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1 **Ultrasound and microbubble-assisted gene delivery: recent**
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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
36 genetic deficiencies and a large variety of acquired diseases. So far, viruses have
37 demonstrated the feasibility of gene therapy and remain the best vehicles to introduce genes
38 into cells. But severe fatal adverse events of viral vectors including acute immune response
39 and insertion mutagenesis observed in gene therapy clinical trials have raised serious safety
40 concerns about their use [1,2].

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

52 However, achieving optimal targeted gene delivery systems based on chemical
53 compounds is still highly challenging. Narrow therapeutic indices and lack of selectivity
54 towards target tissues have been observed. The absence of specificity that causes severe
55 toxicity often hampers the efficacy at the target site. In this respect, innovative delivery
56 strategies that could selectively target tissues of interest are ongoing both in experimental and
57 in clinical research. It is obvious that designing an efficient targeted delivery strategy is the
58 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 μm) 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 ± 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 μm , 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 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 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/cm²) 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 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 W/cm^2 , 50% duty
413 cycle and 10 min exposure time combined with injection of 50 μg 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/cm² 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/cm² 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 μ gpDNA and 5 \times 10⁵BR14 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.

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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/cm² 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/cm², 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/cm², 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.

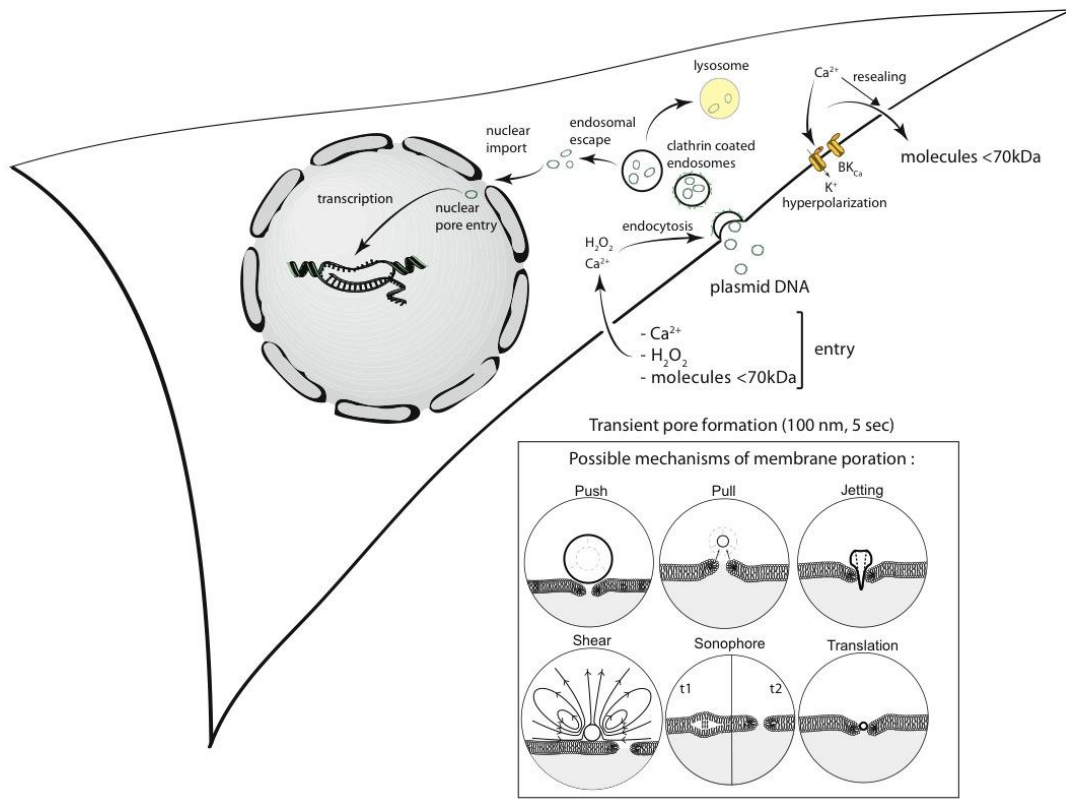
626 - In most case, efficient acoustic conditions have been obtained with 1 MHz with an output
627 ranging from 2 to 5W/cm² and from 20 to 50% duty cycle with various exposure times that
628 seems to be dependent on the type of microbubbles used and insonified tissues.

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.

