Journal of Controlled Release 212 (2015) 1–9

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Focused ultrasound-induced blood-brain barrier opening for non-viral, non-invasive, and targeted gene delivery

Chung-Yin Lin [a], Han-Yi Hsieh [b], William G. Pitt [c], Chiung-Yin Huang [d], I-Chou Tseng [d], Chih-Kuang Yeh [e], Kuo-Chen Wei [d,*], Hao-Li Liu [b,f,**]

[a] Medical Imaging Research Center, Institute for Radiological Research, Chang Gung University/Chang Gung Memorial Hospital, Taoyuan 333, Taiwan
[b] Department of Electrical Engineering, Chang Gung University, Taoyuan 333, Taiwan
[c] Department of Chemical Engineering, Brigham Young University, Provo, UT 84602, United States
[d] Department of Neurosurgery, Chang Gung Memorial Hospital, Linkou Medical Center and College of Medicine, Chang Gung University, Taoyuan 333, Taiwan
[e] Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu 300, Taiwan
[f] Health Aging Research Center, Chang Gung University, Taoyuan 333, Taiwan

ARTICLE INFO

Article history:
Received 20 February 2015
Received in revised form 28 May 2015
Accepted 9 June 2015
Available online 11 June 2015

Keywords:
Focused ultrasound
Gene delivery
Blood-brain barrier opening
Liposomes
Luciferase
GDNF

ABSTRACT

Focused ultrasound (FUS) exposure in the presence of microbubbles can temporally open the blood-brain barrier (BBB) and is an emerging technique for non-invasive brain therapeutic agent delivery. Given the potential to deliver large molecules into the CNS *via* this technique, we propose a reliable strategy to synergistically apply FUS-BBB opening for the non-invasive and targeted delivery of non-viral genes into the CNS for therapeutic purpose. In this study, we developed a gene-liposome system, in which the liposomes are designed to carry plasmid DNA (pDNA, containing luciferase reporter gene) to form a liposomal-plasmid DNA (LpDNA) complex. Pulsed FUS exposure was delivered to induce BBB opening (500-kHz, burst length = 10 ms, 1% duty cycle, PRF = 1 Hz). The longitudinal expression of luciferase was quantitated *via* an *in vivo* imaging system (IVIS). The reporter gene expression level was confirmed *via* immunoblotting, and histological staining was used to identify transfected cells *via* fluorescent microscopy. In a comparison of gene transduction efficiency, the LpDNA system showed better cell transduction than the pDNA system. With longitudinal observation of IVIS monitoring, animals with FUS treatment showed significant promotion of LpDNA release into the CNS and demonstrated enhanced expression of genes upon sonication with FUS-BBB opening, while both the luciferase and GDNF protein expression were successfully measured *via* Western blotting. The gene expression peak was observed at day 2, and the gene expression level was up to 5-fold higher than that in the untreated hemisphere (compared to a 1-fold increase in the direct-inject positive-control group). The transfection efficiency was also found to be LpDNA dose-dependent, where higher payloads of pDNA resulted in a higher transfection rate. Immunoblotting and histological staining confirmed the expression of reporter genes in glial cells as well as astrocytes. This study suggests that IV administration of LpDNA in combination with FUS-BBB opening can provide effective gene delivery and expression in the CNS, demonstrating the potential to achieve non-invasive and targeted gene delivery for treatment of CNS diseases.

1. Introduction

Neurodegenerative diseases, such as ALS, Parkinson's, Alzheimer's, and Huntington's, involve the progressive, loss of neuronal structure or function, resulting in progressive degeneration and/or neuronal cell death. So far, there is no definite treatment approach to prevent or slow neurodegenerative disease progression. For example, in Parkinson's, patients continue to worsen in terms of controlling their movement and other such symptoms, despite progressive therapeutic intervention [1]. Recently, numerous preclinical and clinical studies have demonstrated the potential use of gene therapy for neurodegenerative diseases [2,3]. With the delivery of genes into the CNS, it may be possible to genetically modify neuronal cells that are directly functionally impaired, potentially relieving symptoms or even reversing the progression of the neurodegenerative diseases [4,5].

To deliver therapeutic genes into the CNS, the first obstacle to overcome is the blood-brain barrier (BBB), which effectively blocks delivery to the brain [6,7]. The BBB is formed by the tight junctions between the endothelial cells responsibly for the barrier function, preventing uptake of most therapeutic agents into the brain. To overcome this, current

* Corresponding author.
** Correspondence to: H.-L. Liu, Department of Electrical Engineering, Chang Gung University, Taoyuan 333, Taiwan.
E-mail addresses: kuochenwei@cgmh.org.tw (K.-C. Wei), haoliliu@mail.cgu.edu.tw (H.-L. Liu).

http://dx.doi.org/10.1016/j.jconrel.2015.06.010
0168-3659/

clinical trials have applied local gene delivery through direct injection [5,8], however, the craniotomy/burr-hole installation process prior to gene injection is highly invasive. When attempting non-invasive gene delivery through the circulation, there is the challenge of preventing the rapid degradation of naked DNA by nucleases in circulation and rapid clearance of DNA by the RES systems [9]. Currently to overcome these limitations, numerous nanocarrier-mediated delivery technologies, including viral- and non-viral gene delivery systems, are being developed to more effectively perform gene delivery and gene transfection [10]. Nevertheless, even novel vehicle technology that has been developed to permeate the BBB will encounter the challenge of how to locally express the therapeutic genes. Continued development of novel strategies is necessary to enhance gene transfection efficiency and improve therapeutic treatments of neurodegenerative diseases. Therefore, the two above-mentioned obstacles greatly limit the success of non-invasive CNS gene delivery for brain disease treatment.

To solve the first obstacle, a technology based on targeted focused ultrasound (FUS) exposure in the presence of microbubbles (MBs) has been developed to temporally and locally open the BBB. It has also become one of the most promising strategies to achieve non-invasive and targeted CNS gene delivery [6,7,11–13]. Ultrasound-mediated MB destruction has the potential to open the BBB tight junctions and trigger therapeutic agent deposition at specific sites with non-invasive sonication [14,15]. The interaction of ultrasonic waves with the MBs enhance acoustic cavitation-related microstreaming, sheer stress, and radiation forces directly on capillary endothelial cells, the temporal destruction of tight junctions. The subsequent leaks temporarily increase the endothelial porosity and vascular permeability [16]. Then, to solve the second hurdle, the therapeutic gene can be carried by a plasmid that is incorporated into vehicles to protect it from degradation, such as encapsulation in liposomes, creating a construct called liposomal plasmid DNA (LpDNA). We therefore hypothesized that the combination of LpDNA and FUS-induced BBB opening can be an effective gene delivery system for the brain that could provide a substitute for currently used invasive viral-vector based approaches [17,18].

The aim of this study was to develop a non-viral gene delivery system based on liposomes containing plasmid DNA (LpDNA) and concurrent use of FUS-BBB opening to induce stable CNS transgenic expression. A functionalized liposome containing a plasmid with the luciferase pLuc-N3 and glial cell line-derived neurotrophic factor (GDNF) genes was used to determine whether the developed nanocarrier was effectively delivered to the brain through FUS-induced BBB opening. The approach of using LpDNA in combination with an *in vivo* imaging system (IVIS) and nano-carriers enables semi-quantitative bioluminescent imaging assessment of transgene expression of luciferase in brains. We used IVIS to semi-quantitatively evaluate LpDNA expression in the brains, and immunoblotting analysis was employed to identify luciferase and GDNF expression. The transduced cell types were also confirmed *via* immunohistochemistry staining.

