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Ultrasound and microbubble-assisted gene delivery: recent advances and ongoing challenges

Anthony Delalande, Michiel Postema, Nathalie Mignet, Patrick Midoux, Chantal Pichon

Abstract

Having first been developed for ultrasound imaging, microbubbles are nowadays proposed as tools for ultrasound-assisted gene delivery, too. Their behaviour during ultrasound exposure causes transient membrane permeability of surrounding cells, facilitating targeted local delivery. The increased cell uptake of extracellular compounds by ultrasound in the presence of microbubbles is attributed to a phenomenon called sonoporation. Sonoporation has been successfully applied to deliver nucleic acids *in vitro* and *in vivo* in a variety of therapeutic applications. However, the biological and physical mechanisms of sonoporation are still not fully understood.

In this review, we discuss recent data concerning microbubble-cell interactions leading to sonoporation and we report on the progress on ultrasound-assisted therapeutic gene delivery in different organs. In addition, we outline ongoing challenges of this novel delivery method for its clinical use.

20

21 **Key terms**

22

23 **Sonoporation:** phenomenon by which ultrasonic stimulation transiently alters the
24 permeability of cell plasma membrane, thereby allowing the uptake of extracellular
25 molecules. This phenomenon is amplified by the presence of microbubbles.

26 **Microbubbles:** encapsulated gas bubbles with diameters up to 10 micrometres. Firstly
27 developed as contrast agents for ultrasonic medical imaging, they might be used as carrier
28 vehicles to deliver drugs or genes, aided by sonoporation.

29 **Acoustic cavitation:** formation, oscillation, and collapse of bubbles under sonication.

30 **Ultrasonic and microbubbles-assisted gene delivery:** external-triggered method of plasmid
31 DNA transfer based on sonoporation.

32

Introduction

Gene therapy aims at introducing genetic material into mammalian cells to cure genetic deficiencies and a large variety of acquired diseases. So far, viruses have demonstrated the feasibility of gene therapy and remain the best vehicles to introduce genes into cells. But severe fatal adverse events of viral vectors including acute immune response and insertion mutagenesis observed in gene therapy clinical trials have raised serious safety concerns about their use [1,2].

Therefore, clinical developments still require the use of alternative approaches of high safety, low immunogenicity and easy manufacture. Moreover, the size limitation capacity, weakness of cell targeting and manufacturing issues has boosted efforts to search for non-viral options. During the last decade, many efforts have been carried out to design synthetic or chemical gene delivery systems that incorporate viral-like features required to transfect efficiently cells [3-5]. The final goal of these systems is to deliver a plasmid encoding a therapeutic protein into target cells upon systemic administration. This challenge could be achieved if several extracellular barriers are overcome to reach the target cells where the therapeutic gene has to be efficiently expressed. Tremendous efforts have been made to improve the efficacy of different types of “synthetic viruses” and elegant strategies have been proposed for that as reported in recent reviews [6-8].

However, achieving optimal targeted gene delivery systems based on chemical compounds is still highly challenging. Narrow therapeutic indices and lack of selectivity towards target tissues have been observed. The absence of specificity that causes severe toxicity often hampers the efficacy at the target site. In this respect, innovative delivery strategies that could selectively target tissues of interest are ongoing both in experimental and in clinical research. It is obvious that designing an efficient targeted delivery strategy is the next step to further improve delivery systems and to reduce side effects. Among the delivery

systems found are several physical methods starting from a simple naked DNA injection to much more sophisticated systems such as electroporation and ultrasound-mediated delivery (USMD).

Ultrasound is used for diagnostic clinical applications and for treatment either by itself or in combination with drugs [9,10]. Among the variety of imaging modalities, ultrasonic imaging is a well-established and reliable technique for diagnosis. The development of real-time imaging has allowed for visualizing a variety of anatomical structures non-invasively. These last years, therapeutic applications of ultrasound have gained new interests thanks to the exploitation of ultrasound for delivery. The effect of ultrasound on biological systems was already reported in 1927 [11], but it took until 1987 until the first study on ultrasound-assisted gene delivery was published [12]. Depending on the acoustic settings, ultrasound can induce either thermal or non-thermal effects, each having its own applications. The thermal effects are obtained using high ultrasound intensities and are due to the absorption of acoustic energy by tissue leading to heating. This property of ultrasound is used in techniques like high-intensity focused ultrasound (HIFU) for focussed ultrasound surgery (FUS) or physiotherapy by ultrasound. One of the first applications of HIFU was sonothrombolysis for ischemic stroke treatment. It has been shown that the introduction of microbubbles in the blood pool could act as cavitation nuclei and decrease the energy threshold for thrombolysis by a third [13]. The thermal effects can present a risk on sonicated tissues; the World Federation for Ultrasound in Medicine and Biology Temperature state that an elevation of 1.5°C is considered safe while an elevation of 4-5°C during 5 minutes is potentially dangerous [14]. The non-thermal effects are obtained using lower ultrasound intensities. The main non-thermal effects are inertial cavitation and acoustic streaming. These effects can induce some benefits such as tissue healing or ultrasound-mediated drug or gene delivery.

