A preliminary study of Parkinson’s gene therapy via sono-magnetic sensing gene vector for conquering extra/intracellular barriers in mice

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Article info
Article history:
Received 18 September 2019
Received in revised form 31 January 2020
Accepted 19 February 2020
Available online 24 February 2020

Keywords:
Parkinson’s disease
Gene delivery
Ultrasound
Blood-brain barrier
Superparamagnetic iron oxide

Abstract
Background: Non-virus genetic treatment for Parkinson’s disease (PD) via plasmid glial cell-line derived neurotrophic factor (pGDNF) has shown potential for repairing damaged dopaminergic neurons. However, development of this gene therapy is largely hampered by the insufficient transfection efficiency as a result of the cell membrane, lysosome, and cytoskeleton meshwork.

Methods: In this study, we propose the use of polyethylenimine (PEI)-superparamagnetic iron oxide-plasmid DNA (pDNA)-loaded microbubbles (PSp-MBs) in conjunction with focused ultrasound (FUS) and two-step magnetic navigation to provide cavitation, proton sponge effect and magnetic effects to increase the efficiency of gene delivery.

Results: The gene transfection rate in the proposed system was 2.2-fold higher than that of the commercial agent (TransIT®-LT1). The transfection rate could be boosted ~11%, ~10%, and 6% by cavitation-magnetic hybrid enhanced cell membrane permeabilization, proton sponge effect, and magnetic-assisted cytoskeleton-reorganization, respectively. In vivo data suggested that effective gene delivery with this system results in a 3.2-fold increase in recovery of dopaminergic neurons and a 3.9-fold improvement in the motor behavior when compared to untreated genetic PD mice.

Conclusions: We proposed that this novel FUS-magnetic hybrid gene delivery platform could be integrated with a variety of therapeutic genes for treating neurodegenerative diseases in the future.

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Introduction
Parkinson’s disease (PD) is a progressive neurodegenerative disorder that affects more than 6.2 million people resulting in about 117,400 deaths globally, in which shaking, rigidity, and dementia are the main obvious symptoms [1,2]. PD is pathologically characterized by the loss of dopaminergic neurons within substantia nigra and the ensuing insufficient dopamine (DA) in the striatum [3,4]. Pharmacologic therapies (i.e. levodopa) has been utilized to relieve the symptoms, but might result in complications such as motor fluctuations and dyskinesia [5,6]. Besides, the intensity of complications is related to the dose and duration of levodopa treatment [7]. Treating of PD via surgical procedure has also raised as an alternative when adverse effects of pharmacologic therapy are intolerable or as symptoms do not respond to pharmacologic therapy. The novel surgical options including pallidotomy,
stereotactic thalamotomy, and deep brain stimulation had demonstrated positive well rigidity and/or tremor control response in 80–90% of patients [8,9]. But, such surgical operations might induce several invasive complications such as brain hemorrhage, permanent tissue damage along the electrode insertion path, and possible neural fiber damage which may result in paresis, gait disorder, dystonia or dysarthria. Minimally invasive surgical options could potentially reduce or eliminate such complications. Thereafter, focused ultrasound recently has been proposed to achieve thermal ablative thalamotomy and control tremors for clinical use [10,11].

As the pathology has been clarified by the researchers, the gene therapy is regarded as a promising approach in PD treatment to reduce the side effects that limit the current pharmacological and surgical therapies. The features of gene therapy, including long-term, endogeneity, and the eradication make it more appropriate on progressive PD treatment. Gene therapy using the gene that encodes neurotrophic factor-like glial cell line-derived neurotrophic factor (GDNF) has served as an alternative treatment for PD. Genetic repair of injured dopaminergic neurons can potentially slow or reverse neuregeneration in PD [11]. Unfortunately, the therapeutic outcomes in most of clinical researches have been disappointing. A possible explanation is that the blood–brain barrier (BBB) limits the penetration of gene vectors into the brain parenchyma, thus limiting delivery efficiency and transfection distribution [12,13]. Of note, once the vectors enter the targeted cells, they still need to overcome the intracellular barriers to transport plasmid DNA (pDNA) into the cellular nucleus. These barriers contain the cell membrane, which is composed of a lipid bilayer to hamper the hydrophilic pDNA trafficking into cytosol [14]; the lysosome, which contains hydrolytic enzyme to degrade the xenobiotic pDNA [15,16]; and the cytoskeletal meshwork in the cytoplasm, which impedes pDNA diffusion in the cytoplasm [14,17,18]. Therefore, there is a tremendous need to create a gene delivery strategy that can simultaneously overcome the BBB and intracellular barriers deep in the brain area.

Focused ultrasound (FUS) with microbubbles (MBs) has demonstrated the noninvasive and reversible BBB opening ability in clinical trials [19]. Through the activation of MB cavitation, the MBs exert mechanical stimulations on the vascular wall, temporarily destabilizing tight junctional complexes and permitting the spatially targeted delivery of therapeutic agents [20–22]. Gene delivery conducted by combining FUS–MB-mediated BBB opening with a viral vector has shown synergistic effects in nonhuman primates, resulting in targeted gene expression [23–25]. However, the administration of virus can induce inflammatory responses and off-target effects, making it difficult to apply in the clinic [26,27].

