Expert Opinion

Ultrasound-mediated gene delivery

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Importance of the field: The use of ultrasound with microbubbles raises the possibility of an efficient and safe gene delivery.
Areas covered in this review: This review summarizes the current state of the art of gene delivery by sonoporation under the following topics. First, the basic ultrasound parameters and the characteristics of microbubble in biological systems are discussed. Second, the extensions of sonoporation to other fields of gene delivery such as viral and non-viral vector are briefly reviewed. Finally, recent applications in an animal model for various diseases are introduced.
What the reader will gain: Information and comments on gene delivery by sonoporation or enhanced cell membrane permeability by means of ultrasound.
Take home message: Ultrasound-mediated gene delivery combined with microbubble agents provides significant safety advantages over other methods of local gene delivery.

Keywords: drug/gene delivery, microbubbles, sonoporation, ultrasound

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1. Introduction

Gene therapy is defined as the delivery of nucleic acids into the recipient's cells for therapeutic purposes. Effective gene therapy for a specific disease requires comprehensive understanding of the disease etiology, characteristics of delivered genetic material, and the method of delivery. Delivery strategies have been developed to achieve therapeutic feasibility without side effects. With respect to safety, the simplest approach might be naked DNA delivery without any carriers by either local or systemic administration. In the case of gene delivery to skeletal muscles comprising a substantial percentage (~ 40%) of body weight, the delivery of naked DNA has been reported to demonstrate significant transgene expression [1]. However, the expression level of transgene by the naked DNA delivery often ends up extremely low or undetected. This is mainly owing to rapid degradation by nucleases and fast clearance by the mononuclear phagocyte system [2]. Although viral vector systems have occupied more than half of clinical trials [3], largely because of the high transfection efficiency, the potential of immunogenicity remains a major concern. Moreover, the deleterious consequences caused by the integration of administered DNA into the host chromosome have accelerated the pursuit of a safer system of gene delivery [4]. Non-viral gene delivery methods using polycations or liposomes have been developed to overcome the safety problems of viral vector systems. By enhancing the internalization of DNA into the cytoplasm of the cell, and by circumventing the barriers including DNA clearance and immunologic problems, the efficacy and the safety of non-viral gene delivery systems have been achieved to a certain extent. However, targeting methods and the intrinsic toxicity problems [5,6] of the cationic synthetic gene carriers are still problematic and awaiting further improvements.

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Article highlights.

- One of the main advantages of drug or gene delivery by sonoporation is to achieve site specificity with negligible local and systemic toxicities, by the optimization of parameters of ultrasound and microbubbles.
- The dynamic characteristics of ultrasound that induce cell membrane porosity can be controlled by ultrasonic factors such as frequency, intensity and duration.
- The induction of microbubble cavitation by ultrasound has been considered a major mechanism of delivery, which carries non-permeable macromolecules across the cell membrane.
- Ultrasound can act synergistically with other vector systems in order to have several advantages, such as low cytotoxicity, high target selectivity, low immunogenicity and repeatable application.
- Gene delivery by sonoporation could be a possible therapeutic alternative in current cancer treatment.
- Non-invasive specific gene transfection into the deep seated internal organ is very difficult. Sonoporation might be used for this purpose and the possibility for the application of this technique to the various kinds of diseases would be promising.
- Particularly suited for the various localized diseases and the diseases requiring limited transfection into the deep-seated organs or tissues, sonoporation could be used successfully in clinical practice in the near future.
- Along with its superior safety profile, sonoporation might be regarded as a pioneering technique that could move gene therapy a step closer to clinical medicine.

This box summarises key points contained in the article.

The adoption of physical/mechanical methods for gene delivery is one of the efforts to obtain more established *modus operandi*, which enables improved safety, efficacy and targeting. At present, there are several physical methods, including microinjection, gene gun, electroporation, sonoporation and laser irradiation [7-11].

Of these, sonoporation refers to transient porosities in the cell membranes induced by insonation with ultrasound in the presence of microbubbles and uptake of the drugs or the genes into the cells [12]. One of the main advantages of drug or gene delivery by sonoporation is to achieve site specificity with negligible local and systemic toxicities by the optimization of parameters of ultrasound and microbubbles.