84 The use of ultrasound as an external trigger is one approach that has proven to be
85 effective for drug delivery. The drugs to be delivered can be chemotherapeutic agents for
86 cancer therapy applications or oligonucleotides for gene therapy applications. Ultrasound
87 enables to control both the drug release and the release location by use of ultrasound [15-21].
88

1. Ultrasound

Ultrasound is a form of mechanical vibration of matter with a frequency beyond human audible range, i.e., above 20 kHz. The speed of sound is dependent of the medium: roughly 340m/sec in air, 1480 m/sec in pure water, 1550 m/sec in soft tissue and 3700m/sec in bone [22,23]. The particle excursion in a sound wave is related to the instantaneous local pressure through the wave equation [24]. The wave is damped with propagation distance due to tissue absorption and geometric effects [24]. This behaviour induces mechanical effects in the medium which are amplified when inertial cavitation occurs. Inertial cavitation is the process of formation and subsequent collapse of bubbles driven by an acoustic field [25]. Acoustic cavitation takes place when the peak-negative pressure amplitude of ultrasound waves at given frequency exceeds the so-called cavitation threshold [24]. If so-called cavitation nuclei brought by impurities in the medium are present, the cavitation threshold is lowered. Preformed microbubbles serve as cavitation nuclei and are exploited in ultrasonic contrast imaging. These so-called ultrasound contrast agents consist of micron-size gas-filled bubbles (typical diameter ranged is 1-10 μm) encapsulated by an elastic shell. They oscillate during ultrasound exposure (stable cavitation) and demonstrate highly nonlinear behaviour that ameliorates their detection. Oscillating microbubbles introduced in the blood stream produce a sound field themselves and therefore indicate regions of tissue perfusion [26,27]. The acoustic backscattering of these agents is several orders of magnitude higher than the surrounding tissues.

It has been proposed that microbubble activity plays a key role in therapeutic applications [9]. Stable cavitation refers to the (nonviolent) regime of microbubble oscillation, whereas inertial or transient cavitation corresponds to microbubble oscillation with increasingly large amplitudes until collapsing violently [28]. In the nonviolent regime, fluid streaming around oscillating microbubbles may create enough shears to (transiently) open up

114 cell membranes. Furthermore, the microbubbles themselves may interact with, or even
115 translate through cell membranes [29]. In the inertial regime, jets from collapsing
116 microbubbles have been observed to penetrate cells, inducing permanent damage [30].

117

2. Mechanisms of ultrasound-mediated delivery (USMD)

Several studies have been conducted these last years to delineate mechanisms involved in USMD [31-35]. Many experimental data have shown that exposure to ultrasound increases the cellular uptake of extracellular molecules. The uptake is significantly higher if an ultrasound contrast agent is added [36,37]. However, it is under discussion exactly how cells that are subjected to ultrasound internalize extracellular compounds, and which cellular responses they evoke.

Sonoporation is the ultrasound-assisted transient permeabilization of cell membranes. This permeabilization allows for the transfer of molecules between the intra- and extracellular medium. Several sonoporation mechanisms have been identified. The main hypotheses of microbubble interaction with cells are the push and pull mechanisms, micro-jetting, micro-streaming, and, more recently, translation of microbubbles through cells [29]. It has been proposed that the attraction of oscillating microbubbles by cells is caused by sonophores, tiny cavities inside cell membranes [38]. All these sonoporation mechanisms have been demonstrated with the aid of high-speed photography. These mechanisms of membrane poration have been summarized in Figure 1 (inset).

If a microbubble is touching a cell, the microbubble oscillations induce a cellular massage [39]. This cellular massage mechanically stimulates the plasma membrane. It has been hypothesised, that during the expansion phase of the microbubble the plasma membrane is pushed inward and that during the compression phase the microbubble pulls the plasma membrane outward [31]. Repeated push and pull on the plasma membrane creates weaknesses that might lead to the formation of a pore. In a high-mechanical-index regime, the formation of a microjet can occur. A microjet is a fluid protrusion towards a surface during inertial bubble collapse. Jetting has been observed through cells with laser-generated cavities [30]. However, this mechanism induces high cell toxicity. Several studies conducted *in silico*, *in*

vitro, and *in cellulo* suggest that microbubble jetting behaviour is less likely to be the dominant sonoporation mechanism [40].

Another possibility of pore formation mechanism is the rupture of the cell membrane by the streaming flow created by an oscillating microbubble. Indeed, when an oscillating microbubble is attached to a cell membrane the shear forces induced are strong enough to deform and rupture the cell membrane locally [41,42].

Recently a hypothesis has been presented for cell membrane pore formation after ultrasonic stimulation in the absence of microbubbles[38], which involves bilayer sonophores. In electron microscopy images of fish skin after sonication[43], holes in the inter-cellular space were observed, attributed to cavitation between membrane layers (plasma membrane, nucleus and mitochondria). The hypothesis states that cavitation nuclei could be present between the membrane layers inducing a membrane swelling during the rarefaction phase of ultrasound. This deformation could lead to the formation of a pore. This is consistent with the theory on ultrasound-stimulated liposome membranes[21].

A new mechanism has been discovered recently by observing microbubble interactions with cells at a low mechanical index (MI). It appears that the microbubbles can enter into the cell straight through the membrane. This translation phenomenon has been proven by the use of fluorescent labelled microbubbles[44]. The fluorescence of the microbubble has been found in the cell after the observation of a translating microbubble. The microbubble loses (part of) its shell during translation and dissolves quickly after entering the cell. This new mechanism may trigger new drug delivery strategies.