2. Material and methods

2.1. Materials and reagents

Phospholipid dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG(2000)-amine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Antibodies of goat anti-luciferase, rabbit anti-IBA1, rat anti-GDNF, and mouse anti-GFAP were obtained from Promega Corp. (Madison, WI), Wako Pure Chem. Ind. (Osaka, Japan), R&D Sys. (Minneapolis, MN), and Dako Inc. (Carpinteria, CA), respectively. Goat anti-rabbit antibody conjugated with fluorescence 594 and donkey anti-mouse/anti-goat antibody conjugated with fluorescence 488 were purchased from Molecular Probes, Inc. (Grand Island, NY). Anti-fade reagent with the nuclear marker DAPI was ordered from Calbiochem. (San Diego, CA). Other chemicals, if not specified, were reagent grade from Aldrich-Sigma (St, Louis, MO).

2.2. Plasmid preparation

A single bacterial colony containing a plasmid encoding both the luciferase pLuc-N3 gene (marker gene) and the GDNF gene (therapeutic gene) was selected and inoculated in 500 mL LB medium. The mixture was then incubated for about 24 h at 37 °C with shaking at about 300 rpm. The bacterial cells were harvested by centrifugation at 3000 ×*g* for 30 min at 4 °C. Plasmid DNA (pDNA) was separated by maxiprep according to the manufacturer's instructions and concentrated using ethanol precipitation. The samples were centrifuged at 15,000 ×*g* for 10 min and the supernatant was decanted. Then, 200 μL double-distilled autoclaved water (DDAC) was added to the pellet, followed by 20 μL NaOAc along with 550 μL cold ethanol. The mixture was centrifuged at 4 °C for about 15 min. Finally the supernatant was gently removed and 100 μL of DDAC water was added to the plasmid. A spectrophotometer was used to measure the plasmid concentration. The absorbance at 260- and 280-nm was measured using a Nanodrop (ND-1000, Thermo Fisher Scientific Inc. Waltham, MA) and a ratio greater than 1.8, indicated that the purified pDNA was free of contaminants.

2.3. Liposomal plasmid DNA (LpDNA) formation and characteristics

Liposomes containing DPPC, Chol, DSPE-PEG (2000)-amine, and α-tocopherol in a 3:1:1:0.004 molar ratio were made by the film hydration method [19,20]. Briefly, the lipid mixture dissolved in chloroform was dried onto a flask to produce a homogeneous lipid film. The film was hydrated with a suspension of condensed pDNA at 42 °C until the film dispersed from the bottom of the flask. The suspension was extruded 10 times through 200-nm polycarbonate filters and 10 times through 100-nm filters using an Avanti Mini Extruder (Alabaster, AL), and then passed through a spin column to remove the unencapsulated pDNA. After centrifugation at 12,500 rpm for 15 min and collecting the supernatant, the concentration of LpDNA in the liposomes was determined spectrophotometrically by measuring the absorbance at 260 nm (Hitachi F-7000, Tokyo, Japan). Encapsulation efficiency was calculated as the fraction of original pDNA that was incorporated into the LpDNA vesicles. The size of LpDNA was measured by dynamic light-scattering (DLS) on a Nano-ZS90 particle analyzer (Malvern Instruments, Malvern, Worcestershire, UK). Each run required about 3 min, and 10 runs were averaged. The sample was prepared and imaged by cryogenic-transmission electron microscopy (cryo-TEM).

2.4. In vivo animal model

This study used a total of eighty 8-week-old Balb/c male mice, each weighing about 25 g. The mice were anesthetized with vaporized isoflurane (2%). The experimental procedure met the criteria outlined by the Institutional Animal Care and Use Committee of Chang Gung University (CGU-IACUC), and mice were handled according to the guidelines in *The Handbook of the Laboratory Animal Center*, Chang Gung University.

2.5. Focused ultrasound system

A single-element FUS transducer (Imasonics SAS, France; center frequency = 500 kHz, diameter = 60 mm, radius of curvature = 80 mm, electric-to-acoustic efficiency of 70%) was placed in an acrylic water tank filled with distilled and degassed water. The focus of the ultrasonic field was positioned to the desired region. The signals from the function generator (33120A, Agilent, Palo Alto, CA) were boosted with a power amplifier (150A100B, Amplifier Research, Souderton, PA), measured with an inline power meter (Model 4421, Bird Electronics Corp., Cleveland, OH), and used to drive the FUS transducer as shown in

Fig. 1. The animal was placed directly under a 4×4 cm^2 window of thin polymer film at the bottom of the acrylic tank, in acoustic connection using acoustic transmission gel (Pharmaceutical Innovations, Newark, NJ). The output acoustic pressure was calibrated *via* a calibrated polyvinylidene-difluoride-type (PVDF) hydrophone (Model HNP-0400, ONDA, Sunnyvale, CA, USA).

2.6. Experimental procedure

Fig. 1 shows the FUS-induced BBB opening strategy with the aim of temporally disrupting tight junctions and enhancing LpDNA delivery into the brain. First, LpDNA was injected intravenously through the tail vein. MBs were injected and FUS exposure was immediately applied to the brains to open the BBB, resulting in enhanced delivery of LpDNA across the BBB. Once in the cell, the pDNA eventually enters the nucleus (Fig. 1A and B).

To demonstrate the neuroprotective effect of the liposome, the efficiency of transgenic induction was compared between naked pDNA and LpDNA by making an intracerebral infusion into a localized area of the striatum (with references to bregma −2.12 mm and the interaural 1.68 mm) of 1 μg pDNA/μL (either the naked pDNA or the LpDNA). Then, the day of infusion was marked as day 0, and the brain tissues were collected at daily intervals in order to determine the expression of pDNA in the brain tissues. Six mice were used for each time interval (*i.e.*, on days 1, 2, 3, and 4).

To further study the FUS-induced BBB opening relative to the transduction of LpDNA in the opened region, we applied pulsed FUS sonications with a 10-ms burst length, a 1% duty cycle, a 1-Hz pulse repetition frequency (RPF), and a 60-sec-insonation duration. Microbubbles (SonoVue®, Bracco Diagnostics Inc., Milan, Italy; phospholipid-coated SF6 microbubbles, mean diameter of about 2.5 μm, and microbubble concentration of 2–5 $\times$ 10^8 bubbles/mL) were intravenously injected at a dose of 4 μL/kg through the tail vein after the injection of LpDNA solution. In *in vivo* experiments, three experimental groups consisted of 1) the control mice (no pDNA, no FUS), 2) mice treated with the LpDNA (27 μg pDNA), and 3) mice treated and insonated with three different doses of LpDNA (3, 9, and 27 μg of pDNA) and sonication at 0.5 MPa to study the effect on transfection efficiency in the presence of BBB-opening. In order to confirm the FUS-induced BBB opening, pressures ranging from 0.3–0.8 MPa were employed, while the default acoustic pressure in this study was 0.5 MPa. To stain the BBB-opened brain areas, Evens blue (EB) at 30 mg/kg was administered immediately after the animals were exposed to FUS. The animals were sacrificed approximately 2 h after EB injection. HE staining was used to assess the resulting histological damage.

2.7. In vivo imaging system (IVIS) system

The *in vivo* luminescence imaging was performed with an IVIS Spectrum system (Caliper, Hopkington, MA) on days 1, 2, 3, and 4, respectively. Before imaging, D-luciferin (3 mg/mice) was intraperitoneally injected into mice. Eight minutes after injection, luciferase expression was imaged with IVIS. Then the mice were sacrificed and their brain tissues were collected for later use.

