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Ultrasonic particles: An approach for targeted gene delivery

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Abbreviations: AAA, abdominal aortic aneurysm; AKT, protein kinase B; BDNF, brain-derived neurotrophic factor; BNT162b2, BioNTech COVID-19 mRNA vaccine; C3F8, octafluoropropane; C5F11, decafluorobutane; CD105, endoglin, cluster of differentiation 105; COVID-19, coronavirus disease 2019; CVD, cardiovascular disease; DC-CHOL, dipalmitoylcholine; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; DPPC, dipalmitoylphosphatidylcholine; DSPC-PEG2K-Mal, distearoylphosphatidylcholine-N-[maleimide(polyethylene glycol)-2000]; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000]; DSPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000]; eGFP, enhanced green fluorescence protein; GDNF, glial cell line–derived neurotrophic factor; GFP, green fluorescence protein; IFN-β, interferon-β; I/R, ischemia/reperfusion; LNP, lipid nanoparticle; Man-PEG2000, mannose-binding polyethylene glycol-2000; MB, microbubble; MI, myocardial infarction; miRNA, microRNA; MMP2, matrix metalloproteinase-2; MRI, magnetic resonance imaging; mRNA, messenger RNA; mRNA1273, Moderna COVID-19 mRNA vaccine; NB, nanobubble; Nur1, orphan nuclear receptor; OTC, ornitine transcarbamylase; pDNA, plasmid DNA; PEG, polyethylene glycol; PEGylated-PEI-SH, polyethylene glycol-polyethyleneimine-thiol; PEI, polyethylamine; PMO, phosphorodiamidate morpholino oligomer; PNA, peptide nucleic acid; RNA, ribonucleic acid; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SCF, stem cell factor; SFK, sulfotransferase; siRNA, small interfering RNA; SPIO-NP, fluorinated iron oxide nanoparticle; TA, tibialis anterior; Timp3, tissue inhibitor of metalloproteinase 3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UTGD, ultrasound-targeted gene delivery; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

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Gene therapy has been widely investigated for the treatment of genetic, acquired, and infectious diseases. Pioneering work utilized viral vectors; however, these are suspected of causing serious adverse events, resulting in the termination of several clinical trials. Non-viral vectors, such as lipid nanoparticles, have attracted significant interest, mainly due to their successful use in vaccines in the current COVID-19 pandemic. Although they allow safe delivery, they come with the disadvantage of off-target delivery. The application of ultrasound to ultrasound-sensitive particles allows for a direct, site-specific transfer of genetic materials into the organ/site of interest. This process, termed ultrasound-targeted gene delivery (UTGD), also increases cell membrane permeability and enhances gene uptake. This review focuses on the advances in ultrasound and the development of ultrasonic particles for UTGD across a range of diseases. Furthermore, we discuss the limitations and future perspectives of UTGD.

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1. Introduction

Gene therapy is the introduction of genetic materials into cells to compensate for an abnormal gene or allow the cells to produce beneficial proteins. The potential benefits of gene therapy have attracted major enthusiasm and consequently various gene therapy approaches have been investigated in relation to the treatment of genetic, acquired, and infectious diseases. Gene therapy has traditionally employed viral vectors to correct genetic abnormalities which result in clinical disorders. One of the most successful applications of gene therapy so far has been achieved in patients with severe combined immune deficiency or “bubble boy disease” [1,2]. However, while effective in treating genetic disorders, treatments using viral vectors have been associated with serious adverse events and the development of cancer, so several clinical trials have been stopped [3,4]. Subsequently, non-viral vectors have been proposed as alternatives with the promises of high transfection efficacy and increased gene expression without safety concerns. The current COVID-19 pandemic has supercharged innovation in gene therapy, resulting in the use of messenger RNA (mRNA) as a vaccine for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [5-8]. The use of lipid nanoparticles (LNPs) for the delivery of nucleic acids has several advantages. These include protection of the genetic materials from degradation, prevention of rapid systemic removal, and facilitation of cellular uptake. Comparing LNPs to the conventional viral vectors, the latter have been associated with carcinogenicity or immunogenicity, difficulty in production, and limitations in packaging capacity; therefore LNPs provide safer and more efficient delivery [9-15]. LNPs have been the platform for several gene therapy systems, including the first Food and Drug Administration–approved small interfering RNA (siRNA) therapeutic, Onpattro®, for the treatment of hereditary transthyretin-mediated amyloidosis [16]. LNP platforms are also employed for the delivery of mRNA in the BNT162b2 and mRNA-1273 vaccines for SARS-CoV-2 [5-8,17]. However, most LNPs are delivered via intramuscular or intravenous injection. This is not site-specific for organs or cell types, resulting in off-target delivery. Therefore, LNPs have been further modified to achieve site-specific targeted gene delivery by responding to biological stimuli (ph or enzymes) or being triggered via the incorporation of external stimuli (light or ultrasound) [18]. In this review, we focus on the use of ultrasound and ultrasonic particles for targeted delivery of genetic materials across a range of diseases.

2. Ultrasound imaging

Among the clinical imaging modalities available, ultrasound imaging is the most widely used as this technology offers significant advantages. Ultrasound is safer for patients, as compared to X-ray or nuclear imaging, because it does not involve ionizing radiation. Therefore, the technology is well suited to routine clinical applications where frequent imaging is needed, such as screening and early disease detection. While most imaging modalities, such as magnetic resonance imaging (MRI) and positron emission tomography imaging, require large machines and complex housings, ultrasound scanners are light and highly portable. Over the last two decades, hand-held ultrasound units and laptop systems have become increasingly affordable and gained use in bedside applications, ambulances, and general practitioners’ and other doctors’ clinics. These point-of-care ultrasound scanners are particularly attractive for diagnostic imaging in rural areas and developing countries. More importantly, ultrasound procedures provide inherent real-time imaging and can be completed within minutes. Ultimately, there are no known long-term side effects of ultrasound imaging, while the procedure is painless and rarely causes any discomfort.

2.1. Clinical use of ultrasound

The most common clinical use of diagnostic ultrasound is for obstetric imaging of growth and fetal development during pregnancy. Other common uses include imaging of the abdomen, brain, blood vessels, eyes, glands, heart, muscles, and skin. It is also used for ultrasound-guided procedures such as during tissue collection for biopsies and during needle placement. Another clinical use of ultrasound is high-intensity focused therapeutic ultrasound, where increased levels of acoustic power are focused on specific targets. Further, therapeutic ultrasound is used for pain reduction and improvement in the circulation and mobility of soft tissue, as well as in the treatment of many cancers, such as bone tumors, breast, kidney, and liver cancers, and pancreatic and uterine fibroids.

2.2. Advances in ultrasound technology

Ultrasound imaging has undergone dramatic advances over the last few decades and its use as a diagnostic imaging modality has continued to evolve. While initially ultrasound imaging started...
with brightness-mode systems that produced poor, bi-stable images, it has now progressed to portable hand-held devices that are capable of high-resolution real-time grey-scale imaging, tissue harmonic evaluation, and color-flow Doppler. Improvements in acquisition and analysis of contrast enhancement, motion-derived indices, shear wave elastography, strain, and speckle tracking have added further details for better diagnostic assessment [19-22]. Ultrasound is known to be operator dependent, so many groups have researched artificial intelligence–powered ultrasound to overcome this limitation [23,24].

Conventional diagnostic ultrasound imaging has a high temporal resolution and its spatial resolution can be improved and sharper images obtained by using higher frequency ultrasound waves. A higher frequency of ultrasound results in a shorter wavelength and therefore a shallower depth of penetration [25]. Increased frequency, however, allows the tissues to absorb the energy more readily, therefore producing images that are more faint. At 3.5 MHz imaging depth of 10–20 cm is possible, while at 50 MHz the depth is limited to<1 cm [26]. Clinically, high-frequency ultrasound is most useful in areas such as skin imaging and imaging during minimally invasive surgery where resolution is critical but penetration requirements are small [26]. However, high-frequency ultrasound is also a valuable tool for preclinical imaging, in particular the imaging of small animals such as mice and zebrafish [27-31], unlocking many opportunities for drug and gene therapy research (Fig. 1).