Size of the pore

The formation of a pore during sonication is well-established. Several groups have detected the presence of pores on cell membranes by electron microscopy after sonoporation [34,45,46]. Mehier-Humbert *et al.* were the first to study the poration size. They showed the presence of 100 nm-sized pores in the cell membrane by electron microscopy. The use of

169 fluorescence nanospheres of specific sizes allowing for the measuring of the uptake in
170 MATBIII cells by flow cytometry. They concluded that sonoporation induced a poration of
171 the cell membrane facilitating the transfer of 75-nm-sized spheres. However this study has
172 been done with a specific type of microbubble making the comparison with other
173 studies difficult. More recently, Zhou *et al.* used an electro-diffusion model to measure the
174 size of the pore during sonoporation [47]. This technique based on transmembrane current
175 measurements by voltage clamp [48] permitted to evaluate the size of the pore in live during
176 sonoporation, the pore size measured was 110 ± 40 nm. This value is consistent with the results
177 obtained by electron microscopy. Although several studies have shown the presence of a pore
178 after sonication, we still do not know if these pores are responsible of the drug entry. The cell
179 membrane permeabilization and viability is highly dependent on the ultrasound parameters
180 [49]. Juffermans *et al.* have reviewed the cellular effects of ultrasound [50]. Ultrasound has
181 been shown to induce a transient intracellular calcium entrance only in the presence of
182 microbubbles [48,51-56]. This calcium entrance could promote pore closure [48] and
183 endocytosis [57,58]. The closure time of the pore has been estimated at 5 seconds after
184 ultrasound stimulation [34,47]. It has been reported that sonoporation induces the production
185 of hydrogen peroxide (H_2O_2), which plays an important role in calcium entrance and
186 consequently in pore formation [59]. This production of H_2O_2 has been shown by others and
187 is thought to be related to inertial cavitation [55,60,61]. A hyperpolarisation of sonicated cells
188 has been recorded on different cellular types (Xenopus oocyte, MDA-MB-231, NIH3T3,
189 H9c2) in the presence of microbubbles [48,62,63] and in absence of microbubbles [55]. This
190 phenomenon is directly related to a mechanical stress of the cell membrane. A similar
191 hyperpolarization was observed applying a mechanical pressure on the membrane using a
192 glass probe [64]. The hyperpolarization is attributed to the opening of the BK_{Ca} channels
193 [62,63]. The hyperpolarization and the calcium signaling could increase the uptake of

macromolecules by endocytosis or macropinocytosis. Moreover, we have found sonoporation can also induce an outward transport of small intracellular molecules that likely due to membrane destabilization [33]. The permeabilization engenders a transient release of small molecules such as enhanced green fluorescent protein (eGFP) from the cytosol of HeLa cells stably expressing eGFP gene while preserving cell viability. These results reinforce the theory that the pore formation is a transient mechanism. Active sonoporation is not likely to be the only mechanism for increased uptake, because endocytosis mechanism might also be involved [56]. It is still not clear if the type of mechanism(s) involved could be both dependent on the microbubble chemical composition and on the type of tissue.

Importance of the size of the cargo and endocytosis

The presence of a pore during sonoporation allows for the passive transfer of molecules between the extracellular and the intracellular medium. Hence, the size of the pore limits the size of the cargo to deliver. The use of fluorescent dextran has shown a completely different localization profile depending of the molecular size of dextran after sonoporation [56]. A small dextran molecule (3 kDa) would be localized and diffused inside the cytoplasm and in the nucleus, a medium-sized dextran molecule (70 kDa) would be present in the cytoplasm only, and large dextran molecule (150 and 500 kDa) would be found as patchy structures in the cytoplasm. Endocytosis plays an important role in the USMD process, indeed it has been shown that endocytosis inhibitors could decrease the drug delivery efficiency by sonoporation [55,56]. In these studies, the authors presented a colocalization of the drug to deliver and the coating endosomes protein, clathrin. Meijering *et al.* used dextrans as reporter cargos. They observed that the inhibition of endocytosis pathways (clathrin, caveolin and macropinocytosis) inhibited the delivery. The size of pDNA corresponds to the MDa weight range (a 6 kb plasmid average molecular weight would be 3.6 MDa). The important size of plasmid DNA (even folded) implies that the entrance of the plasmid in the

cell through a pore is not the main event. Paula *et al.* have observed a colocalization between pDNA and clathrin suggesting that pDNA can be routed to the clathrin-mediated endocytosis machinery [56]. The link between endocytosis and drug delivery by sonoporation seems to be well established. Yet, when the plasmids reach the endocytosis machinery, they have to escape from the endosomes in order to avoid lysosomal degradation and be available for the import to the nucleus for gene expression. To our knowledge, the trafficking of the plasmid DNA after sonoporation is still ill known. The mechanisms of gene uptake after sonoporation are summarized in Figure 1.

The gene transfer efficiency is dependent on acoustic pressure, frequency, duty cycle, sonication time, the concentration of microbubbles and local plasmid DNA (pDNA), and cell type used [33,49,65]. The use of luciferase reporter plasmid has permitted to identify the optimal parameters for an efficient gene delivery *in vitro*. Without experimental setup, the optimal parameters found were 1 MHz, 150 kPa, 40% duty cycle (ratio of run time to total cycle time), 60 sec of sonication time and 0.3% (v:v) of microbubble. Under these conditions, less than 10% of cytotoxicity has been measured (Delalande *et al.*, unpublished results).

3. Microbubbles features for improved efficiency in gene delivery

Most microbubbles used as commercial ultrasound contrast agents have a biodegradable shell composed of phospholipids and a heavy gas core, to prevent them from rapid dissolution. Their diameters are typically below 10 μm , to allow them to pass through the capillary system.

The usual production techniques are based on emulsion by shaking or sonication. They induce a wide size distribution of the microbubble size. Moreover, the use of ultrasound in this technique generates high pressures and temperatures which could lead to a degradation of the shell components. Getting a narrow distribution of the microbubble population with

these methods is challenging and requires the use of centrifugation and filtration steps. However other microbubble production methods using microfluidic T-junction chambers or coaxial electrohydrodynamic atomisation are under development and could be able to produce microbubbles of a specific diameter size [66].

