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

3

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5

6 **Abstract**

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

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

19

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

33 **Introduction**

34  
35

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

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

62         Ultrasound is used for diagnostic clinical applications and for treatment either by  
63 itself or in combination with drugs [9,10]. Among the variety of imaging modalities,  
64 ultrasonic imaging is a well-established and reliable technique for diagnosis. The development  
65 of real-time imaging has allowed for visualizing a variety of anatomical structures non-  
66 invasively. These last years, therapeutic applications of ultrasound have gained new interests  
67 thanks to the exploitation of ultrasound for delivery. The effect of ultrasound on biological  
68 systems was already reported in 1927 [11], but it took until 1987 until the first study on  
69 ultrasound-assisted gene delivery was published [12]. Depending on the acoustic settings,  
70 ultrasound can induce either thermal or non-thermal effects, each having its own applications.  
71 The thermal effects are obtained using high ultrasound intensities and are due to the  
72 absorption of acoustic energy by tissue leading to heating. This property of ultrasound is used  
73 in techniques like high-intensity focused ultrasound (HIFU) for focussed ultrasound surgery  
74 (FUS) or physiotherapy by ultrasound. One of the first applications of HIFU was  
75 sonothrombolysis for ischemic stroke treatment. It has been shown that the introduction of  
76 microbubbles in the blood pool could act as cavitation nuclei and decrease the energy  
77 threshold for thrombolysis by a third [13]. The thermal effects can present a risk on sonicated  
78 tissues; the World Federation for Ultrasound in Medicine and Biology Temperature state that  
79 an elevation of 1.5°C is considered safe while an elevation of 4-5°C during 5 minutes is  
80 potentially dangerous [14]. The non-thermal effects are obtained using lower ultrasound  
81 intensities. The main non-thermal effects are inertial cavitation and acoustic streaming. These  
82 effects can induce some benefits such as tissue healing or ultrasound-mediated drug or gene  
83 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

## 89 **1. Ultrasound**

90 Ultrasound is a form of mechanical vibration of matter with a frequency beyond human  
91 audible range, i.e., above 20 kHz. The speed of sound is dependent of the medium: roughly  
92 340m/sec in air, 1480 m/sec in pure water, 1550 m/sec in soft tissue and 3700m/sec in bone  
93 [22,23]. The particle excursion in a sound wave is related to the instantaneous local pressure  
94 through the wave equation [24]. The wave is damped with propagation distance due to tissue  
95 absorption and geometric effects [24]. This behaviour induces mechanical effects in the  
96 medium which are amplified when inertial cavitation occurs. Inertial cavitation is the process  
97 of formation and subsequent collapse of bubbles driven by an acoustic field [25]. Acoustic  
98 cavitation takes place when the peak-negative pressure amplitude of ultrasound waves at  
99 given frequency exceeds the so-called cavitation threshold [24]. If so-called cavitation nuclei  
100 brought by impurities in the medium are present, the cavitation threshold is lowered.  
101 Preformed microbubbles serve as cavitation nuclei and are exploited in ultrasonic contrast  
102 imaging. These so-called ultrasound contrast agents consist of micron-size gas-filled bubbles  
103 (typical diameter ranged is 1-10  $\mu\text{m}$ ) encapsulated by an elastic shell. They oscillate during  
104 ultrasound exposure (stable cavitation) and demonstrate highly nonlinear behaviour that  
105 ameliorates their detection. Oscillating microbubbles introduced in the blood stream produce  
106 a sound field themselves and therefore indicate regions of tissue perfusion [26,27]. The  
107 acoustic backscattering of these agents is several orders of magnitude higher than the  
108 surrounding tissues.