2.8. Western blotting

Fresh frozen brain tissues were homogenized in lysis buffer, kept overnight, and then centrifuged at 7000 $\times g$ for 30 min. After centrifugation to remove tissue debris, the protein contents of the tissue homogenates were determined according to the Bradford method [21]. The supernatant with 50 μg protein was dissolved in a sample buffer, separated by 7.5% SDS-PAGE, and then transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was further incubated with a primary goat anti-luciferase antibody or rat anti-GDNF antibody at a 1:1000 dilution and a secondary donkey anti-goat antibody at a 1:5000 dilution. To measure the optical density of the positive bands, the film was scanned using the BioSpectrum Imaging System (UVP LLC, Upland, CA).

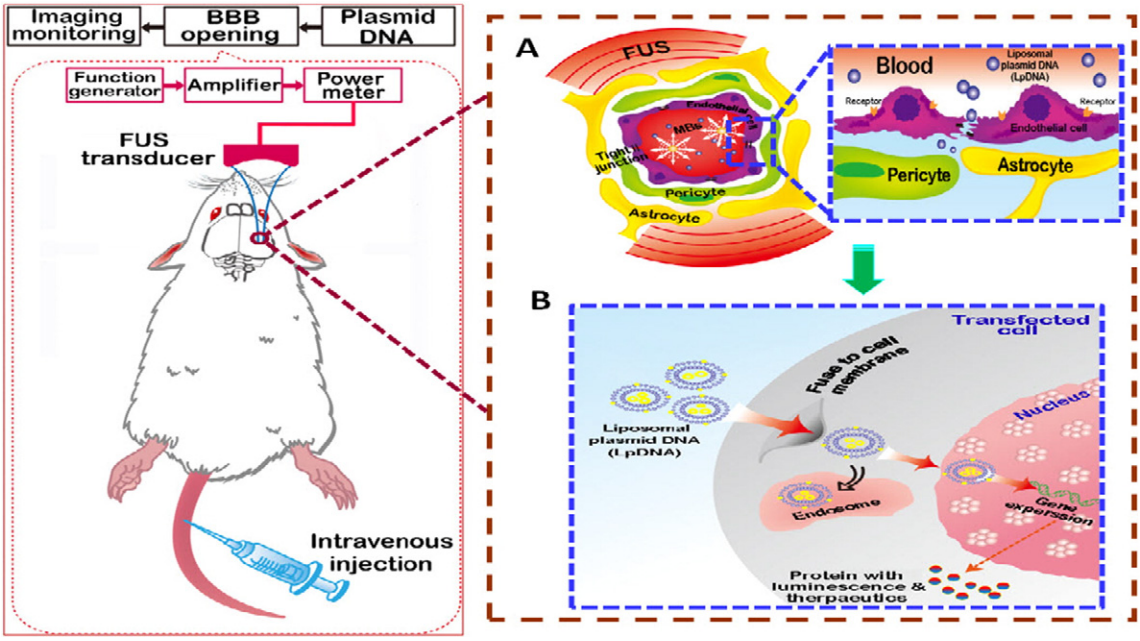

Fig. 1. Schematic representation of gene delivery enhanced through FUS-induced BBB opening. LpDNA was injected intravenously through the tail vein first, followed by immediate FUS exposure at the brain after MBs were injected intravenously. Ultrasonic waves induce MB oscillation in the blood vasculature, causing stable cavitation. The bubble oscillation and the stable cavitation may induce disruption of the tight junctions, resulting in an enhanced delivery of LpDNA into the brain. The liposomal character of LpDNA increases its ability to fuse cell membranes, transfect cells, and induce transgene expression of the reporter or therapeutic genes. FUS, focused ultrasound; BBB, blood-brain barrier; LpDNA, liposomal plasmid DNA; MBs, microbubbles.

2.9. Immunohistochemistry staining

Brain tissues were prepared and sectioned according to standard procedures. The tissues were removed from a −80 °C freezer to a −20 °C Leica CM3050 S Cryostat (Meyer, Inc., TX). The brain tissues were sectioned into 8-μm-thick sections on SuperFrost® glass slides (Thermo Fisher Scientific, Braunschweig, Germany). For immunohistochemistry, tissue sections were stained overnight at 4 °C with the following primary antibodies: anti-luciferase (1:200), anti-Iba1 (1:1000), and anti-GFAP (1:1000). After rinsing in PBS, the sections were incubated in secondary antibody with goat anti-rabbit fluorescence 594 or donkey anti-mouse fluorescence 594 (1:500, for GFAP or Iba1) or with donkey anti-goat fluorescence 488 (1:500, for luciferase) for 1 h at room temperature. After rinsing in PBS, coverslips were applied on slides with anti-fade reagent with the nuclear marker DAPI. Finally, the sections were imaged by a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany).

2.10. Statistics

All the data are presented as mean ± standard deviation (SD). Statistical analysis was performed on a personal computer using SPSS version 16.0 (SPSS Inc., Chicago, IL) statistical software. Statistical differences were assessed using ANOVA combined with post-hoc testing or Mann–Whitney U testing. Statistical significance was denoted as "*" when $p < 0.05$ and denoted as "**" when $p < 0.01$.

3. Results

3.1. Characterization of liposomal plasmid DNA (LpDNA)

To formulate liposomes containing plasmid DNA (LpDNA), we encapsulated pDNA into liposomes, as illustrated in Fig. 1. After encapsulating pDNA within liposomes and removing the unencapsulated pDNA by spin column, the amount of encapsulated pDNA was calculated from the absorbance of pDNA in the resulting LpDNA. Fig. 2A shows the absorbance intensity of pDNA in liposomes, from which one can calculate the weight percentage of pDNA entrapped in liposomes. The LpDNA had an entrapment efficiency of 72.8%, and the desired amount of pDNA could be added to the liposomes for the LpDNA formulation. Fig. 2B shows the size distribution of LpDNA with a mean diameter in 105 nm. Fig. 2C shows a cryo-TEM image of LpDNA. Apparently, the DPPC-based liposomes can trap hydrophilic plasmids and interact with plasmids to form the LpDNA.

3.2. FUS-induced blood-brain barrier (BBB) opening

The feasibility of using focused ultrasound exposure to induce targeted BBB opening is demonstrated by staining of exposed brain sections by Evans blue (EB) extravasation and HE staining. Fig. 3 illustrates the gross inspection profiles of brain tissues after 500-kHz FUS sonication at 0.3, 0.5, and 0.8 MPa exposure level, respectively. The exposure was delivered to the left hemisphere and EB extravasation was localized to a small region with blue staining. A successful BBB opening at the exposure site was achieved at 0.5 MPa, but not 0.3 MPa. In contrast, 0.8 MPa showed a more broad flux area with hemorrhage damage in the exposure regions. The 0.5 MPa exposure level was therefore selected for the following examinations.

3.3. In vivo optical imaging of localized transgenic expression

To detect the bioluminescence generated by transgene transduction and expression, the transferred pDNA was encoded with the luciferase reporter gene. In order to compare the transduction efficiency of LpDNA, we conducted two positive control experiments to directly inject pDNA and LpDNA into the brains and the luciferase expression level was measured *via* IVIS. Fig. 4A and B show that luciferase activity was induced with increasing time from days 1 to 4, respectively, and the relative intensity of luciferase activity is presented as percentage activity increase relative to that of the control (set to 100%) (Fig. 4B, experiments were repeated 6 times for each group, and each point is the mean of at least six measurements). Weak luminescence slightly above the control was detected in brain tissues when directly injecting naked pDNA and LpDNA, but luciferase expression in the directly injected LpDNA group consistently produced a relatively higher and more stable expression level during the monitoring duration (from days 1 to 4). These results suggest that gene transduction *via* the LpDNA system produces a more efficient gene transduction and expression efficiency than the DNA plasmid alone, and could be associated with enhanced liposome-related cellular fusion or enhanced cell-endosomal activity such as endocytosis.