While the size distributions of microbubbles used is appropriate for imaging, they are nevertheless too wide and present a polydispersity unsuitable for therapeutic applications [67], for extensive reviews see [21,36,37,68]. The thickness and the elasticity of the shell determine microbubbles stability. A microbubble having a very soft shell would be disrupted at small pressure variations. However, a microbubble with a very hard shell would not be able to oscillate. The most elastic shells are made of phospholipids while the stiffer shells are made of polymers or proteins. To reduce the softness of the phospholipid-based microbubble shell, polymers are usually added. Custom microbubbles are mainly made with classical phospholipids, perfluorocarbon gas and pegylated lipids [69-72]. The presence of the polyethylene glycol (PEG) on the lipid plays the role of emulsifying agent [73]. The development of acoustically active microbubbles has been optimized (for a review [68]) but efforts have been mainly focused to get microbubbles with an optimized acoustic response. These microbubbles are highly echogenic but again they are made with classical phospholipids without any specific feature. Recently, more complexes molecular architecture of liposome-based microbubble have been described: liposomes bubbles consisting of PEG-modified liposomes that encapsulate perfluoropropane gas enclosed in PEG-lipid micelles [74,75] and a hybrid particle made with microbubble loaded with liposomes that are made of thousands of small unilamellar biotinylated liposomes attached *via* avidin molecule to biotinylated microbubble [76]. Of note, the big size, the complexity of architecture and the use of biotin and avidin in those systems are not convenient for *in vivo* use.

To achieve improvements in sonoporation, the development of new microbubbles able to reach specifically the target and deliver the nucleic acid locally is needed. Targeted microbubbles like Target-Ready Micromarker® (VisualSonics™) can be found on the market, the targeting consists of the conjugation of antibodies linked to the microbubble by biotin-streptavidin interaction (Figure 2). Targeted-microbubbles with antibodies can interact with a specific antigen present on a cell membrane allowing the binding of the targeted microbubble even when they are in flow [77]. For example, microbubbles can be targeted to the P-selectin to analyze the endothelium inflammation [78] or to the scVEGF have been developed to analyze tumor angiogenesis [79]. These microbubbles are mainly used in molecular imaging, their development was only based on the acoustic properties and they are not able to carry nucleic acids.

Two main strategies have been proposed to prepare microbubbles able to bind nucleic acids: (i) the use of cationic lipids (DOPE, DOTAP) in the composition of the microbubble shell allowing electrostatic interactions between the nucleic acid and the microbubble, (ii) Compacting the nucleic acid using polymers or liposomes linked to the microbubble by biotin-streptavidin interaction (Figure 2). The use of cationic microbubbles to directly complex pDNA is being more and more reported [80-84]. In these studies, gas-filled cationic liposomes were made with neutral classical (DMPC or DSPC) and cationic lipids as (1,2-distearoyl-3-trimethylammoniumpropane). There are different manners to couple liposomes or polymers on microbubbles. Amongst them are the deposition of single [85] or ii) multi-layers [86] of cationic polymer on the microbubble shell to complex pDNA and a covalent linking of nucleic acids-nanoparticle carriers [87]. These strategies offer the possibility of having the DNA complexes and the microbubble at the same location and likely at the same time. As an example, Sirsi *et al.* have coupled PEI on the surface of lipid-based microbubbles via polyethylene glycol lipid [88].

Despite a good acoustic response of these microbubbles, the level of *in vivo* gene transfer obtained with them was rather low may be due to different limitations of the use of large bubbles size (limited or no extravasation). These last years, Maruyama and colleagues have developed bubble liposomes (BLs) which have smaller size ($< 1\mu\text{m}$) than conventional microbubbles. These structures combine the liposomes features and the acoustic activity of microbubbles. They are composed of DSPC and DSPE-PEG2000-OMe phospholipids filled with perfluoropropane gas [89]. BLs were efficient to deliver gene *in vitro* and *in vivo*. The optimal parameters used for *in vitro* delivery are 2 MHz ultrasound frequency, duty cycle of 50%, 2.5 W/cm^2 of intensity and 10 sec of exposure time. Whilst for *in vivo* (solid or ascites tumors), the best parameters are 1 MHz ultrasound frequency, duty cycle of 50%, 1 W/cm^2 of intensity and 1 to 2 min of exposure time [90-93]. Taking into account the particular features of these BLs (small size, gas particles embedded in liposomes). It will be of interest to know if the different proposed events leading to pore formation can be applied for such structures (figure 1).

4. Applications of US-mediated delivery for *in vivo* gene delivery

The potentiality of US-mediated delivery (USMD) applied for gene transfer is attested by the growing number of related publications for both *in vitro* and *in vivo* use [94-96]. The minimal invasiveness and the high targeting capacity of USMD render the technique potential for clinical transfer. Efficient gene transfer by sonoporation has been obtained when transmitted ultrasound frequencies used are close to those used clinically and extend from 0.5 to 4 MHz. Molecules having variable molecular weights ranging from plasmids [35], oligonucleotide [97] to radioactive tracers [39] were successfully incorporated into cells by sonoporation. In the first studies, pDNA were used alone in presence of ultrasound without microbubbles. Significant results have been obtained *in vitro* as well as *in vivo* with focused

ultrasound [98-100]. Microbubbles have been added to enhance the sonoporation efficiency. However, the level of gene expression is still not higher than that obtained with chemical vectors even though it can be 1 or 2 orders of magnitude more than the level obtained with pDNA alone [94,95]. One of the main difficulties in the field of ultrasound-assisted gene delivery is the lack of homogeneity in sonication set-ups and acoustic conditions used yielding comparisons rather hard to make. Indeed, ultrasound parameters applied are variable in terms of frequency (from 1 to 4 MHz), of acoustic power (from 0.5 to 5 W/cm²) of pulse mode (from 10% to continuous wave) and of stimulation time (from 10 sec to 30 min). The association of ultrasound with gas microbubbles is necessary to get an optimal transfection even though application of ultrasound alone induces a weak molecule transfer into cells [46]. The majority of studies conducted on USMD has been performed *in vitro* with a variety of cell types including primary cells [95]. The main conclusion that can be drawn is the necessity to optimize ultrasound parameters, microbubble and pDNA concentrations for every cell types. Concerning the type of microbubbles, one critical feature is their stability considering the positive correlation between microbubble lifetime and the sonoporation efficiency [101]. Moreover, the stability of gas microbubbles is dependent on both its shell and gas composition [65,101].

