Cavitation-facilitated transmembrane permeability enhancement induced by acoustically vaporized nanodroplets

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ABSTRACT

Ultrasound-facilitated transmembrane permeability enhancement has attracted broad attention in the treatment of central nervous system (CNS) diseases, by delivering gene/drugs into the deep site of brain tissues with a safer and more effective way. Although the feasibility of using acoustically vaporized nanodroplets to open the blood–brain-barrier (BBB) has previously been reported, the relevant physical mechanisms and impact factors are not well known. In the current study, a nitrocellulose (NC) membrane was used to mimic the multi-layered pore structure of BBB. The cavitation activity and the penetration ability of phase-changed nanodroplets were systemically evaluated at different concentration levels, and compared with the results obtained for SonoVue microbubbles. Passive cavitation detection showed that less intensified but more sustained inertial cavitation (IC) activity would be generated by vaporized nanodroplets than microbubbles. As the results, with a sufficiently high concentration (~5 \times 10^8/mL), phase-changed nanodroplets were more effective than microbubbles in enabling a fluorescent tracer agent (FITC, 150 kDa) to penetrate deeper and more homogeneously through the NC membrane, and a positive correlation was observed between accumulated IC dose and the amount of penetrated FITC. In vivo studies further confirmed acoustically vaporized nanodroplets performed better than microbubbles by opening the BBB in rats’ brains. These results indicated that phase-changed nanodroplets can be used as a safe, efficient and durable agent to achieve satisfactory cavitation-mediated permeability enhancement effect in biomedical applications.

1. Introduction

The blood–brain-barrier (BBB) is a highly specialized structure of blood vessels and capillaries in the central nervous system (CNS). It is composed of the arachnoid membrane, brain capillary endothelial cells (ECs) and choroid plexus [1]. These lamellar cell structures constitute tight junctions (TJs), also known as vascular occlusions [2]. By forming an almost impermeable barrier to prevent the diffusion of macromolecules and hydrophobic molecules, the BBB protects the normal brain parenchyma from toxic foreign substances, but it also makes the CNS unavailable for effective diagnosis and potential medication under disease conditions [3]. Therefore, the normal physiological function of the BBB needs to be temporarily interrupted to allow macromolecular (>400 Da) diagnostic or therapeutic drugs to diffuse into the brain tissue [4]. With ultrasound (US) irradiation, microbubble-mediated BBB opening is a feasible prospect for drug delivery in the treatment of CNS diseases, including Alzheimer’s, Parkinson’s diseases and brain cancer [5]. This non-invasive [6], temporary, and reversible method is capable to achieve safe [7] and effective delivery of drugs /genes through the BBB. Negishi et al. delivered plasmid DNA into the brain using lipid microbubbles full of C2F6 gas and the application of ultrasound [8]. Ting et al. used microbubbles loaded with 1,3-bis(2-chloroethyl)-1-

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nitrosourea to treat glioblastoma tumors, which reduced the systemic drug toxicity [9]. The major mechanism of BBB opening induced by US combined with microbubbles is thought to be via stable or inertial cavitation [10]. In stable cavitation (SC), the microbubbles repeat a process of volumetric oscillation while expanding and contracting under ultrasound stimulation. The expansion of the microbubbles separates the inner-layer ECs, while the contraction results in invaginations in the vascular lining [11]. The push–pull effect and steady microstreaming could be induced by stable cavitation microbubbles. As the results, a strong shear force will be exerted on the surrounding ECs and TJJs to interrupt their integrity [12] and enhance the permeability of ECs could be induced by stable cavitation microbubbles. As the results, a strong shear force will be exerted on the surrounding ECs and TJJs to interrupt their integrity [12] and enhance the permeability of ECs [1,13]. Inertial cavitation (IC) generally occurs under strong ultrasound excitation, causing the microbubbles to collapse suddenly and producing strong mechanical stress, microstreaming, and microjets around the microbubbles [2,14]. Instant microjets and shock waves can enhance cell membrane penetration and blood tissue permeability, even causing erythrocyte extravasations or micro-damage to the ECs.