3.4. In vivo optical imaging of ultrasound-triggered transgenic expression

To evaluate the synergistic enhancement of gene transduction and transfection from the combination of IV-administered LpDNA and FUS-BBB opening, we conducted experiments with LpDNA administration alone and LpDNA administration combined with FUS-BBB opening (control group is defined as both LpDNA- and FUS-absent). Also, to compare the effect of LpDNA titer, the plasmid concentration in LpDNA was 1 μg pDNA/μL, and administrations of 3, 9, and 27 μg were made. FUS exposure with 0.5 MPa for 60 s was delivered for each LpDNA group.

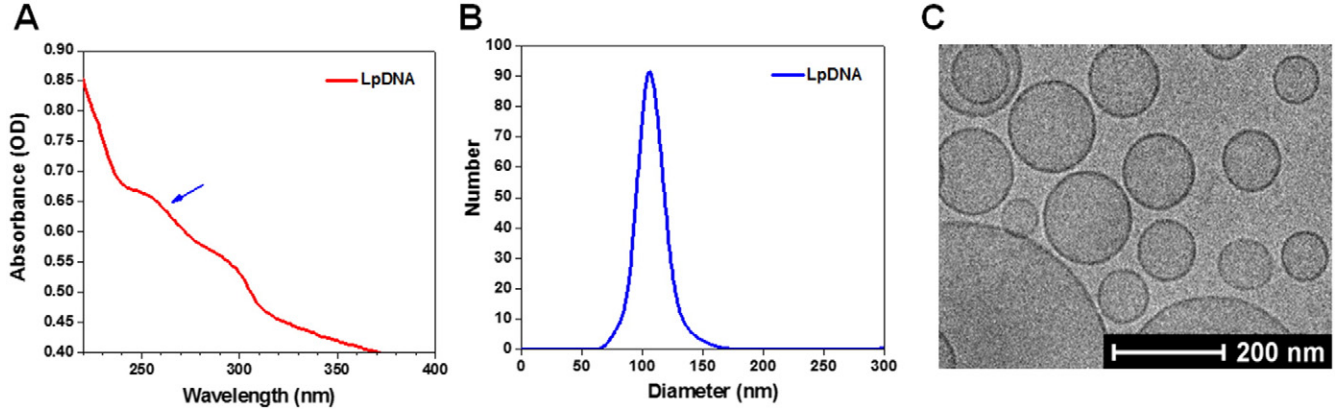

Fig. 2. Characteristics of the liposomal plasmid DNA (LpDNA) used in this study. The LpDNA had both a reporter gene and a therapeutic gene. (A) The UV-Vis absorbance spectra of LpDNA. (B) Particle size distribution of LpDNA measured by dynamic light scattering. (C) Cryo-TEM image of the LpDNA.

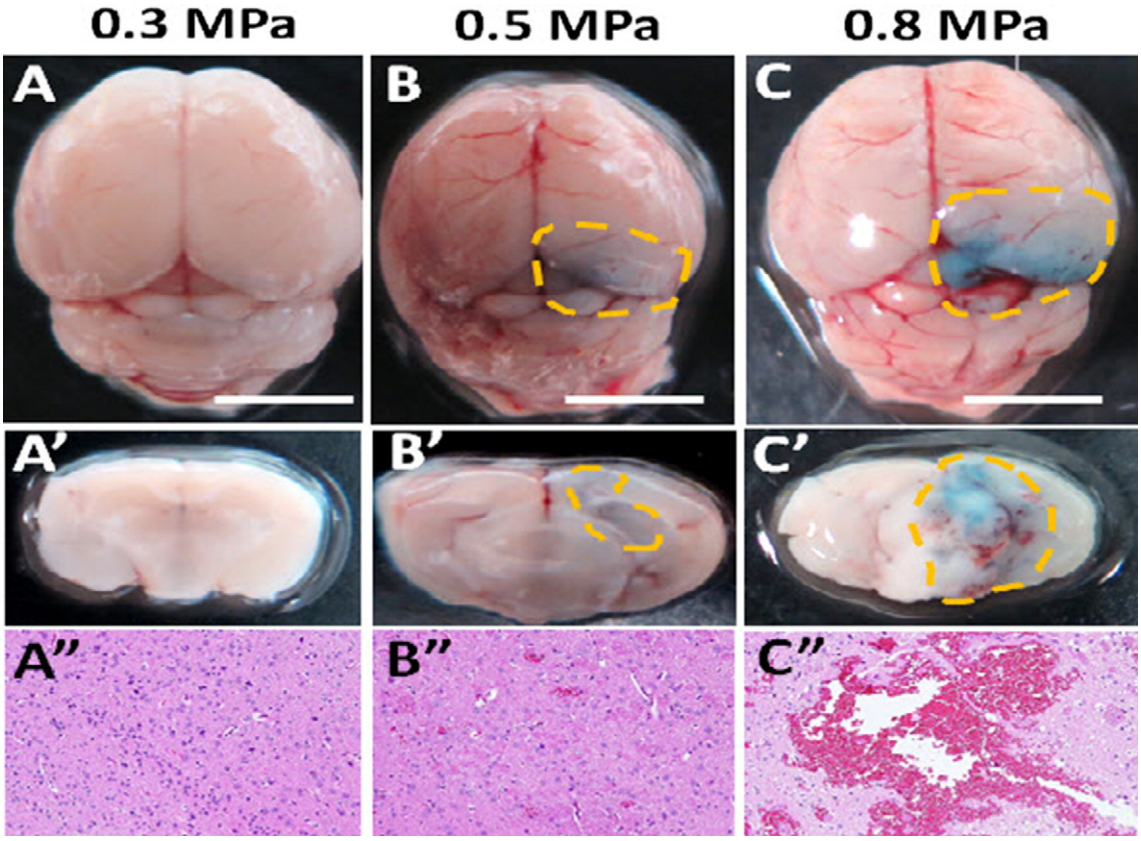

Fig. 3. Effect of FUS-induced blood-brain barrier opening on Evens blue (EB) extravasation and HE staining with acoustic pressure of A) 0.3 MPa; B) 0.5 MPa; and C) 0.8 MPa, respectively. Brain sections characterized from the middle of A′) 0.3 MPa; B′) 0.5 MPa; and C′) 0.8 MPa. Bar = 5 mm. Histological morphology is shown from the bottom of A″) 0.3 MPa; B″) 0.5 MPa; and C″) 0.8 MPa. Section magnification = 200×.

Following FUS exposure, we performed longitudinal measurements of luciferase activity across all experimental groups *via* IVIS. Fig. 5A shows that images of luciferase activity induced over time from days 1 to 4, respectively, and Fig. 5B presents the relative intensity of luciferase activity as percentage activity relative to the control (expressions of 27-μg LpDNA administration only was defined as 100%; experimental repetition at least 6 for each group). We observed that LpDNA administration alone did not transduce the gene into the brain or induce luciferase expression. When concurrently applied with FUS exposure to open the BBB, LpDNA successfully penetrated the CNS at the target hemisphere and induced luciferase expression ($p < 0.05$) for all monitored durations. In addition, LpDNA combined with FUS system showed dependence on the dose of LpDNA administered, and we observed that higher LpDNA dosage indeed increased luciferase expression ($p < 0.01$). Maximal luciferase expression was observed on day 2, and a nearly 5-fold increase in expression level (495%) was induced in the 27 μg LpDNA group.