This review limits discussions to the following issues. First, the basic concepts of ultrasound parameters and the characteristics of microbubbles in biological systems are addressed. Second, the extensions of sonoporation to other fields of gene delivery such as viral and non-viral vectors are briefly reviewed. Finally, recent applications in animal models for various diseases are introduced with detailed ultrasound conditions.

2. Gene delivery by sonoporation

For gene delivery by sonoporation, microbubble collapse during acoustic cavitation is required, which releases energy that temporarily increases the internalization of exogenous molecules into living cells. Acoustic cavitation refers to the rupture of liquids and its associated effects. In ultrasound-mediated gene delivery, cavitation may be subdivided into two categories: microbubble oscillating (stable cavitation) or microbubble collapsing cavitation (inertial or transient cavitation) [13]. The threshold of microbubble collapse may vary according to the composition of the microbubble, such as gas, shell, and so on. The mechanism of gene entry across the plasma membrane is not fully known. So far about three candidate mechanisms have been suggested; i) active transport by means of endocytosis; ii) passive transport through transient, nanometer pores in the plasma membrane; and iii) uptake through repairable 'wounds' in the plasma membrane. Of these, the mechanism through repairable wounds has been reported recently [14].

For practical application of ultrasound to gene delivery, commercial or customized sinusoidal probes at megahertz frequencies are usually used. The dynamic characteristics of ultrasound that induce cell membrane porosity can be controlled by ultrasonic factors such as frequency, intensity and duration.

2.1 Ultrasound

Ultrasound is defined as cyclic sound pressure with frequency range > 20 kHz. The nature of the ultrasound wave is longitudinal, which makes pressure variations in the medium. The ultrasound exposure parameters related to the increase in the cell membrane permeability are classified as follows: pulse center frequency, intensity and duration.

2.1.1 *Frequency*

Suslick *et al.* [15] provided three distinct sets of ultrasound conditions based on frequency range and applications.

- *High frequency*: ultrasound with frequency ranging between 3 and 10 MHz (or over) is used in clinical imaging.
- *Medium frequency*: therapeutic ultrasound with frequency ranging between 0.7 and 3.0 MHz is used in physical therapy.
- *Low frequency*: the conventional type of ultrasound, which is used for lithotripsy, cataract emulsification, liposuction, cancer therapy, dental descaling and ultrasonic scalpels (18 – 100 kHz).

Although it is still not clear which frequency is best suited for ultrasound-mediated gene delivery, megahertz (1.0 – 2.0 MHz) frequencies have been utilized as 'conventional' therapeutic ultrasound. ter Haar [16] and Rokhina *et al.* [17] summarized ultrasonic parameters and their biological effects. For an effective gene delivery by sonoporation, it is important to acquire the pressure threshold of microbubbles under a constituent frequency of ultrasound. Chen *et al.* [18] demonstrated the thresholds of three different microbubbles under various acoustic conditions. Optison™ (GE Healthcare, Inc., Princeton, NJ, USA), Sonazoid™ (GE Healthcare, Buckinghamshire, UK) and

biSphere™ (POINT Biomedical, San Carlos, CA, USA) had fragmentation threshold at 0.13, 0.48 and 0.96 MPa at 1.1 MHz, respectively.

2.1.2 Intensity

Acoustic intensity can be defined as the average flux of acoustic energy per unit area per unit time (watts per square centimeter). As the acoustic intensity is proportional to acoustic pressure square and has a positive correlation with the power and energy of the sound, the higher intensity indicates higher accumulation of energy at the target area. As acoustic intensity fluctuates according to space and time, the description of acoustic intensity is often accompanied by spatial or temporal dimension. Commonly used are ISPTA (spatial peak, temporal average intensity), ISATA (spatial average, temporal average intensity) and ISPPA (spatial peak, pulse average intensity). Therapeutic ultrasound can be broadly divided into two categories: 'low' intensity (0.125 – 3 W/cm^2) and 'high' intensity (> 5 W/cm^2) [19].

