Ultrasound-Mediated Perfluorocarbon Microbubbles
Bursting for Gene Transfection

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Abstract
Ultrasound contrast agents (microbubbles) have been extensively applied as acoustic cavitation nuclei for drug and gene delivery. During microbubble bursting, several physical impacts such as shock waves can transiently disrupt cell membranes, and thereby facilitating the cellular uptake of genetic materials. Instead of using commercial microbubble (e.g., Optison or Definity), perfluorocarbon microbubbles with phase-shifting capability were harnessed in this study to examine their efficacy for gene delivery. Using ultrasonic homogenization technique, nonechogenic liquid perfluoropentane (C₅F₁₂) microdroplets were stabilized by human serum albumin in the cell culture medium. Because the boiling point of perfluoropentane is 29°C, the liquid emulsions shifted to echogenic microbubbles at 37°C and served as cavitation nuclei. NIH 3T3 fibroblasts were exposed to ultrasound in the presence of perfluoropentane microbubbles and luciferase-encoded plasmid DNA. Our results showed that under 5-min mega-hertz ultrasonic exposure in the presence of perfluoropentane microbubbles, the level of luciferase gene expression increased up to 2-fold, in comparison with the one without adding microbubbles.

Keywords: Perfluoropentane, Microbubble, Ultrasound, Nonviral transfection, Gene delivery

Introduction
Although high efficiency of gene delivery can be achieved by viral gene carriers (e.g., retroviruses, adenoviruses, and lentiviruses), a serious of defects, such as immunogenicity [1] and mutagenicity as a result of viral sequence integration [2], hamper the development of viral approach. Therefore, even though limited by its low efficiency of transfection, nonviral methods have increasingly gained attention due to safety concern of using viral vectors. One of them is ultrasound-mediated nonviral transfection which has been developed since 1980s [3]. The mechanism involved in forcing nonviral vectors into cells was recognized as ultrasound-induced cavitation by numerous studies [4-6]. Cavitation refers to the formation and destruction of microbubbles in acoustic fields. These bubbles oscillate and gain pass through a process know as rectified diffusion. As bubbles grow, they may quickly reach resonant diameter and then be destroyed. Such destruction can concentrate the intensity of the acoustic field up to 11 orders of magnitude [7], thereby enhancing cell permeabilization [8] and allowing cellular uptake of nucleic acids [9,10], dextran molecules [11,12], and chemotherapeutic agents [13].

Besides applying ultrasound alone, most recently it was associated with microbubbles (ultrasound contrast agents) and has been widely applied for gene delivery in vitro [14] and in vivo [15]. This is because microbubbles can reduce the threshold of cavitation by ultrasonic energy [16]. Generally speaking, the shells of microbubbles are made by nontoxic biodegradable materials (e.g., albumin or lipids), and encapsulated with low-solubility and non-reactive gases (e.g., air or perfluorocarbon). In addition to acting as cavitation nuclei, microbubbles could be served as target/delivery vehicles via incorporation of microbubble shell with targeting ligand and plasmid DNA [17-19]. The aim of this study was to investigate nonviral transfection efficiency using ultrasound aided with phase-shift perfluorocarbon microbubbles. Instead of using C₃F₈ or C₄F₁₀ (e.g., Optison, and MP1950), perfluoropentane (PFP; C₅F₁₂)-based microbubbles were prepared and examined for the efficacy of gene delivery. Although PFP microbubbles has been commercially made (Echogen; Sonus Pharmaceuticals, Bothell, WA) and investigated in imaging of blood flow, and delineation and perfusion of tissue or organ [20,21], no study was reported to use PFP microbubbles as ultrasound cavitation nuclei for gene delivery. Unlike the Echogen microbubbles stabilized by artificial surfactants (PEG Telomer B), human serum albumin