At present, commercial or self-made gas microbubbles used for ultrasound-induced BBB opening generally have an average radius at the scale of micrometers (1–8 μm), which is large enough to prevent the microbubbles from penetrating into deeper blood vessels or tissues, while may also limit the efficiency of BBB opening mediated by cavitation microbubbles [15]. In addition, the gas microbubbles circulating in the body may suffer from relatively short half-life (typically only a few minutes), and their stability might be easily affected by physiological temperature and environmental pressure. As the results, additional US excitation and microbubble injection must be given if most bubbles have been damaged before satisfied diagnostic/therapeutic purposes are achieved, which could be dangerous if the injected bubble volume exceed clinical drug doses [16,17].

The application of phase-changed nanodroplets has the potential to solve these problems. The liquid in the core of nanodroplets will vaporize to a gaseous state under ultrasonic excitation, causing the droplet to expand to about 5–6 times its original diameter, termed acoustic vaporization [18]. The gaseous microbubbles formed by these droplets can functionize as contrast agents in US diagnosis [19]. They also can undergo more violent oscillation or even cavitation activities under the excitation of therapeutic ultrasound [20]. Due to their small size, phase-changed droplets can easily penetrate microvessels via the enhanced permeability and retention effect in the tumor environment and accumulate in the target region to achieve therapeutic purposes [21]. Yeh et al. [22] used camptothecin-loaded acoustically activated nanodroplets to inhibit tumor growth for up to six weeks. Kennedy et al. [23] found that, by applying 1-MHz ultrasound radiation for 3 min, the vaporization and cavitation of phase-changed droplets can be induced to produce good therapeutic effects with paclitaxel in animal models of breast and pancreatic cancer. In the CNS, the nanodroplets are able to naturally penetrate the BBB and work in deeper regions. For instance, Green et al. [24] achieved a rapid effect by loading small propofol molecules on nanodroplets for the treatment of acute epilepsy in rats.

Although acoustically vaporized nanodroplets have demonstrated promising potentials in the treatment of CNS diseases by improving the efficiencies of BBB opening and drug delivery, it is still lack of comprehensive investigation on the difference in the cavitation behaviors and transmembrane permeability enhancement effects generated by regular ultrasound contrast agent (UCA) microbubbles and acoustically vaporized nanodroplets, especially under in vivo situations. The present work was conducted to use the respectively long-lasting perfluoropropane (PPF) [25] as the core of the phase-changed droplets, and then compare the cavitation activity, transmembrane permeability enhancement and BBB opening effects induced by PPF nanodroplets with those generated by UCA microbubbles, under both in vitro and in vivo conditions. Generally, at the first-stage, the multilayer-porous nitrocellulose (NC) membrane was used to mimic the physical structure of BBB, and both the IC dose (ICD) and the penetration performance of the two type of agents were explored at different concentration levels. Subsequent in vivo experiments were conducted at the optimal agent concentrations determined according to the in vitro studies, so that the BBB opening effect induced by PPF nanodroplets and SonoVue microbubbles could be further compared in the rat’s model. The current work will help us better understand the physical mechanism underlying the enhancement of transmembrane permeability induced by acoustically vaporized nanodroplets, which may provide useful knowledge for the development of safer and more effective phase-changed nanodroplet application strategies.