For high-intensity focused ultrasound (HIFU), temperatures in excess of 56°C are achieved in tissues by raising focal peak intensities to 1500 W/cm^2 at 1.7 MHz for 1 – 2 s [16]. The high temperature is used for the shattering of kidney stone or ablation of tumor or fibroid.

Low-intensity ultrasound has been used for clinical treatment of bone healing, sonopophoresis, gene therapy and sonothrombolysis [16]. Thermal effects are less likely to be involved when applying low-intensity ultrasound.

The mechanical index (MI) is a standard measure of the transfection efficiency. The MI value can indicate the possibility of mechanical damage to the tissue as a result of acoustically driven bubbles or gas bodies. The MI is defined as peak rarefactional pressure of ultrasound longitudinal wave propagation in a uniform medium divided by the square root of the center frequency of the transmitted ultrasound pulse. A MI of 1.6 indicates sixfold superior to one of 1.2. Higher levels of energy can increase the permeability of the endothelial barrier, with possible side effects such as hemorrhage [20].

2.1.3 Duration

When continuous wave ultrasound or pulsed ultrasound exposures are used for sonoporation, the duration of ultrasound is critical for drug or gene delivery. The ratio of duration is expressed as duty cycle, which is the proportion of time during ultrasound insonification. One per cent of duty cycle represents 1 s of ultrasound insonification out of 100 s. Using *Xenopus oocyte* as a model, Pan and co-workers demonstrated that duration was highly related to the cell survival rate at a fixed ultrasound frequency (0.98 MHz). They found that the membrane resealing initiates at the end of ultrasound application with a recovery time constant on the order of 2 – 8 s. In their study, the authors indicated the importance of spreading of energy over a longer duration, which helps to avoid irreversible sonoporation. They observed that a 1 s tone-burst exposure under the same conditions (0.4 MPa and 5% Optison concentration) always causes irreversible membrane disruption and immediate cell death [17,21-24].

2.1.4 Biological effects of ultrasound and microbubble

Many studies have been made of biological effects of ultrasound and microbubble. Dalecki *et al.* [25] demonstrated that ultrasound with air-filled albumin microbubble (Albunex) induced massive hemorrhage in all organs of mice exposed to a lithotripter field with peak amplitude of 2 MPa. Miller *et al.* [26] emphasized that the presence of microbubbles may have an effect on tissue heating by oscillating nonlinearly to dissipate acoustic energy as heat.

Stratmeyer *et al.* [27] reviewed extensively the potential biological effects of diagnostic ultrasound. In the case of DNA delivery by sonoporation, there are several reports on the biological effects, including secondarily generated processes between ultrasound and tissues. Free radicals are generated through breakdown of microbubbles by ultrasound. The collapse of the microbubble produces shock waves and free radical reactive oxygen species (ROS) in the ultrasound-irradiated medium. ROS formation by the ultrasound results mainly from the inertial cavitation, which is a potential cause of endothelial damage only after long exposure times. Lionetti *et al.* [28] demonstrated that the transient exposure of HUVEC (human umbilical vein endothelial cell) to ultrasound with high mechanical index (MI = 1.2) increased endothelial permeability through a caveolae-dependent endocytosis partially mediated by ROS. Ultrasound treatment was reported to induce the reparable cell membrane change, which increases calcium uptake for intensities from 0.5 to 1.0 W/cm^2, with a maximum increase of 18% after a 5 min exposure in embryonic chick 3T3 fibroblasts [29]. Erythrocytes exposed to ultrasound (1.55 MHz, continuous wave) were induced to form agglutinates. The agglutination or aggregation was reversible. Bulk fluid movement due to ultrasound irradiation was the major cause and transient cavitation appeared not to be the mechanism involved [30]. Ultrasound is also reported to induce DNA damage, including DNA single-strand break, sister chromatid exchange and increased mutation frequency [31]. Although low-intensity pulsed ultrasound is adopted for clinical applications such as the repair of soft tissue damage, further research on the detailed mechanisms of ultrasound-mediated gene delivery is still required.