3. Ultrasound contrast agents and ultrasonic particles

The sound-scattered signal from blood is similar to that from tissue; therefore, measurement of blood perfusion with ultrasound is difficult [32]. This challenge can now be overcome by the use of ultrasound contrast agents, also known as microbubbles (MBs). These agents were introduced to human ultrasound in 1968 by Gramaik and Shah, who observed that the reflection of ultrasound in the aortic root after injection of little air bubbles was noticeably enhanced [32,33]. MBs have a different density and compressibility to those of the blood and surrounding tissue [34]; therefore, when administered in the blood pool or a cavity, they provide efficient backscattering of sound waves which results in enhancement of ultrasonic signals [35]. Ultrasound contrast agents are administered into the venous system, where they are rapidly distributed by blood flow to the imaging site, and the contrast enhancement is typically apparent within seconds [32]. These agents include MBs, echogenic liposomes, and perfluorocarbon droplets [34]. These highly compressible objects can resonate in the sound field, producing a non-linear acoustic response that enables detection strategies to differentiate between the echoes from the ultrasound contrast agent and those of the blood or tissue [34-40].

3.1. Development and use of ultrasound contrast agents

The properties of MBs are the result of both the material used for their shell and their gas core. Initially, these contrast-enhancing agents started with normal saline, which was agitated. Such bubbles had extremely limited storage stability and hence had to be prepared in the immediate vicinity of the patient [41]. The generation of these crude MBs was achieved through the agitation of blood and saline; consequently they lacked a shell and their gas core was simply air. Since these air-filled microspheres had high solubility in blood and were rapidly cleared by the lungs, they could only be visualized for a few seconds after intravenous administration. They were therefore not ideal for opacification of the left heart [42].

Subsequent improvements to the formulation of MBs have increased their stability and functionality. A thin shell comprising albumin or galactose palmitic acid enables the MBs to pass through the pulmonary capillary bed; however, they are incapable of recirculation in the bloodstream because they cannot resist arterial pressure gradients [36]. A thick shell provides stability but impairs their ability to resonate, causing a weak acoustic backscatter response [36]. Currently, the MB shell, designed to enhance in vivo stability, can be made of proteins, lipids, or biopolymers [43], with shell thickness varying from 10 to 200 nm [32]. Many lipid-shelled agents have polyethylene glycol (PEG) incorporated into the shell to enhance stability and reduce immune-system recognition [32]. More importantly, second-generation MBs are produced using water-insoluble gases such as perfluorocarbon octafluoropropane \([C_4F_8]\), decafluorobutane \([C_4F_{10}]\), and sulfur hexafluoride \([SF_6] \) in order to prevent gas diffusion, which improves their survival and stability under pressure [32,43].

3.2. Clinical use of ultrasound contrast agents

Several MBs have been approved for clinical use, mainly for applications such as perfusion imaging, characterization of liver lesions, and blood pool enhancement [43]. Optison \(^\text{TM}\) (GE Healthcare) is generated by sonication of human albumin with octafluoropropane, a perfluorocarbon gas. Luminson \(^\text{TM}\) (Bracon) is a contrast agent stabilized with a lipid shell and filled with \(SF_6\). The MBs are reconstituted by mixing saline with lyophilisate and are stable in the vial for approximately 6 h at room temperature after reconstitution. Sonazoid \(^\text{TM}\) (GE Healthcare) are lyophilised MBs that encapsulate \(C_4F_{10}\) in a lipid membrane. Once it has been reconstituted, Sonazoid \(^\text{TM}\) should be used within 2 h. A similar product is Definity \(^\text{TM}\) (Lantheus Medical Imaging), a lipid shell with \(C_4F_{10}\) gas, which has been shown to be stable for 12 h post activation of the vial. The current clinical indications for the administration of MBs are for patients who produce technically limited, suboptimal echocardiograms. For cardiac imaging, MBs are employed to improve visualization of the endocardia, enable assessment of the left ventricular structure/function, confirm or exclude echocardiographic diagnosis of left ventricular structural abnormalities, and assist in the detection and classification of intracardiac masses [44,45].

3.3. Preclinical use of ultrasound contrast agents and ultrasonic particles

In addition to anatomical imaging, advancement of micro-/nano-particles via material selection and technological development in ultrasound imaging has extended their functions for molecular imaging in preclinical settings [27,28]. By conjugating these contrast agents to ligands that target biomarkers, they can be used for direct visualization of diseases [46]. Furthermore, developments in biosensing of micro-/nano-bubbles have allowed for ultrasound imaging of reactive oxygen species production [47] and detection of pH changes in vivo [48]. Recent advances in nanotechnology and material sciences have led to the use of MBs as drug carriers for targeted drug and gene delivery [29,49,50].

4. Acoustic pressure and ultrasonic particles

By altering the acoustic pressure, the properties of these ultrasonic particles can be fine-tuned [51]. A low acoustic pressure will result in stable oscillation of ultrasonic particles, known as stable cavitation (Fig. 2). Alternatively, a higher acoustic power setting will lead to the bursting or destruction of the MBs (Fig. 3) [52]. Both stable and
Fig. 1. Ultrasound imaging of a mouse using a clinical scanner and a preclinical high-frequency machine. A. Use of a 15 MHz clinical transducer for ultrasound imaging of the heart, aortic arch, and carotid artery resulted in unclear visualization of the anatomy. B. Use of a 55 MHz high-frequency transducer for ultrasound imaging of the heart, aortic arch, and carotid artery resulted in clear visualization of the anatomy and definitive vascular structures. **Left**: Brightness mode images. **Right**: Images with annotation of the anatomy. IA, innominate artery; CCA, common carotid artery; SA, subclavian artery.
inertial cavitation increase cell permeability, which in turn aid the delivery of drugs and transfection of genetic agents.

5. Delivery of genetic material with microbubbles and ultrasonic nanoparticles

Although the uptake of genetic materials can be enhanced by ultrasound on its own, there are several disadvantages: 1. Off-target effects have been observed when the material is administered systemically. 2. Nucleases in the circulation cause rapid degradation of the genetic materials. 3. There is rapid clearance of the genetic materials from the reticuloendothelial system [53,54]. The use of MBs, liposomes, and other ultrasonic LNPs protects genetic materials from degradation, increases the packaging of materials, and facilitates cellular uptake. MBs usually range between 1 µm and 8 µm in diameter, while most other ultrasonic nanoparticles range between 1 nm and 1 µm. The large size of MBs makes them more suitable for vascular targets because of their difficulty in entering deeper tissues. The development of ultrasonic nanoparticles, including nanoliposomes, nanobubbles (NBs), nanodroplets, and micelles (Fig. 4), allows them to exit vascular confinements and to enter leaky microvasculature and perivascular areas. In terms of their classification, NBs have a gas core, while nanodroplets have a liquid core. Nanodroplets usually encapsulate a low-boiling-point perfluorocarbon or perfluoropentane liquid [55]. Under high acoustic pressure, these nanodroplets are vaporized and become MBs. These ultrasonic particles have been employed for drug and gene delivery over the last two decades.
6. Ultrasound-targeted gene delivery

The addition of ultrasound-focused techniques activates these ultrasonic particles and achieves targeted gene delivery to the site of exposure, thereby overcoming some of the issues of off-target delivery. This enhancement of drug and gene uptake by the cells is due to the increase in membrane permeability resulting from the combination of ultrasound exposure and ultrasonic nanoparticles [37,56-59]. This method allows for a direct, site-specific transfer of genetic materials into the organ/site of interest and is also termed ultrasound-targeted microbubble destruction, ultrasound-mediated gene delivery, and ultrasound-targeted gene delivery (UTGD). More recently, the employment of these ultrasonic particles, as dual-function contrast agents and therapeutic carriers, has unlocked the development of theranostic strategies (concurrent diagnosis and therapy) [29,60,61]. In addition, conjugation targeting of ligands onto ultrasonic particles allows selective binding of biomarkers to further enhance cell and disease specificity (Fig. 5).

A range of nucleic acids, including plasmid DNA (pDNA), micro-RNA (miR), mRNA, short hairpin RNA (shRNA), and siRNA, have been used for gene therapy, with most proof-of-concept studies using nucleic acids encoding enhanced green fluorescence protein (eGFP) or luciferase (Table 1 and Table 2). Pioneering research started simply with co-administration of commercial MBs with these nucleic acids followed by ultrasound application. A study noted no difference in UTGD efficacy using two different commercially available MBs, SonoVue and Definity™ [62]. A high concentration of unprotected nucleic acids is required because their permeation of the cell membrane is hampered by their negative electrostatic charge and size [63,64]. Unprotected nucleic acids are also recognized as pathogens by the reticuloendothelial system, therefore they face rapid degradation and clearance from the circulation [65].