109 It has been proposed that microbubble activity plays a key role in therapeutic  
110 applications [9]. Stable cavitation refers to the (nonviolent) regime of microbubble oscillation,  
111 whereas inertial or transient cavitation corresponds to microbubble oscillation with  
112 increasingly large amplitudes until collapsing violently [28]. In the nonviolent regime, fluid  
113 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



## 118 **2. Mechanisms of ultrasound-mediated delivery (USMD)**

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

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

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

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

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

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

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

#### 164 **Size of the pore**

165         The formation of a pore during sonication is well-established. Several groups have  
166 detected the presence of pores on cell membranes by electron microscopy after sonoporation  
167 [34,45,46]. Mehier-Humbert *et al.* were the first to study the poration size. They showed the  
168 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  $110\pm 40$  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

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

### 203 **Importance of the size of the cargo and endocytosis**

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

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

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

234

### 235 **3. Microbubbles features for improved efficiency in gene delivery**

236 Most microbubbles used as commercial ultrasound contrast agents have a  
237 biodegradable shell composed of phospholipids and a heavy gas core, to prevent them from  
238 rapid dissolution. Their diameters are typically below 10  $\mu\text{m}$ , to allow them to pass through  
239 the capillary system.

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

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

248           While the size distributions of microbubbles used is appropriate for imaging, they are  
249 nevertheless too wide and present a polydispersity unsuitable for therapeutic applications  
250 [67], for extensive reviews see [21,36,37,68]. The thickness and the elasticity of the shell  
251 determine microbubbles stability. A microbubble having a very soft shell would be disrupted  
252 at small pressure variations. However, a microbubble with a very hard shell would not be able  
253 to oscillate. The most elastic shells are made of phospholipids while the stiffer shells are  
254 made of polymers or proteins. To reduce the softness of the phospholipid-based microbubble  
255 shell, polymers are usually added. Custom microbubbles are mainly made with classical  
256 phospholipids, perfluorocarbon gas and pegylated lipids [69-72]. The presence of the  
257 polyethylene glycol (PEG) on the lipid plays the role of emulsifying agent [73]. The  
258 development of acoustically active microbubbles has been optimized (for a review [68]) but  
259 efforts have been mainly focused to get microbubbles with an optimized acoustic response.  
260 These microbubbles are highly echogenic but again they are made with classical  
261 phospholipids without any specific feature. Recently, more complexes molecular architecture  
262 of liposome-based microbubble have been described: liposomes bubbles consisting of PEG-  
263 modified liposomes that encapsulate perfluoropropane gas enclosed in PEG-lipid micelles  
264 [74,75]and a hybrid particle made with microbubble loaded with liposomes that are made of  
265 thousands of small unilamellar biotinylated liposomes attached *via* avidin molecule to  
266 biotinylated microbubble[76]. Of note, the big size, the complexity of architecture and the use  
267 of biotin and avidin in those systems are not convenient for *in vivo* use.

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

279 Two main strategies have been proposed to prepare microbubbles able to bind  
280 nucleic acids: (i) the use of cationic lipids (DOPE, DOTAP) in the composition of the  
281 microbubble shell allowing electrostatic interactions between the nucleic acid and the  
282 microbubble, (ii) Compacting the nucleic acid using polymers or liposomes linked to the  
283 microbubble by biotin-streptavidin interaction (Figure 2). The use of cationic microbubbles to  
284 directly complex pDNA is being more and more reported [80-84]. In these studies, gas-filled  
285 cationic liposomes were made with neutral classical (DMPC or DSPC) and cationic lipids as  
286 (1,2-distearoyl-3-trimethylammoniumpropane). There are different manners to couple  
287 liposomes or polymers on microbubbles. Amongst them are the deposition of single [85] or ii)  
288 multi-layers [86] of cationic polymer on the microbubble shell to complex pDNA and a  
289 covalent linking of nucleic acids-nanoparticle carriers [87]. These strategies offer the  
290 possibility of having the DNA complexes and the microbubble at the same location and likely  
291 at the same time. As an example, Sirsiet *al.* have coupled PEI on the surface of lipid-based  
292 microbubbles via polyethylene glycol lipid [88].