2.2 Microbubble and mechanism of DNA entry by sonoporation

Over two decades, microbubbles have been used as contrast agents for ultrasound imaging. Since Bao *et al.* [8] first observed fluorescein isothiocyanate (FITC)-dextran uptake and luciferase expression in Chinese hamster ovary cells by sonoporation with 2.25 MHz frequency with 10% air-filled microbubble (Albunex), microbubbles subjected to ultrasound have been recognized as therapeutic tools for drug and gene delivery.

Stride [32] classified microbubbles into four groups. The coated microbubbles are composed of coating materials and gas such as perfluoropropane and hexafluoride (e.g., Albunex, Definity™ (Bristol-Myers Squibb, North Billerica, MA, USA), Optison™, Sonazoid and SonoVue® (Bracco International BV, Amsterdam, Netherlands)). The phase shift emulsion is typically composed of a stabilized emulsion of volatile liquid droplets that evaporate to form microbubbles either on injection or following exposure to ultrasound of sufficient intensity (e.g., EchoGen®; Sonus Pharmaceuticals, Bothell, WA, USA). The echogenic liposome consists of phospholipid bi-layers encapsulating a mixture of liquid and gas. The multilayered microbubble provides a means of increasing the effective surface area and hence the quantity of material that can be attached (e.g., biSphere). Of these, SonoVue, Definity and Optison are microbubble agents approved for human use by FDA.

Microbubble-enhanced sonoporation is a dynamic process. Ferrara *et al.* [33] recently explained the characteristics of microbubbles and their application in gene delivery and imaging. The induction of microbubble cavitation by ultrasound has been considered to be a major mechanism of delivery that carries non-permeable macromolecules across the cell membrane. Recently, Forbes *et al.* [34] suggested that the effect is related to linear or nonlinear oscillation of microbubbles occurring at pressure levels below the inertial cavitation threshold in Chinese hamster ovary cell line (CHO).

Schlicher *et al.* [14] suggested that the intracellular delivery of drug or gene occurs by sonoporation through membrane resealing. In the process of resealing, vesicle exocytosis requiring Ca^{2+} was mechanistically related to the ultrasound-mediated uptake. As they utilized only low-frequency ultrasound (24 kHz, 10% duty cycle) in the experiments, the mechanism they suggested might not be applicable to other ranges of ultrasound. However, it is not deniable that wound repair is one of the mechanisms by which cells uptake macromolecules by sonoporation.

3. Applications of sonoporation

For non-viral gene delivery using carrier molecules, plasmids containing therapeutic gene must go through cellular space to enter the nuclear compartment. Once internalized by endocytosis, plasmid DNA undergoes sorting and recycling via the early and late endosomes. The released DNA is diffused towards the nuclear pore complex (NPC) for transcription [35].

Ultrasound can act synergistically with other vector systems in order to have several advantages, such as low cytotoxicity, high target selectivity, low immunogenicity and repeatable application.

3.1 Viral vector

Chen *et al.* [36] demonstrated high levels of luciferase gene expression using systemic intravenous infusion of adenovirus encoding luciferase gene driven by a cytomegaly virus (CMV) promoter using microbubbles under ultrasound in anesthetized Zucker rats. In their experiment, targeted gene delivery without the profound liver uptake of adenovirus was implied and the possibility of organ specificity through sonoporation was suggested at low transmission frequency (1.3 MHz). Naka *et al.* [37] reported increased transduction efficiency of retrovirus with ultrasound exposure in human embryonic kidney cell line (293T). Recently, Taylor *et al.* [38] showed ultrasound-targeted gene delivery to a restricted area of the cell using envelope-deficient retrovirus-loaded cationic microbubbles under 1 MHz ultrasound for 5 s. These experiments demonstrate the potential applications of sonoporation with viral gene therapy for increasing the tissue or organ selectivity.