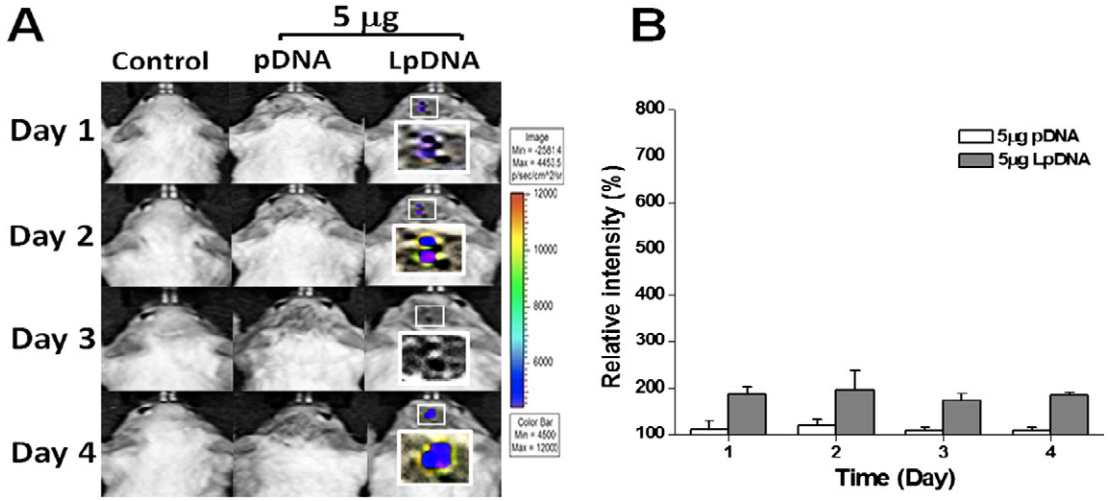

Fig. 4. A. Representative *in vivo* imaging system (IVIS) images of mice following injection with 5 μg of naked pDNA or LpDNA expressed at days 1, 2, 3, and 4, respectively. The bioluminescence signal (p/s/cm2/sr) as a measure of transfection efficiency was commenced 1 day after intracerebral infusion. B. Relative bioluminescence intensity (%) compared to the control. Bar = 1 cm.

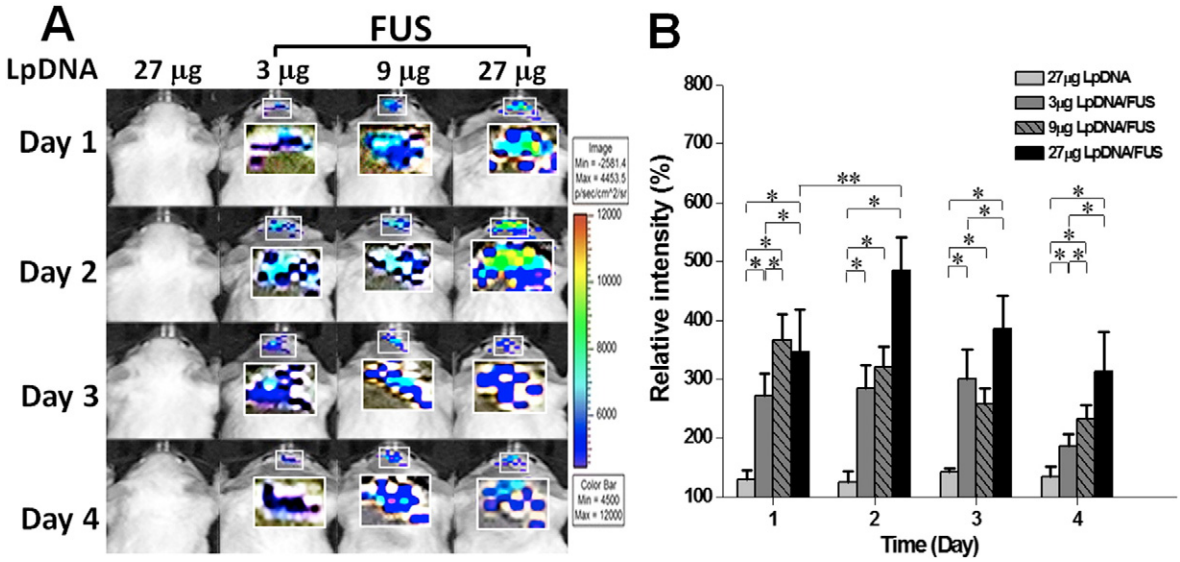

Fig. 5. A. Representative *in vivo* imaging system (IVIS) images of mice observed after different treatment conditions: LpDNA only and LpDNA under FUS-induced BBB opening with different pDNA doses expressed at days 1, 2, 3, and 4, respectively. The bioluminescence signal (p/s/cm2/sr) as a measure of transfection efficiency was commenced 1 day after gene delivery. B. Relative bioluminescence intensity (%) compared to the control. Columns, mean; bars, SD, and *: $p < 0.05$; **: $p < 0.01$ (Mann–Whitney U test, each condition, $n \geq 6$). Bar = 1 cm.

Although induced luciferase expression was significantly elevated in the BBB-opened brain region ($p < 0.05$, $n \geq 6$) after 2 days, there was an apparent drop in activity at 3 and 4 days, showing the peak transduction and expression occurred at day 1 or 2. These data suggest that combing FUS-induced BBB opening with LpDNA administration is an effective and stable approach for achieving gene delivery into the CNS.

3.5. The effect of LpDNA expressed after FUS-induced BBB opening

To demonstrate the efficacy in gene delivery and ability to achieve stable expression for future therapeutic purpose, a plasmid encoding the luciferase pLuc-N3 gene was also encoded with glial cell line-derived neurotrophic factor (GDNF) gene for concurrent luciferase and GDNF expression in this system. Fig. 6A presents the results of Western blotting analysis, which show that expression of both luciferase and GDNF protein was enriched after FUS sonication-enhanced vascular permeability procedures. We demonstrated that concurrently applied FUS-BBB opening and LpDNA IV administration indeed induced luciferase proteins and increase GDNF protein levels, with the quantitative analysis showing statistical significance when compared to the sham control and LpDNA IV-administration alone groups (both $p < 0.05$). Luciferase expression in LpDNA + FUS group was also significantly higher than the LpDNA-administration group, which is consistent with the findings shown in Fig. 5 and supplementary S1. On the other hand, LpDNA administration alone (27 μg) failed to increase GDNF expression in the CNS, but can be successfully increased in GDNF expression when combining with FUS-induced BBB opening and seems to be LpDNA dose dependent (Fig. 6B; all $p < 0.05$ when compared with control).