293 Despite a good acoustic response of these microbubbles, the level of *in vivo* gene transfer  
294 obtained with them was rather low may be due to different limitations of the use of large  
295 bubbles size (limited or no extravasation). These last years, Maruyama and colleagues have  
296 developed bubble liposomes (BLs) which have smaller size ( $< 1\mu\text{m}$ ) than conventional  
297 microbubbles. These structures combine the liposomes features and the acoustic activity of  
298 microbubbles. They are composed of DSPC and DSPE-PEG2000-OMe phospholipids filled  
299 with perfluoropropane gas [89]. BLs were efficient to deliver gene *in vitro* and *in vivo*. The  
300 optimal parameters used for *in vitro* delivery are 2 MHz ultrasound frequency, duty cycle of  
301 50%,  $2.5\text{ W/cm}^2$  of intensity and 10 sec of exposure time. Whilst for *in vivo* (solid or ascites  
302 tumors), the best parameters are 1 MHz ultrasound frequency, duty cycle of 50%,  $1\text{ W/cm}^2$  of  
303 intensity and 1 to 2 min of exposure time [90-93]. Taking into account the particular features  
304 of these BLs (small size, gas particles embedded in liposomes). It will be of interest to know  
305 if the different proposed events leading to pore formation can be applied for such structures  
306 (figure 1).

307

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

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



318 ultrasound [98-100]. Microbubbles have been added to enhance the sonoporation efficiency.  
319 However, the level of gene expression is still not higher than that obtained with chemical  
320 vectors even though it can be 1 or 2 orders of magnitude more than the level obtained with  
321 pDNA alone [94,95]. One of the main difficulties in the field of ultrasound-assisted gene  
322 delivery is the lack of homogeneity in sonication set-ups and acoustic conditions used  
323 yielding comparisons rather hard to make. Indeed, ultrasound parameters applied are variable  
324 in terms of frequency (from 1 to 4 MHz), of acoustic power (from to 0.5 to 5 W/cm<sup>2</sup>) of pulse  
325 mode (from 10% to continuous wave) and of stimulation time (from 10 sec to 30 min). The  
326 association of ultrasound with gas microbubbles is necessary to get an optimal transfection  
327 even though application of ultrasound alone induces a weak molecule transfer into cells  
328 [46]. The majority of studies conducted on USMD has been performed *in vitro* with a variety  
329 of cell types including primary cells [95]. The main conclusion that can be drawn is the  
330 necessity to optimize ultrasound parameters, microbubble and pDNA concentrations for every  
331 cell types. Concerning the type of microbubbles, one critical feature is their stability  
332 considering the positive correlation between microbubble lifetime and the sonoporation  
333 efficiency [101]. Moreover, the stability of gas microbubbles is dependent on both its shell  
334 and gas composition [65,101].

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

342

### 343 **Cardiovascular applications**

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

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

362 USMD applications for therapeutic angiogenesis have been widely investigated in  
363 myocardial infarction and in hind limb ischemia models in rodents. In a rabbit hind limb  
364 ischemic model, the transfer of pDNA encoding the Hepatocyte Growth Factor (HGF) by  
365 ultrasound associated with Optison™ microbubbles has allowed to get a better angiogenesis  
366 compared to transfer of pDNA alone or pDNA with ultrasound [46]. In a rat model of  
367 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

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

395           Ultrasound application away from injection site of pDNA and microbubbles can also  
396 induce a sufficient gene delivery. The injection of pDNA encoding the luciferase combined  
397 with cationic lipid microbubbles by intra-muscular (IM), intra-venous (IV) or intra-arterial  
398 (IA) routes and with an insonation of rat hind limb skeletal muscles has also been assessed  
399 [82]. Luciferase activity detected in limb muscles following IA injection was similar to that  
400 obtained following IM injection and was 200-fold greater than achieved after IV  
401 administration. Overall, these studies demonstrate the strength of this method for future  
402 cardiovascular disease therapies.