These last years, more and more studies report the successful use of ultrasound-enhanced gene transfer *in vivo* both with reporter and therapeutic genes. In the next step, we will focus more on advances in USMD for *in vivo* gene delivery applications and especially for cardiovascular applications following systemic injection. Then we will describe studies relative to USMD application following local injection of pDNA in musculoskeletal tissues. Next, some recent significant data obtained by combining USMD and others delivery systems will be reported.

Cardiovascular applications

It is not surprising that one of the first application of ultrasound and microbubble for gene delivery concerns cardiovascular imaging system. Since, imaging of cardiovascular system has been highly improved by contrast enhanced ultrasound thanks to the wide development of microbubbles in order to detect cardiovascular perfusion pathologies. Combination of the visualization of heart structures and gene delivery has been obtained by exploiting USMD in rats. Commercial and custom microbubbles were tested to deliver pDNA encoding luciferase in the left ventricle [102]. Triggered 1.3 MHz insonation applied every 4 heartbeat gave higher gene expression compared to insonation under continuous mode. The organ specificity is validated by the restriction of gene expression mainly to the heart. The absence of toxicity and the safety of the method (no significant modification in host genes regulation) permit a repeated treatment enhancing the duration of gene expression [103].

Taniyama and colleagues have reported a gene therapy trial on rat restenosis after angioplasty model [46]. A long-term benefit effect of angioplasty is limited by restenosis phenomenon occurring in 40% of patients. This process is produced by an abnormal smooth muscle cell proliferation of intima. The delivery of pDNA encoding p53 anti-oncogene protein in the presence of ultrasound (2.5 W/cm^2) and Optison™ microbubbles has produced an overexpression of p53 in smooth muscle cells leading to an inhibition of intima cells proliferation on rat carotid artery [46].

USMD applications for therapeutic angiogenesis have been widely investigated in myocardial infarction and in hind limb ischemia models in rodents. In a rabbit hind limb ischemic model, the transfer of pDNA encoding the Hepatocyte Growth Factor (HGF) by ultrasound associated with Optison™ microbubbles has allowed to get a better angiogenesis compared to transfer of pDNA alone or pDNA with ultrasound [46]. In a rat model of infarction, a treatment combining ultrasound and microbubbles with pDNA encoding the

368 HGF reduced significantly the scar and left ventricle weight with an increased number of
369 capillaries compared to control groups comprising treatment with pDNA alone or pDNA with
370 ultrasound and untreated rats [104]. More recently, the efficiency of USMD was investigated
371 in a severe chronic ischemia model [105]. The treatment consisting of VEGF-165 gene
372 delivery was performed 2 weeks after the induction of unilateral hind limb ischemia in rats.
373 Upon intravenous injection, of 500 µg pDNA encoding VEGF-165 coupled to these
374 microbubbles were injected intravenously (IV), the muscle blood flow assed by contrast-
375 enhanced ultrasound and the vessel density investigated by fluorescent microangiography
376 were improved in ischemic adductor muscles exposed to ultrasound and with minimal
377 changes in control groups. The expression of VEGF persisted for 4 weeks. Even though the
378 amount of pDNA used was quite high, this study demonstrates the potentiality of USMD for
379 gene delivery since it was expected that microbubbles would have a low transit through
380 ischemic skeletal muscle. The same research group has compared the efficacy of the VEGF
381 gene delivery by USMD after IV injection to intramuscular (IM) injection [106]. They found
382 that the best increase of the microvascular blood flow and volume was obtained with USMD
383 despite the low transfection efficiency. This superiority could be attributed to the wider
384 localization of transgene expression in the vascular endothelium of capillaries and arterioles
385 of ischemic adductor muscle whilst it was mainly localized to perivascular regions and
386 myocytes of injection site area after IM injection. Along the same line, Fuji and colleagues
387 have delivered VEGF gene or Stem Cell Factor gene, another angiogenic gene, to the murine
388 myocardium seven days after coronary artery ligation [107]. DNA delivery was performed in
389 presence of Definity® (Lantheus medical imaging) MB and US at 8 MHz with a mechanical
390 index of 1.6 directed to the heart during 20 min with an intermittent mode of 1 burst every
391 500 msec. Two weeks post-treatment, an improved capillary and arterial density, myocardial
392 function and infarct morphometry was obtained in treated animals. The cardiac repair proved

by echocardiography and myocardial perfusion, was further improved when multiple treatments were performed (1, 3 or 6 at 2 days interval) [108].

Ultrasound application away from injection site of pDNA and microbubbles can also induce a sufficient gene delivery. The injection of pDNA encoding the luciferase combined with cationic lipid microbubbles by intra-muscular (IM), intra-venous (IV) or intra-arterial (IA) routes and with an insonation of rat hind limb skeletal muscles has also been assessed [82]. Luciferase activity detected in limb muscles following IA injection was similar to that obtained following IM injection and was 200-fold greater than achieved after IV administration. Overall, these studies demonstrate the strength of this method for future cardiovascular disease therapies.