2. Materials and methods

2.1. Nanodroplets and ultrasound contrast agent microbubbles

The nanodroplets used in the study were lipid shells that encapsulated PFP droplets. The lipid shells consisted of 60% DSPC powder (850365P, Avanti Polar Lipids, Inc., AL), 25% DSPE2000 powder (880120P, Avanti Polar Lipids, Inc., AL), and 15% DPPG powder (840455P, Avanti Polar Lipids, Inc., AL). Chloroform and methanol were added at a 2:1 ratio. After shaking to a uniform state in a 500 mL flask, the lipid was heated and evaporated at 45°C about 6 h to a membrane shape using a rotary evaporator, and the resultant lipid film was resuspended using phosphate-buffered saline (PBS, Gibco Inc., Carlsbad, CA, USA). Finally, 10 mL of the resuspended lipid solution was sonicated together with 800 μl perfluoropentane (Strem Chemicals Inc., MA) for a total duration of 20 s in a 50 mL centrifuge tube under ice bath using an ultrasonic processor (VCX 750, Vibra-Cell Processors, Sonic & Materials, Inc., CT) at 30% output setting (20 kHz, 225 W output power approx.) [26]. After the contrast agents were produced, the size distribution of droplets was measured using a particle sizer based on dynamic light scattering (Range: 0.3 nm–6 μm, Nanobrook 90 Plus Zeta, Brookhaven Instruments, NY). The droplets were placed into a gel phantom composed of acrylamide [27]. After sonication, the gel was sliced and then the state of the droplet particles was observed under a microscope (IX51, Olympus, Shinjuku, Tokyo, Japan).

SonoVue microbubbles, whose mean radius is about 2.5 μm [28], were purchased from Bracco (Bracco diagnostics Inc., Geneva, Switzerland). Before the experiments, the vial of SonoVue would be vented with a sterile needle (18-gauge), followed by the injection of PBS to make SonoVue microbubble solutions. Evans blue was purchased as powder (E6135, WanQing, NanJing, China).

2.2. Animal preparation

Adult SPF (specific pathogen free) male Sprague-Dawley rats (Charles River Laboratories, Beijing, China) weighing 180–200 g were used in this study. Animals were housed in groups of five in plastic cages under controlled temperature (22 ± 3 °C), humidity (50%–60%), ventilation, and lighting (12 h light/dark cycle) and were allowed free access to food and water. All experimental protocols were approved by Institutional Animal Care and Use Committees of Affiliated Hospital of Nanjing University of Chinese Medicine, China.

2.3. In vitro assessments of US-activated cavitation behaviors and nitrocellulose membrane penetration performance

A device similar to a Franz diffusion cell (FDC) [29], including two compartments, was designed to compare the cavitation-facilitated penetration performance induced by two types of agents (viz., PFP nanodroplets and SonoVue microbubbles). The NC membrane (YA1711, Solarbio, Beijing, CN) with an average pore diameter of 450 nm was placed between the donor (150462, Thermo Fisher Scientific, Waltham, MA, USA) and the receptor compartments. The receptor compartment under the NC membrane was filled with PBS at a thermostatic temperature of 37 °C. A magnetic stirrer was used to distribute the delivered
As shown in Fig. 1, a single-element planar probe (center frequency: 1.07 MHz; diameter 67 mm; WED-310, Weld, Shenzhen, CN) was driven by an ultrasonic sonicator (YDT-0101, WED-310, Weld, Shenzhen, CN). The probe was immersed in the donor compartment containing the sample solution. During sonication, the ultrasound driving parameters were set as follows: 700-kPa acoustic pressure, 80-μs pulse length, 400-Hz pulse repetition frequency (PRF), 3.2% duty ratio and 5-min irradiation time. A hydrophone (HNA, ONDA, Sunnyvale, CA, USA) was mounted at 45° to the ultrasonic beam to passively detect the broadband noises scattered from cavitation bubbles in real time. An oscilloscope (54810, Agilent, USA) displayed the waveform and recorded the data. According to the method described elsewhere [30], a MATLAB (Mathworks, Natick, MA, USA) program was used to post-process the acquired waveforms and quantify the IC doses (ICDs) generated by acoustically vaporized nanodroplets and SonoVue microbubbles.