Engineered nanomedicines are attractive candidates as gene vectors because they can increase delivery efficiency by overcoming biological barriers [28–30]. However, the off-target effects and the low accumulation in the target area are largely limited in the application of nanomedicines. Therefore, a strategy to yield delivery of a high local gene concentration is to develop a vector composed of materials with FUS-sensitive properties and high gene payload. In pursuit of this strategy, we focused on superparamagnetic iron oxide (SPIO) with a coating of cationic polyethyleneimine (PEI) to create a gene vector (PSPiO). PSPiO has demonstrated selective intracellular deposition by magnetic targeting and protection of pDNA from lysosome degradation through the proton sponge effect [31]. Moreover, the interaction between intracellular PSp and magnetic force potentially induces a cytoskeleton orientation that facilitates the transport of pDNA into the nucleus, turning PSp-based nanomedicine into a promising candidate for advanced gene delivery [32,33].

Here, we report on a non-viral sono-magnetic sensing vector that comprises PSp-pDNA (PSp) nanomedicine and lipid-based MBs (PSp–MBs). The modification of PEI enables immobilization of pDNA onto PSp and facilitates pDNA escape from the lysosome [34–36]. With loading PSp onto MBs, we hypothesized that BBB opening and intracellular gene delivery could be achieved simultaneously (Fig. 1(A)). Moreover, we could target the PSp–MBs by applying an external magnetic field. The magnetic navigation (MN) significantly increased local gene concentration and promoted pDNA intracellular trafficking into the nucleus, thus improving gene delivery efficacy (Fig. 1(B)). We constructed an efficient gene delivery system by successfully taking advantage of both physical (FUS and magnetism) and chemical (PEI) strategies that overcome obstacles in current non-virus gene delivery.

Materials and methods

Materials

The lipid monolayer shell was composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Distearyl-sn-glycero-3-phosphoglycerol (DSPG), and 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (poly (ethyleneglycol))-2000] (DSPE-PEG2000), which were purchased from Avanti Polar Lipids (AL, USA). Iron oleate complex, oleic acid, and PEI were obtained from Sigma-Aldrich (MO, USA) for PSpion synthesis. pGFP and pGDNF were purchased from Origene (MO, USA), and the transgene expression was controlled by the CMV promoter.

Synthesis of PSp nanomedicine and PSp–MBs

The PSpion nanoparticles were synthesized by SPIO and PEI with a ligand exchange process [31]. The PSp nanomedicine was fabricated by adding PSp solution into pDNA (50:50 vol%). The gel retardation assay was employed to evaluate the condensation ability between PSpion and pDNA. Naked pDNA and PSp nanomedicine were mixed with O’SAFE Red 6X Loading Dye (Omics bio, USA) and subjected to the agarose gel electrophoresis (0.9%). The size distribution and zeta potential of PSp nanomedicine were measured with Zetasizer (Zetasizer Nano ZSP, Malvern Instruments, Worcestershire, UK).

The thin film hydration method was used to fabricate the anionic MBs which were composed of DPPC, DSPG and DSPE-PEG2000 at a molar ratio of 10:4:1, in which the negative charge was provided by the DSPG. The lipid powder was dissolved in the chloroform for lipid mixture. The organic solvent was evaporated by an evaporator (Rotavapor R-210, Büchi Labortec Ag, Flawil, Switzerland) overnight. Then, 0.5% glycerol phosphate-buffered saline (PBS) was added to the dried lipid film for lipid resuspension with a sonicator (Model 2510, Branson, NY, USA) and the air in the vial was replaced with perfluoropropane (C3F8). Finally, the MBs were formed by intense shaking on an agitator at 65 °C for 45s. To remove the free lipid, the solution was centrifuged at 2000 rcf for 1 min and the top foam cake was collected and resuspended with PBS.

To fabricate the PSp–MBs, the MBs were diluted to 2×10^8 MB/mL and added into the PSp nanomedicine suspension. The mixture was incubated at the –4 °C for 20 min and the conjugation between MBs and PSp nanomedicines were facilitated via electrostatic force.

Characterization of PSp–MBs

The mean size and concentration of the prepared PSp–MBs were measured with a coulter counter equipped with a 30 μm sensor orifice (Multisizer 3, Beckman Coulter, FL, USA). The solution was
centrifuged at 500 rcf for 5 min to remove the free PSp nano-medicine in the pellet, which was quantified to determine the PSp loading efficiency by the potassium thiocyanate method [32].

The feasibility of magnetic-mediated PSp-MBs collection by magnetic navigation was investigated by injecting the PSp-MBs into a cellulose tube (inner diameter of 200 μm; Spectrum Laboratories, Inc., CA, USA) with a syringe pump (velocity: 40 mm/s; KDS100, KD Scientific, PA, USA) (Fig. 2(A)). Meanwhile, a permanent 0.37 T magnet (18 mm × 5 mm, Alnico magnet, LSC Magnet, Taipei, Taiwan) was placed at the left of tube to collect the PSp-MBs in the flow field condition. The process was recorded by microscopy (IX-71, Olympus, Melville, NY, USA).

The ultrasound sensing capability of PSp-MBs was assessed by passive cavitation detection method [37]. The PSp-MB solution was injected into a homemade 2% agarose phantom with tunnel (diameter: 5 mm) by the syringe pump at 40 mm/s (Fig. 2(B)). Then, the PSp-MBs were sonicated by a 1-MHz FUS transducer (model V303, Olympus, MA, USA, cycle number: 5000, pulse repetition frequency: 1 Hz, acoustic pressure: 100–600 kPa) and the broadband signal was received by a 15-MHz FUS transducer (model V324, Olympus). Note that the focal zone of 1-MHz FUS transducer and 15-MHz FUS transducer was aligned in the tunnel. The 1-MHz FUS transducer was triggered by a power amplifier (Model 150A100B, Amplifier Research, Hazerswoude-Dorp, Netherlands) and a function generator (AFG3251, Tektronix, OR, USA). The broadband signal was processed by the Fourier transform and the signal between 10 MHz and 20 MHz was integrated to evaluate the activity of the inertial cavitation, which as an indicator for MBs disruption. The acoustic pressures used in this study were measured using a polyvinylidene difluoride type hydrophone (model HGL-0085, ONDA, CA, USA; calibration range = 1–40 MHz) in a water tank that was filled with distilled and degassed water at 25 °C.