Bone, muscle, intervertebral discs and tendon

Osteoinduction is required in the field of orthopedics when there are high bone defects caused by fracture, joint related surgery and congenital anomalies. The improved knowledge of genes involved in bone formation has facilitated the development of new therapeutic applications for bone repair and bone regeneration [109]. Amongst them, Bone Morphogenetic Proteins (BMPs) are known for their ability to induce bone formation. BMP11 transfection has been successfully achieved in canine teeth *in vivo* by sonoporation, this transfection has allowed a reparative dentin formation [110]. In 2007, a pilot study made by Gazit's group showed that sonoporation is able to induce bone formation in hindlimb muscle of mice [111]. Acoustic parameters consisting of 1 MHz ultrasound at 5 W/cm², 50% duty cycle and 10 min exposure time combined with injection of 50 µg pDNA encoding rhBMP-9 mixed to 5% Optison™ gave the highest efficiency for ectopic bone formation. It is interesting to note that compared to electroporation, the volume of bone formed using sonoporation in muscles was lower but more dense. This difference in the volume density obtained by sonoporation could be attributed to the therapeutic benefit effect of ultrasound.

Indeed, it is known that low-intensity pulsed ultrasound (LIPUS) has a positive effect on bone regeneration [112].

Another study related to the BMP-2 gene transfer by repeated sonoporation treatments confirmed the promise of USMD for bone induction [113]. BMP-2 has the ability to induce the differentiation of non-osteogenic cells into osteoblasts [114]. Sonoporation has been done at 1 MHz, 4 W/cm² and a 50% duty cycle in presence of 75 µg pDNA and SonoVue® microbubbles. X-ray imaging, histochemical analysis and biochemical evaluation were performed to assess the osteo-induction. Data show that when the transcutaneous sonoporation was repeated 7 times with a 24-hour interval, a cartilage and immature bone were detected in the treated area after 14 days. On day 21, radiographies show denser opacities than on day 14 and muscle fibers revealed the presence of bone matrix with bone marrow and many osteoblasts concomitantly with high level of biochemical markers (calcium and alkaline phosphatase).

These last decades, the aging population led to an upsurge of age-related pathologies as degenerative diseases. In many spinal disorders, degeneration of the intervertebral discs is an underlying etiology that causes pain and morbidity [115,116]. Nishida *et al.* have been the first who reported the application of USMD on intervertebral discs [117]. They have mixed microbubbles with pDNA encoding the GFP and the firefly luciferase before local injection into rat coccygeal intervertebral discs followed by ultrasound exposure on the surface of injected discs. Ultrasound application induced an improvement of the gene transfer efficiency by 11-fold over pDNA alone. The method has been also successful to transfer siRNA in intervertebral discs [118]. Local injection of siRNA (20pmol) mixed with Optison™ followed by ultrasound exposure (1 MHz, 2 W/cm² and 60 sec ultrasound exposure time) has resulted in long-term expression lasting up to 24 weeks. This expression led to long-term down-regulation of exogenous reporter gene in rat discs *in vivo*. This unusual long period of RNA

inhibition in intervertebral disc *in vivo* may be due to the long quiescent state of highly differentiated disc cells.

We have also observed such lasting expression in Achilles tendons. Tendons are also composed of tenocytes that divide very slowly. Our recent data show remarkably that it is possible to get an efficient localized gene expression in Achilles tendons which is sustained up to 100 days by using ultrasound and BR14 lipid shelled microbubbles[119,120]. Optimized gene transfer was obtained with 1 MHz ultrasound frequency, 200kPa and 40% duty cycle in the presence of 10 μ gpDNA and 5 $\times$ 10⁵BR14 microbubbles. The level of gene transfer was 130-fold more efficient than that obtained with naked pDNA. Note that this sustained gene expression is relied on the presence of microbubbles since no effect of ultrasound or microbubble alone was obtained. The level of gene expression obtained here was as good as with adenoviral vectors in tendons highlighting the potential of this system [121]. Most importantly, this approach has permitted the restoration of fibromodulin gene expression fibromodulin KO mice. Ultrastructural analysis of these tendons revealed that collagen fibrils diameter distribution and circularity were similar that of wild type mice indicating that the fibromodulin expression was enough to restore the collagen fibrils phenotype one week post-transfection.

The effectiveness of this method in tendons could be due to the presence of standing wave which is produced when the reflected ultrasound beam from any sort of interface and the progressive ultrasound beam merge together [150]. Tendons being structures close to bone, a standing wave can be created. Therefore, the exact acoustic power that exactly applied to tendons is hard to know because of the standing wave. This could explain the need of long exposure time as 10 min to achieve high and sustained tendon gene transfer.

Cancer gene therapy

The USMD feasibility has also been examined for cancer gene therapy. Several studies have been performed to evaluate its efficiency on different cancer cells in vitro (for a review see [94,95,122]). Intra-tumoral injection of anti-tumor gene or systemic injection gene delivery followed by a percutaneous application of ultrasound on the tumor region has also been reported [122-125]. One recent example concerns the production of interleukin (IL)-12 protein through the injection of pDNA encoding IL-12 either in skeletal muscular fibers or directly delivered to the targeted tumor or tissue by blood vessels close to it [92]. Intra-tumor administration is still the best route giving efficient gene expression and in most cases repeated administration is required to reduce the tumor growth [126-128]. One explanation could be the high dilution of pDNA after injection in the blood pool.

From these studies, one can conclude that efficient acoustic conditions have been obtained with 1 MHz with an output of 2 to 5W/cm² and 20 to 50% duty cycle with various exposure times that seems to be dependent on the microbubbles used. In most case, *in vivo* ultrasound-mediated gene therapy resulted in a 55% cure rate in tumor-bearing animals. This efficacy is comparable to that obtained with an electrotransfer-based approach but with the benefit of the non-invasiveness of the method.