The experiments were generally divided into four groups: control group (viz., natural diffusion without US exposures); PBS group exposure to US (US Only); SonoVue group exposure to US; and PFP nanodroplet group exposed to US. To explore the correlation between the penetration performance and IC [31] activity generated by the two types of agents, the nanodroplets and SonoVue microbubbles were dissolved in the PBS solution at two different concentration levels (viz., ~10^8/mL and ~5 × 10^5/mL, correspondingly referred as low concentration and high concentration respectively). After 5 min of US exposure, a 1 mL sample solution was retrieved from the receptor compartment. The FITC extravasation of the solution was quantified via an ELISA microplate reader (ELx800, BioTek, VT, USA; 460-nm excitation and 534-nm emission) by a linear regression standard curve derived from fourteen reader (ELx800, BioTek, VT, USA) was mounted at 45°- to the ultrasonic beam to passively detect the broadband noises scattered from cavitation bubbles in real time. An oscilloscope (54810, Agilent, USA) displayed the waveform and recorded the data. According to the method described elsewhere [30], a MATLAB (Mathworks, Natick, MA, USA) program was used to post-process the acquired waveforms and quantify the IC doses (ICDs) generated by acoustically vaporized nanodroplets and SonoVue microbubbles.

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![Fig. 1. Schematic diagram of the in vitro experimental system.](image1)

![Fig. 2. Schematic diagram of the in vivo experimental system.](image2)
The distribution of the mean hydrodynamic diameter of PFP(a). Sample microscopic images of PFP nanodroplets (b) without US exposure and (c) after 1-min US exposure.

3. Results

3.1. Acoustically vaporization of nanodroplets

The nanodroplets used in the experiment were in translucent milky white color, with an average hydrodynamic diameter of 470 nm measured by a particle size analyzer (Fig. 3a). To verify that the lipid nanodroplets could be effectively vaporized under the experimental US parameters, a gel phantom was used as the carrier to encapsulate PFP nanodroplets. As can be seen in Fig. 3b, the original nanodroplets are barely visible under 400× magnification. After US exposure (700-kPa acoustic pressure, 80-μs pulse length, 400-Hz pulse repetition frequency, 3.2% duty ratio) for 1 min, some of the nanodroplets become large enough to be clearly observed (Fig. 3c), indicating that they were responsive to the US irradiation and could be acoustically vaporized. Five minutes after US exposure, there were traces of particle fragmentation, which indicated that inertial cavitation might occur to result in the collapse of vaporized droplets and make them become invisible again.

3.2. In vitro cavitation assessments based on Franz diffusion cell system

A PCD system was used to evaluate the cavitation activities generated during in vitro transmembrane studies, for both PFP nanodroplets and SonoVue microbubbles with two different concentrations. After repeated testing (three times per group), the root mean square (RMS) FFT amplitude of IC signals measured for each sample over time were obtained, as shown in Fig. 4a. In general, there is no significant cavitation signal was detected for PBS under the current US parameter settings. The SonoVue microbubbles responded strongly to US and apparently reached very high levels in initial 40–60 s of US irradiation, but gradually decreased to PBS levels at the late stage. Different from SonoVue microbubbles, PFP nanodroplets responded stably to US activation and sustained almost the entire US exposure process without obvious decrease. The ICD values accumulated during the US treatment processes were also quantitatively evaluated for individual groups. As shown in Fig. 4b, treated by the same US parameter settings, the PBS groups gives the lowest ICD level. Comparing the result gotten for PBS with the ICDs assessed for individual microbubble/nanodroplet groups, it is noticed that all the 4 groups produced a certain degree of IC activities under US exposure, and for both agents, ICD levels increase with the enhancement of their concentrations. It is also interesting to observed in Fig. 4b that, at relatively low concentration, the PFP group produces weaker IC activities than the SonoVue group, while at high concentration the nanodroplets demonstrate a significant enhancement of the IC activity than all the other groups by giving the highest ICD value.