3.2 Polyethylenimine and liposome

Deshpande and Prausnitz [39] tested the combination of ultrasound and polyethylenimine (PEI) to increase DNA transfection *in vitro*. In human prostate cancer cell line (DU145), the combination of ultrasound and PEI synergistically increased DNA transfection efficiency up to 200-fold compared with the control group, which resulted in reporter gene expression by 34% of cells. Ultrasound and PEI have each separately been shown to increase DNA transfection efficiency. This study proved the hypothesis that the combination of ultrasound and PEI could have a synergistic effect to increase DNA transfection. They showed that the simultaneous sonoporation with PEI much enhanced the intracellular uptake of PEI–DNA complexes without the DNA damage revealed by gel electrophoresis. Although the mechanisms of how ultrasound enhances the transfection efficiency of PEI–DNA nanoparticles need further explanation, they postulated that the formation of transient pores of nanometer to micrometer dimensions by ultrasound might cause increased intracellular delivery of the polyplexes into the cells.

Chumakova *et al.* [40] reported that a combination of polycation agents such as poly(lactic-*co*-glycolic acid) (PLGA) and PEI with ultrasound may yield a powerful tool for efficient cancer therapy. In their experiment, PLGA was used as microbubble. They estimated the transfection efficiency of PLGA/PEI/DNA complex nanoparticles with ultrasound in tumor slices from nude mice injected with DU145 cells. According to their observation, the oscillating PLGA nanoparticles stimulated tissue transfection, resulting in at least eightfold higher efficiency of gene transfer *in vivo*. Lentacker *et al.* [41] showed that the sonoporation delivered PEGylated lipoplexes into the cell cytoplasm without the endocytotic uptake using a metastatic human melanoma cell line (BLM).

These experiments using simultaneous ultrasound and cationic gene carriers might have very important implications. The high charge densities of cationic gene carriers are always mandatory for packaging the DNA. It would be very difficult to separate the toxicities of cationic carriers from their

transfection efficiencies because both phenomena are closely related with the charge densities of cationic substances. In this sense, the fact that the combination of ultrasound could synergistically enhance the transfection efficiency means that the amount of cationic carrier for gene transfection might be reduced, decreasing the toxicity. Further studies are necessary to clarify this issue.

3.3 Short interfering RNA

Ultrasound can also be used for short interfering RNA (siRNA) delivery for the targeted silencing of specific genes in various cells. Vandenbroucke *et al.* [42] evaluated the effect of siRNA delivery using PEGylated siPlexes (PEG-siPlexes) by the decrease of luciferase activity in a human hemochromatotic cell line (HUH7) that stably expressed eGFP-luciferase. In this new delivery system, they utilized ultrasound (1 MHz; 2 W/cm^2; 10% duty cycle) with perfluorobutane-filled microbubbles. They showed the controlled release of PEGylated siRNA–liposome complexes in response to ultrasound radiation. Otani *et al.* [43] demonstrated naked siRNA delivery by sonoporation in mesenchymal stem cells (MSC) and adipose tissue-derived stromal cells (ASC), and that the intracelluar delivery of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) resulted in significant knockdown of PTEM mRNA expression and activation of PKB/Akt cell survival signaling. In their experiments, Sonanzoid consisting of perfluorobutane and hydrogenated egg phosphatidyl serine was used as a microbubble (1.2×10^9 microspheres/ml).

The main action mechanism of siRNA is the interference of specific gene expression to modify the course of diseases. The delivery of siRNA should be organ- or tissue-specific to minimize the untoward side effects. In this sense, local injection of siRNA in the disease area followed by ultrasound irradiation could be developed as a clinically useful siRNA delivery technique in many kinds of localized disease in the near future.

3.4 Minicircle DNA

The transfection efficiency of sonoporation can be improved by modification of the plasmid structure to be delivered. Minicircle DNA is supercoiled dsDNA comprised of promoter, gene of interest, and polyadenylation signal without bacterial-originated sequences such as replication origin, or antibiotic resistance [44]. The small size of minicircle DNA in comparison with the conventional plasmid containing the bacterial backbone sequences may show better diffusion *in vivo*. The reduced number of unmethylated CpG motifs in minicircle DNA triggers less immune response. In the authors' experiment for the wound healing of streptozotocin-diabetic mice, ultrasound-mediated minicircle-VEGF^{165} (vascular endothelial growth factor) delivery demonstrated a 2.5-fold higher transfection efficiency in NIH3T3 cells. Administration of minicircle-VEGF^{165} followed by insonication of ultrasound (1 MHz; 2.0 W/cm^2; 20% duty cycle; 30 s) showed reasonable wound closure and restored diabetic wound microarchitectures to their normal state [45].