The pDNA within the vesicles of PSp-MBs was verified by gel retardation assay. The sample (pDNA group, Psp group, Psp-MBs group, Psp-MBs + FUS group) were incubated with DNase I (Thermo-Fisher, MA, USA) in the reaction buffer at 37 °C for 2 h to prevent the interference of free pDNA. Finally, the solution was subjected to the 0.2% agarose gel electrophoresis.

**In vitro experiment**

**Cell culture**

The cellular gene transfection efficiency was verified in SH-SY5Y because many studies had indicated that SH-SY5Y cell line is a popular cell model for PD research with several characteristics of dopaminergic neurons [38]. For example, these cells express tyrosine hydroxylase and dopamine-beta-hydroxylase, as well as the dopamine transporter. SH-SY5Y cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco).
A total of $3 \times 10^4$ cells were seeded in a 24-well plate in 500 $\mu$L medium and cultured at $37 \degree C$ overnight. The experimental setup and flowchart were illustrated in Fig. 2(C) and Fig. S1, respectively. For the experiment, PSp-MB (concentration: $2 \times 10^8$ bubbles/mL; PSp: 8 $\mu$g) solution was added to the medium and the magnet was placed under the 24-well plate to perform the magnetic navigation (1st MN). After magnetic navigation for 3 min, 1-MHz FUS (cycle number: 5000, pulse repetition frequency: 1 Hz, duration: 1 min, acoustic pressure: 100–500 kPa) was exposed to facilitate the cavitation and PSp release. Note that the magnetic navigation was concurrently performed.

The cell membrane permeabilization was determined by propidium iodide (PI) dye staining. Before starting the experiment, PI dye (5 $\mu$g/mL) was added to the culture medium and observed by inverted fluorescence microscopy (Eclipse Ti, Nikon, Tokyo, Japan). The nucleus was stained by adding 2 $\mu$L Hoechst dye (10 mg/mL).

To observe the cellular uptake of PSp nanomedicine, the pDNA was previously labeled with red fluorescence by using the Label IT.
Rhodamine kits (Mirus, WI, USA) to form PSp nanomedicine. The labeling protocol was followed the procedure recommended from manufacturer. When treatment was finished, the cells were fixed by 4% paraformaldehyde and observed by inverted fluorescence microscopy. Cell viability was determined by the Alamar Blue (AbDSerotec, Oxford, UK) indicator.

**Verification that PSp nanomedicine escaped the lysosome**

To evaluate the lysosomal escape resulting from PEI, lysotracker (LysoTracker™ Green DND-26, Thermo-Fisher) was employed to label the lysosome before treatment. The location between the PSp nanomedicine and was observed by inverted fluorescence microscopy. The co-localization rate was calculated using NIS Elements AR software, starting from 60 s before FUS sonication, and lasting for a total of 8 h.

**Enhanced intracellular trafficking of pDNA by magnetic-mediated cytoskeleton re-organization**

Twenty-four hours after the treatment, the magnet was placed at the left side of culture dishes to perform magnetic navigation (2nd MN) for 1 h. After finishing the experiment, the cells were fixed with 4% formaldehyde and stained with F-actin staining dye (Thermo-Fisher) and Hoechst dye to label cytoskeletal structures and cellular nuclei. The alignment of the cytoskeleton and deposition of pDNA within the cellular nuclei were observed by the inverted fluorescence microscopy. The amount of pDNA within the

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**Fig. 3.** Properties of PSp-MBs. (A) Structure illustration of PSp-MBs. (B) Microscopic bright-field and fluorescence images of PSp-MBs. (C) The size distribution of PSp-MBs after loading various amount of PSp measured by Multisizer. (D) The PSp payload and loading efficiency of PSp-MBs. (E) The zeta potential of PSp-MBs measured by Zetasizer. Data were analyzed by ANOVA (post hoc test: Bonferroni) and presented as mean ± standard deviation (n = 6 per group).
cellular nuclei was estimated using the NIS Elements AR software (Nikon, Tokyo, Japan).

**Quantification of cellular gene transfection**

The cells were cultured after 2nd MN for another 24 h. The pGFP transfection was analyzed and observed by flow cytometry (FACS-calibur, BD Biosciences, CA, USA) and the inverted fluorescence microscopy, respectively. A total of 100,000 events were recorded for each sample during flow cytometry examination. The positive transfection events were defined according to the negative control (cell only) group, which had a transfection rate below 5%. For comparison, the pGFP transfection also conducted using a commercialized transfection reagent TransIT®-LT1 (Mirus).

**In vivo experiment**

**Animal preparation**

All procedures of animal experiments were followed the guidelines of the National Tsing-Hua University animal committee (IACUC approval number: NTHU107034). Healthy C57BL/6 J mice (20–30 g, National Laboratory Animal Center, Taipei, Taiwan) were employed to evaluate the degree and safety of BBB opening resulting from PSp-MBs, FUS, and magnetic navigation. For the PD model, we utilized MitoPark mice (age: 12 week) because previous studies had reported that MitoPark mouse model had mitochondrial dysfunction in dopamine neurons that could cause recapitulate several features of PD in humans, including degeneration of nigrostriatal dopamine circuitry and progressive neurodegeneration [39–41]. The MitoPark mice were obtained by National Laboratory Animal Center (Taipei, Taiwan) from a colony maintained at the NIDA (NIH) Intramural Program. Before experiments, the mixture of Zoletil 50 (Virbac, Carros, France) and Rompun 2% (Bayer HealthCare, Leverkusen, Germany) (50:50 vol%) was injected intraperitoneally to anesthetize the animals.