3.3. In vitro cavitation-facilitated transmembrane studies

In vitro experiments were performed here to compare cavitation-facilitated transmembrane capabilities generated by PFP nanodroplets and SonoVue microbubbles with different concentrations. A Franz diffusion cell system was adopted in the present work and the NC membrane system was used to approximately mimic biological BBB. As illustrated in Fig. 1, the 150-kDa FITC could be delivered into the donor compartment through the NC membrane with the help of the cavitation effect generated by nanodroplets or microbubbles. The significant penetration depth of FITC in the NC membrane could be determined using the confocal microscope. As shown in Fig. 5a, the image taken for the control group (viz., the passive diffusion group with US sham) shows that FITC particles basically stayed on the surface, whereas all the US-treated groups provided more FITC penetration into the NC membranes with the facilitation of cavitation effect. It is noticed in Fig. 5a that, for both SonoVue and PFP agents, the FITC penetration depth is improved with the increasing agent’s concentration. But under the current US parameter settings, only the group containing PFP nanodroplets with high centration allowed the FITC particles to fully penetrate through and open the entire cross-section of the NC membrane, so that the fluorescent green color appears to evenly distribute all over the NC membrane for the PFPHIGH group. Quantitative analyses were performed to assess the fluorescence intensities for the samples taken from individual groups. Fig. 5b demonstrates that all the US-treated groups demonstrate an enhancement effect of FITC penetration in NC membranes comparing with the US sham group. It is also noticed that the high concentration groups exhibit superior transmembrane capability than the low concentration ones, and the best FITC penetration performance is observed in the PFPHIGH group.

There are some FITC particles can eventually pass through the NC membrane and get into the receptor compartment of the FDC. Therefore,
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the solution samples of individual groups were collected after the experiments, and the absorbancy of each sample was assessed by an ELISA microplate reader. All the measurement data are plotted in Fig. 6. The results suggest that the addition of SonoVue bubbles combined with US exposure does be helpful for the FITC particles to diffuse into FDC receptor. Consistent with the results shown in Fig. 5b, the high-concentration PFP group causes the most FITC particles to pass through the NC membrane (~40 times that of control group). However, no significant difference can be identified between control group and the PFP group with low concentration.

3.4. In vivo BBB opening analysis

The results of in vitro transmembrane experiments suggested that, at relatively high concentration, acoustically vaporized PFP nanodroplets can even perform better than SonoVue microbubbles. Therefore, in vivo experiments were performed to evaluate the BBB opening capability for the following 4 groups: control (US sham), US only, SonoVue and PFP, and relatively high concentration (~5 × 10^9/mL) was selected in subsequent experiments.

The presence of the EB in the animal brain was regarded as a representative indicator to assess the degree of effective BBB opening effect. As shown in Fig. 7, the presence of EB can be easily observed in the rat brain treated with US exposure combined with the addition of nanodroplets or microbubbles, but it is not easily observable in the control and US only groups. Comparing with the SonoVue group, the EB blue color in the rat’s brain of the PFP group is much darker (Fig. 7a) and diffused much deeper inside the brain tissue (Fig. 7b).

The total amount of EB in the brain tissue of rats was extracted and the absorbancy was quantitatively measured using an ELISA microplate reader. Specifically, the measured absorbancy value of each group was compared with the average value of the control group. A series of relative absorbance comparison values were obtained to reflect the amount of EB in the rat brains. As can be seen in Fig. 7c, the relative amount values of the EB of the four groups are 1, 1.43, 2.78, and 5.91. It indicates that the scheme of nanodroplet combined with US can deliver more macromolecular drugs into the brain parenchyma, with a high level of statistical significance (p < 0.0001) compared with the other three groups. In other words, the results further confirm that acoustically vaporized PFP nanodroplets can achieve much more significant BBB opening effect than SonoVue microbubbles.