Minicircle DNA could be used in all kinds of non-viral gene delivery system, including naked DNA delivery, gene gun, electroporation and cationic carriers, which traditionally used plasmid DNAs as the carrier of therapeutic DNA sequences, to improve the transfection efficiencies of each method. The main problems limiting the wider use of minicircles are the cost and the technical difficulties for mass production, because minicircles do not contain the sequence of 'origin of replication' that is critical for the simple amplification in the *Escherichia coli* culture system. Technical advances will solve this problem in the near future.

4. Ultrasound-mediated gene or drug delivery

4.1 *In vivo* sonoporation studies

Li *et al.* [46] demonstrated prolonged functional luciferase gene expression with the 40 kHz pulse ultrasound in mouse hind leg muscle and in tumors. Microbubble–plasmid mixtures were injected into the hind leg muscle of 8-week-old C3H/HeN mice or radiation-induced fibrosarcoma tumor (RIF-1) on the dorsum of the animals, reaching a volume of 150 – 200 mm^3 by means of intratumoral injection. Following ultrasound-mediated transfection, *in vivo* imaging was performed to assess the luciferase expression using bioluminescence without killing. They found that the signal remained elevated for a period of up to 84 days.

Gene delivery by sonoporation could be a possible therapeutic alternative in current cancer treatment. As listed in **Table 1**, several recent efforts using sonoporation have been made for the treatment of cancer. Chen *et al.* [47] reported gene delivery by sonoporation rendering RNA interference (RNAi) could be applied to cervical cancer treatment. They demonstrated the level of survivin, the inhibitor of apoptosis protein family, was significantly downregulated by the sonoporation of short hairpin RNA (shRNA) in tumor xenograft tissues of BALB/c mice.

Besides cancer treatment, studies of gene delivery by sonoporation to other tissues have increased. Schratzberger *et al.* [48] observed augmented neovascularization of ischemic hindlimb muscles of New Zealand White rabbits. They delivered naked VEGF DNA using ultrasound at 1 MHz, 100 W/cm^2, 6% duty cycle and 5 min exposure time. Inagaki *et al.* [49] tested the effect of naked p53 cDNA on neointimal formation in rat ballon injury. The naked DNA was delivered into blood vessels with Optison microbubble by means of ultrasound for 2 min (2.5 W/cm^2). The expression level of p53 was detected and neointimal formation was significantly inhibited in blood vessels. For the prolonged graft survival, antisense oligodeoxyribonucleic acid against NFκB (NFκB-decoy) was delivered to the donor kidney in a rat renal allograft model by sonoporation with Optison. They reported decreased expression of NFκB-regulated cytokines and adhesion molecules, including interleukin-1 (IL-1), inducible NO synthase (iNOS), monocyte chemoattractant

Table 1. Summary of recent studies of gene delivery by sonoporation *in vivo*.

