels was plotted over multiple laser pulses.

2.4 In vivo blood brain barrier opening using laser-activated PFHnDs

All animal studies were conducted under the protocol approved by the Institutional Animal Care and Use Committee at Georgia Institute of Technology. Prior to anesthesia, an injection of sustained released buprenorphine (IP, 0.8 mg/kg) was administered to each animal (C57BL/6 mouse). Mice were anesthetized using a combination of isoflurane (2%) and oxygen (0.6 L/min). Mice were positioned in a stereotax in the prone position, on a heating pad (Stoelting Co). Hair from the scalp was removed through shaving and depilatory cream. Proparacaine (0.5%, Henry Schein) was applied to the eyes and a retro-orbital injection of 50 µL 3% w/v of sterile filtered Evans Blue (EB) (Sigma Aldrich) was administered with or without a co-injection of 50 µL PFHnDs (~10⁸ droplets, measured using a Malvern NanoSight NS300), depending on the animal group. The animal groups included mice irradiated with 1064 nm light and co-injected with EB dye and PFHnDs (experimental group, n = 10), mice irradiated with 1064 nm light and injected with EB dye only (i.e., no PFHnDs, control group, n = 3), and mice co-injected with EB dye and PFHnDs but not irradiated (another control group, n = 3). Animals were positioned beneath an unfocused 1.5 mm core diameter optical fiber (0.39 NA, Thorlabs, Inc.) to enable irradiation of the right side of the brain. Irradiation was performed using a Vibrant laser (10 Hz, 5-7 ns pulse length, Opotek Inc.) with a wavelength of 1064 nm and a fluence of 70 mJ/cm². Irradiation was performed for 60 seconds, falling within a similar time range to microbubble induced FUS BBB opening sonication [3,23]. After laser irradiation, mice were observed for 4 hours, with no gross behavioral damage observed. Animals were then sacrificed and perfused with 1X PBS (pH 6.8) followed by 4% paraformaldehyde (PFA). Heads were removed and post-fixed overnight in 4% PFA solution. After 24 hours, brains were excised and whole brain photographs were taken.

2.5 Ex vivo ultrasound and photoacoustic imaging

Fixed, excised mouse brains were imaged using a photoacoustic and ultrasound imaging system (Vevo LAZR, FUJIFILM VisualSonics Inc.) with a 40 MHz ultrasound and photoacoustic imaging probe (LZ-550, FUJIFILM VisualSonics Inc.). The system’s tunable ND:YAG laser (20 Hz, 5-7 ns pulse length) was operated at a wavelength of 1064 nm and at a fluence of 10-12 mJ/cm², and specimens were imaged at a USPA frame rate of 5 frames per second. Tissue samples were placed on top of an 8% gelatin base in a container filled with degassed water. Images were acquired by performing coronal and sagittal 3D USPA imaging with a distance step size of 0.1 mm. 3D USPA imaging at 1064 nm was performed both for the experimental and two control groups.
2.6 Histology and immunohistochemistry

After remaining in 4% PFA solution for 24 hours at 4°C, brains were transferred to a solution of 30% sucrose and stored at 4°C for 5 days. Brains were snap frozen, and 20 µm coronal sections were cut using a cryostat (Leica CM 1860, Leica Biosystems). For each brain, 72 sections were cut and analyzed, spanning a total volume of 1.44 mm. Standard hematoxylin (Sigma Aldrich, Gill No.2) and eosin (VWR) (H&E) staining was performed. H&E photomicrographs were captured using the bright field mode of a Zeiss AxioObserver Z1 Microscope. IHC was also performed using DAPI (4',6-diamidino-2-phenylindole, Invitrogen) and a secondary antibody of goat anti-mouse IgG (H + L) tagged with Alexa Fluor 488 (Invitrogen). IHC photomicrographs were captured using a Zeiss Laser Scanning Confocal Microscope 700.