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Figure 1. (a) Photograph of the transducer. The piezoelectric transducer was mounted on the end of a borosilicate glass rod with a hollow channel in the center. The wires soldered on both sides were connected to the ultrasonic equipment. (b) Schematic diagram for the setup of acoustic apparatus. The ultrasound exposure was performed in continuous sinusoidal waves with 1 MHz and 0.02 W/cm² for various time periods. After wrapped by polyvinyl membrane and sterilized using 70% of ethanol, the transducer was submerged in the medium containing PFP microbubbles (○) and plasmid DNA (•) and kept 4 mm above NIH 3T3 cell monolayer (□) growing on the bottom of culture well. To avoid the generation of standing waves, a rough silicone mat was placed under the experimental plate to minimize acoustic reflection.

was used to stabilize PFP emulsions. After warmed at 37°C, PFP microspheres changed from nonechogenic liquid microdroplets to echogenic microbubbles due to low boiling point of PFP (i.e., 29°C). To achieve the most efficacious usage, it is important to convert the maximum number of microdroplets to echogenic microbubbles. Our results showed that 30 min was the optimal time to obtain large amounts of PFP microbubbles prior to their use for gene transfection. After 5-min ultrasonic exposure in the presence of PFP microbubbles, the level of luciferase gene expression in NIH 3T3 cells increased up to 2-fold compared to the one without the aid of microbubbles.

Materials and Methods

Cell culture

NIH 3T3 fibroblasts (ATCC; American Type Culture Collection, Manassas, VA) were cultured in 35 mm diameter plastic Petri dishes (Falcon, San Jose, CA) with 2 mL of Dulbecco’s modified Eagle’s medium (DMEM; Mediatech Inc., Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Irvine Scientific, Santa Ana, CA) and 100 U/mL penicillin-streptomycin (Irvine Scientific). The cells were maintained at 37°C and balanced with 5% CO₂ in a 100% humidified incubator.

Preparation of PFP microbubbles

1 % w/w PFP (Oakwood Products Inc., West Columbia, SC) was added into 15 mL aqueous solution containing 0.67% w/w human serum albumin (HSA; RDI Research Diagnostics, Inc., Concord, MA). The mixture was emulsified using a sonicator (Misonix Inc., Farmingdale, NY) performed in a pulse wave at 20 kHz and 550 W of output energy for 2 min. All steps were performed in a 0°C ice bath to avoid PFP evaporation during the preparation. 15 mL of pre-chilled phosphate-buffered saline solution (PBS; Irvine Scientific) was added in order to decrease the viscosity of emulsion solution. To generate PFP echogenic microbubbles shifted from nonechogenic liquid microdroplets, the HSA-stabilized liquid PFP emulsions were heated in a 37°C water bath to obtain PFP microbubbles. The mean size of microbubbles was measured by the Coulter Counter (Coulter Corp., Miami, FL) after heating from 0 to 70 min.

Ultrasonic apparatus setup and pressure measurement

1-MHz ultrasound continuous sinusoidal wave was emitted from a piezoelectric transducer composed of 25-mm diameter and 2-mm thickness lead zirconate titanate disc (PZT5800; Channel Industries Inc., Santa Barbara, CA). The transducer was mounted on the end of a borosilicate glass rod (O.D. = 25 mm, I.D. = 5 mm, and length = 200 mm) with a hollow channel in the center. The wires soldered on both sides of the transducer was connected to the ultrasonic equipment (Figure 1(a)). The oscillation signal was generated from a function generator (HP 3326A Two-Channel Synthesizer; Hewlett-Packard Corp., Palo Alto, CA) and augmented by an amplifier (75A250; AMPLIFIER RESEARCH®, Souderton, PA) with an impedance matching transformer (MT-56, Krohn-Hite Corp., Brockton, MA). In this study, ultrasound exposure was performed at continuous sinusoidal waves with 1 MHz and 40 Vp-p output voltage amplitude. Prior to its use, the transducer was covered by a polyvinyl membrane filling with glycerol as the coupling agent, and sterilized by 70% ethanol. Then, the cylindrical glass rod glued with the transducer disc was submerged into the medium containing PFP microbubbles and plasmid DNA, and kept 4 mm above NIH 3T3 cell monolayer settled down on the bottom of the culture well. To guarantee the temperature of the culture medium higher than...
Gene Transfection Using PFP Microbubble