	Model system	Nucleic acid	Microbubble	Ultrasound conditions	Comments on effect
Li *et al*. [46]	C3H/HeN mice (8 weeks) radiation-induced fibrosarcoma (RIF-1) tumors	Luciferase CMV promoter (pEPI-1-Luc) 20 μg	Sonidel MB101 or SonoVue®	1 MHz; 1.9 W/cm^2; DC 25%; 3 – 6 min	The expression of luciferase was increased to a maximum by day 6 and the signal remained relatively constant between days 6 and 15
Chen *et al*. [47]	BALB/c nude mice (4 – 6 weeks) inoculated s.c. with cervical cancer cells (HeLa)	Survivin short hairpin RNA (shRNA) 50 μg	SonoVue®	3 MHz; 2 W/cm^2; DC 20%; 2 min.	Survivin shRNA facilitated by sonoporation caused knock down surviving gene expression, induced cell apoptosis, and inhibited proliferation *in vivo*
Suzuki *et al*. [62]	ddY mice (4 weeks old) inoculated with mouse sarcoma (S-180 cells)	Luciferase pCMV-luc 10 μg	Optison	0.7 MHz; 1.2 W/cm^2; DC 50%; 2 min	The plasmid DNA was delivered to a specificarea of the abdomen or solid tumor tissue by local exposure to ultrasound
Nie *et al*. [63]	C57BL/6J mice (4 weeks old) inoculated s.c. with Hepa 1 – 6 tumor cell	KDR-tk gene 60 μg	SonoVue®	1 MHz; 2.0 W/cm^2; DC 50%; 5 min	The expression and antitumor effect mediated by US alone was originally weak, but the addition of SonoVue resulted in sufficient and specific high expression of transfected genes
Duvshani-Eshet *et al*. [64]	C57/black mice (4 – 5 weeks old) inoculated s.c. with PC2 cells	GFP or luciferase (pGL3-Luc or pIRES-EGEP 100 μg)	Optison	1 MHz; 2 W/cm^2; DC 30%; 20 or 30 min	The level of expression by sonoporation was also significantly higher compared to other non-viral method, such as Lipofectamine

CMV: Cytomegalovirus; DC: Duty cycle; GFP: Green fluorescent protein; KDR: Kinase domain-containing receptor; s.c.: Subcutaneously; tk: Thymidine kinase.

protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and intercellular adhesion molecule-1 (ICAM-1).

Lu *et al.* [50] tested the safety of ultrasound combined with Optison microbubble in C57B10 mouse skeletal muscle. They reported that ultrasound at a moderate power (1 MHz, 3 W/cm^2; 20% duty cycle; 60 s) exposure did not elicit tissue damage or the inflammatory reactions. Yang *et al.* [51] reported *GFP* gene delivery to skin by sonoporation in the dorsum of female BALB/cAJc1-nu nude mice. Transfected cells survived, moving from the basal layer to the stratum corneum, until day 14 post-transfection. The applied ultrasound frequency was 2 MHz at an intensity of 8 W/cm^2 for 60 s.

Nishida *et al.* [52] reported a long-term gene expression by sonoporation in coccygeal intervertebral discs of Sprague–Dawley rats. The *in vivo* gene expression of marker DNA (GFP or luciferase) sustained up to 24 weeks without any side effects. Takahashi *et al.* [53] reported a 25-fold increase of spinal luciferin bioluminescence 1 day after ultrasound irradiation in BALB/c mice. The expression of luciferase protein was mostly limited to the dorsal meningeal cells, which were insonified with ultrasound. In the spinal gene delivery, the site limitation of transgene expression with minimal toxicity is critical for repeated applicability. Spinal cord is a very delicate structure with topographical collections of numerous neurons with different functions, so widespread gene expressions or the toxicities might cause terrible clinical adverse events. These experiments could be regarded as showing the advantages of sonoporation for accurate and safe local gene delivery.

Hynynen *et al.* [54] reported that non-invasive and reversible disruption of the blood–brain barrier (BBB) was using focused ultrasound bursts in conjunction with an ultrasound contrast agent. The exact mechanisms of this BBB opening, however, are not well understood. Ultrasound-induced BBB disruption is thought to occur from both enhanced molecular transportation through the endothelial cells and disruption of the tight junction. Sheikov *et al.* [55] suggested that the mechanism includes the tight junction opening, vesicular transcellular trafficking, and endothelial cell fenestration and channel formation.

Sonoporation using ultrasound at 1 MHz frequency has recently been recognized as a safe method of transiently altering BBB permeability for drugs or gene delivery [56-58]. Xie *et al.* [59] showed that microbubbles and ultrasound could increase BBB permeability in pigs. Two different kinds of costumed microbubble (ipid-encapsulated microbubble [LEMB] and perfluorocarbon-exposed sonicated dextrose albumin [PESDA], ImaRx Therapeutics, Inc.) were used to deliver drug across the BBB. From the MRI data, significantly quantitative differences in BBB permeability were observed by treatment of a 1 MHz ultrasound with microbubbles. The clinical implications and the safety of the method shown in this report require further investigation.