#### 403 **Bone, muscle, intervertebral discs and tendon**

404           Osteoinduction is required in the field of orthopedics when there are high bone  
405 defects caused by fracture, joint related surgery and congenital anomalies. The improved  
406 knowledge of genes involved in bone formation has facilitated the development of new  
407 therapeutic applications for bone repair and bone regeneration [109]. Amongst them, Bone  
408 Morphogenetic Proteins (BMPs) are known for their ability to induce bone formation. BMP11  
409 transfection has been successfully achieved in canine teeth *in vivo* by sonoporation, this  
410 transfection has allowed a reparative dentin formation [110]. In 2007, a pilot study made by  
411 Gazit's group showed that sonoporation is able to induce bone formation in hindlimb muscle  
412 of mice [111]. Acoustic parameters consisting of 1 MHz ultrasound at  $5 \text{ W/cm}^2$ , 50% duty  
413 cycle and 10 min exposure time combined with injection of 50  $\mu\text{g}$  pDNA encoding rhBMP-9  
414 mixed to 5% Optison™ gave the highest efficiency for ectopic bone formation. It is  
415 interesting to note that compared to electroporation, the volume of bone formed using  
416 sonoporation in muscles was lower but more dense. This difference in the volume density  
417 obtained by sonoporation could be attributed to the therapeutic benefit effect of ultrasound.

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

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

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

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

445 We have also observed such lasting expression in Achilles tendons. Tendons are also  
446 composed of tenocytes that divide very slowly. Our recent data show remarkably that it is  
447 possible to get an efficient localized gene expression in Achilles tendons which is sustained  
448 up to 100 days by using ultrasound and BR14 lipid shelled microbubbles[119,120].  
449 Optimized gene transfer was obtained with 1 MHz ultrasound frequency, 200kPa and 40%  
450 duty cycle in the presence of 10 $\mu$ g pDNA and 5 $\times$ 10<sup>5</sup> BR14 microbubbles. The level of gene  
451 transfer was 130-fold more efficient than that obtained with naked pDNA. Note that this  
452 sustained gene expression is relied on the presence of microbubbles since no effect of  
453 ultrasound or microbubble alone was obtained. The level of gene expression obtained here  
454 was as good as with adenoviral vectors in tendons highlighting the potential of this system  
455 [121]. Most importantly, this approach has permitted the restoration of fibromodulin gene  
456 expression fibromodulin KO mice. Ultrastructural analysis of these tendons revealed that  
457 collagen fibrils diameter distribution and circularity were similar that of wild type mice  
458 indicating that the fibromodulin expression was enough to restore the collagen fibrils  
459 phenotype one week post-transfection.

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

466

467

## 468 **Cancer gene therapy**

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

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

## 485 **Combination of USMD and other delivery systems**

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

489 An improvement of viral adenoviral gene transfer has been reported in rat cardiomyocytes in  
490 vivo following heart exposure with ultrasound. The treatment was consisting of viral particles  
491 injection into apical myocardium of the ventricle and application of 1 MHz ultrasound, 1.5  
492 W/cm<sup>2</sup>, continuous wave during 5 min before and after injection. This condition has enhanced

493 the percentage of transfected myocytes from 1.7% to 13.2%[129]. Another study reported by  
494 Horward *et al* .concerns the encapsulation of adenoviral vectors inside Imagent® microbubble  
495 injected via tail vein [130]. This has enhanced the specificity of the transgene expression in  
496 vitro as well as *in vivo* to the target organs. Their results elegantly demonstrate that when  
497 Imagent® microbubbles were reconstituted in presence of adenoviral vectors, it allows  
498 preventing their destruction from complement system activation. The improved transduction  
499 in the targeted area was generated upon application of acoustic pressures less than 500kPa.  
500 More recently, a combination of adenoviral vector (Ad5 serotype) that encodes for MDR-1  
501 associated with Albumin-coated perfluoropropane gas microbubbles filled with fluorocarbon  
502 has been found to be efficient to transduce rabbit bone marrow mononuclear cells [131]. The  
503 improvement of gene expression observed was almost 3-fold (8.5% with Ad-5 alone versus  
504 24.5% with Ad-5 combined with ultrasound and microbubble) with no alteration of the cell  
505 viability. The presence of microbubbles did not enhance significantly the gene transfer  
506 efficiency compared to ultrasound alone. This might be due to the stability of the microbubble  
507 used. Indeed, it has been found that microbubble stability is one of the requirements for  
508 optimal sonoporation efficiency. A comparison between Optison™, SonoVue® and  
509 Sonazoid® has shown that stability, more than size and shell, crucially influenced gene  
510 therapy (Alter et al., 2009). Optison™ and Sonazoid® have a similar efficiency in heart that  
511 is superior to that of SonoVue®, this latter being less stable.