3. Results

Fig. 1. Characterization of perfluorohexane nanodroplets (PFHnDs) (A) Size distribution of droplets (340 nm ± 170 nm). (B) Normalized absorbance spectra of droplets containing a near-infrared (NIR) dye with a peak absorption at 1064 nm and Evans Blue dye with a peak optical absorption at 600 nm, both of which are used for in vivo experiments of blood brain barrier opening. (C) Zeta Potential of the fluorosurfactant shell of the PFHnDs, with a peak at −21 mV. (D) Normalized ultrasound signal produced by PFHnDs as they repeatedly vaporize in response to pulsed laser irradiation and then immediately recondense after each laser pulse.

The results of PFHnD characterization including size, absorbance, charge, and dynamic ultrasound behavior are shown [Fig. 1]. The synthesized PFHnDs had a diameter of 340 nm ± 170 nm [Fig. 1(A)], an order of magnitude smaller than typical microbubbles used for BBB opening [1]. Due to optical NIR dye embedded into PFHnDs, the droplets exhibit peak optical absorption at around 1064 nm [Fig. 1(B)]. We specifically selected the dye absorbing near the 1064 nm wavelength because of the deep penetration of light in tissue at this wavelength, readily available from an energy-efficient nanosecond pulsed laser system [13]. Evans Blue had a peak optical absorption of 600 nm, thus there was minimal optical interaction between the 1064 nm light from the Nd:YAG laser and Evans Blue dye. A fluorosurfactant shell was
used to encapsulate liquid PFH and the NIR dye, resulting in a droplet with a negative zeta potential when measured in PBS [–21 mV, Fig. 1(C)].

The synthesized PFHnDs produced a repeatable vaporization and recondensation response to pulsed laser irradiation [Fig. 1(D)]. This liquid-gas-liquid phase change dynamic is shown via the linear ultrasound intensity of a PFHnD over time and for multiple laser pulses, with dashed lines indicating time between acquisitions. In the presence of laser irradiation, PFHnDs quickly change from liquid to gas, denoted by an increase in ultrasound amplitude. During this phase change process, PFHnDs expand to 4-5 times their droplet size, making these particular droplets 1-2 µm in size in their gaseous state [11]. After 10 milliseconds, the PFHnD recondenses, resulting in a lower observed ultrasound intensity. This vaporization and recondensation cycle is repeated every 0.1 seconds, which corresponds to the laser PRF of 10 Hz. As a result, laser activation of PFHnDs enables the creation of repeatable, transient microbubbles, which can be observed via their ultrasound behavior.

![Laser and droplets](A) ![Droplets, no laser](B) ![Laser, no droplets](C)

Fig. 2. Photographs of Evans Blue (EB) dye extravasation into tissue (A-B) Whole brain (top view) image and coronal cross-section of brain showing EB dye leakage into brain tissue post-irradiation. Rounded dashed contour highlights area of EB dye extravasation and the horizontal dashed line indicates the location of the coronal cross section displayed in panel B. (C-D) Control: whole brain image and coronal cross section of brain with EB dye and droplets administered but no laser irradiation applied. Without laser irradiation, EB dye is unable to extravasate across the blood brain barrier, preventing tissue staining. (E-F) Control: whole brain image and coronal cross-section of brain with laser irradiation applied and EB dye administered but no droplets injected. Without droplets, EB dye is unable to extravasate across the blood brain barrier, preventing tissue staining.

Validation of BBB opening was confirmed through visual examination of EB dye staining, USPA imaging, and histology. EB dye extravasation was evaluated through inspection of the whole brain followed by inspection of coronal cross-sections for each animal group [Fig. 2]. The differences in brain tissue coloration results from the lighting in which images were taken. EB dye is a commonly used substance for determining BBB opening, as it attaches itself to albumin and will only cross the BBB when the barrier has been opened [24]. When Evans Blue dye has extravasated, it is visibly evident in the tissue. After laser irradiation, EB dye was allowed to extravasate for four hours prior to animal perfusion, as the BBB likely begins to close at this time point [3,25]. For the experimental group, when EB dye was co-injected with PFHnDs and the laser irradiation was applied, EB dye extravasation into tissue is apparent in both whole brain and coronal cross section images [Fig. 2(A-B)]. This staining is located on the right side where the pulsed laser irradiation was applied. It appears greatest at the top of the brain corresponding to where laser fluence is the highest. However, when EB dye and PFHnDs were co-injected and no laser irradiation was applied, the brain tissue remains unstained [Fig. 2(C-D)]. Finally, the combination of laser
irradiation and EB dye administration but no injection of PFHnDs also results in tissue with no EB dye extravasation [Fig. 2(E-F)] Thus, these results demonstrate that PFHnDs combined with laser irradiation are capable of opening the BBB and delivering substances to the brain.