**FUS-mediated BBB opening and brain damage evaluation**

Evans blue (EB) dye (20 μL, 75 mg/kg, Sigma-Aldrich) solution was retro-orbitally injected and circulated for 10 min. PSp-MBs...
solution (100 μL, 1×10⁸ MB/mL, PSp: 40 μg) was then retro-orbitally injected and the magnet was placed at the left side of the brain to perform the 1st magnetic navigation for 3 min. The 1-MHz FUS (cycle number: 5000, pulse repetition frequency: 1 Hz, duration: 1 min, acoustic pressure: 100–500 kPa) was transcranially delivered to the SN of mice with a home-made 25-MHz sonography guided system (Fig. 2(D)) [42]. Mice were sacrificed after 30 min of circulation for EB extravasions and perfused with normal saline (0.9%). Brains were then removed and stored at −20 °C. The frozen brains were sliced into coronal sections. Hematoxylin and eosin (H&E) staining was then employed to diminish the occurrence of erythrocyte extravasation. The degree of brain injury was classified using a three-point scale as following [43,44]: grade 0 = no occurrence of erythrocyte extravasation; grade 1 = few erythrocyte extravasations without neuronal loss; grade 2 = occurrence of severe erythrocyte extravasations. The percentage of each brain injury was counted in 10 nonoverlapping microscopic fields (×400) in the sonicated area from 3 mice.

The extravasation of EB was quantified to evaluate the degree of BBB opening. The EB within tissue was extracted by trichloroacetic acid (50%, Thermo-Fisher) for 24 h. The mixture was then centrifuged at 2000g for 10 min to remove the tissue and collect the suspension. The absorbance was read at 570 nm by the Plater reader (Spark™ 10 M, Tecan Trading AG, Switzerland). Finally, the value of absorbance was converted to the concentration of EB according to the calibration line.

**PD mouse treatment and behavior assessments**

MitoPark mouse was treated from the age of 12 weeks. The PSp-MB solution was retro-orbitally injected after anesthetization and the magnet was placed at the left side of brain to perform 1st magnetic navigation for 3 min. Then, the SN of the mouse was sonicated by transcranial 1-MHz FUS (cycle number: 5000, pulse repetition frequency: 1 Hz, duration: 1 min, acoustic pressure: 300 kPa) with magnetic navigation. After FUS exposure, the magnet treatment was continued for another 20 min to facilitate the deposition of PSp at the BBB opening site. The wound was sutured and disinfected by the iodine. After 24 h of treatment, the mouse was anesthetized and the magnet was placed at the left side of brain to perform the 2nd MN for 1 h.

The motor behavior and balance were assessed to verify the treatment outcome by beam walking test [45]. The beam was 80 cm in length, 1 cm in width and placed 50 cm above the bench surface. The mice were previously trained to traverse the beam for 3 days before treatment. Then, the mice received consecutive trials once a week 8 times and the latency to traverse was recorded for each trial. The time was normalized to the value recorded before treatment to reduce the individual difference.

The motor willingness was evaluated by open field test [46]. The mice were placed in a homemade black cube with 40 cm length and allowed to move freely for 10 min. Meanwhile, the movement was recorded by a camera. Then, the videos were analyzed by the software (Smart, Cybertracker, USA) to calculate the total distance in 10 min. The distance was normalized to the value recorded before treatment to reduce the individual difference.

**Western blot analysis and immunohistochemical staining**

To identify the presence of GDNF and tyrosine hydroxylase (TH), the Western blot analysis was employed. The brain tissues were incubated with NP40 to isolate the total cellular proteins. Then, the total cellular proteins were isolated with 10% SDS-PAGE and transferred to the nitrocellulose membranes with a blocking buffer for 1 h. The membrane was next incubated with TH antibody or GDNF antibody (1:1000, GeneTex Inc., TX, USA) overnight at 4 °C and washed with TBST for 10 min. The horseradish peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L, 1:10000; Jackson ImmunoResearch, PA, USA) was added and incubated for 1 h at room temperature. The membrane was washed with TBST and TBS three times over a 10 min interval. Finally, nitrocellulose membranes were visualized using an enhanced chemiluminescence method and the expression level was quantified using Image J (Version 1.51f, LSU Health Sciences Center, New Orleans, LA, USA).

In immunohistochemical (IHC) staining, the tissue sections were incubated with a blocking buffer to prevent the non-specific staining for 1 h. The sections were then incubated with anti-GDNF antibody (1:1000, GeneTex) or anti-TH antibody (1:1000, Genetex) overnight. After rinsing with PBS, the sections were incubated in secondary antibody with goat anti-rat fluorescence 488 (1:1000, GeneTex) or Dylight 594 conjugated anti-rabbit secondary antibody (1:500; GeneTex) for 20 min. Finally, the sections were washed with PBS for observation.

**Statistics**

All results are represented as the mean ± standard deviation. All statistical evaluations were carried out with unpaired two-tailed Student’s t-tests or ANOVA with Bonferroni post hoc test. A p value of less than 0.05 was referred to a significant difference.

