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Sonoporation: From the lab to human clinical trials

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Abstract—Therapeutic ultrasound has been in use for over 70 years but has primarily been a thermal modality. Sonoporation, the use of ultrasound and stable gas microbubbles in the size range of 2–10 μm to form transient pores in cell membranes, has been of great interest in the past 15 years. This technique could be used to improve the delivery of current drugs in very localised regions. There are several phenomena behind sonoporation that all occur non-exclusively: push, pull, jetting, inertial cavitation, shear and, translation. Pre-clinical work has shown that sonoporation can be used to reduce primary tumour burden and inhibit metastatic development. Our clinical trial showed that ultrasound in combination with microbubbles and chemotherapy can effectively double the number of chemotherapy cycles patients can undergo, meaning that the patients were healthier for a longer period of time. Nevertheless, sonoporation is still in its infancy and there is vast room for improvement in both the areas of microbubbles and ultrasound.

I. INTRODUCTION

Therapeutic ultrasound has been in use since the early 1940s [1], primarily as method to heat deep tissue to promote healing. Nevertheless, in the past 15 years, due to advancements in technology, new ultrasound-based therapeutic modalities have surfaced; specifically High Intensity Focused Ultrasound (HIFU) and sonoporation. HIFU is used to heat targeted tissue in very specific regions to such a high temperature that thermal necrosis occurs. This is often done with the aid of Magnetic Resonance Imaging to visualise the target tissue and measure the thermal deposition [2]. Sonoporation on the other hand aims to solve the problem of time and space controlled drug delivery [3]. One of the major barriers in effective drug delivery is the cell membrane; it acts as wall selectively choosing what to allow in and out of the cell [4]. Sonoporation can be defined as the *the transient formation of pores in a cell membrane due to ultrasound or a combination of ultrasound and microbubbles*. By forming pores in the cell membranes we can effectively bypass this barrier that is the cell membrane, resulting in highly efficient targeted drug delivery. In our work we explore the phenomenon of sonoporation, from the lab bench all the way to the World’s first clinical trials.

II. MICROBUBBLES IN A SAFE CLINICAL SETTING

The first clinical use of microbubbles was reported in 1969 [5]. Microbubbles in the blood pool are used to improve the signal-to-noise ratio or contrast of blood when imaged

TABLE I
CLINICALLY APPROVED ULTRASOUND CONTRAST AGENTS.

Contrast agent	Shell	Gas	Size range $\varnothing(\mu\text{m})$	Manufacturer
Optison [™]	Albumin	C ₃ F ₈	2.2–4.5	GE Healthcare
SonoVue [®]	Lipid	SF ₆	2.5	Bracco Int.
Definity [®]	Lipid	C ₃ F ₈	1.1–3.3	Lantheus Medical Imaging
Sonazoid [®]	Lipid	C ₄ F ₁₀	1.9–2.4	Daiichi Pharmaceutical Co.

using B-mode sonography. Hence, these microbubbles are also known as ultrasound contrast agents. Their sizes range between 2–5 μm , typically with over 95% below 10 μm so that the microbubbles can pass through the lung capillaries. Microbubbles, like most physical objects, have a resonant frequency; a frequency where its oscillation amplitude is maximal. Similar to a bell, when a force strikes it, the microbubble will volumetrically oscillate and generate sound at its resonance frequency. For a free gas bubble this frequency can be approximated by [6]:

$$f_r \approx \frac{6.5}{D}, \quad (1)$$

where f_r is the resonant frequency and D is the diameter of the bubble. From this we can see that a 2.5 μm gas bubble will resonate at approximately 2.6 MHz, a typical diagnostic ultrasound frequency. Free gas microbubbles are inherently unstable, and dissolve almost instantly. For this reason clinical diagnostic ultrasound contrast agents typically have a lipid or albumin shell with a slowly diffusing gas core, increasing stability, allowing for efficient clinical imaging. Table I summarises clinically approved ultrasound contrast agents and their contents.