USPA imaging of perfused, excised murine brains was also performed to evaluate BBB opening [Fig. 3]. The synthesized PFHnDs contain a dye absorbing at 1064 nm, which produces a PA signal at that wavelength. Thus, if droplets containing the dye empty some of their cargo in the process of vaporization, this dye has the potential to extravasate across the BBB. Once extravasation has occurred, PA signal at 1064 nm should be present, allowing for USPA imaging of the tissue. US imaging provides an anatomical map, while PA imaging provides the localized signal of extravasated dye. In the experimental group [Fig. 3(A)], where PFHnDs and laser irradiation were present during the BBB opening procedure, PA signal is present on the right side of the brain in a coronal cross section that corresponds to the location of EB staining as seen in the whole brain and cross section photographs [Fig. 2(A-B)]. To confirm that the PA signal comes from the 1064 nm absorbing dye extravasated post-laser irradiation, control groups were also USPA imaged at 1064 nm. One control group involved the administration of droplets but no laser irradiation during the BBB opening procedure [Fig. 3(B)]. The second control group included laser irradiation but no administration of droplets [Fig. 3(C)]. USPA imaging of these brains demonstrates that extravasation of dye only occurs if droplets are present and have been irradiated because no PA signal at 1064 nm is produced otherwise [Fig. 3(B-C)]. Thus, USPA imaging can be used to evaluate BBB opening when laser-activated PFHnDs are used.

Fig. 3. Ex vivo photoacoustic and ultrasound (USPA) imaging of murine brains (A) USPA imaging of an animal with blood brain barrier opening and release of near-infrared (NIR) dye from PFHnDs after irradiation. After opening, the NIR dye has extravasated across the blood brain barrier and provides signal when imaged at 1064 nm. (B) Control: USPA imaging demonstrating that without laser irradiation to vaporize droplets, no NIR dye has extravasated, resulting in no PA signal produced at the imaging wavelength. (C) Control: USPA image demonstrating that without droplets to open the blood brain barrier, no NIR dye has extravasated, resulting in no PA signal produced at the imaging wavelength.
BBB opening was further examined on the cellular level using histological and immunohistochemical methods [Fig. 4]. For these studies, larger sized PFHnDs were synthesized (837 nm ± 229 nm) to ensure significant BBB opening and enable definitive extravasation of biological particles across the BBB. When activated via laser irradiation, these PFHnDs should expand to 3-4 μm in size in their gaseous state [11]. As a result, these larger bubbles will enable greater BBB opening and illicit a larger biological response [26]. For the studies involving laser irradiation [Fig. 4(A-C), (G-I)], irradiation was performed for 60 seconds at a wavelength of 1064 nm and a fluence of 70 mJ/cm². To examine the extravasation of nanosized constructs, fluorescence images captured the extravasation of mouse immunoglobulin G (IgG), which should only cross the BBB when the barrier is opened, due to its width being 13.7 nm [Fig. 4(A-B), (D-E), (G-H)] [27]. In the experimental group where opening took place (laser irradiation and droplets), staining of IgG is clearly present [Fig. 4(A-B)]. In control animals where no laser irradiation was applied but droplets were administered [Fig. 4(D-E)], no extravascular IgG is observed. However, there appears to be low levels of IgG signal in the lateral ventricles in the fluorescent image of the whole brain [Fig. 4(D)], but when a high magnification view of the tissue is examined [Fig. 4(E)], no IgG is present. In the case of laser irradiation but no droplet injection, low levels of IgG are also observed in both the whole brain image and magnified view [Fig. 4(G-H)]. From the IHC analysis, it appears that some IgG may be present in the control groups, however, delivery of
IgG across the BBB is most obvious in the experimental group containing both laser irradiation and PFHnDs.