3.6. Immunohistochemical detection in BBB-opened brain region

Fig. 7 shows a typical example of cells immunohistochemically labeled for luciferase expression to identify which cells were transduced (Fig. 7A–D: sham control; Fig. 7E–H: LpDNA administration alone, 27 μg; Fig. 7I–L: LpDNA administration + FUS-BBB opening, 3 μg). Double-labeled IHC (IBA-1 to identify microglia and DAPI to mark all cell nuclei) was used to identify microglia cell expression (shown in rows 1–3; obtained at day 2). Moreover, we employed double-labeled IHC (GFAP to identify astrocytes, and DAPI to mark all cell nuclei) to identify astrocytes (shown in row 4; obtained at day 2). The LpDNA combined with FUS sonication (Fig. 7I–K) showed that luciferase was also expressed in microglia cells, with neural cell enrichment around the BBB-opened brain regions *via* IBA-1/DAPI staining (see Fig. 7I–K). With separate GFAP/DAPI staining, we observed that luciferase can be largely expressed within the brain tissue region with astrocyte neuron-enriched regions (see Fig. 7L; astrocytes were identified by their spatial cell shapes and long synapses). It is also interesting that

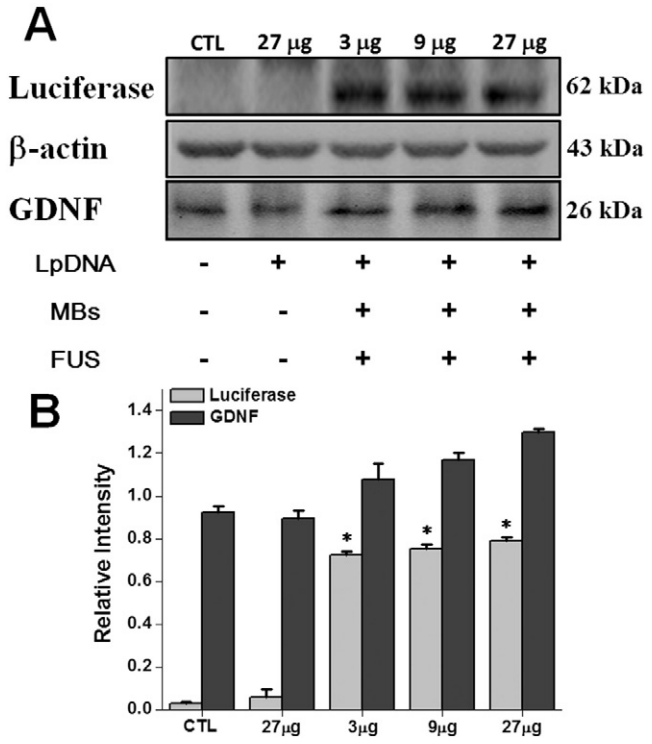

Fig. 6. Results of Western blot analysis of BBB-opened brain regions and its luciferase and GDNF protein expression after 2 days of transduction. The following conditions were studied: CTL (control), LpDNA only, and LpDNA plus FUS sonication with different pDNA doses. Brain tissue lysates (2 μg/lane) were isolated on a 7.5% SDS-PAGE gel and immunoblotted with anti-luciferase pAb, anti-GDNF antibody, or anit-β-actin. The bands reflected strong expression of luciferase and GDNF after FUS sonication. Actin functioned as a control for protein loading. Statistical significance compared with the control. "*" represents $p < 0.05$.

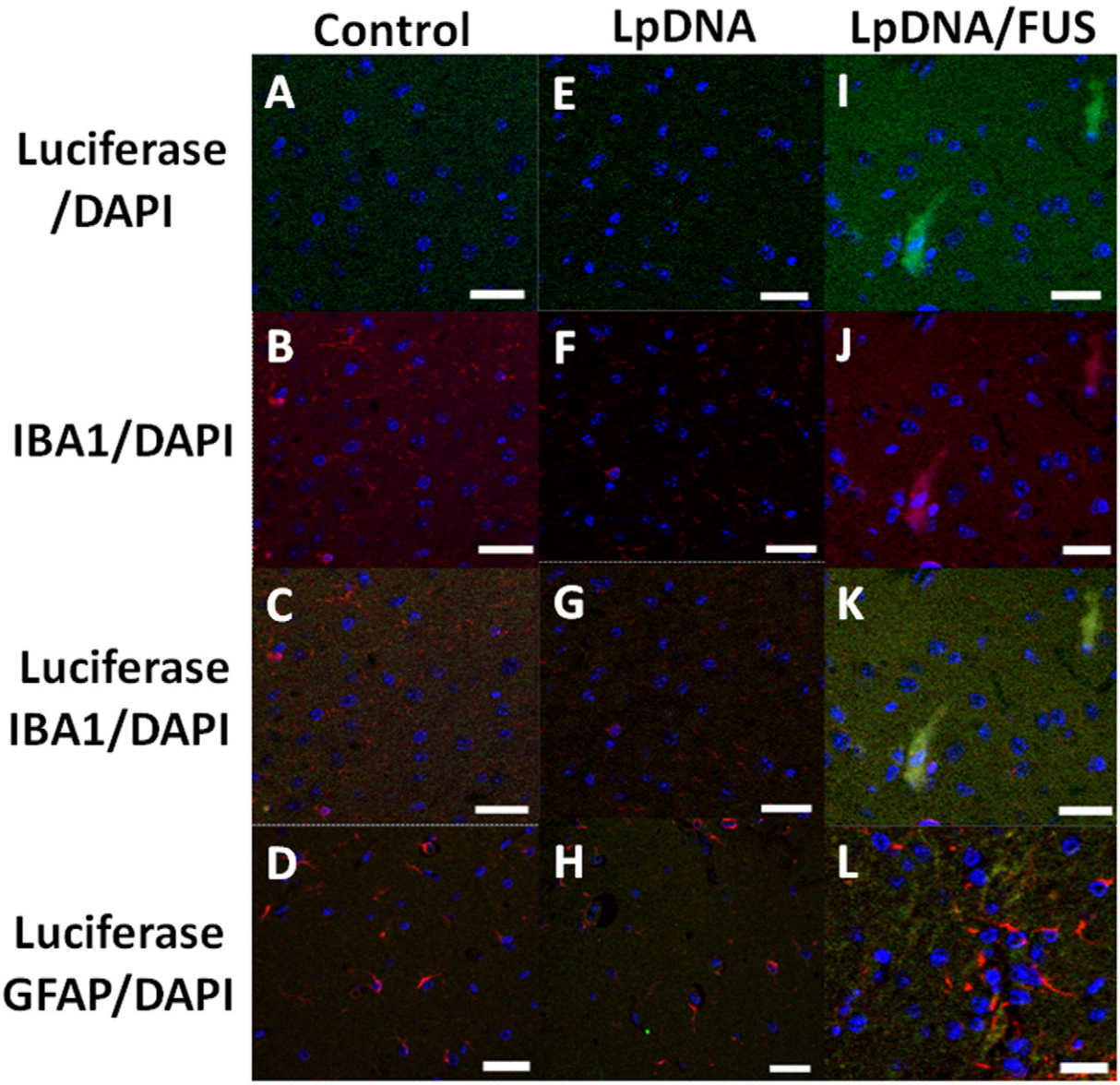

Fig. 7. Immunohistochemistry of *in vivo* transfection in glia or neuronal cells after gene delivery. Fluorescence staining of brain tissues revealed expression of luciferase, IBA1, and GFAP in the BBB-opened brain region 2 days after transduction. Transfected luciferase/DAPI, IBA1/DAPI, and luciferase/GFAP/DAPI for the control (A–D), LpDNA only (E–H), and LpDNA with FUS sonication (I–L, a typical presented in 3 μg pDNA dose). Scale bar = 30 μm. DAPI, 4′,6-diamidino-2-phenylinodole; IBA1, ionized calcium binding adapter molecule 1; GFAP, glial fibrillary acidic protein.

not all luciferase-positive cells were also GFAP-positive, and some luciferase-positive cells may have neuronal morphology.

4. Discussion

In this study, we successfully encapsulated plasmid DNA (pDNA) into liposomes (LpDNA), which effectively delivered protected plasmids to the brain. In all experiments, the expression of reporter genes containing luciferase and GDNF was significantly greater from LpDNA than from free pDNA. With the concurrent use of FUS-induced BBB opening with LpDNA, we confirmed that both luciferase and GDNF were successfully expressed at the targeted sites, with the gene expression been significantly amplified. We demonstrated that the transduction observed 2 days after BBB opening had a relative intensity that was 5-fold greater than that of the LpDNA only. The *in vivo* enhancement of transgene expression in the BBB-opened brain was consistent with our previous results that showed drugs and genes can be deposited at higher concentrations into targets by FUS-induced BBB opening [12,15], demonstrating the stability and repeatability of this FUS-BBB opening technique. We have proven that LpDNA with FUS sonication produces sufficient plasmid deposition to express both luciferase and GDNF production (Fig. S1), suggesting that the combined use of LpDNA intravenously administered with FUS-BBB opening can achieve non-invasive and targeted gene delivery for future clinical application in CNS disease treatment.