6.1. Loading nucleic acids onto ultrasonic particles

Further work looked into different formulations of particle shells and cores to package and encapsulate nucleic acids. There are two main strategies to load nucleic acids onto ultrasonic particles: 1) direct conjugation onto the surface or 2) packaging onto cationic polymers nanocomplexes, as a secondary carrier, which is then attached onto the particles. Loading of nucleic acids are performed by exploiting the electrostatic interaction between negatively charged nucleic acids and positively charged lipids, polymers or peptides [66-73]. This coupling improves the stability of the nucleic acid cargo by reducing degradation and removal from circulation, as well as increasing cellular interaction and uptake [58,74].

Using direct coupling of nucleic acids to cationic lipids that form the membrane of the ultrasonic particles, studies have shown increased in vitro and in vivo UTGD transfection using cationic MBs, as compared to neutral MBs [75,76]. The most commonly used cationic lipids for UTGD include DPPC, DSPC, DOTAP and DOTMA [67,77-79]. While cationic lipids have permanent positive charged head groups, recent studies have also employed ionizable lipids, which exhibit positive charge at low pH and are neutral at physiological pH [80]. Ionizable lipids exhibit better biocompatibility through minimal interactions with blood cells and are the key component of Onpattro, the first FDA approved siRNA drug [80-82].

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In vivo proof-of-concept studies

In vitro studies. Others have employed cationic polymers, such as polyethylenimine (PEI) to capture the nucleic acids and form nano-complexes [69,70,83-85]. These complexes are then conjugated on ultrasonic particles for UTGD. The cross-linking of PEI with fluorine-containing alkyl chains to form fluorinated polymers as the outer membrane of nanodroplets [72,73,86], has also been shown gated on ultrasonic particles for UTGD. The cross-linking of PEI with fluorine-containing alkyl chains to form fluorinated polymers as the outer membrane of nanodroplets [72,73,86], has also been shown

Table 1
In vitro studies.

| Cells | Ultrasonic particles (shell and gas core) | Nucleic acids/gene | Ultrasound parameter | Outcome | References |
|-------|-----------------------------------------|-------------------|---------------------|---------|------------|
| HUH7 cells with stable expression of eGFP & luciferase | DPPC & DSPE-PEG2000-biotin C12F10 | siRNA against luciferase | 1 MHz 2 W/cm² 10% duty | Higher gene silencing resulting in loss of luciferase signal | Vandenbroucke et al. [103] |
| 3 T3-MDEI, C2C12 & CHO cells | Pluronic block copolymers | pDNA encoding eGFP | 1 MHz 1 W/cm² 20% duty | Increased transfection efficiency | Chen et al. [105] |
| Dendritic cells | DPPC, DSPE-PEG-biotin C12F10 | mRNA encoding luciferase or eGFP | 1 MHz 2 W/cm² 50% duty | Highest luciferase expression observed at 8 h post transfection | De Temmerman et al. [104] |
| COS-7 cells | DPPC, PEG2000, DOTAP C12F6 | siRNA against luciferase | 2 MHz 2 W/cm² 50% duty | Downregulation of luciferase expression | Endo-Takahashi et al. [77] |
| BLM melanoma cells | DPPC, DSPE-PEG-biotin C10F10 | AAV encoding pDNA eGFP | 1 MHz 2 W/cm² 10% duty | Increased internalization of AAV-pDNA into the cytosol but not into the nuclei | Geers et al. [171] |
| HUVECs | DPPC, DSPEPEG2000-Om, DSPE-PEG2000-Mal C12F6 | pDNA luciferase | 2 MHz 0.1 W/cm² 50% duty | Significantly higher luciferase expression using AG73 peptide (targeting tumor angiogenic endothelium) particles for UTGD | Negishi et al. [172] |

Table 2
In vivo proof-of-concept studies

| Disease type | Ultrasonic particles (shell and core) | Nucleic acids/gene | Ultrasound parameter | Outcome | References |
|--------------|--------------------------------------|--------------------|---------------------|---------|------------|
| Liver imaging, assessing ultrasound kinetics (murine model) | Optison MBs (GE Healthcare) | pDNA luciferase | 1 MHz 0–4.3 MPa | Gene enhancement optimum during pressure range of 2–3 MPa | Shen et al. [112] |
| Liver – long-term gene expression (murine model) | DMAPAP, PEG2000, CHOL C12F10 | pDNA luciferase | 1 MHz 930 kPa 20% duty | Increased luciferase expression of up to 180 days post UTGD | Manta et al. [108] |
| Kidney tumor experiments (murine model) | DSPC, DSPC-PEG2K, DSPC-PEG2K-Mal C12F10 | pDNA luciferase, eGFP | 2 Hz burst 1 MHz 2 W/cm² 10% duty | 10-fold higher bioluminescence of tumor region | Sirsi et al. [70] |
| Breast cancer (murine model) | Halobacterium NRC-1 (Halo), PEI air | pDNA luciferase, eGFP | 2 Hz burst 1 MHz 2 W/cm² 10% duty | Increased luciferase expression | Tayier et al. [85] |
| Radiation-induced fibrosarcoma-1 – xenograft (murine model) | Sonovue (Bracco) Sonidel MB101 (Sonidel) | pDNA luciferase | 10% duty 3 and/or 6 min | Expression of luciferase observed throughout the lifetime of the tumor. | Li et al. [121] |
| Intralymphatic imaging (canine model) | DPPC, DSPE-PEG3400 C12F6 | mRNA luciferase | Clinical scanner | Unsuccessful delivery of mRNA. Higher power may be needed | Dewitte et al. [173] |
| Skeletal muscle (murine model) | Optison MBs (GE Healthcare), PEI C12F6 | pDNA eGFP | 1 MHz 3 W/cm² 20% duty | Increased eGFP expression, especially in older (6-month-old) mice | Lu et al. [83] |
| Skeletal muscle (murine model) | DSPC, DPPE-PEG2000 palmitic acid C12F10 | pDNA luciferase | 1 MHz 2 W/cm² 50% duty | Cationic MBs displayed better transfection than neutral MBs | Panje et al. [67] |
| Retina (rodent model) | Sonovue (Bracco), PEI C12F6 | pDNA eGFP | 1 MHz 1 W/cm² 50% duty | Increased eGFP expression in the retina | Wan et al. [69] |
6.2. Materials and techniques to generate ultrasonic particles

Cationic lipids or polymers have been associated with cytotoxicity, which may be due to their electrostatic interactions with anionic serum plasma proteins [64]. Biocompatible, neutral or helper phospholipids have been incorporated in these particles to reduce toxicity [15,70,80,88-91]. These phospholipids aid in their stability, fusion with the cell membrane, and in promoting the release of nucleic acid in the cytoplasm [92,93]. The incorporation of cholesterol helps stabilize the lipid particle formulation by increasing the packing and reducing the mobility of phospholipid molecules [94].

In addition to the choice of the lipid/polymer layer, many groups have incorporated polyethylene glycol (PEG) into their ultrasonic particles to form a hydrated layer and provide steric stabilization [94]. PEG-particles have been shown to have high biocompatibility and improve in vivo dynamics by increasing circulation time [58]. Studies have shown that PEGylated coatings prevent aggregation and reduce phagocytic uptake of the nanoparticles [95]; therefore the half-life of PEGylated nanoparticles can be prolonged from 30 min to 5 h in vivo [96].

The internal gaseous or aqueous core of ultrasonic particles also determines their circulating half-life in vivo. The use of gaseous or liquid perfluorocarbons is advantageous because of their resistance to biochemical breakdown and their incorporation leads to improved stability. Most micro/nanobubbles are generated using low-solubility perfluorocarbon gases because their low diffusion coefficient and solubility in the blood contribute to longer circulation duration in vivo, as compared to particles with an air-filled core [97]. Alternatively, nanodroplets are generated by encapsulating liquid perfluorocarbons and have been demonstrated to be less susceptible to mechanical stress and pressure disparities [97,98]. Echogenicity on ultrasound imaging is also dependent on the size of the ultrasonic particles. Microbubbles of approximately 2 μm provide the optimal acoustic backscatter for imaging [43]. Under ultrasound simulation, these nanodroplets undergo ultrasound-induced droplet vaporization and transition into microbubbles, which result in contrast enhancement and visualization [97]. Nanodroplets, generated with cationic lipids or fluorinated-PEI outer shells [72,73,86], have been shown as ideal theranostic agents because they demonstrate ultrasonic contrast properties and can be triggered for efficient gene delivery in vitro and in vivo [87,99].