Delivery of larger constructs, specifically red blood cells (RBCs), was also examined via H&E staining [Fig. 4(C),(F),(I)]. As seen in the experimental group [Fig. 4(C)], RBCs extravasated across the blood brain barrier and are highlighted by the tissue area stained red. For both controls, however, no RBCs extravasated [Fig. 4(F),(I)]. Because RBCs were able to extravasate in the experimental group, the result indicates that laser-activated PFHnDs were able to create openings in the BBB that allowed for 6 µm diameter particles to cross the blood brain barrier [28].

4. Discussion

In this work, successful delivery of various sized constructs such as RBCs, Evans Blue (EB) visible dye bound to albumin, NIR dye, and IgG was delivered across the BBB using laser-activated perfluorohexane nanodroplets (PFHnDs). The delivery of these particles was verified and evaluated through different methods including EB dye staining, USPA imaging, and histological tissue analysis. Thus, droplet parameters play a key part in determining BBB opening. Specifically, droplet size, PFC species, and photoabsorbers selected all play a unique role in the effective size and spread of BBB opening and therefore delivery of substances into the tissue. For example, larger sized PFmDcs can cause an increase in BBB opening volume and potentially damage to the tissue. A lower boiling point perfluorocarbon that does not repeatedly vaporize and recondense such as perfluoropentane may not open the BBB to a similar extent as PFH. Further, a lower wavelength photoabsorber could prevent deeper activation of PFmDcs due to reduced light penetration in the tissue. Thus, by selectively choosing the appropriate droplet components, droplets can be used in and tailored to a diverse set of applications, including delivery of contrast agents and therapeutics to the brain. Furthermore, laser parameters also contribute to BBB opening and can also be adjusted based on droplet parameters to achieve the desired volume and location of BBB opening in the brain tissue.

In our reported studies, PFmD characteristics were chosen to achieve effective BBB opening. Specifically, PFHnDs were synthesized such that upon laser irradiation, the size of resulting microbubbles would be similar to those that have been used in FUS BBB opening [Fig. 1(A)] [3]. A NIR dye absorbing near 1064 nm was used not only for increased depth penetration of light but also to avoid optical absorption by the Evans Blue dye co-injected with PFHnDs. Evans Blue has a peak absorption of 600 nm, and at 1064 nm, EB absorption is negligible [Fig. 1(B)]. Therefore, the negligible interaction of EB and the 1064 nm laser light will not cause BBB opening, and extravasation of the EB across the BBB will only occur when droplets are both present and exposed to laser irradiation. In addition, low EB absorption at 1064 nm indicates that the captured USPA signal is produced from NIR dye delivered to the tissue. The USPA imaging of the NIR dye in comparison to Evans Blue photographs also suggests that different sized particles are able to cross the BBB using laser-activated PFHnD opening [Fig. 3(A)].

Furthermore, the PFC species used in these droplets plays a key role in successful BBB opening. The ability of PFHnDs to repeatedly vaporize under pulsed laser excitation and then recondense potentially makes a PFH droplet core more attractive than lower boiling point PFCs such as perfluorobutane or perfluoropentane [20]. The reactivation of PFHnDs over multiple laser pulses suggests that in combination with an increased number of laser pulses used, lower concentrations of PFHnDs could be used to open the BBB, reducing the injected PFHnD dose. This concentration comparison may also extend to microbubble concentrations used in FUS BBB opening. Based on the rapid expansion and recondensation of PFHnDs, it is supposed that the phase changing droplets interact with the BBB similar to the way microbubbles do in a FUS field [Fig. 1(D)]. As PFmDcs have a longer circulation time than microbubbles (i.e. hours vs. minutes, respectively), fewer PFHnDs may be needed to achieve
effective BBB opening [16]. As a result, PFC choice plays a key role when designing laser-activated PFCnDs for BBB opening.