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



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

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

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

542 latter is complexed with PEI. The combination of ultrasound and Optison™ microbubbles  
543 with PEI/pDNA complexes increased the transfection up to 200-fold resulting in the  
544 transfection of 34% of the cells. Qiu and colleagues have assessed the correlation between  
545 acoustic cavitation and sonoporation in ultrasound-mediated gene transfection with PEI *in*  
546 *vitro* [142]. The study was done on MCF-7 cells and with 1MHz ultrasound frequency in the  
547 presence of microbubbles. Data indicate that the transfection efficiency initially increased  
548 linearly with the acoustic cavitation, reaching saturation when the acoustic cavitation is too  
549 high. There was a high correlation between the measured acoustic cavitation, the sonoporation  
550 pore size assessed by electron microscopy, and the cell viability. Xenariou *et al.* have  
551 evaluated the effect of sonoporation on gene delivery mediated by a cationic lipid GL67  
552 complexed with pDNA and PEI/pDNA in lungs [145]. Upon nasal instillation of 100µg of  
553 pDNA in mice, their chests were exposed with ultrasound. Despite the possible ultrasound  
554 waves attenuation when travelling through air, sonoporation(1 MHz, 3 W/cm<sup>2</sup>, 20% duty  
555 cycle and 20 min of exposure time) increases the transfer of naked pDNA when mixed with  
556 microbubbles (Optison™), but no enhancement of the transfection efficiency of GL67/pDNA  
557 or PEI/pDNA occurred. Moreover, lung hemorrhages were observed with the optimal  
558 acoustic parameters. These data suggest the necessity of optimizing the acoustic conditions  
559 and the type of microbubbles used to prevent ultrasound bio-effects without altering the  
560 benefit on the gene delivery. The main aim will be to develop original microbubbles for safe,  
561 non-invasive and target controlled drug delivery by sonoporation in order to enhance the  
562 delivery of their payload at a specific location.