Combination of USMD and other delivery systems

USMD has also been used in combination with viral or non-viral vectors in order to enhance gene transfer efficiency. The benefit of this combination is the potentiality to enhance the delivery of the gene in a vicinity of ultrasound and microbubble action.

An improvement of viral adenoviral gene transfer has been reported in rat cardiomyocytes in vivo following heart exposure with ultrasound. The treatment was consisting of viral particles injection into apical myocardium of the ventricle and application of 1 MHz ultrasound, 1.5 W/cm², continuous wave during 5 min before and after injection. This condition has enhanced

the percentage of transfected myocytes from 1.7% to 13.2%[129]. Another study reported by Horward *et al* .concerns the encapsulation of adenoviral vectors inside Imagent® microbubble injected via tail vein [130]. This has enhanced the specificity of the transgene expression in vitro as well as *in vivo* to the target organs. Their results elegantly demonstrate that when Imagent® microbubbles were reconstituted in presence of adenoviral vectors, it allows preventing their destruction from complement system activation. The improved transduction in the targeted area was generated upon application of acoustic pressures less than 500kPa. More recently, a combination of adenoviral vector (Ad5 serotype) that encodes for MDR-1 associated with Albumin-coated perfluoropropane gas microbubbles filled with fluorocarbon has been found to be efficient to transduce rabbit bone marrow mononuclear cells [131]. The improvement of gene expression observed was almost 3-fold (8.5% with Ad-5 alone versus 24.5% with Ad-5 combined with ultrasound and microbubble) with no alteration of the cell viability. The presence of microbubbles did not enhance significantly the gene transfer efficiency compared to ultrasound alone. This might be due to the stability of the microbubble used. Indeed, it has been found that microbubble stability is one of the requirements for optimal sonoporation efficiency. A comparison between Optison™, SonoVue® and Sonazoid® has shown that stability, more than size and shell, crucially influenced gene therapy (Alter et al., 2009). Optison™ and Sonazoid® have a similar efficiency in heart that is superior to that of SonoVue®, this latter being less stable.

Combining viral vectors with ultrasound and microbubble gives the possibility to downscale by at least one order of magnitude the amount of viral vectors required for an efficient transduction as shown by Muller *et al*.[132]. In their work, AAV vectors encapsulated in liposomes-based bubbles upon systemic injected have been efficiently targeted into rat hearts upon ultrasound application which is a noninvasive technique in

contrast to direct intramyocardial or intracoronary. Of note, all studies have demonstrated that no relevant cardiac adverse events occurred following these treatments [133,134].

Recombinant adeno-associated virus (rAAV) vectors thought being demonstrated as useful tool for gene delivery into eye[135], are hard to scale up rendering their use for clinical applications limited [136]. The easy accessibility of ocular surface has opened the use of physical method as electroporation to transfer therapeutically genes [137]. USMD can deliver pDNA into retinal ganglion cells safely and effectively *in vitro* and *in vivo*. An enhancement of rAAV transduction into retinal ganglion cells of rats was obtained after intravitreal injection. Recombinant AAV serotype 2 infection combined with ultrasound has led to an efficient, stable and safe transfection of the retina [138]. Gene transfer in retina using USMD has been tested *in vitro* and *in vivo*. The rAAV serotype 2 was chosen to assess the feasibility, efficiency, and safety of the transfection of rAAV2 into RGCs *in vivo* by USMD. When examining the retinal flat mounts, data prove that eGFP expression in the AAV2-eGFP and USMD-treated group was the strongest and the number of transfected retinal ganglion cells was higher (19.48% versus 3.23%) compared to control groups. Gene transfer into retinal ganglion cells has a tremendous application because of its potentiality to treat glaucomatous optic neuroprotection by preventing apoptosis [139].

A combination of USMD and chemical vectors has been also proposed in different recent studies. Sonoporation has been found to enhance the efficiency of polyethyleneimine (PEI) *in vitro* [140-142]; as well as *in vivo*[143,144]. Deshpande and Prausnitz have reported that the combination of ultrasound and PEI has a synergistic effect to increase pDNA transfection efficiency[141]. They examined the influence of ultrasound and PEI:pDNA complexation on transfection of human aortic smooth muscle cells and human prostatic carcinoma DU145 cells with GFP and luciferase reporter genes. Ultrasound stimulation improves the transfection by up to 18-fold relative to naked DNA and by 90-fold when this

latter is complexed with PEI. The combination of ultrasound and Optison™ microbubbles with PEI/pDNA complexes increased the transfection up to 200-fold resulting in the transfection of 34% of the cells. Qiu and colleagues have assessed the correlation between acoustic cavitation and sonoporation in ultrasound-mediated gene transfection with PEI *in vitro* [142]. The study was done on MCF-7 cells and with 1MHz ultrasound frequency in the presence of microbubbles. Data indicate that the transfection efficiency initially increased linearly with the acoustic cavitation, reaching saturation when the acoustic cavitation is too high. There was a high correlation between the measured acoustic cavitation, the sonoporation pore size assessed by electron microscopy, and the cell viability. Xenariou *et al.* have evaluated the effect of sonoporation on gene delivery mediated by a cationic lipid GL67 complexed with pDNA and PEI/pDNA in lungs [145]. Upon nasal instillation of 100µg of pDNA in mice, their chests were exposed with ultrasound. Despite the possible ultrasound waves attenuation when travelling through air, sonoporation(1 MHz, 3 W/cm², 20% duty cycle and 20 min of exposure time) increases the transfer of naked pDNA when mixed with microbubbles (Optison™), but no enhancement of the transfection efficiency of GL67/pDNA or PEI/pDNA occurred. Moreover, lung hemorrhages were observed with the optimal acoustic parameters. These data suggest the necessity of optimizing the acoustic conditions and the type of microbubbles used to prevent ultrasound bio-effects without altering the benefit on the gene delivery. The main aim will be to develop original microbubbles for safe, non-invasive and target controlled drug delivery by sonoporation in order to enhance the delivery of their payload at a specific location.