**Results**

**Characterization of PSpIO-pDNA nanomedicine**

To fabricate the nanomedicine, we synthesized ~15.0 nm SPIO to provide magnetic properties and stabilize the pDNA. To achieve the immobilization of pDNA, we modified the SPIO with PEI, which served as cationic scaffold for condensing DNA. pDNA-loaded (green fluorescent protein plasmid, pGFP) PSpIO had an averaged hydrodynamic diameter of 191.0 ± 2.3 nm, which is similar to that of PSpIO (182.9 ± 4.2 nm) (Fig. S2(A)). The PSp exhibited a slightly lower positive zeta potential than PSpIO (41.0 ± 2.3 nm) vs. 47.3 ± 2.8 mV) due to the successful condensing of DNA (1 μg) shown by gel retardation assay (Fig. S2(B–C)).

**Sono-magnetic sensing gene vector integrated with PSpIO-pDNA nanomedicines and lipid-shell MBs**

To integrate PSp with MBs, we chose the anionic lipid component as the shell of MBs, allowing PSp to be naturally immobilized onto the MB surface by electrostatic interaction (Fig. 3(A)). The high co-localization between PSpIO (blue spot, stained by Prussian blue), pDNA (red spot, labeled by rhodamine), and the MB contour in the microscopic images revealed that the PSp nanomedicines were successfully embedded in the shells of the MBs (Fig. 3(B)). The size distribution of PSp-MBs increased with the payload of PSp (8 μg: 2.7 ± 0.2 μm; 40 μg: 2.7 ± 0.1 μm; 80 μg: 3.2 ± 0.3 μm) due to the electrostatic neutralization that results from the excess of PSp, which serves as the cross-linker that bridges MBs into clusters (up to 9 μm) (Fig. 3(C)). The loading efficiency dropped with the increase of PSp loading (8 μg: 98 ± 10%; 40 μg: 80 ± 10%; 80 μg: 65 ± 15%; Fig. 3(D)). A previous report has indicated that the size of the circulating particles exceeds 6 μm, which may induce gas embolism in mice [47]. Therefore, PSp-MBs formed with 40 μg of PSp were used in the following experiments. The zeta potential of the PSp-MBs was only slightly increased after loading PSp, (−58.4 ± 3.2 mV to −49.9 ± 6.2 mV; Fig. 3(E)), ensuring biocompatibility in blood circulation.
Sono-magnetic sensing property of PSp-MBs

In vessel-mimic phantom, the PSp-MBs could achieve a significant accumulation under magnetic guidance (0.37 T) with a flow rate of 40 mm/s (0 min: 0 a.u.; 1 min: 0.7 ± 0.2 a.u.; 2 min: 2.9 ± 0.2 a.u.; 3 min: 3.5 ± 1.7 a.u.; 4 min: 6.2 ± 2.1 a.u.; 5 min: 7.4 ± 5.2 a.u.; Fig. 4(A)), indicating the concentration could potentially be locally improved within the brain arteries [48]. The acoustic stability of PSp-MBs remained relatively high until 60 min (0 min: 18.8 ± 0.5 dB; 30 min: 17.2 ± 0.8 dB; 60 min: 17.0 dB ± 0.6 dB, Fig. S3). The PSp-MBs started to transit into nanosized vesicles at 300 kPa of FUS sonication (2.7 μm−240 nm, similar with PSp) (Fig. 4(B)), as the translation efficacy increased with sonication pressure, suggesting the feature of ultrasound sensitivity. The size of these vesicles was probably causing leakage into the brain tissue from the BBB opening area (<290 nm) [42]. The verification of PSp within the vesicles was evaluated by incubating the vesicles with DNase I. Normally, the free pDNA would be degraded by DNase I, leading to complete loss of...

Fig. 5. (A) Upper: PI cellular uptake after treatment in microscopic images; lower: quantification of PI-positive cells and cell viability evaluated by flow cytometry and Alamar blue assay. Data were analyzed by ANOVA (post hoc test: Bonferroni) and presented as mean ± standard deviation (n = 6 per group). All of the data were compared with cell only group. (B) The cellular uptake of the rhodamine-labeled PSp with different conditions. Left: microscopic images; right: quantification data measured by flow cytometry. Original magnification: 20×. * p < 0.05. Data were analyzed by ANOVA (post hoc test: Bonferroni) and presented as mean ± standard deviation (n = 6 per group).
bands of pDNA in electrophoresis tests (Fig. 4(C)). We noticed that the band of pDNA still appeared in the PSp-MB + FUS group, similar to observations in the PSp group and PSp-MB group, suggesting the vesicles contained pDNA (Fig. 4(D)). Besides, the PSp could release pDNA under intracellular-mimic redox environment (10 mM of Glutathione, GSH) because of GSH induced PEI degradation (Fig. S4). Taken together, these features indicate that PSp-MBs could effectively be targeted to the desired site by magnetic navigation and for remote control of cargo release by FUS.

**US-magnetic hybrid interaction enhanced pDNA-PSPIO cellular deposition**

Next, we used PSp-MBs in conjunction with FUS and two-step magnetic navigation to provide the cavitation, proton sponge effect, and magnetic effects to increase gene delivery efficiency (Fig. S1). In other words, we considered that the intracellular accumulation of PSp complexes would be enhanced by concurrently performing US and step I magnetic navigation (1st MN). Second, the PEI enabled the escape of PSp complexes from lysosome degradation. Finally, step II magnetic navigation (2nd MN) was applied to promote transport of the pDNA into the nucleus by taking advantage of magnetic-mediated cytoskeleton re-organization.