A homogenous shell membrane is essential for ultrasonic particles and is commonly achieved via thin-film hydration, reverse-phase or detergent-depletion methods. The thin-film hydration and reverse-phase evaporation methods are used to dehydrate the lipids from their organic solvent [58,100]. The dried film is then rehydrated with physiological buffers containing the substance for encapsulation [58,100]. The generations of ultrasonic particles require mechanical agitation to develop unilamellar and homogeneous particles. Techniques such as sonication, extrusion and high-pressure homogenization are employed to further generate sized-controlled ultrasonic particles [27,58,101].

It is important to note that most ultrasonic particles have good gene delivery capabilities on their own and may also achieve low-level genetic transfer without ultrasound stimulation. The addition of ultrasound stimulation will avoid off-target gene delivery by providing a direct, site-specific transfer with increased transfection efficiency. Therefore in this review, we focus on UTGD of nucleic acids via ultrasonic particles across a range of diseases.

7. Ultrasound-targeted gene delivery in vivo

To achieve an increase in transfection efficiency, the Sanders group first created large biotinylated MBs and small biotinylated cationic liposomes coated with fluorescent-labeled nucleic acids; these were then bridged with avidin to form lipoplexes [102,103]. Using pDNA that encoded luciferase, the group demonstrated successful delivery, transfection, and expression post ultrasound exposure in vitro [102]. Using siRNA for gene silencing in cells that expressed luciferase, the authors observed a significant reduction in luciferase expression after the cells were exposed to UTGD [77,103]. UTGD methods were used for the transfection of mRNA encoding luciferase and eGFP into dendritic cells in vitro [104]. Using MBs based on pluronics (polymer blocks with customizable lengths), Chen et al. showed increased transfection efficiency of pDNA into fibroblasts, myoblasts, and endothelial cells when combined with ultrasound in vitro [105]. Other in vitro work by Yang et al. silenced P-glycoprotein using shRNA coated on doxorubin-encapsulated NBs via UTGD and demonstrated increased cytotoxicity of human breast cancer cells with adriamycin resistance [106]. Overall, in vitro UTGD has been well established and is frequently used as a proof of concept for the properties of ultrasonic particles and their transfection efficacy. However, the transition into in vivo applications requires many optimization steps, specific to the anatomical location of the tissue/organ and its composition.

8. Ultrasound-targeted gene delivery in vivo

8.1. Safety and specificity

The safety of UTGD has been evaluated in several studies. In vivo models have also been employed to determine whether the destruction and cavitation of ultrasonic agents lead to tissue damage. Many of the studies chose to use pDNA encoding luciferase to determine gene expression and to measure changes in liver enzymes as an indicator of hepatic damage. While some studies have raised concerns in relation to elevated enzymatic measurements [107], most studies have indicated no or minimal tissue damage [108-111].

Noble et al. documented increased luciferase expression in the liver in an in vivo canine study [107]. In this proof-of-concept study, the authors found that luciferase expression in some tissue sections produced up to an 1800-fold enhancement compared with the sham-treated animals [107]. The authors noted minor liver damage in areas that were exposed to therapeutic ultrasound via elevated liver enzyme levels and on histological studies. However, the central lobe, which was exposed to diagnostic ultrasound, was not affected [107]. However, the nine dogs used in this study, each varied in relation to the amount of MBs administered, the route or time of injection, the choice of transducer or its peak negative pressure, and the total treatment time. Therefore, it is important to note that for each specific therapy, there was only one sample. There is clearly a need to perform more experiments to determine the safety of their UTGD approach in vivo before we can draw a conclusion.

In a murine study using Optison MBs, Shen et al. noted that the optimum peak negative pressure ranged between 2 and 3 MPa, and resulted in an 85-fold increase in luciferase expression [112]. However, the authors did not look at possible liver damage in this study. Manta et al. observed prolonged luciferase expression in the liver for 180 days, but noted that UTGD caused damage to the liver cells in the first 2 days [108]. However, the liver enzymatic levels returned to normal by day 7 [108]. These findings highlight the potential of using UTGD for long-term therapy. Nevertheless, increased amplitudes have been associated with higher degrees of tissue damage [113], therefore there is a need to optimize the ultrasound pressure amplitudes required. Many groups have also investigated the use of optical UTGD and have reported
no histological tissue damage to the retina or the sub–conjunctival tissues, or any other adverse effects [69,114-116].

8.2. Liver diseases

Ultrasonic imaging is already commonly used for diagnosis of liver disease, including fatty liver and fibrosis. As a result, UTGD for liver diseases is widely studied. In a rodent model of liver ischemia/reperfusion (I/R) injury where Yan et al. co-injected siRNA against heat shock protein 72 with MBs, ultrasonic irradiation resulted in a smaller degree of liver injury [117]. Using in vivo models of liver fibrosis, UTGD of hepatocyte growth factor has been shown to produce an anti-fibrosis effect, preserve the lobule structure, and result in smaller amounts of fibrous septum as compared to controls [118,119]. Furthermore, Jiang et al. showed that this therapy directly resulted in a significant reduction in liver enzyme levels [118]. Zhang et al. demonstrated in the same rodent model that UTGD of hepatocyte growth factor and transforming growth factor β improved liver function, reduced the severity of hepatic fibrosis, and promoted the regeneration of liver cells [120]. These studies indicated that UTGD provided liver protection without tissue damage. Together with the frequent usage of ultrasound for liver imaging, these preclinical studies provide substantial proof of concept data for future clinical translation of UTGD in the therapy of hepatic conditions.

8.3. Muscular diseases

The murine hindlimb skeletal muscle model is well established and widely used for the investigation of gene transduction. Using this in vivo model, Panje et al. investigated the transfection efficiency of firefly luciferase pDNA using either cationic or neutral MBs [67], indicating that ultrasound exposure and surrounding MBs were sufficient to facilitate transfection. A separate experiment showed that UTGD resulted in prolonged gene expression in vivo in the hindlimb for up to 84 days after a single intramuscular injection of pDNA and MBs [121], demonstrating the potential for sustained therapeutic benefits. Since the simple co-administration of MBs and nucleic acids has been associated with cellular damage, Lu et al. demonstrated that there was less tissue damage with incorporation of the cationic polymer PEI [83].

Using UTGD on the tibialis anterior (TA) muscle, after intramuscular injection of acoustic liposomes and 10 ug of pDNA encoding luciferase Wantanabe et al. observed much higher in vivo bioluminescence signal post therapy [122]. Using a gamma counter to measure the uptake of the iodine-124 isotope radiotracer, biodistribution studies indicated localization of gene expression in the TA muscle. Regions received pDNA encoding sodium/iode symporter genes, acoustic liposomes, and ultrasound exposure [122]. The successful transfection of this pDNA and its expression were further confirmed 4 days post therapy using positron emission tomography imaging to image the uptake of the sodium-124 isotope in the TA muscle [122].

Duchenne muscular dystrophy, a fatal condition and the most common pediatric neuromuscular disease, has been of major interest in relation to genetic therapy approaches (Table 3). Using bubble liposomes coupled with phosphorodiamidate morpholino oligomer (PMO), Negishi et al. demonstrated successful gene therapy in vivo in a murine model of Duchenne muscular dystrophy [123,124]. After intramuscular injection into TA muscle tissue and targeted ultrasound, the group observed an increase in the number of dystrophin-positive fibers via immunofluorescence microscopy [123]. However, in this article controls for the bubble liposomes + PMO without ultrasound as well as the PMO + ultrasound were not presented. Therefore it is difficult to determine the efficacy of the combination without relevant comparison to the needed controls. Direct intramuscular injections and UTGD have shown successful gene transfection to the muscle of interest. Although further research must be conducted to ensure effectiveness and safety, the simplicity of the UTGD on skeletal muscle makes this technology ideal for the treatment of Duchenne muscular dystrophy and may lead to an early prospect for clinical translation.