5. Considerations and future perspectives

Exciting results from recent clinical trials demonstrate without doubt the promise of gene therapy. The advances made shows that the field is now surely moving from the

conceptual technology to more and more clinical translational. The majority of these advances have been obtained with viral vectors. Therefore, there is still room for non viral methods to be developed since they are more secure in terms of safety. But, improving their efficacy is mandatory for a widespread use. The combination of the ultrasound trigger effect with targeted gas microbubbles as drug/gene carrier holds great promise by offering a double targeting controlling both gene release and gene transfer location [15-21]. The non-invasiveness of this system renders it superior to other physical methods as electroporation. However, some challenges must be overcome to ensure its efficiency and data consistency. Below are some points that could be considered:

- Rigorous characterization of acoustic fields and description of the experimental configurations: One has noticed that there is lack of consistency regarding the ultrasound exposure configurations in many studies. Researchers in the field are encouraged to describe the set-up used and to make such measurements. This will make the comparison between different studies easier and will help to the identification of critical points which in turn will allow to fast forwarding the technology. Recently, the Safety Committee of the British Medical Ultrasound Society has published a useful set of recommendations that could be easily followed [146].

- Combining a judicious choice of microbubbles composition and plasmid DNA sequence with regard to gene delivery in a specific cell type: It is now well admitted that the gene transfer efficacy of any non viral system is highly dependent on the cell or organ type. So far in the case of USMD, cationic microbubbles that complex pDNA did not improve the efficiency as much as expected. This could be due to the fact that if an endocytosis process occurs during sonoporation, internalized pDNA has to overcome cellular barriers similar to those encountered by chemical vectors [147]. Therefore, pDNA escape from endosomes and

its diffusion and entry inside the nucleus must be improved. This could be tackled by fine tuning microbubble composition and reducing their size.

The composition of pDNA backbone and its length has to be chosen with care depending on the type of gene expression needed (transient or long-term expression). Today, enough experience has been gained in the field of gene delivery to define pDNA constructs that bear specific sequences facilitating nuclear import and long-term gene expression [147-149].

- An improved knowledge of microbubble-cell interactions and how they affect the cell and the impact on the gene delivery is still required to exploit this method in a safe and efficient way. A better understanding of underlying mechanisms that are induced at the plasma membrane and inside the cells during the sonoporation would establish a rational determination of ultrasound exposure conditions. Compilation of new observations and identifying molecular signaling mechanisms induced by sonoporation could help to optimize rationally this delivery method.

- The development of ultrasound platform that allow monitoring in real time a concomitant image of gene delivery could be useful. In this case, it would be necessary to design original microbubbles that could serve as platforms to develop targeted microbubble for both imaging and delivery applications.

Executive summary:

Gene therapy potentiality to cure inherited and acquired diseases is well established. Non-viral methods offer a good alternative for gene therapy due to safety reasons. Unlike other physical delivery methods, ultrasound-mediated delivery allows to combine the possibility of reaching deeper organs with a non-invasive manner and to restrict the delivery at a specific area.

Sonoporation mechanism

- Sonoporation uses ultrasound activation of microbubbles to increase the permeability of plasma membrane of cells.

-Gas microbubbles oscillations under ultrasound activation induce membrane alteration including formation of transient pores and endocytosis process.

Applications for gene delivery

- Ultrasound and microbubbles-assisted delivery can be exploited to efficiently deliver genes *in vitro* as well as *in vivo* in several organs after systemic or local administration. The optimal ultrasound parameters used are highly dependent on the microbubble, the type of cell or the tissue.

- In most case, efficient acoustic conditions have been obtained with 1 MHz with an output ranging from 2 to 5W/cm² and from 20 to 50% duty cycle with various exposure times that seems to be dependent on the type of microbubbles used and insonified tissues.

- Ultrasound and microbubble-assisted delivery combined with viral or chemical vectors has a synergetic effect to improve the gene delivery in the vicinity of ultrasound and microbubble action.

Future challenges

-Designing targeted microbubbles that are able to carry the gene of interest could also reduce the amount of nucleic acid needed.

635 - More knowledge on the impact of microbubbles-cell interactions and the intracellular
636 routing of microbubbles and pDNA has to be gained to fully exploit this methodology. This
637 will allow to fine tune microbubbles composition and to design adequate equipment
638 according to the target organs and to translate the technology for clinical use.
639

Figures

Figure 1. Schematic description of mechanisms occurring during sonoporation.

Upon ultrasound exposure, the microbubble is interacting with cell membrane leading to the formation of a pore. Here are summarized the six possible mechanisms of membrane poration (inset): the pushing or pulling of the membrane by a microbubble; the microbubble jetting toward the cell membrane; the mechanical shear force; the sonophore formation within the membrane layers and the translation of a microbubble to a cell. The membrane poration is a transient mechanism (5 sec) leading to the passive entry/exit of ions and small molecules. Calcium and H_2O_2 entry enhances the endocytosis process allowing extracellular molecule uptake. Hyperpolarization produced by BK_{Ca} channels activation caused by calcium entry could positively impact the endocytosis. The pDNA was found mainly uptaken via clathrin-mediated pathway. The pDNA has to escape from endosomes to avoid lysosomes and it must be imported into the nucleus for its expression.

Figure 2. Representation of two possible strategies for microbubble targeting and functionalization for gene delivery.

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