Figure 2. Formation and evolution of PFP microbubbles during heating at 37°C ranging from 0 to 70 min. Photomicrographic images were taken using an inverted microscope from the formation of PFP liquid emulsions immediately after ultrasonic homogenization in the ice bath. During heating process, liquid emulsions shifted to gas-filled microbubbles, and the mean size of microbubble increased due to evaporation of PFP. The maximum diameter could reach 5 µm measured from the Coulter Counter. Microbubble attenuation revealed significantly due to loss of encapsulated gas into aqueous solution after exposing to 37°C for 40 min. Bar = 3 micron.

PFP boiling point (i.e., 29°C), the experimental culture plate was placed in a 37°C water bath as shown in Figure. 1(b). In addition, a silicone mat served as an acoustic absorber was placed under the experimental plate to minimize acoustic reflection and concomitant standing wave formation. The acoustic pressure was measured according to the formula:

\[ P = \frac{p^2}{2\rho C} \]  

(1)

Here, \( \rho \) is the water density, and \( C \) is the sound speed in water. The acoustic equivalent area of the transducer (\( A \)) was determined by mapping the acoustic field using a capsule-type hydrophone (HGL-0200, ONDA Corp., Sunnyvale, CA) mounted on a three-way micropositioner. The vertical intensity (\( I \)) transmitted from the PZT transducer was measured using a radiation force balance (Sartorius CP 224S; Precision Weighing Balances, Bradford, MA). Various acoustic pressures were estimated under 6, 10, 15, 20, 25, 30, and 40 V\(_{pp}\) output voltage amplitude.

Preparation of plasmid DNA

The luciferase reporter vector used in the study was pGL3-Control vector (Promega Corp., Madison, WI). To generate sufficient amount of DNA, the reporter vector was amplified by JM-109 bacteria transformation and isolated from the bacteria using Qiagen Mini Kit-25 (Qiagen, Valencia, CA). The purification and concentration of isolated plasmid DNA were determined by measuring absorption at 260 (\( A_{260} \)) and 280 nm (\( A_{280} \)) wavelength using UV spectrophotometry. The ratio of the \( A_{260} \) to \( A_{280} \) was between 1.7 and 2.0, indicating the purified plasmid DNA was free of remaining contaminants.

Cell viability after ultrasound exposure with PFP microbubbles

NIH 3T3 cells were cultivated in a 6-well culture plate at 37°C. When reaching 80% confluence, 10% (v/v) of solution containing PFP microbubbles was added to each well with NIH 3T3 cells, followed by ultrasound exposure performed at 1 MHz and 40 V\(_{pp}\) for 0, 1, and 5 min. Then cells were harvested by trypsinization and evaluated cell viabilities immediately after ultrasound exposure and post 24-h incubation using a hemocytometer with trypan blue exclusion method.

Sonoporation assay

One day before sonoporation, cells were trypsinized and transferred to a 6-well culture plates with 2.5x10^5 cells per well and cultivated at 37°C. After 24-h incubation, 2 µg of luciferase plasmid DNA and pre-heated PFP microbubbles were added into each well. Prior to their use, PFP microspheres were heated at 37°C for a given time to reach an optimized bubble size. Cells were then exposed to acoustic fields with 1 MHz and 40 V\(_{pp}\) of output voltage amplitude in the presence or absence of 10% v/v PFP microbubbles for 0, 1, and 5 min. After additional 4-h incubation, culture media were replaced with fresh growth media for each set, followed by incubation at 37°C for another 48 h. The transfection efficiency exhibited by the level of luciferase expressed in 3T3 cells was determined using luciferase assay kit (Promega Corp., Madison, WI). Briefly, cells were harvested using a lysis buffer. After centrifugation at 12000 rpm for 30 sec, the supernatant was mixed with luciferase assay reagent. Then, the luciferase activity was detected by a luminometer (LS 6000 IC; Beckman Coulter Inc., Fullerton, CA).