Our results demonstrated that membrane permeabilization occurred when the acoustic pressure was higher than 300 kPa, as confirmed by extracellular PI dye diffusion into neuron-like SH-SY5Y cells (24.6 ± 4.8%). The membrane permeabilization was a result of the mechanical interaction between FUS and PSp-MBs, called the cavitation effect (Fig. 5(A)). The portion of PI-positive cells could be increased 1.8-fold by concurrently applying 1st MN (43.4 ± 10.1%). Increasing the acoustic pressure up to 500 kPa increased the number of PI-positive cells, but reduced the cell viability from 97.4 ± 4.0% to 87.0 ± 6.0% because of cavitation-induced cell death [49,50], suggesting that 300 kPa was more suitable for the transportation of PSp. In the meantime, the intracellular deposition of PSp also benefited from FUS sonication in conjunction with magnetic navigation according to the ratio of PSp-positive cells (PSp-MBs only: 10.8 ± 2.7%; PSp-MBs + FUS: 31.8 ± 1.5%; PSp-MBs + FUS + 1st MN: 40.3 ± 4.0%), because the magnetic navigation promoted the high concentration of PSp-MBs near the cells and facilitates the release of PSp across the cellular membrane (Fig. 5(B)). Taken together, these results showed that the cavitation and magnetic force could provide a synergistic effect to significantly increase the efficiency of cellular drug delivery. On the other hand, previous studies have demonstrated that 4 h is needed to completely transport the pDNA into the cytosol via endocytosis [51]. In contrast to endocytosis-mediated delivery, our proposed delivery system provided more rapid transport of PSp into the cytoplasm.

**The lysosome escape of PSPIO-pDNA**

Once PSp entered the cell, we assessed the capability of lysosomal escape by determining the co-localization ratio between lysosomes (green fluorescence) and PSp (red fluorescence) with time-lapsed live-cell microscopic imaging. After FUS sonication, most of the PSp was co-localized with the lysosomes (0.8 ± 0.1) (Fig. 6(A)). Encouragingly, this value progressively decreased with time (4 h: 0.7 ± 0.1; 8 h: 0.5 ± 0.2) by proton sponge effect. In contrast, the co-localization ratio did not change in pure MBs + FUS + pDNA group over the course of the study (0 h: 0.9 ± 0.1; 4 h: 0.9 ± 0.1; 8 h: 0.9 ± 0.1). This indicated that while the majority of intracellular PSp were immediately trapped by lysosomes after FUS sonication, the presence of PEI could prevent 50% of pDNA from lysosomes degradation. These results were...
consistent with those from a previous report in which chitosan was employed to facilitate lysosome escape, and there was a 20% decrease in the co-localization ratio between pDNA and lysosome [52]. After that, the pDNA detached from the PSp by intracellular redox environment-induced PEI degradation.

Magnetic-mediated cytoskeleton orientation to facilitate pDNA trafficking in the cytoplasm

For non-viral gene vectors, the trafficking of pDNA within the cytoplasm is thought to be the limiting step to achieving successful gene delivery, since a variety of cytoskeletal components (e.g., microtubules, intermediate filaments, and microfilaments) within the cytoplasm form a disordered structure that largely hampers the diffusion of large molecules [18,53]. It has been reported that the cytoskeleton is aligned in the direction of the intracellular magnetic force due to the self-defense mechanisms of cells [32,33]. Therefore, we tried to boost the pDNA trafficking inside cells by taking advantage of cytoskeleton alignment. After FUS sonication for 24 h, step II magnetic navigation (2nd MN) was performed by incubating the cells on the left side of the magnet for 1 h, yielding an intracellular magnetic force with the residual PSp nanomedicines. Interestingly, the randomly distributed F-actin fibers within cells were more aligned in the direction of the magnetic force following 2nd MN (Fig. 6(B)). Subsequently, the accumulation of pDNA at the perinuclear site was improved 2.8-fold by magnetic force (Fig. 6(C)). These data suggested that the gene transfection rate could be improved through high concentration of the pDNA in the cellular nuclei.

Enhanced gene transfection rate in neuron-like cells

An effective pGFP transfection would result in expression of detectable green fluorescence signals (Fig. 7(A)). We therefore used flow cytometry to evaluate how ultrasound-magnetic hybrid interaction affected the gene transfection rate of pGFP. The transfection rate of the PSp-MBs groups (6.7 ± 2.1%) was obviously lower than that of the group of commercial transfection agent TransIT®-LT1, PSp, and PSp + 1st MN (10.3 ± 0.7%, 8.9 ± 2.6%, and 10.2 ± 1.5%) because the non-targeting transfection could be avoided by loading of MBs (Fig. 7(B)). This value increased upon FUS sonication (10.5 ± 2.7%), due to cavitation-enhanced cell membrane permeabilization. Simultaneous administration of FUS and magnetic navigation improved the transfection rate 2.5-fold (16.4 ± 0.4%), due to the local concentration of PSp-MBs and the transport and release of PSp into the cytoplasm. Furthermore, the presence of PEI and the magnetic-mediated cytoskeleton orientation boosted the transfection rate by 1.6-fold (6.1 ± 2.1 vs. 10.3 ± 2.7%) and 1.4-fold (22.7 ± 6.3%), respectively. In summary, our proposed ultrasound-magnetic hybrid gene delivery platform could improve gene delivery efficiency and gene transfection rate by overcoming existing intracellular barriers.