4. Discussion

As a promising technology for noninvasive transcranial drug delivery, the studies on the feasibility of opening BBB with acoustically vaporized nanodroplets have attracted broad interests of researchers. Many previous studies have shown the effectiveness of this method. However, most of them focused on the impacts of US parameters (i.e., sound pressure, pulse length and PRF) or drugs properties on the effectiveness of this therapy [35]. In the present work, systemic in vitro and in vivo experiments were performed to better understand the mechanisms underlying the effects of acoustically vaporized nanodroplet cavitation on enhanced transmembrane permeability and BBB opening capability.

First, an in vitro FDC system was designed to characterize the mechanical effect of acoustically vaporized nanodroplets on the structure of NC membrane. The NC membrane is selected to mimic the physical structure of biological, because it is easy to obtain and has much less risk of contamination than in vitro cultures of brain tissue endothelial cells. Similar to the BBB, the NC membrane itself has a layered network structure with tiny holes randomly distributed on the cross-section of each layer. The cavitation dosages generated by US-irradiated PFP nanodroplets with two different concentrations were evaluated based on PCD measurements. Confocal microscopy assessments revealed the penetration effect of fluorescent dye inside the NC membrane, and then an ELISA microplate reader analyses provided quantitative information about the relative amount of FITC fully passing through the NC membrane. Meanwhile, all the observation and measurement results for the PFP nanodroplets were compared with those obtained for SonoVue microbubbles. The results demonstrated in Figs. 4 and 5 suggest that, at relatively low concentration, acoustically vaporized nanodroplets exhibit less ICD and weaker penetration ability than SonoVue microbubbles, while high-concentration PFP can generate much stronger ICD to result in much superior permeability enhancement effect on the NC membrane than microbubbles treated with the same parameters. Due to the similarity in the variation trends of ICD and the fluorescence intensity of FITC penetrating inside the NC membrane, the correlation between measured ICD and fluorescence intensity were further evaluated for the pooled data, and a significant positive correlation can be easy observed in Fig. 8, which is consistent with pervious observations on improved sonoporation effect induced by US-activated microbubble cavitation [36,37].

Although the accumulated ICDs exhibited similar variations for both
PFP nanodroplets and SonoVue microbubbles, it should be noticed in Fig. 4a that the temporal evolution IC FFT amplitudes demonstrate significantly different behaviors. Regardless the concentration level, the FFT amplitude of SonoVue microbubbles always has a much higher peak value than PFP nanodroplets, although it will drop quickly after reaching the peak value. On the contrary, the cavitation activity of PFP nanodroplets behaves more moderately and can sustained throughout entire US exposure period. A reasonable explanation for this observation is that, before stimulating cavitation activity, part of incident acoustic energy needed to be utilized to trigger the phase change of PFP nanodroplets first [38], so that the peak IC intensity generated by nanodroplets is always lower than that of microbubbles. It could be a shortcoming for the application of PFP nanodroplets with low concentration because the relatively low ICD might not be sufficient to achieve satisfactory transmembrane effect. But at sufficiently high concentration, PFP should perform much better in the circumstance when stable IC activity is required over relatively long time, because most SonoVue microbubbles could be destroyed by excessively violent IC activity so that it might be lack of enough nuclei to maintain sustained IC activity during the following US irradiation time.

Above observations indicated that a favorable transmembrane effect could be achieved by utilizing high-concentration PFP nanodroplets combined with US irradiation [39]. In order to verify this speculation, scanning electron microscopy was used to observe the morphological changes in the surface of the NC membrane treated under four different conditions. Typical SEM images are provided in Fig. 9. In the control group (US sham; Fig. 9a), some tiny pores with an average diameter of ~ 450 nm are randomly distributed on the membrane surface. Simple US exposure without the addition of either nanodroplets or microbubbles barely changes the membrane surface morphology (Fig. 9b). The combination of US and SonoVue microbubbles with high concentration may generate significant cavitation effect to enlarge the pore size on the surface of NC membrane (Fig. 9c), while the treatment generated by acoustically-vaporized PFP nanodroplets can not only increase the pore diameter but also make the holes penetrate deeper inside the NC membrane (Fig. 9d). The observation indicates that, comparing with SonoVue microbubbles, the nano-scale size of PFP droplets can firstly penetrate deeper into the NC membrane and then be vaporized and cavitated by incident US to achieve much better transmembrane effect.