563

## 564 **5. Considerations and future perspectives**

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

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

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

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

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

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

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

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

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609

610 **Executive summary:**

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

616 ***Sonoporation mechanism***

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

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

621 ***Applications for gene delivery***

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

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

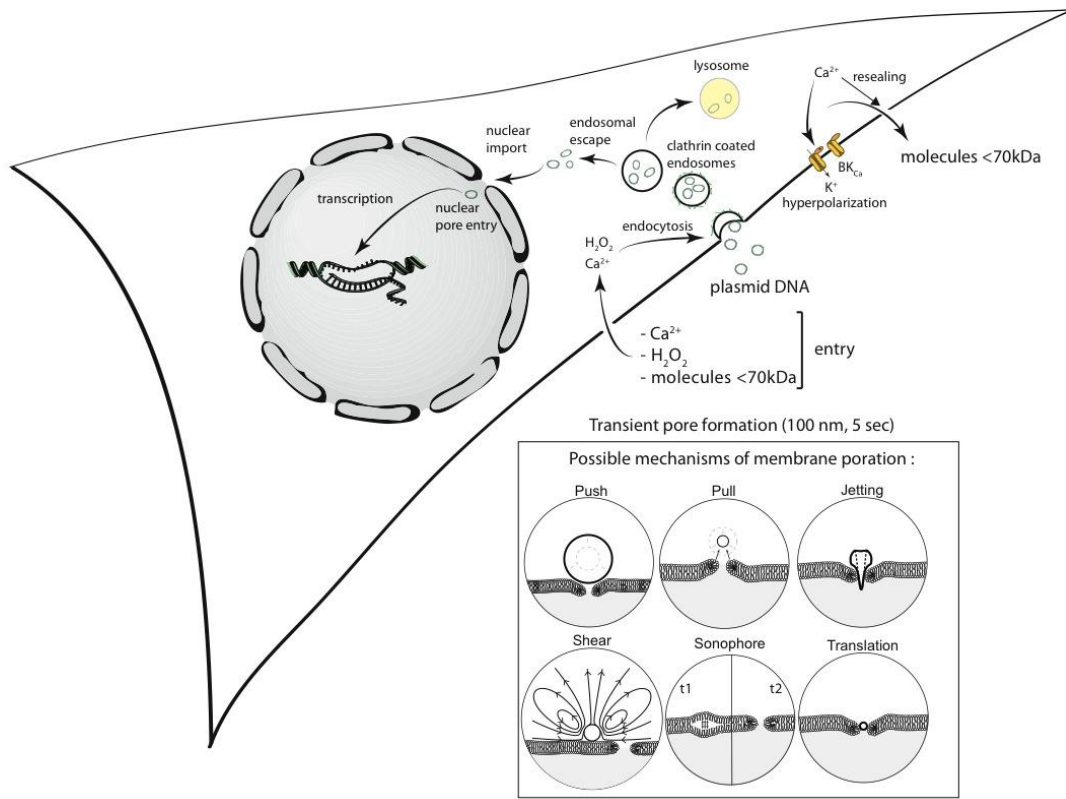
629 - Ultrasound and microbubble-assisted delivery combined with viral or chemical vectors has a  
630 synergetic effect to improve the gene delivery in the vicinity of ultrasound and microbubble  
631 action.