Statistical analysis

All of the experimental data were obtained in triplicate and presented as mean ± standard deviation. Statistical comparison by the analysis of variance was done at a significance level of \( p < 0.05 \) based on the Student’s t-test.

Results

Evolution of PFP microspheres

Photographic images given in Figure 2 displayed the evolution of PFP microspheres during 70-min heating at 37°C.
Figure 3. Acoustic pressure as a function of output voltage amplitude ($V_{pp}$). The voltages used were 6, 10, 15, 20, 25, 30, and 40 $V_{pp}$. The corresponding pressures were calculated by Eq. (1) in association with measured vertical intensities and acoustic equivalent area of the transducer.

![Figure 3](image)

Figure 4. Viability of NIH 3T3 cells after various ultrasound exposure times in the presence of 10% v/v PFP microbubbles. Ultrasound was performed in continuous sinusoidal waves at 1 MHz and 0.02 W/cm$^2$ of acoustic intensity for 0, 1, 5 min, respectively. Cell viabilities were determined after 24-h incubation, using hemocytometry with trypan blue staining. Each bar represents the mean of three independent experiments. Error bars are the standard deviation of cell viability ($P < 0.05$).

![Figure 4](image)

Figure 5. Effect of ultrasound exposure time on DNA transfection efficiency. NIH 3T3 cells were transfected with luciferase-encoding plasmid DNA with (●) and without (□) the presence of 10% v/v PFP microbubbles. The ultrasound exposure was performed in continuous sinusoidal waves at 1 MHz and 0.02 W/cm$^2$ of acoustic intensity for 0, 1, 5 min, respectively. The DNA transfection rates were then measured by detecting the level of luciferase expression using a luminometer after 48-h incubation at 37°C. Error bars are the standard deviation of counted photons per minute (CPM) obtained from three independent experiments ($P < 0.05$).

![Figure 5](image)

In the beginning, the PFP liquid emulsions with 0.5 µm of mean size were homogeneously distributed in the medium. When heating at 37°C, PFP started to evaporate and formed gas-filled microbubbles. Due to the substantial decrease in density of PFP as a gas, the average diameter of the microbubbles increased to about 5 µm after 30-min heating. Then, PFP microbubbles attenuation occurred due to the leaking of PFP gas. The mean size decreased to 1 µm after heating for 70 min.

**Measurement of acoustic pressure**

The acoustic equivalent area ($A$) transmitted from the PZT transducer was measured as 239.58 mm$^2$. The average vertical intensities under various output voltage amplitudes were assessed by a radiation force balance, and obtained as 1.9, 3.9, 6.8, 18, 22.3, 29.1, and 51 mW. The corresponding spatial-average peak pressure was calculated by Eq. (1) respectively, and the linear relation was shown in Figure 3.

**Cell viability and DNA transfection efficiency**

To determine the influence of ultrasound irradiation period on NIH 3T3 cells in the presence of PFP microbubbles, the cell viabilities were examined under 0, 1, and 5-min ultrasound exposure performing at 1 MHz and 25.3 kPa of acoustic pressure. Before ultrasound activation, the PFP liquid microdroplets were heated at 37°C for 30 min, followed by adding in the acoustic chamber with the concentration of 10% (v/v). The cell viability decreased to 80% when the exposure time was increased to 5 min as illustrated in Figure 4.

With the assurance of cell viability after ultrasound exposure in the presence of PFP microbubbles, nonviral transfection efficiency was examined using NIH 3T3 fibroblasts and luciferase-encoding plasmid DNA. As shown in Figure 5, the level of luciferase intensity in transfected NIH 3T3 cells was augmented with the ultrasound exposure time with or without adding PFP microbubbles. Moreover, when cells exposed to PFP microbubble-mediated ultrasound at 5 min, it led to a 2-fold increment of luciferase activity compared with the group without PFP microbubbles.