8.4. Fetal diseases

A common X-linked genetic disorder of the urea cycle in infants is related to ornithine transcarbamylase (OCT) deficiency. Since OTC is a single-gene defect, researchers have been investigating whether the repair and/or replacement of this defective gene might offer a therapeutic alternative to liver transplantation [125]. Using OTC-deficient female mice, Oishi et al. generated heterozygous pregnant mice. On day 16 of gestation, an incision

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**Table 3**

| Disease type | Ultrasonic particles (shell and core) | Nucleic acids/gene | Ultrasound parameter | Outcome | References |
|-------------|--------------------------------------|-------------------|---------------------|---------|------------|
| Huntington’s disease (murine model) | SonoVue MBs (Bracco) | pDNA | 1 MHz | Significant neuroprotective effect and improved motor ability | Lin et al. [127] |
| Huntington’s disease (murine model) | DSPC, Bio-DSPE-PEG2000 | pDNA | 1 MHz | Improved behavior scores and immunohistochemical staining showed increased levels of tyrosine hydroxylase and dopamine transporter | Yue et al. [130] |
| Parkinson’s disease (rodent model) | DPPC, DSPE-PEG2000, DPTAP | pDNA & Nurr1 | 2 W/cm² 20% duty | Neuroprotective effect in mice with restored behavior function | Fan et al. [129] |
| Duchenne muscular dystrophy (murine model) | C3F8 | Antisense PMO | 1 MHz | Increased PMO-mediated exon-skipping efficiency and enhanced dystrophin expression | Negishi et al. [123] |
| Duchenne muscular dystrophy (murine model) | DSPE-PEG2000, DPPC | Antisense PMO | 2 W/cm² 50% duty | Recovered dystrophin expression in the targeted skeletal muscle | Negishi et al. [124] |
| Spinal cord injury (rodent model) | DPTAP, DPPC, DSPE-PEG-COOH | pDNA | 1.5 W/cm² 5 min | Significant neuroprotective effect on the injured spinal cord with decreased level of apoptosis | Song et al. [126] |
was made to the abdominal to expose the uterus, where the authors directly injected the liver of the fetus with 5 μg of pDNA encoding OTC and 1.25 μl of Sonazoid MBs [125]. Following the injection, UTGD was performed directly on the fetal liver through the uterus wall at 1 W/cm² at 50% duty for 30 s. The authors observed decreases in the blood ammonia level and the urinary orotic acid:creatinine ratio in the treatment group of hemizygous males at 3 days after birth [125]. It was also noted that the ex vivo liver specimens that underwent gene therapy using sonoporation measured an increase in OTC activity at pH 9.5, as compared to pH 7.7, although the mechanism or reasoning for this association is uncertain [125]. One possible explanation suggested was that UTGD might induce structural changes which resulted in more efficient functionalisation at higher pH levels [125]. Delivering therapeutics to the developing fetus requires consideration of both mother and fetus, as well as to account for any immunological responses that may occur. While more research into their safety is required, these challenges may be overcome by UTGD to facilitate targeted treatment directly to the fetus without off-target effects on the mother.

8.5. Spinal cord and neurodegenerative diseases

Another emerging area for gene therapy is the field of spinal cord injury and neurodegenerative diseases (Table 3). Song et al. generated MBs that were targeted to the neuron-specific molecular marker microtubule-associated protein 2 for the delivery of a plasmid-encoding brain-derived neurotrophic factor (BDNF) [126]. Using an acute spinal cord injury rodent model where the contusion injury was induced on the 10th thoracic segment, the rats underwent 5 min of UTGD every 12 h for 3 days. The authors demonstrated increased BDNF gene and protein expression post UTGD treatment in vitro and in vivo [126]. Furthermore, the rats that underwent this treatment exhibited normal morphology, increased regenerative axons, and increased values on the Basso, Beattie, and Bresnahan locomotor rating scale for behavioral consequences, as well as decreased neuronal necrosis and smaller lesion cavity areas [126]. Overall, this study demonstrated UTGD successfully provided neuroprotection on the injured spinal cord.

The brain is a complex target for most therapies because of the difficulty in penetrating the blood–brain barrier. To rectify this, groups have investigated the use of MB-facilitated focused ultrasound in an attempt to generate temporary openings [127,128]. By employing pDNA encoding glial cell line–derived neurotrophic factor (GDNF) coupled onto liposomes, Lin et al. demonstrated MBs opening the blood–brain barrier and achieved gene therapy in the brain in a mouse model of Huntington’s disease in vivo [127]. They observed significant improvement in motor ability on the Basso, Beattie, and Bresnahan locomotor rating scale for behavioral consequences, as well as decreased neuronal necrosis and smaller lesion cavity areas [126]. Overall, this study demonstrated UTGD successfully provided neuroprotection on the injured spinal cord.

8.6. Ophthalmic and retinal diseases

Genetic therapy is ideal for a variety of ophthalmic and retinal diseases because of its accessibility and favorable immunological properties in relation to being immune privileged. In a proof-of-concept study, Sonoda et al. performed UTGD by simultaneously using commercially available MBs (Optison) and pDNA encoding eGFP in vitro in cultured rabbit corneal epithelial cells and in vivo by co-administering them to New Zealand albino rabbits [132]. The authors observed more eGFP-positive cells in the targeted regions of the corneal stroma of animals receiving UTGD than those that received plasmid injections alone, received Optison alone, or were just subjected to ultrasound bursts [132].

Wan et al. generated PEI-conjugated eGFP pDNA and demonstrated increased eGFP expression in cultured human retinal pigment epithelial cells in vitro when delivered using commercially available MBs (Sonovue) and UTGD [69]. The authors also observed a strong positive eGFP signal after co-administering MBs with PEI-conjugated eGFP pDNA and UTGD in Sprague-Dawley rat retinas in vivo [69]. In another study, Sonoda et al. investigated transfer of the eGFP plasmid via intravitreal ultrasound irradiation to the retina in vivo using pDNA-coated PEG-liposomes containing perfluoropropane gas [84]. The authors observed a significant increase in the number of eGFP-positive cells in the retinas of the rabbits, exclusive to the area exposed to ultrasound [84]. The probe used in this study was approximately the size of a 19-gauge needle [84], indicating that this intravitreal UTGD may be more selective than most other methods. The same group also demonstrated an increase in eGFP signal in the conjunctiva of rats in vivo after UTGD [114].

Importantly, these groups found no adverse effects and no histological tissue damage on the retina or the sub–conjunctival tissues [69,114]. However, while UTGD may be a safe procedure for ophthalmic and retinal diseases, most of these studies have only used nucleic acids encoding for eGFP or luciferase. Therefore it is still unknown whether this approach may provide a clinical benefit to vision.

8.7. Malignant diseases

Genetic therapy for malignant diseases has been heavily explored (Table 4). Using Balb/c-nu Slc nude mice, Sakakima et al. treated subcutaneously implanted hepatocellular carcinoma solid tumors with pDNAs encoding eGFP, interferon-β (IFN-β), and β-galactosidase (LaCZ) by ultrasound MB-targeted delivery [133]. In in vitro assays, the authors showed only 24% transfection efficacy when using sonoporation of commercially available MBs and 10 μg of eGFP pDNA on SK-Hep1, human hepatic adenocarcinoma cells [133]. The group further observed that 60% of the nodules (12/20 nodules) had a reduction in tumor size in vivo 4 weeks post UTGD using MBs and 50 μg of IFN-β pDNAs [133]. In end point histology, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was used to demonstrate apoptotic induction in subcutaneous tumors that underwent sonoporation, further indicating that the anti-tumor effect of the IFN-β gene may have inhibited tumor growth [133].