To examine BBB opening, EB staining was evaluated grossly and supported the hypothesis that BBB opening would only occur where both laser irradiation and PFHnDs are present [Fig. 2]. Ex vivo USPA imaging was also performed to co-register PA signal with the area of EB staining, both localized to the right side of the brain [Fig. 3]. USPA contrast from delivered NIR dye indicates the potential for future in vivo USPA imaging of laser-activated PFCnDs during BBB opening and delivery of PA contrast agents. Finally, histological staining analysis was completed to examine the effect of laser-activated PFHnD opening on the molecular level. Overall, extravasation was greatest in the experimental group [Fig. 4(A-C)], further demonstrating that BBB opening can be achieved using laser-activated PFHnDs. However, in both control groups [Fig. 4(D-E),(G-H)], IgG signal is present. For the control group where PFHnDs were injected but no laser irradiation was applied, IgG signal was visible in the lateral ventricles but not in a magnified view of the brain tissue, suggesting that the signal most likely corresponds to an incomplete perfusion. In the control group with laser irradiation and Evans Blue dye but no PFHnDs administered, there are a few possibilities as to why IgG may be present. Both deoxygenated hemoglobin and oxygenated hemoglobin absorb in the NIR wavelength range and as a result, interaction of the blood with laser energy could cause small openings in the BBB that would allow for IgG to cross the BBB [29]. Additionally, incomplete perfusion of the tissue could also cause a false positive fluorescence in the sample, as there appears to be IgG signal coming from the lateral ventricles [Fig. 4(G)] in addition to fluorescence signal seen in the magnified view of the pictomicrograph [Fig. 4(H)]. Despite the fluorescence signal seen in both control samples, IgG signal is greatest in the experimental group showing that localized BBB opening is effective when PFHnDs are irradiated via pulsed laser excitation. Overall, the results of the methods used to evaluate BBB opening (i.e., EB staining, USPA imaging, and histological tissue analysis), support the hypothesis that laser-activated PFHnDs are capable of opening the BBB.

Not only can droplets cause opening of this particular biological barrier, but they also have the potential to be implemented to open other biological barriers that may be inhibited otherwise and are preventing effective, non-invasive treatment. In particular, another barrier that poses challenges in noninvasive delivery is the blood spinal cord barrier (BSCB) [30]. Recently, the BSCB has been opened using FUS and microbubbles, so investigation into the ability of PFCnDs to open the BSCB is warranted [30,31]. For opening of both the BBB and BSCB, laser and droplet parameters should be evaluated for safety and efficacy, as opening of these barriers with parameters not fully optimized has the potential to damage tissue.

In addition, droplets can be synthesized to as small as 100-200 nm and, therefore, have the potential to extravasate themselves when the BBB has been opened [32,33]. Thus, through the administration of one set of small, cargo carrying droplets and a set of larger droplets used for BBB opening, delivery of substances can be contained within cargo carrying PFCnDs until they have reached their desired location. Because droplets can be produced in various sizes and compositions, they are capable of performing as a multiplexed system, enabling subpopulations of droplets to perform different tasks. This multiplexed ability can be harnessed not only via size, but by also using photoabsorbers of different peak wavelengths, different core perfluorocarbons, and different shell compositions.

Consequently, laser-activated droplet adaptability enables a platform for opening of the BBB and delivery of both therapeutics as well as imaging contrast agents to the brain. Furthermore, laser-activated droplets produce localized, temporally modulated ultrasound and photoacoustic contrast and therefore in the future could provide the opportunity for image-guidance of BBB opening as well as image-guidance of delivery of cargo to the tissue [18,19,22,34,35]. Because the process of optimized BBB opening is transient and reversible, localized delivery of cargo to the brain is reproducible, enabling the potential to treat or image longitudinally. From opening barriers to providing delivery and imaging contrast, the
presented studies provide an initial demonstration of the capabilities and potential of laser-activated PFCnDs in neurological applications.

5. Conclusion

This work demonstrated the ability of laser-activated perfluorocarbon nanodroplets (PFCnDs) to open the blood brain barrier (BBB). BBB opening was evaluated using ultrasound-guided photoacoustic imaging techniques, examining Evans Blue dye extravasation, and performing histology and immunohistochemistry. The results indicate that laser-activated PFCnDs are capable of providing a localized, noninvasive BBB opening as well as delivery of agents across the barrier. These initial studies provide the foundation for future work involving the harnessing of laser-activated PFCnDs for use in BBB opening, imaging, and delivery. Overall, laser-activated PFCnDs have the potential to become a versatile tool for neurological applications.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.