The SEM observations may provide a good perspective to interpret the FITC penetration results shown in Fig. 5a. In the control group with US sham, these tiny holes are small enough to block the passive diffusion

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**Fig. 5.** Cavitation-facilitated transmembrane effects induced by PFP nanodroplets and SonoVue microbubbles with different concentrations. (a) Confocal microscope images illustrating the penetration depth induced by individual sample groups sonicated by US with the follow parameters: 700-kPa acoustic pressure, 80-μs pulse length, 400-Hz PRF, 3.2% duty ratio and 5-min irradiation time; (b) Quantitative assessments for the fluorescence intensity of FITC penetrated into the NC membrane in individual groups. Significant differences are statistically identified with p < 0.05 (***, p < 0.001, **: p < 0.01, *: p < 0.05).
of FITC particles with relatively large molecular weight. Treated by high-concentration SonoVue microbubbles with US exposures, the membrane could be stretched out and bigger holes can be opened on the upper surface of the NC membrane that allowed some FITC particles to get into the membrane [22]. However, complete penetration of the NC membrane required comparably large pores on each layer. As can be seen in Fig. 9d, more layers of the fine mesh structure are destroyed so that larger and deeper holes can be observed in the NC membrane treated by acoustically-vaporized PFP nanodroplets at high concentration. This may be due to the small size of the nanodroplets, which gradually penetrated into these fine pores and reached deeper than the microbubbles before US exposure [40]. Even more, after the initial penetration, the nanodroplets continued to grow and create more holes due to the acoustic energy. Fig. 6. Light absorbancy assessments to evaluate the amount FITC particles penetrated through the NC membrane and get into the receptor compartment of FDC in individual groups. Significant differences are statistically identified with $p < XXX$ (ns: no significance, **: $p < 0.01$, *: $p < 0.05$).

Fig. 7. The comparison of effective BBB opening effect generated in individual groups. The US parameters were set as 700-kPa acoustic pressure, 80-μs pulse length, 400-Hz PRF, 5-min exposure time and 3.2% duty ratio. (a) Typical pictures of the presence of EB in rats’ brain after the treatment; (b) EB distribution in the section slices of rat’s brain treated by acoustically activated PFP nanodroplets or SonoVue microbubbles; and (c) Quantitatively assessment of the amount of EB extravasation in rat’s brain treated by acoustically activated PFP nanodroplets or SonoVue microbubbles. Significant differences are statistically identified with $p < 0.01$.

Fig. 8. The correlation between FITC fluorescence intensity penetrating inside the NC membrane and the ICD generated by US contrast nanodroplets/microbubbles.
penetration of nanodroplets, the remaining non-phase-changed droplets might penetrate through the porous NC membrane again and be vaporized by US in the following time [41]. As the results, the FITC can diffuse wider and penetrate farther inside the membrane under the combined treatment of high-concentration nanodroplets and US sonication (Fig. 5a). Thus, the results suggest that, with sufficiently high concentration, nanodroplets could be used as a more favorable agent to improve the membrane permeability or opening BBB to enhance drug delivery efficiency in the regions exposed to US [42]. Subsequent in vivo experiments validated the results of in vitro experiments. With a high

![Fig. 9. Transmission electron microscopic images taken for the NC membranes treated under the following conditions: (a) US sham, (b) US only, (c) SonoVue microbubbles with high concentration exposed to US, and (d) PFP nanodroplets with high concentration exposed to US.](image)