Similarly, UTGD was employed to deliver pDNA encoding interleukin (IL)-27 in vivo and showed significant tumor size reduction (50–75%) across three different models of immune-competent prostate adenocarcinoma [134]. Suzuki et al. used 1,2-distearoyl-sn-glycero-3-phosphorylcholine (DSPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[polyethylene glycol-2000] (DSPE-PEG2000) to form the lipid shell of liposomes via sonication
| Disease type | Ultrasonic particles (shell and core) | Nucleic acids/gene | Ultrasound parameter | Outcome | References |
|--------------|--------------------------------------|-------------------|----------------------|---------|------------|
| Breast cancer – xenograft (murine model) | DPPC, DSPE-PEG2000-Biotin, DSPE-PEG2000, DC-CHOL C5F8 | pDNA CD105 (endoglin) | 1 MHz 1 W/cm² 50% duty 30 s | Significantly smaller tumor size with decreased level of angiogenesis | Zhou et al. [76] |
| Breast cancer – xenograft (murine model) | PEGylated species DPPC and DSPE C5F8 | siRNA cell-penetrating peptides | 1 MHz 1 W/cm² 10 s sonication + 10 s pause for a total of 60 s | c-Myc silencing and inhibition of tumor growth | Xie et al. [142] |
| Adriamycin-resistant breast cancer – xenograft (murine model) | mPEG-PLGA-PLL, PEAL water | siRNA against ABCG2 | 1 MHz pulse of 100 Hz | UTGD with siRNA that silenced breast cancer resistance protein (ABCG2), together with adriamycin, resulting in stronger inhibition of tumor growth | Bai et al. [145] |
| Hepatocellular carcinoma – xenograft (murine model) | BR-14 MBs (Bracco) | pDNA IFN-β | 1 MHz 2 W/cm² 50% duty | UTGD of IFN-β resulted in decreased tumor size | Sakakima et al. [133] |
| Hepatocellular carcinoma – xenograft (murine model) | miPEG-NH2, C5F8-C5F5-NH2 Perfluoro-n-pentane | miRNA miR122 | 1 MHz 1.2 W/cm² 20% duty | Suppression of tumor growth and proliferation | Guo et al. [141] |
| Hepatocellular carcinoma – xenograft (murine model) | DPPC, DSPE, DSPE-PEG2000 C5F8 | pDNA HSV-TK (suicide gene) | 1 MHz 2 W/cm² 5 min | Significantly higher apoptosis of cancer cells, improved anti-tumor effects and survival | Zhou et al. [137] |
| Hepatocellular carcinoma – xenograft (murine model) | SonoVue MBs (Bracco) | pDNA HSV-TK (suicide gene) | 1 MHz 2 W/cm² 5 min | UTGD together with ganciclovir treatment increased apoptosis index, reduced tumor growth and improved survival | Nie et al. [138] |
| Hepatocellular carcinoma – xenograft (murine model) | Egg PC, DSPE, DSPE CHOL 0° | pDNA HSV-TK or Timp3 genes | 1 MHz 3 min | Individual gene therapy with HSV-TK or Timp3 genes resulted in 45% suppression of tumor growth and increased survival. Further 30% improvement was achieved with co-delivery | Yu et al. [174] |
| Hepatocellular carcinoma – xenograft (murine model) | DPPC, DSPE, DSPE-PEG2000, PEI SF6 | shRNA against survivin | 1 MHz 1.1 W/cm² 50% duty | Reduced tumor volume with decreased survivin expression | Li et al. [175] |
| Doxorubicin-resistant hepatocellular carcinoma – xenograft (murine model) | BRI8 MBs (Bracco) with PLGA NP | miRNA miR122, anti-miR21 | Clinical transducer | Synergistic treatment with doxorubicin resulted in ~ 27% apoptosis in resistant tumors, 6-fold greater than using doxorubicin alone | Mullick Chowdhury et al. [146] |
| Metastatic melanoma (rodent model) | DSTDAP, DSPE, NH2-PEG2000-DSPE or mang-PEG2000 C5F8 | pDNA pUb-M murine melanoma GP-100 & TRP-2 pDNA p16 (tumor suppressor gene) | 1.045 MHz 1 W/cm² 50% duty 10 Hz burst rate 2 min | Enhanced secretion of Th1 cytokines (IFN-γ and TNFα) was observed in splenic cells. Suppression of pulmonary metastatic tumors post induction was achieved | Un et al. [149] |
| Colon or pancreatic cancer – xenograft (rodent model) | PLGA Core not specified | pDNA p16 (tumor suppressor gene) | Color Doppler mode with a mechanical index of 1.5 | Reduced doubling time of tumors | Hauff et al. [136] |
| Human cervical cancer – xenograft (murine model) | DPPE, DSPE, DSPE PEI C5F8 | siRNA against X-linked inhibitor of apoptosis protein | 1 MHz 1 MPA 50% duty | Increased gene-silencing effect with decreased cancer cell density and increased pro-apoptotic components | Wang et al. [71] |
| Human cervical cancer – xenograft (murine model) | SonoVue MBs (Bracco) | shRNA against human survivin gene | 3 MHz 2 W/cm² 20% duty | Successful inhibition of survivin after UTGD via shRNA resulting in cancer cell apoptosis | Chen et al. [111] |
| Prostate cancer – xenograft (murine model) | DPPC, DSPE-PEG2000-COOH, DC-CHOL 0° C5F8 | siRNA against forkhead box M1 | 1 MHz 1 W/cm² 50% duty 45 V 2 Hz on specified intervals 500 kHz 2 W/cm² 10 min | Inhibited tumor growth and prolonged survival rate | Wu et al. [147] |
| Prostate cancer – xenograft (murine model) | SonoVue MBs (Bracco) | pDNA IL-27 | 1 MHz 1 W/cm² 50% duty 45 V 2 Hz on specified intervals 500 kHz 2 W/cm² 10 min | Reduction in cell viability, with 50% to 75% reduction in tumor growth | Zolochewska et al. [134] |
| Drug-resistant testicular cancer (rodent model) | Not specified | siRNA against MDR1 gene | UTGD of siRNA against MDR1 gene, together with daunorubicin, significantly reduced testicular tumor volumes | He et al. [144] |
and constructed ultrasonic liposomes via supercharging with perfluoropropane gas. UTGD using these ultrasonic particles to deliver pDNA encoding IL-12 resulted in effective tumor suppression, while in 80% of the mice it achieved complete regression [135]. No anti-tumor effects were noted for the control groups treated with the DNA alone, with ultrasonic particles, with ultrasound, or with a commercially available transfection agent, lipofectamine 2000 [135]. In addition, UTGD with pDNA encoding the tumor suppressor gene p16 or the herpes simplex virus thymidine kinase suicide gene has also significantly slowed tumor growth and increased survival rates in vivo [136-139].

In a recent study, Dong et al. fabricated sponge-loaded magnetic nanodroplets for the delivery of microRNAs (miRNAs). These targeted the miR-515 family, resulting in an in vivo therapeutic effect of tumor shrinkage post simulation with focused ultrasound [140]. These nanodroplets were produced by dispersing fluorinated iron oxide nanoparticles (SPIO-NPs) in a perfluorocarbon-coated cationic lipid shell and coating them with miRNA sponges. For therapy, the placement of a magnet on top of the tumor enabled magnetism-assisted targeting of the non-biomarker-targeted nanodroplets. After more than 6 h of magnetism-assisted targeting, focused ultrasound was performed on xenograft tumors. In addition to the reduction in tumor size, immunohistochemistry also demonstrated an increase in expression of anti-oncogenes in the cancer cells [140]. Using miR122 and nanodroplets, Guo et al. also demonstrated that UTGD successfully suppressed tumor growth and inhibited proliferation in vivo [141].

In addition to lipid-based ultrasound particles, some groups have incorporated the cationic polymer PEI into their formulation for nucleic acid delivery [70,111]. Sirsi et al. coupled luciferase pDNA to PEI-coated MBs and demonstrated in vivo successful transfection into tumors that were implanted in the kidneys of mice [70]. Using PEGylated-PEI-SH, the thiol group was covalently conjugated onto the MB shell using the maleimide group on DSPC-PEG25K-Mal lipid. Ultrasound was performed directly on the kidney region [70]. Post transfection, in vivo bioluminescence imaging showed an over 10-fold higher signal from the tumor region compared to untreated tissue [70].

A study by Xie et al. conjugated siRNA against the human c-Myc gene to cell-permeable peptides. These were then entrapped in NBs and addition of ephrin mimetic peptide enabled targeting to EphA2-positive human breast adenocarcinoma cells [142]. Post UTGD, increased transfection was observed within the tumor region, resulting in further strong anti-tumor effects in vivo [142]. CD105, endoglin transmembrane glycoprotein, is highly expressed within endothelial cells in breast cancer and hence were the target for UTGD. This therapy resulted in a 24.7-fold increase in transfection efficacy in vitro and successful delivery of pDNA encoding human endostatin, which inhibited tumor growth in vivo [76]. UTGD and siRNA have also been used to silence the isocitrate dehydrogenase 1 gene and the multidrug-resistant protein 1 gene in vivo, resulting in significant reductions in the sizes of glioma and testicular tumors respectively [143,144]. Since multidrug resistance presents an issue in cancer therapy, UTGD’s ability to silence or alter these resistant cells may also aid their effectiveness in vivo [144-146].