**Discussion**

Ultrasound-induced cavitation has been widely developed for gene therapy since Fechheimer et al. [3] exposed cells and plasmid DNA to ultrasound by immersing the tapered sonicator microtip into the suspension. Quite a few ultrasound contrast agents including air-based Albunex [9]; Levovist [22], and perfluorocarbon-based Optison [23] have been extensively applied in association with ultrasound to enhance cell permeabilization. In this study, PFP-based phase-shift microbubbles were harnessed to facilitate gene delivery efficiency in vitro. Under 37°C heating, the volume of PFP encapsulated in microspheres varied. Due to the increment of PFP volume during phase shift from liquid to gas, the size of microspheres expanded up to 5 micron after 30-min heating (shown in Figure 2). However, microbubble swelling will increase the permeability of the HSA shell membrane, resulting in the leak of encapsulated PFP gas. Therefore, numbers of microbubbles were decreased after heating over 40
min. According to the result of formation and evolution of PFP microbubbles during heating at 37°C, 30 min was determined to be the optimal heating period to obtain the optimal number and size of gas-filled echogenic microbubbles used for ultrasound-mediated gene transfection.

It was demonstrated during ultrasound exposure, shock waves and shear forces generated from microbubble bursting could enhance cell permeabilization, and thereby increasing cellular uptake for foreign DNA or other macromolecules. However, those physical forces were also harmful to cells. To examine the effect of PFP microbubbles on cells under ultrasound exposure, cell viability was measured under various exposure times (i.e., 0, 1, and 5 min). As a result, cell viabilities were all over 80% within the ultrasonic timeframe, indicating cell necrosis caused by ultrasound irradiation in the presence of PFP microbubbles were not prominent like other published data using sonoporation [11]. It is noteworthy that low acoustic intensity (0.02 W/cm²; 25.3 kPa) was applied to avoid quick collapse of PFP microbubbles and large percentage of cell death. The gene transfection efficiency might be enhanced further over than 2-fold as reported here if the acoustic intensity is increased. However, this could lead to low cell viability. In any case, it will be useful to perform a series of study using a wide range of acoustic pressures to optimal the transfection rate with a tolerable trade-off (i.e., cell viability).

In addition to the advantage of simple preparation, the property of phase-shift PFP microbubble offers other potentials for application. For example, microbubble and DNA molecule can be employed as separate entities for in vitro study. However, it is difficult to co-delivery them into the targeted cells and trig the burst of microbubbles unless they are integrated together. In other words, the nucleic acids have to be encapsulated inside the microbubble [25] or loaded on the surface of microbubble [19]. However, it is a challenge to achieve sufficient high amount of DNA plasmids incorporated with microbubbles. To maximize the yield of DNA incorporation with microbubble, use of water-in-oil-in-water (W/O/W) double emulsion for DNA encapsulation could be feasible utilizing the phase-shift feature of PFP. To achieve that, the primary homogenization, fabricating water-in-PFP emulsions, can be used to trap all of the DNA plasmids in the water phase using an appropriate fluorosurfactant. Then, the second homogenization makes the previous water-in-PFP emulsions encapsulated in water microdroplets (i.e., the water-in-PFP-in-water double emulsions). Following heating at 37°C, PFP will be evaporated and form bubble-like particles containing DNA plasmids. After targeting cells in vivo, ultrasound cavitation will burst the PFP bubble and eject its payload (i.e., DNA plasmids) into cells with porated membrane.

In conclusion, PFP microbubble-mediated ultrasound irradiation can facilitate nonviral transfection efficiency up to 2-fold within 5-min exposure. To further extend the phase-shift feature of PFP, integrating DNA molecules into PFP microbubbles using double emulsion method could open a new avenue of research.

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