![Fig. 7. Qualitative and quantitative evidence that pGFP was transfected into SH-sy5y cells with the different protocols. (A) Upper: the fluorescence distribution of GFP-positive cells measured by the flow cytometry; lower: microscopic images of the SH-sy5y cells after pGFP transfection. Original magnification: 60×. (B) The quantification of transfection efficiency under different conditions was measured by flow cytometry. Data were analyzed by ANOVA (post hoc test: Bonferroni) and presented as mean ± standard deviation (n = 6 per group).](image-url)

![Fig. 8. Parameter optimization for BBB opening. (A) Histologic section and H&E staining were employed to estimate the degree of BBB opening level and safety in brain tissue under different FUS parameters and the 1st magnetic navigation. The degree of BBB opening was determined based on the EB extravasation (blue area). The SN area (red dash line) was further investigated by microscopy. (B) The quantification of damage after BBB opening with different treatment protocols. (C) The quantification of EB extravasation after BBB opening by 300 kPa of FUS and with or without magnetic navigation. Data were analyzed by t-test and presented as mean ± standard deviation (n = 4 per group).](image-url)
Magnetic navigation enhances FUS + PSp-MB-induced BBB opening

To examine whether FUS plus PSp-MBs could open the BBB at the substantia nigra (SN) in mice, we use EB dye as an indicator for BBB opening. The SN regions are regarded as the pathogenic region in PD and are thus researched extensively [54–56]. The result showed that PSp-MBs carrying pGDNF with FUS produced BBB opening at 300 kPa, and the degree of opening increased with acoustic pressure (Fig. 8(A)). Moreover, we found that concurrently applying magnetic navigation, PSp-MBs, and FUS resulted in enhancement of BBB opening compared with the PSp-MBs + FUS group. Fig. 8(B) demonstrated that the prevalence of grade 0 lesions decreased linearly with increasing peak pressure level. However, the percentage of mild (grade = 1) or severe erythrocyte extravasation (grade = 2) increased proportionally to the peak pressure level. At 300 kPa, mild hemorrhage (grade = 1) occurred in 22% of cases in Psp-MBs + FUS + 1st MN group, slightly higher than Psp-MBs + FUS group (17.4%). Severe erythrocyte extravasation (grade = 2) became apparent at 500 kPa, especially in Psp-MBs + FUS + 1st MN group (38% vs. 26%). Altogether, our proposed ultrasound-magnetic hybrid platform achieved 2.8-fold enhancement of BBB opening compared with conventional MBs + FUS technique (8.3 ± 1.7 μg/mg vs. 22.9 ± 1.3 μg/mg, respectively) without noticeable erythrocyte extravasation because magnetic force yields a higher local MB concentration in brain circulation (Figs. 8(C), Fig. 4(A)). Compared with the previous study, our technique achieved the same level of EB extravasation only using one-tenth of the MB concentration due to a higher local MB concentration [57]. In the meantime, this phenomenon resulted in more local MB-released cargo accumulation, potentially improving brain drug delivery.

The GDNF expression was also evaluated by Western blot at 5 days and 6 weeks following the treatment to verify whether enhanced gene expression is transient or sustained. At 5 days after treatment, Psp-MBs + FUS + 1st MN + 2nd MN group provided the most profound pGDNF transgene expression (3.3 fold increase compared to that of normal healthy mice), which is superior to those of the Psp-MBs + FUS + 1st MN group and Psp-MBs + FUS group (2.7 fold and 2.2 fold, respectively, compared to the normal healthy mice) (Fig. 9(A)). In addition, at 6 weeks after treatment, we found that Psp-MBs + FUS + 1st MN + 2nd MN group still had higher pGDNF expression than other groups (2.4 fold; Psp-MBs + FUS + 1st MN group: 1.7 fold; Psp-MBs + FUS group: 2 fold; Pure MBs + FUS + pGDNF group: 1.2 fold, compared with normal healthy mice). However, the overall pGDNF transgene expression at 6 weeks in each group were lower than that at 5 days, probably suggesting the gene expression was a transient effect. Additionally, IHC fluorescence staining identified co-localization between the BBB opening area and GDNF expression region (Fig. 9(B)). These data indicate that the proposed strategy could produce more profound gene expression in vivo due to the improvement of gene delivery efficiency.

Ultrasound-magnetic hybrid gene delivery improves PD syndrome

To examine whether Psp-MBs, FUS, and magnetic navigation could reverse neurodegeneration in mice, we selected the MitoPark mice with progressive degeneration of dopaminergic neurons as the experimental PD model [39]. To verify the treatment outcome, the beam walking test and open field test were employed weekly to quantify the balance ability and motor willingness in animal behavior, respectively. As shown in Fig. 10(A–B), decreased balance ability and rearing behavior during the experimental period (5 weeks) in untreated PD mice (13.4 ± 2.7 s to 42.1 ± 5.5 s, 6410.2 ± 851.9 cm to 14815.5 ± 551.7 cm) confirmed the parkinsonian phenotype. The balance ability of PD mice was almost restored after treatment with Psp-MBs + FUS + 1st MN + 2nd MN during the experimental period (12.9 ± 1.4 s to 10.6 ± 1.9 s). Although treating PD mice with Psp-MBs + FUS + 1st MN also showed a reduction of the abnormal balance in the 5th week (11.5 ± 1.4 s to 16.7 ± 2.3 s), the balance ability still worsened after treatment was finished (4th week). Psp-MBs + FUS group showed a slight therapeutic effect compared with untreated PD mice at week 5 (11.0 ± 1.3 s to 22.1 ± 5.9 s), but the effect was not significant in the 1st week (10.2 ± 1.5 s). The pure MBs + FUS + pGDNF group, FUS only group, Psp-MBs only group, and pure MBs + FUS group did not provide any noticeable effect to limit the development of PD-like syndrome (10.9 ± 3.8 s to 31.2 ± 9.2 s; 11.7 ± 2.3 s to 35.6 ± 4.2 s; 11 ± 2.0 s to 31.2 ± 4.8 s; 10.7 ± 1.7 s to 42.6 ± 5.8 s; Fig. S5).