Wang et al. generated siRNA micelles by coupling the siRNA with a cationic diblock copolymer to increase encapsulation efficiency and to protect them from exposure to ribonuclease [71]. Prior to UTGD, these cationic micelles were directly incorporated onto MBs by incubation. The authors used siRNA against the X-linked inhibitor of apoptosis protein to investigate the anti-cancer effect on tumor-bearing mice via direct intratumoral injection on days 1, 4, 7, and 10 [71]. Mice treated with UTGD of siRNA-conjugated MBs showed a decrease in tumor size and increased survival [71]. The authors reported that 16.7% of mice in this treatment group died from loss of weight, a phenomenon that was not reported from their other control groups [71]. They stated that the reason for these deaths was unclear, so more rigorous investigation might be needed to determine safety.

An increase in survival was also observed in an in vivo study using gene silencing of forkhead box M1 transcription factor [147]. The group loaded siRNA onto cationic NBs and conjugated them with A10–3.2 aptamers which targeted the prostate-specific membrane antigen expressed on human prostate cancer cells [147]. A substantially slower tumor growth rate was noted in tumor-bearing mice which underwent these UTGD treatments every 3 days for a total of 7 times [147]. Another interesting approach to UTGD is the utilization of bacteria-produced, gas-filled, proteinaceous nano-compartment as biosynthetic NBs. Incubation of these biosynthetic NBs with cationic PEI allowed for electrostatic loading of egFP and/or luciferase gene reporter pDNA [85]. In a proof-of-concept in vivo subcutaneous xenograft murine model, Tayier et al. observed increased bioluminescence signals in the tumor areas post UTGD treatment [85].

Gene therapy has also been frequently studied in relation to treating chronic inflammation. Un et al. generated mannose-binding polyethylene glycol–2000 bubbles (Man-PEG2000 lipoplexes) for the delivery of luciferase- or ovalbumin-expressing pDNA [148]. Since mannose receptors are abundantly expressed on antigen-presenting cells, these Man-PEG2000 lipoplexes selectively targeted hepatic non-parenchymal cells and splenic dendritic cells. Using lymphoma cells expressing ovalbumin (EG7-OVA), the authors demonstrated anti-tumor activity by increasing cytotoxic T lymphocytes via ovalbumin-coated Man-PEG2000 lipoplexes and ultrasound exposure [148]. The group then performed 3 doses of immunization at weeks 0, 2, and 4 prior to EG7-OVA cell tumor induction in vivo in week 6, and demonstrated an increase in survival rate and a decrease in tumor size [148]. Using a similar immunization timeline, as well as the same Man-PEG2000 lipoplexes and ultrasound settings, the group investigated an in vivo metastatic murine model using a pDNA co-expressing murine melanoma glycoprotein-100 and tyrosinase-related protein-2 [149]. Suppression of pulmonary metastatic tumor post-induction with B16B16 melanoma cells was observed [149]. It is worth noting that a high volume of 400 μL of lipoplexes was injected into each mouse for the in vivo gene transfection study.

Clinically, the vast number of mechanisms by which cancers may arise complicate their treatment. The above preclinical studies demonstrate the clear potential for UTGD treatment across a multitude of solid cancers and metastases. Notably, the studies showcase the flexibility of the ultrasonic particles and the choice of gene therapy available, as well as the use of UTGD and their potential to be employed for personalized medicine to develop treatment strategies tailored to individual cancer cases.

8.8. Inflammatory diseases

During inflammation, Kupffer cells and hepatic endothelial cells also express the mannose receptor, so these Man-PEG2000 lipoplexes can be repurposed for anti-inflammatory therapy. Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract, may be suited for gene therapy. In another proof-of-concept study using TNFαARE mice, which are an animal model of inflammatory bowel disease–like disorders, Tlaxaca et al. demonstrated successful gene transfection using MAcCAM-1 or VCAM-1 targeted MBs [150]. These MBs were loaded with luciferase gene pDNA resulting in an increased bioluminescence signal in the gut post UTGD [150]. Inflammation is the root cause of many diseases, including allergy, asthma, atherosclerosis-related cardiovascular diseases and multiple sclerosis [46,151,152], therefore, further
investigation of UTGD for inflammation and their targets may offer therapy to a broad range of downstream medical issues.

8.9. Cardiovascular diseases

Cardiovascular diseases (CVDs) such as ischemic heart disease, heart failure, stroke, and vascular diseases are the largest cause of death worldwide [153]. Therefore many groups have investigated the use of UTGD for long-term therapy (Table 5). For patients who are suffering end-stage heart failure, often their only chance of survival is a heart transplant. However, acute cardiac rejection results in 20% mortality in the first year post heart transplant [154]. Using antagomir-155 delivered via MBs and UTGD, Yi et al. observed an attenuation of acute cardiac rejection in a mouse in vivo setting [78]. In this study, the MBs were synthesized via the sonication method using distearoylphosphatidylcholine (DSPC), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and DSPE-PEG2000 with perfluoropropane gas. UTGD resulted in targeted delivery of antagomir-155 into the murine allograft hearts, downregulated miRNA-155, and the downregulation of several cytokines and inflammatory markers [78].

Similar findings were reported in a recent rodent study using UTGD of galectin-7 siRNA on days 1, 3, 5, and 7 post cardiac transplantation [154]. Wang et al. observed reduced cardiomyocyte apoptosis, attenuated inflammatory infiltration and myocyte damage, and minimal immune rejection in the targeted therapy group [154]. Compared to currently used clinical strategies using broad-scale immunosuppressant therapeutics and their associated side effects, UTGD shows great promise in reducing organ rejection with reduced risk.

In addition to finding ways to prevent rejection, there is a need to discover new therapeutic and prophylactic approaches to CVDs. Zhang et al. demonstrated that UTGD of shRNA, which silences the oxygen-dependent prolyl hydroxylase-2, resulted in a better outcome after I/R in a rodent model [155]. The MBs were produced with dipalmitoylphosphatidylcholine (DPPC), DC-cholesterol (DC-CHOL), and DSPE-PEG2000 by sonication with octafluoropropane gas, followed by coupling of 18 µg of pDNA per 5 × 10^8 MBs. Using a rodent myocardial I/R model where the left anterior descending coronary artery was ligated for 10 min, the MBs were administered to rats on day 0 and day 4 for UTGD. On histology, the short-term outcome (48 h post treatment) showed fewer apoptotic cells in the infarct area, while the long-term outcome (4 weeks post treatment) showed decreased infarct size [155].

Wang et al. showed increased expression of vascular endothelial growth factor (VEGF) protein and angiogenesis in the myocardium of rats that underwent myocardial I/R injury, after treatment with UTGD of VEGF-coupled MBs [156]. However, in this study the authors only looked at the histological endpoint and it is unclear if there was improvement to the function of the heart. The improvement of cardiac function after myocardial I/R injury has been reported by several groups post gene therapy via UTGD in vivo. Fuji et al. demonstrated that a single dose of gene therapy with pDNA encoding for either VEGF or stem cell factor (SCF), given 7 days after full ligation of the left coronary artery, both resulted in increased ejection fraction as measured via echocardiography [157]. However, 2 years later the group followed up with a myocardial I/R model that required 6 sessions of gene therapy using pDNA encoding for SCF and stromal cell–derived factor-1α to achieve increased vascular density, increased ejection fraction, and decreased infarct size [158]. The difference between the number of sessions needed for the 2 studies was not discussed; however, it may be worth noting that the former study was in mice, whereas the latter study was in rats. It is also unclear why Tropomin I, a clinical marker of MI, was not increased in the blood of the rodents post I/R injury in the latter study.