Studies have also used nanoscale vesicle design for gene delivery [17,18,22]. Most strategies have utilized viral vectors to deliver therapeutic genes through BBB opening [11,12]. However, there is little information available on delivery to the brain using non-viral vectors, the relevance of plasmid sequestration in liposomes on the localized transgene expression, or the effect of this treatment on transduction of genes. We hypothesized that during FUS-induced uptake of LpDNA, the ultrasound pressure waves interact with the MBs, causing bubble expansion, followed by bubble growth, oscillation, deformation, and perhaps even stable cavitation. These latter cavitation phenomena may shear and disrupt the LpDNA bilayer membranes or disrupt cell membranes to

enhance transgene transport within or into brain cells [23,24]. This study demonstrated a new gene-carrying vehicle design using plasmids encapsulated inside DPPC-based liposomes. We demonstrated that a relatively high encapsulation efficiency (approximately 72.8% of encapsulation) by the hydration method can be achieved. These LpDNA were relatively stable in the plasma, and the lipid shell appeared to reduce the enzymatic degradation of the plasmid. We expect that LpDNA remains intact in the blood circulation [22]. We also confirmed that this mixture is stable in the robust lipid bilayer of the DPPC. We believe that the proposed gene-carrying LpDNA is one of the most effective and efficient vehicles for gene delivery.

The hydration of dry lipid film containing zwitterionic phospholipids and cholesterol with an aqueous suspension of pDNA (pLuc-N3 DNA encoded GDNF) produced stable liposomes, as shown by the DLS and cryo-TEM data. Conventional liposomes (like the LpDNA used herein) in biological systems exhibit several benefits for therapeutic delivery, such as high-stability formulations incorporating a constant amount of drug and long-circulating properties leading to successful crossing of vascular barriers [25]. Compared with previous publications showing an encapsulating efficiency of approximately 40%–55% in conventional liposomes [26–28], LpDNA had a relatively high entrapment efficiency (72.8%). We specially formulated our liposomes to be positive in charge by using DSPE-PEG-(2000)-amine and the positively charged choline groups of DPPC in order to entrap negatively-charged plasmids. Our results demonstrate that stronger functional group interaction can be induced to improve the encapsulation efficiency of pDNA into LpDNA. LpDNA forms lipid particles (Fig. 2C) that condense and interact through the electrostatic interaction of the negative charges on nucleic acids and the positive charges on amine groups of DSPE-PEG (2000) and choline groups of DPPC. We concluded that DPPC/PEG(2000)-amine, a lipid formulation with a positive surface charge, can reliably interact with pDNA to form a stable bilayer structure and contribute to high plasmid-entrapping efficiency.

In Fig. 4, the LpDNA system had a 2-fold superior transfection efficiency compared to pDNA delivered *via* direct intracerebral injection, and out-performed free luciferase-encoded plasmid, which demonstrated a nearly undetected luciferase expression level. We hypothesize that the lipid bilayer is key in protecting the plasmid from DNase I digestion [22]. The peak expression duration of luciferase expression when compared with pDNA and LpDNA was identical at day 2, but only with the expression level difference, implying that the DNA encapsulated in liposomes enhances the transfection rate, but does not delay transfection cycles.

We studied the effects of FUS-induced BBB opening on LpDNA CNS delivery *via* 500-kHz focused ultrasound exposure. It has been reported that an acoustic pressure of 0.5 MPa can induce apparent acoustic cavitation at 500-kHz, which is almost identical to the exposure level we applied in this study [14], and can support the exposure level selection to induce effective BBB opening. At this exposure level, the luciferase and GDNF gene encoded into the LpDNA system can be stably expressed. In addition to the BBB-opening effect, gene transduction may be enhanced through various acoustic mechanisms such as the interaction of FUS with MBs producing bubble vibrations that lead to cavitation, microstreaming and production of radiation forces, and increased cellular membrane permeability. A higher FUS level exposure could improve gene transfection and result in higher gene expression since the BBB-opened scale can be further enlarged. However, high-level exposure may have safety issues because it increases the risk of large-scale erythrocyte extravasation. During reporter gene expression monitoring *via* IVIS, we frequently observed an enhanced and detectable luciferase activity above the background level from the animal's back. This discrepancy in enhancement may originate from: (a) the interference of the high absorption photons in the presence of hemoglobin, melanin and other pigmented macromolecules [29], (b) attenuation and light scattering by tissues [30], and (c) locally inhomogeneous light-scattering by cell and organelle membranes [31].

Fig. 6 shows the luciferase and GDNF protein expression from brain tissues 2 days post-injection (as well as the sham group), and we observed the BBB opening and transgene expression of LpDNA combined with FUS can last 4 days. By Western blotting analysis, luciferase appeared as a major band at approximately 62 kDa, while the GDNF band was observed at 26 kDa. The amount of protein produced in tissues after FUS exposure was enhanced, particularly with injections of 27 μg of LpDNA, showing FUS treatment significantly enhanced protein expression (supplementary S1).

Fig. 7 shows the immunofluorescence staining for LpDNA expression in tissues from control, LpDNA only, and LpDNA (3 μg dose) with FUS after 2 days of transduction. The immunofluorescence results showed that the transgene expression was colocalized primarily to glial cells, and that some astrocyte cells initially (at day 2) could express luciferase. Interestingly, luciferase pLuc-N3 DNA-encoded GDNF plasmid also showed strong expression in glial cells. GDNF could be expressed in both neurons and astrocytes [32]. Increased GDNF protein expression in astrocytes is believed to be neuroprotective *in vivo* for motor neurons and dopaminergic neurons. Our results showed that the BBB-opened brain regions at the given parameters were associated with enhanced GDNF production, suggesting that the proposed CNS gene delivery approach has potential for application to a number of neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's.

5. Conclusions

This study demonstrated the development of a novel liposomal DNA plasmid system that can be used in a synergistic combination with FUS-BBB opening to achieve efficient and reliable CNS gene delivery. We also showed that these LpDNA carriers are biocompatible and have potentials for use as effective carriers in ultrasonically triggered gene delivery. We propose that LpDNA can be formulated to carry genes and protect them from serum nucleases. In addition to gene delivery by BBB opening, there may be a number of gene therapeutics that can be loaded and delivered to the cell nuclei in other specific locations targeted by FUS. The results of this study suggest that IV administration of liposomal plasmid-DNA with the combination of FUS-BBB opening can enable effective gene delivery and expression in the CNS, and provide non-invasive and targeted gene delivery for treatment of CNS diseases.

Acknowledgments

This work was supported by the Ministry of Science and Technology, Taiwan, with grant nos. 103-2120-M-001-CC1, 104-2325-B-182-001, 101-2221-E-182-002-MY3 and 102-2221-E-182-020-MY3, National Health Research Institutes, Taiwan, with grant no. NHRI-EX103-10004NI, Chang Gung University, Taiwan, with grant no. EMRPD1D0951E, and Chang Gung Memorial Hospital, Taiwan, with grant nos. CORPD3E0071, CMRPD2A0033, CMRPG3D0571, CIRPD2E0051, CMRPD2D0111-3, CMRPD2A0033, and CIRPD3E0131. We are particularly grateful to Dr. Chiung-Mei Chen and Dr. Kuo-Hsuan Chang for their assistance and facilities in helping this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2015.06.010.