![Fig. 10. H&E detections of the SonoVue and PFP groups were compared at 20 and 200 magnification times after in vivo experiments. Black arrow points to red blood cell extravasations. (US parameters: 700-kPa acoustic pressure, 80-μs pulse length, 400-Hz PRF, 5-min exposure time and 3.2% duty ratio) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.](image)
concentration level, PFP droplets performed better than the microbubbles in terms of both the amount and depth of EB. Fig. 7 shows that the EB-dyed brain tissue of the microbubble group has a smaller volume with lighter color than in the nanodroplet group. This may be because, for in vivo experiments, the nano-sized droplets might easily diffused through blood vessels to get into the rat’s brain tissues before US exposure while the micron-sized bubbles were stuck in the vessels, which indicated that the nanodroplets group could provide more cavitation nuclei in deep brain tissues to be vaporized after US was turn on. Secondly, as shown in Fig. 4a, the IC activity generated in the microbubble group is less stable than that of the nanodroplets group, so that IC-facilitated BBB opening effect generated by microbubbles was hard to sustained after 60-s US irradiation, especially at the high concentration level. As the results, more sustained IC activity could be generated in the high-concentration nanodroplets group to results in more effective BBB opening effect in larger and deeper regions.

To verify the safety of cavitation-facilitated nanodroplets, the brain tissues were paraffin embedded and sectioned at 6 μm in thickness. Hematoxylin and eosi (H&E) staining was performed for histological analysis. The results of pathological examination of both SonoVue and PFP groups are shown in Fig. 10. It is obvious that red blood cell extravasations are found in the SonoVue group while almost no damage is observed in the PFP group, which indicates that with appropriate parameters the combination of US and PFP might be safer to perform effective BBB opening treatment.

Inevitably, despite the ability of the NC membrane to mimic in vivo BBB in the experiments, there are substantial differences between the two. Specifically, the NC membrane could not repair itself, and the porosity of each layer was relatively uniform, whereas the BBB contains a variety of cell parts in each section, the hardness and thickness of which are not always consistent with the NC membrane. Therefore, although this method is suitable for exploring the physical mechanism of ultrasound combined with the administration of contrast agents, it does not provide sufficient information about biochemical experiments such as delivering targeted bubbles loaded with proteins or gene drugs to specific treatment points. From the results of the in vivo experiments, planar US may transmit the macromolecules to relatively shallow depths over a wide area than focused US waves [43,44], but is hard for the planar US to accurately locate and irradiate a smaller area when requiring precise treatment. In the future, experiments should be conducted to compare the advantages and disadvantages of nanodroplet-mediated BBB opening treatment under focused and non-focused situations.

5. Conclusion

In summary, the main purpose of the present work was to elucidate the physical mechanisms underlying the transmembrane permeability enhancement effect generated by acoustically vaporized nanodroplets. In vitro NC membrane penetration experiments were performed to evaluate the relationship between the IC activity and enhanced membrane permeability induced by PFP nanodroplets and Sonovue microbubbles at different concentrations. The results illustrated that, for the pooled data, a positive correlation was observed between the ICD and the total amount of penetrated FITC particles. In general, the nanodroplets would generate relatively moderate but more sustained IC activity than the Sonovue microbubbles, which made the phase-changed nanodroplets with sufficiently high concentration penetrate farther and wider in the multilayer porous NC membrane. The following in vivo studies further confirmed safer and more efficient BBB-opening effect could be achieved by acoustically vaporized nanodroplets than Sonovue microbubbles. The current work revealed the excellent penetration ability of PFP nanodroplets through the membrane and its correlation with the cavitation dose, which may enable more opportunities for the development and optimization of phase-changed nanodroplet applications in the biomedical areas.

CRediT authorship contribution statement

Renjie Song: Data curation, Formal analysis, Writing – original draft. Chunbing Zhang: Data curation, Formal analysis. Fengmeng Teng: Data curation, Formal analysis. Juan Tu: Conceptualization, Funding acquisition, Writing – review & editing, Writing – original draft. Xiasheng Guo: Writing – original draft. Zheng Fan: Writing – original draft. Yinfai Zheng: Conceptualization, Funding acquisition, Writing – review & editing. Dong Zhang: Conceptualization, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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