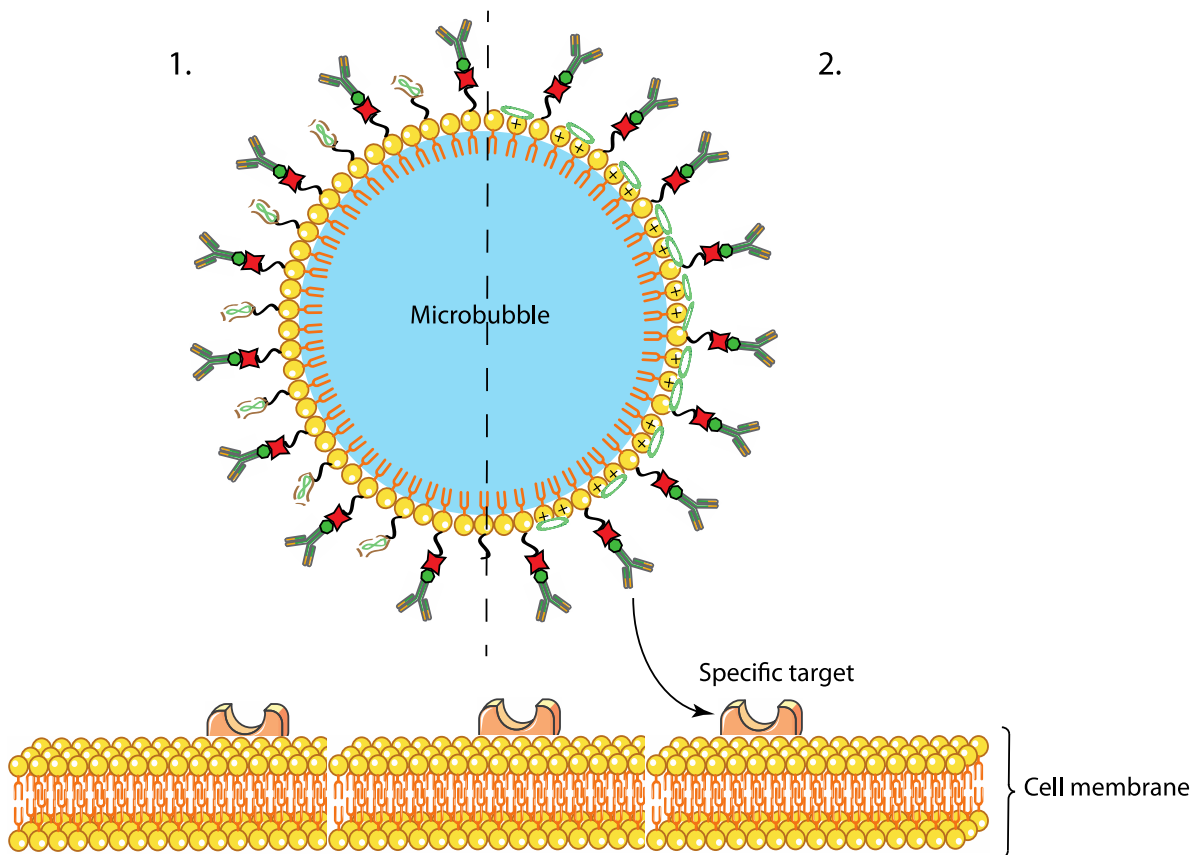
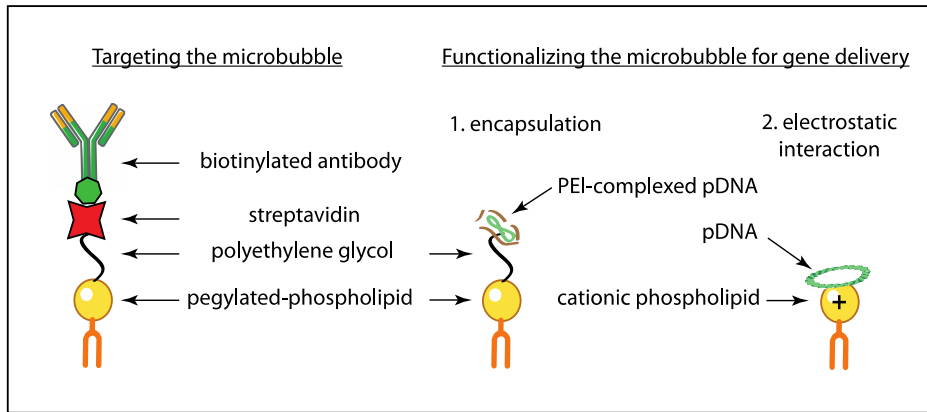
635 - More knowledge on the impact of microbubbles-cell interactions and the intracellular
636 routing of microbubbles and pDNA has to be gained to fully exploit this methodology. This
637 will allow to fine tune microbubbles composition and to design adequate equipment
638 according to the target organs and to translate the technology for clinical use.
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642

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_2O_2 entry enhances the endocytosis process allowing extracellular molecule
 651 uptake. Hyperpolarization produced by BK_{Ca} 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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