References

[1] H. Braak, E. Braak, D. Yilmazer, C. Schultz, R.A. de Vos, E.N. Jansen, Nigral and extranigral pathology in Parkinson's disease, J. Neural Transm. Suppl. 46 (1995) 15–31.
[2] M. Politis, Neuroimaging in Parkinson's disease: from research setting to clinical practice, Nat. Rev. Neurol. 10 (2014) 708–722.

[3] B. Huang, Y. Tabata, J.Q. Gao, Mesenchymal stem cells as therapeutic agents and potential targeted gene delivery vehicle for brain diseases, J. Control. Release 10 (2012) 464–473.
[4] A. Siderowf, M.B. Stern, Premotor Parkinson's disease: clinical features, detection, and prospects for treatment, Ann. Neurol. 64 (2008) s139–s147.
[5] P.G. Coune, B.L. Schneider, P. Aebischer, Parkinson's disease: gene therapies, Cold Spring Harb. Perspect. Med. 2 (2012) a009431.
[6] H. Yao, K. Wang, Y. Wang, S. Wang, J. Li, J. Lou, L. Ye, X. Yan, W. Lu, R. Huang, Enhanced blood-brain barrier penetration and glioma therapy mediated by a new peptide modified gene delivery system, Biomaterials 37 (2014) 345–352.
[7] K. Hynynen, Ultrasound for drug and gene delivery to the brain, Adv. Drug Deliv. Rev. 60 (2008) 1209–1217.
[8] S. Nagabhushan Kalburgi, N.N. Khan, S.J. Gray, Recent gene therapy advancements for neurological diseases, Discov. Med. 15 (2013) 111–119.
[9] Y. Takakura, M. Nishikawa, F. Yamashita, M. Hashida, Development of gene drug delivery systems based on pharmacokinetic studies, Eur. J. Pharm. Sci. 13 (2001) 71–76.
[10] W.J. Bowers, X.O. Breakefield, M. Sena-Esteves, Genetic therapy for the nervous system, Hum. Mol. Genet. 20 (2011) 28–41.
[11] S. Wang, O.O. Olumolade, T. Sun, G. Samiotaki, E.E. Konofagou, Noninvasive, neuron-specific gene therapy can be facilitated by focused ultrasound and recombinant adeno-associated virus, Gene Ther. 22 (2015) 104–110.
[12] P.H. Hsu, K.C. Wei, C.Y. Huang, C.J. Wen, T.C. Yen, C.L. Liu, Y.T. Lin, J.C. Chen, C.K. Shen, H.L. Liu, Noninvasive and targeted gene delivery into the brain using microbubble-facilitated focused ultrasound, PLoS One 8 (2013) e57682.
[13] F. Wang, Y. Shi, L. Lu, Y. Cai, H. Zheng, X. Liu, F. Yan, C. Zou, C. Sun, J. Shi, Y. Chen, Targeted delivery of GDNF through blood-brain barrier by MRI-guided focused ultrasound, PLoS One 7 (2012) e52925.
[14] A. Burgess, K. Hynynen, Drug delivery across the blood-brain barrier using focused ultrasound, Expert Opin. Drug Deliv. 11 (2014) 711–721.
[15] H.L. Liu, M.Y. Hua, H.W. Yang, C.Y. Huang, P.C. Chu, J.S. Wu, I.C. Tseng, J.J. Wang, T.C. Yen, P.Y. Chen, K.C. Wei, Magnetic resonance monitoring of focused ultrasound/magnetic nanoparticle targeting delivery of therapeutic agents to the brain, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 15205–15210.
[16] W.Y. Chai, P.C. Chu, M.Y. Tsai, Y.C. Lin, J.J. Wang, K.C. Wei, Y.Y. Wai, H.L. Liu, Magnetic-resonance imaging for kinetic analysis of permeability changes during focused ultrasound-induced blood-brain barrier opening and brain drug delivery, J. Control. Release 192 (2014) 1–9.
[17] M.L. Rogers, R.A. Rush, Non-viral gene therapy for neurological diseases, with an emphasis on targeted gene delivery, J. Control. Release 157 (2012) 183–189.
[18] J.M. Bergen, I.K. Park, P.J. Horner, S.H. Pun, Nonviral approaches for neuronal delivery of nucleic acids, Pharm. Res. 25 (2008) 983–998.
[19] C.Y. Lin, M. Javadi, D.M. Belnap, J.R. Barrow, W.G. Pitt, Ultrasound sensitive eLiposomes containing doxorubicin for drug targeting therapy, Nanomedicine: NBM 1 (2014) 67–76.
[20] J.R. Lattin, W.G. Pitt, D.M. Belnap, G.A. Husseini, Ultrasound-induced calcein release from eLiposomes, Ultrasound Med. Biol. 38 (2012) 2163–2173.
[21] M.M. Bradford, A rapid and sensitive methods for quantification of microgram quantities of protein utilizing the principle for protein-dye binding, Anal. Biochem. 7 (1976) 248–254.
[22] E.S. Ghaly, J. Wang, E.S. Ghaly, Mediated liposomes for gene delivery to mice brain Part I. Design and characterization of liposome-DNA complexes, Glob. J. Med. Res. 13 (2013) 1–9.
[23] A. Delalande, S. Kotopoulis, M. Postema, P. Midoux, C. Pichon, Sonoporation: mechanistic insights and ongoing challenges for gene transfer, Gene 525 (2013) 191–199.
[24] R. Bekeredjian, P.A. Grayburn, R.V. Shohet, Use of ultrasound contrast agents for gene or drug delivery in cardiovascular medicine, J. Am. Coll. Cardiol. 45 (2005) 329–335.
[25] S.C. Dinda, G. Pattnaik, Nanobiotechnology-based drug delivery in brain targeting, Curr. Pharm. Biotechnol. 14 (2013) 1264–1274.
[26] M. Fresta, R. Chillemi, S. Spampinato, S. Sciuto, G. Puglisi, Liposomal delivery of a 30-mer antisense oligodeoxynucleotide to inhibit proopiomelanocortin expression, J. Pharm. Sci. 87 (1998) 616–625.
[27] S. Li, L. Huang, Targeted delivery of antisense oligodeoxynucleotides formulated in a novel lipidic vector, J. Liposome Res. 8 (1998) 239–250.
[28] R.I. Pakunlu, Y. Wang, W. Tsao, V. Pozharov, T.J. Cook, T. Minko, Enhancement of the efficacy of chemotherapy for lung cancer by simultaneous suppression of multidrug resistance and antiapoptotic cellular defense: novel multicomponent delivery system, Cancer Res. 64 (2004) 6214–6224.
[29] B.J. Tromberg, N. Shah, R. Lanning, A. Cerussi, J. Espinoza, T. Pham, L. Svaasand, J. Butler, Non-invasive in vivo characterization of breast tumors using photon migration spectroscopy, Neoplasia 2 (2000) 26–40.
[30] C.H. Contag, M.H. Bachmann, Advances in in vivo bioluminescence imaging of gene expression, Annu. Rev. Biomed. Eng. 4 (2002) 235–260.
[31] S.S. El-Amouri, P. Cao, C. Miao, D. Pan, Secreted luciferase for in vivo evaluation of systemic protein delivery in mice, Mol. Biotechnol. 53 (2013) 63–73.
[32] A. Drinkut, Y. Tereshchenko, J.B. Schulz, M. Bahr, S. Kugler, Efficient gene therapy for Parkinson's disease using astrocytes as hosts for localized neurotrophic factor delivery, Mol. Ther. 20 (2012) 534–543.