In another myocardial I/R rodent model, UTGD of a protein kinase B (AKT) plasmid was investigated by Sun et al. using both commercially available Definity MBs and octafluoropropane

| Disease type                                      | Ultrasonic particles (shell and core) | Nucleic acids/gene | Ultrasound parameter | Outcome                                      | References          |
|--------------------------------------------------|--------------------------------------|-------------------|----------------------|---------------------------------------------|---------------------|
| Hindlimb ischemia (rodent model)                  | PEG-40 steatate, DSPC, DSTAP         | miRNA             | 1.3 MHz              | Significant improvement in microvascular perfusion | Cao et al. [163]    |
|                                                  | C14 F10                               |                   | 0.9 W/cm²           |                                             |                     |
|                                                  | BSA, sucrose                          |                   | 5 s interval         |                                             |                     |
|                                                  | O2 & C14 F10                           |                   | 1 MHz                | Reduced smooth muscle cell proliferation    | He et al. [162]     |
|                                                  |                                      |                   | 1.5 W/cm²           |                                             |                     |
|                                                  |                                      |                   | 6 min                |                                             |                     |
| Iliac artery intimal proliferation (rabbit model) | Sonovue MBs (Braeco)                 | siRNA             | 2.5 MHz              | Reduced liver injury and necrosis in treatment group, with lower plasma levels of ALT, HSP72, and TNF-α | Yan et al. [117]    |
|                                                  |                                      |                   | 1 MHz MI 1.0         |                                             |                     |
| Liver I/R (rodent model)                         |                                      | miRNA             | 2 MHz                | Attenuation of acute cardiac rejection and increased survival time | Yi et al. [78]      |
|                                                  |                                      |                   | 2 W/cm² 50% duty     |                                             |                     |
|                                                  |                                      |                   | 2 min                | Significant reductions in inflammatory infiltration and myocyte damage. Prevented acute cellular rejection | Wang et al. [154]   |
| Allograft hearts (marine model)                  |                                      | miRNA             | 2 MHz                | Reduced infarct size and increased neovascularization | Zhang et al. [155]  |
|                                                  |                                      |                   | 2 W/cm² 50% duty     |                                             |                     |
|                                                  |                                      |                   | 2 min                |                                             |                     |
| Myocardial I/R (rodent model)                    |                                      | Short hairpin RNA (shRNA) against PHD2 | Clinical scanner | Reduced myocardial apoptosis, increased vascular density and better cardiac function | Sun et al. [68]     |
|                                                  |                                      |                   | 1 MHz                |                                             |                     |
|                                                  |                                      |                   | 2 W/cm² 50% duty     |                                             |                     |
| Myocardial I/R (rodent model)                    |                                      | pDNA AKT          | Clinical scanner with second harmonic mode | Clinical scanner with second harmonic mode | Yan et al. [79]     |
| Myocardial I/R (rodent model)                    |                                      |                   | 2 min                |                                             |                     |
| Myocardial I/R (rodent model)                    |                                      | pDNA MMIP2 & Timp3 | Clinical scanner with mechanical index of 1.6 | Increased vascular density, increased ejection fraction and decreased infarct size | Fuji et al. [157]   |
| Acute MI (murine model)                          |                                      | pDNA VEGF and SCF | Clinical scanner with mechanical index of 1.6 |                                             |                     |
| Acute MI (rabbit model)                          |                                      | pDNA Ang-1, ICAM-1 | Clinical scanner with second harmonic mode | Improved angiogenesis and heart function | Zhou et al. [159]   |

Table 5

Ischemic and cardiovascular diseases
cationic MBs [68]. UTGD therapy with AKT plasmid was performed on day 5 post I/R injury. Outcomes highlighted that their cationic MBs had a higher binding capacity than the commercially available MBs, increased levels of downstream proteins resulting from AKT phosphorylation and activation, reduced myocardial apoptosis, and increased myocardial vascular density, cardiac function, and perfusion [68].

For active targeting, the group further looked at using a matrix metalloproteinase-2 (MMP2) antibody conjugated to cationic MBs to deliver pDNA encoding tissue inhibitor of metalloproteinase (Timp3), a strong inhibitor of MMP2 and matrix metalloproteinase-9 [79]. A single therapy performed 3 days post injury resulted in an improvement in ejection fraction on echocardiography and reduced cardiac scarring on histology at day 21 [79].

In post myocardial I/R injury rabbits, a similar improvement in cardiac function was achieved using active targeting of the intercellular adhesion molecule-1 to deliver pDNA encoding angiopoietin-1 gene into infarcted heart tissue, promoting angiogenesis [159]. The success of these preclinical studies indicates that gene therapy and UTGD can be used for complex cardiovascular diseases. One of the main hurdles for gene therapy in complex diseases is off-target effects. The addition of active targeting to biomarkers will help overcome this limitation; therefore antibody-targeting of other cardiovascular biomarkers [160,161] may further improve the treatment for myocardial I/R injury via UTGD.

Vascular dysfunction is the starting point of atherosclerosis and will ultimately lead to MI, so the ability to reduce vascular inflammation will hinder atherosclerotic progression. In a rabbit model of
iliac artery intimal proliferation, He et al. demonstrated inhibition of smooth muscle cell proliferation and reduced intimal thickness in the animals treated with albumin-based MBs and targeted ultrasound treatment [162]. In an attempt to resist neceses, He et al. used peptide nucleic acids (PNAs), a DNA analog where the natural nucleic acid’s sugar phosphate backbone is replaced by a synthetic peptide backbone [162]. Unlike most other groups, which use the different charges between their carrier and the nucleic acids, the authors directly generated the albumin-based MBs together with PNAs [162].

Three in vivo studies investigated UTGD of miR-126, an endothelial-specific miRNA for VCAM-1 [50,163,164]. In an ischemic hindlimb rodent model, UTGD resulted in an improvement in normalized microvascular perfusion [163]. Endo-Takahashi et al. have also reported that miR-126-loaded UTGD led to the induction of angiogenic factors and the improvement of blood flow in their ischemic hindlimb murine model [164]. AAA is one of the 10 most common causes of mortality, responsible for 2% of all deaths. AAA is monitored using ultrasound and invasive therapy is the only option to prevent a rupture. Wang et al. conjugated single-chain antibody against VCAM-1 onto MBs to achieve targeting of the inflamed endothelium layer on the vessel wall of AAA for dual-targeted delivery of miR-126 [50]. After the targeted MBs were bound to the AAA region, ultrasound bursts were applied to provide UTGD to the abdominal aorta. Using an angiotensin-II infusion murine model of gradual AAA development, the authors vide UTGD to the abdominal aorta. Using an angiotensin-II infusion murine model of gradual AAA development, the authors showed amelioration of AAA in miR-126 mimic treated animals via 3D ultrasound imaging (Fig. 6) [50]. Further histological data from the study proved downregulation of VCAM-1 expression, as well as successful reduced plaque and aneurysm size [50]. This dual-targeting and site-specific ultrasound trigger may be particularly important for treatments of vascular diseases because the ultrasound particles will naturally disperse throughout the circulatory system. Overall, preclinical UTGD approaches have shown promising results in gene delivery and potential to provide a targeted medical option for patients.

9. Limitations

Nevertheless, there are some hurdles and considerations before UTGD can be translated to clinical practise. Ultrasound-associated tissue heating with increased frequency has presented a concern and so the frequency and length of imaging should be carefully considered. To further prevent heating, the intensities used are usually between 0.3 and 3 W/cm² for drug or gene delivery. Furthermore, an increase in mechanical index, which is the peak negative pressure (MPa), is directly proportional to increased cavitation; therefore the mechanical index typically ranges between 0.2 and 1.9. Some studies have shown that prolonged or repetitive use of ultrasound-targeted MB destruction has been linked to damage to microvasculature integrity, cardiac arrhythmias, and hemolysis [165–170]. While most studies have reported UTGD to be safe, some have also shown tissue damage, especially when subjected to high mechanical indices [107,108,113]. Ultimately, future research must be conducted, especially in large animals, using human scanners to determine the optimal frequency and intensity of ultrasound. There is also a need to investigate the suitability, toxicology, and circulating half-life of the ultrasonic particles and their efficacy in gene translation. In addition, according to the disease of interest, there is a need to determine the suitable nucleic acids and particle platforms. While a single-gene delivery approach may be suitable for genetic diseases, it may not be as useful in chronic diseases, in particular inflammation and CVDs.

10. Conclusions

The recent successes of LNP platforms for nucleic acid delivery in the high-price clinical approval of multiple mRNA vaccines for COVID-19 have set the stage for future gene therapy. Research in gene therapy has developed from a delivery of a single gene approach to the site-specific delivery of multiple nucleic acids over repeated treatments. To avoid unnecessary uptake of nanoparticles and nucleic acids, UTGD has been successfully employed to specifically deliver genetic materials to diseased areas across a range of diseases. In particular, this review covers the successful use of UTGD to directly deliver genetic materials to the diseased area, thereby eliminating any off-target effects, for CVDs and cancer. With further advancement in gene therapy, development of ultrasonic particles and improvement in ultrasound technology, the potential for clinical translation of UTGD will benefit patients across a broad spectrum of diseases.

Declaration of Competing Interest

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

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