Next, in the open field test, Psp-MBs + FUS + 1st MN + 2nd MN group also presented the most profound improvement in motor willingness (6348.6 ± 995.2 cm to 2927.1 ± 186.7 cm, 54.9%
The PSp-MBs + FUS group and PSp-MBs + FUS + 1st MN group showed 43.6% (5162.6 ± 554.3 cm to 2907.9 ± 300.3 cm) and 46.5% (4970.1 ± 558.0 cm to 2656.8 ± 882.4 cm) reduction in the 5th week, respectively. The pure MBs + FUS + pGDNF group failed to restore the motor willingness at all (6497.2 ± 163.0 cm to 1630.4 ± 259.1 cm). The most dramatic PD symptom relief in PSp-MBs + FUS + 1st MN + 2nd MN group was likely due to the most profound dopaminergic neuron recovery as assessed by TH expression in this group (PD group: 0.21; pure MBs + FUS + pGDNF group: 0.34; PSp-MBs + FUS group: 0.52; PSp-MBs + FUS + 1st MN group: 0.56; PSp-MBs + FUS + 1st MN + 2nd MN group: 0.68) (Fig. 10(C)).

To histologically confirm dopaminergic neuron degeneration reduction of the proposed strategy, we used tyrosine hydroxylase (TH)-immunohistochemistry staining to observe the dopaminergic structures in the SN TH-absence was observed at the both hemisphere brain in untreated PD mouse (left brain: 45.1 ± 5.5 vs. right brain: 39.8 ± 8.2) because the mitochondrial transcription factor TFAM is selectively removed in dopamine neurons of Mito-Park mouse, resulting in progressively degeneration of dopamine neurons (Fig. 11(A)). However, PSp-MBs + FUS + 1st MN + 2nd MN treatment successfully recovered dopaminergic neurons characterized by dense TH stains observed at the sonicated site (303 ± 29.1, Fig. 10(B), whereas recovery of the dopaminergic neurons was not observed in non-sonicated site (45.1 ± 6.8). PSp-MBs + FUS treatment and PSp-MBs + FUS + 1st MN treatment also provided treatment efficiency (197.4 ± 31.5 and 180.7 ± 25.3). Pure MBs + FUS + pGDNF treatment only provided slightly therapeutic effect (left brain: 49.2 ± 11.5 vs. right brain: 23.5 ± 6.6). These data also support the observed treatment effect was contributed by the improvement of TH neurons number.

**Fig. 10.** PD treatment outcomes by different conditions. (A) Motor ability measured by the beam-walking test. Times to cross 80 cm beam were recorded. (B) The motor willingness measured by the open field test. Left: The total moving distance in 10 min, and the distance was normalized to week 0 and defined as deterioration. (C) The content of TH was determined by the Western blot analysis. Data were analyzed by ANOVA (post hoc test: Bonferroni) and presented as mean ± standard deviation (n = 4 per group).
Conclusion

In this study, we developed a versatile sono-magnetic sensing gene vector to overcome the existing obstacles for in vivo gene delivery by combining ultrasound, magnetic force, and cationic polymer to improve transgene expression. The intracellular accumulation of PSp nanomedicines was enhanced by concurrently performing FUS and magnetic navigation. The PEI allowed PSp complexes to escape lysosomal degradation. Subsequently, the amount of pDNA entering the nucleus could be further increased by magnetic-mediated cytoskeletons re-organization with the residual PSp nanomedicines. Finally, the treatment outcome in animals strongly suggested that this gene vector offers an extendable platform with great clinical translation potential for treating neurodegenerative diseases. This work encourages further exploration of an ultrasonic-magnetic hybrid strategy for improving the efficiency of gene/drug delivery. However, the PD animal model (MitoPark mice) used in this study is still preliminary because the PD symptoms might not really be similar to primate model or humans. Therefore, the future work of this study will need to be needed in other animal model before transferring this technology into clinical usage. Besides, in current framework, the Psp-MBs did not have the function of targeting dopaminergic neurons. The next steps include: (1) increasing neuronal targeting by changing the promoter of GDNF plasmid (i.e. synapsin I promoter for neuronal cell; tyrosine hydroxylase TH promoter for dopaminergic neuron) [58–60]; (2) increasing targeting ability of MBs by modifying targeting ligand on to the surface of Psp-MBs (i.e. fragment of tetanus toxin for neuronal cell; dopamine for dopaminergic neuron) [61,62].

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Chun-Yao Wu: Data curation, Formal analysis, Investigation, Writing - original draft. Rih-Yang Huang: Formal analysis, Methodology. En-Chi Liao: Formal analysis, Methodology. Yu-Chun Lin: Funding acquisition, Methodology, Resources. Yi-Ju Ho: Visualization, Writing - review & editing. Chien-Wen Chang: Resources. Hong-Lin Chan: Resources, Ying-Zu Huang: Funding acquisition, Methodology, Resources. Tsung-Hsien Hsieh: Funding acquisition, Methodology, Resources. Ching-Hsiang Fan: Conceptualization, Data curation, Project administration, Validation, Writing - original draft. Chih-Kuang Yeh: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Acknowledgments

The authors also thank the Center for Advanced Molecular Imaging and Translation, Chang Gung Memorial Hospital, Linkou for their assistance in the experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brs.2020.02.024.

References