4.2 *In vivo* sonoporation into the deep organs

Sonoporation can also be used for gene therapy into the deep-seated organs. After intravenous systemic injection of therapeutic gene and microbubble, timed external ultrasound irradiation to the specific organs, for example heart or pancreas, results in successful transfection of the irradiated organs [60]. Chen *et al.* [61] demonstrated that the injection of plasmid and microbubble into the rat jugular vein followed by sonoporation to the pancreas resulted in successful transfection into the rat pancreatic β-cells. They used a rat insulin promoter (RIP) for the expression of reporter gene specifically at the β-cells.

Non-invasive specific gene transfection into the deep-seated internal organ is very difficult. Sonoporation might be used for this purpose and the possibility for the application of this technique to the various kinds of diseases would be promising.

5. Conclusions

Ultrasound-mediated gene delivery is in its infancy. A great deal of research still remains to be done concerning the acoustic parameters to be used. Further understanding of gene delivery mechanisms by means of sonoporation is required. Nevertheless, the safety and efficacy of the technology will aid in the treatment of debilitating diseases. Particularly suited for the various localized diseases and the diseases requiring limited transfection into the deep-seated organs or tissues, sonoporation could be used successfully in clinical practice in the near future.

6. Expert opinion

The most important advantage of sonoporation is the proven safety profile as compared with other gene delivery techniques. The principal reason for the slow transition of gene therapy techniques from the bench to the bedside might be safety issues rather than the efficacy. Ultrasound has been widely used in clinical practice for the diagnosis and treatment of various diseases, and microbubble agents have also been used in clinical practice for diagnostic imaging purposes.

Sonoporation is a method of localized gene delivery. At the beginning of the gene therapy research, the main targets were the malignant cancers and the hereditary disorders. These two categories require systemic gene delivery techniques. Sonoporation could be used for the other kinds of localized disease, as well as systemic diseases caused by localized pathologies. This means that the vast majority of diseases could be the targets of sonoporation.

The relatively weak transfection efficiency of sonoporation could be overcome by using minicircle technology. Minicircle DNA is a small super-coiled circular DNA composed only of expression cassette, that is, promoter, therapeutic DNA and polyadenylation signal. Its small size and the paucity of immunogenic sequences in the minicircle are clear advantages

over the conventional plasmid DNA for improving the transfection efficiency.

The applications of sonoporation in the deep-seated internal organs are very promising. By using organ-specific promoters, nearly all the internal organs could be successfully transfected by sonoporation. In the case of the diseases involving heart, for example myocardial infarction, the introduction of the therapeutic genes has conventionally been done by a direct approach using very long intravascular catheters, which might cause fatal ventricular arrhythmias owing to the direct irritation on the unstable myocardium. Type 1 diabetes mellitus is caused by the immunologic destruction of insulin-secreting β-cells in the pancreas. To change the pathophysiology of type 1 diabetes, many attempts have been made to transfect the β-cells with various kinds of therapeutic gene. Most of the attempts were performed by the ERCP (endoscopic retrograde cholangiopancreaticogram) technique, which is done by direct injection into the main pancreatic duct through an endoscopically introduced catheter. The most dangerous clinical complication of this technique is fatal necrotizing pancreatitis resulting from the mechanical irritation of the pancreas. Sonoporation into the heart or pancreas could easily be done by systemic injection of DNA and microbubbles and the timed external ultrasound irradiation into the target organs, which would enable us to avoid mechanical irritations. This procedure would be more simple, non-invasive and less expensive. This might be a very important advantage in actual clinical practice.

Along with its superior safety profile, sonoporation might be regarded as a pioneering technique that could move gene therapy a step closer to clinical medicine. Sonoporation could have significant safety advantages over other methods of local gene delivery. Wider application to the various kinds of disease will be required in the near future.

Declaration of interest

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