632 ***Future challenges***

633 -Designing targeted microbubbles that are able to carry the gene of interest could also reduce  
634 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.  
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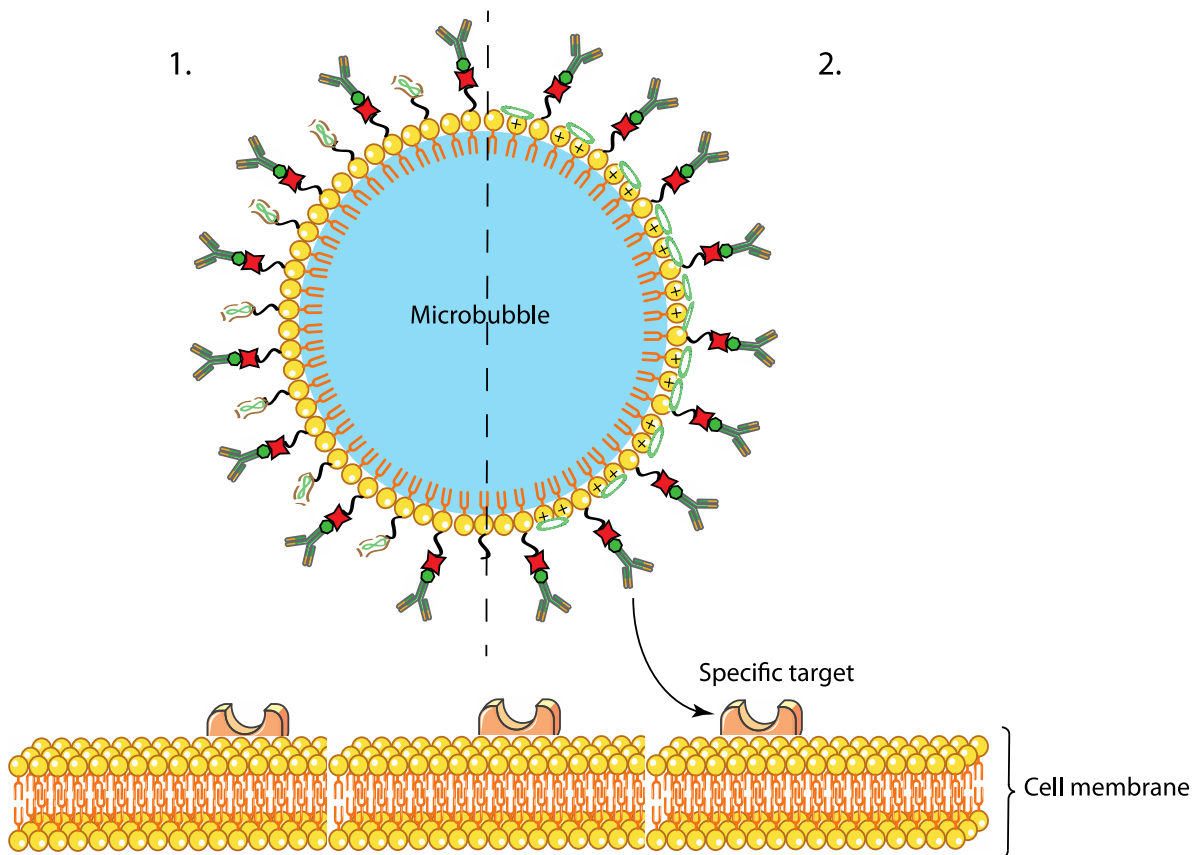
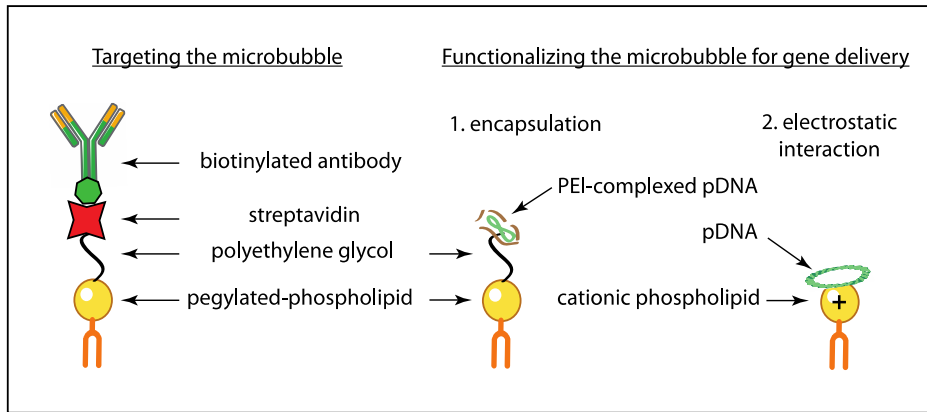
640 **Figures**



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643 **Figure 1. Schematic description of mechanisms occurring during sonoporation.**

644 Upon ultrasound exposure, the microbubble is interacting with cell membrane leading to the  
 645 formation of a pore. Here are summarized the six possible mechanisms of membrane poration  
 646 (inset): the pushing or pulling of the membrane by a microbubble; the microbubble jetting  
 647 toward the cell membrane; the mechanical shear force; the sonophore formation within the  
 648 membrane layers and the translation of a microbubble to a cell. The membrane poration is a  
 649 transient mechanism (5 sec) leading to the passive entry/exit of ions and small molecules.  
 650 Calcium and H<sub>2</sub>O<sub>2</sub> entry enhances the endocytosis process allowing extracellular molecule  
 651 uptake. Hyperpolarization produced by BK<sub>Ca</sub> channels activation caused by calcium entry  
 652 could positively impact the endocytosis. The pDNA was found mainly uptake via clathrin-  
 653 mediated pathway. The pDNA has to escape from endosomes to avoid lysosomes and it must  
 654 be imported into the nucleus for its expression.



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658 **Figure 2. Representation of two possible strategies for microbubble targeting and**

659 **functionalization for gene delivery.**

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