In clinical diagnostic ultrasound imaging, the Mechanical Index (MI) gives an indication of the mechanical damage possible due to inertial cavitation when an ultrasound contrast agent is present. It is defined by:

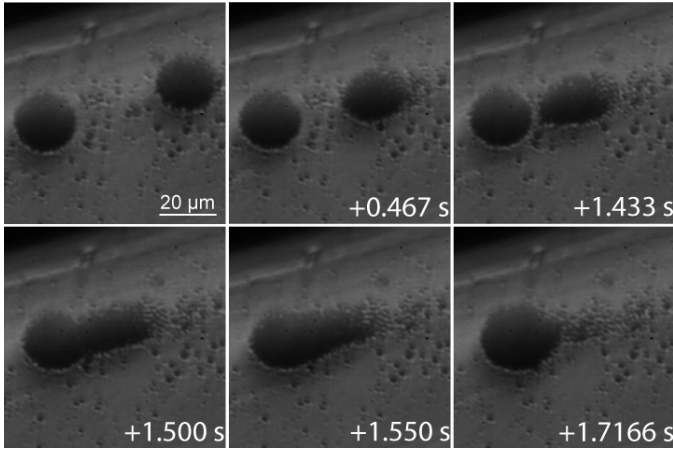


Fig. 1. Photo micrographs depicting clusters of Definity[®] microbubbles attracting and merging in under 2 seconds.

$$MI = \frac{p^-}{\sqrt{f_c}}, \quad (2)$$

where p^- is the peak-negative acoustic pressure in-situ measured in MPa, and f_c is the centre frequency in MHz. An $MI < 0.3$ is considered safe, an MI between 0.3 and 0.7 is considered moderately safe as there is a risk of damage to neonatal lung tissue or intestine, and an $MI > 0.7$ there is a high risk of inertial cavitation of the ultrasound contrast agents, and a theoretical risk of cavitation formation without ultrasound contrast agents present [7]. For this reason, in all our work, we attempt to work at $MI \leq 0.2$, ensuring no destructive and currently uncontrollable inertial cavitation is present and allowing for faster transition from lab to clinic.

The first step in being able to induce localised sonoporation was to attempt to control the location of the microbubbles [8]. A custom made experimental setup incorporating a 2.2 MHz ultrasound transducer, a synthetic $\varnothing 200\text{-}\mu\text{m}$ capillary and a high-speed camera was built on top of an inverted microscope. Continuous-wave ultrasound with centre frequencies of 2-MHz and 7-MHz with peak-to-peak acoustic pressures of 20 kPa were used in conjunction with Definity[®] ultrasound contrast agent. Upon insonation, the microbubbles were seen to attract each other forming small spherical clusters of microbubbles. These clusters of 1–2 thousand microbubbles would form within several seconds, and were spaced $\frac{1}{4}\lambda$ apart. As the attracted microbubbles were oscillating in phase, when continuing sonication, the clusters were attracted to each other, forming even larger clusters (*c.f.* Fig. 1). When increasing the acoustic pressure the clusters could be radiated towards the membrane wall. These results indicated that if necessary, microbubbles could be accumulated to specific regions and radiated toward a vascular wall.

III. *In-vitro* WORK

In-vitro work has been the hallmark for evaluating cellular effects for years. In our work, we attempted to understand why

specific acoustic conditions were optimal for increased drug delivery.

As can be expected, injecting a gas bubble into the blood stream, that generates a detectable acoustic force, may have some inherent side-effects. In 1997, upon studying such side-effects [9] and [10] showed that ultrasound in combination with microbubbles could increase the uptake of fluorescent genes.

Following this exciting discovery, several research groups, both experimental and theoretical rushed to understand the physical behaviours behind this increased uptake. It accepted that the oscillatory behaviour of the microbubbles under sonication forms small transient pores in the cell membrane, yet the question of *how* still remained.

Hence, the next step was to attempt to understand the physical mechanisms of microbubble-cell interaction using optimised acoustic conditions. Whilst a vast majority of high-speed imaging is performed at several million frames per second, we attempted to record at slower frame rates giving us longer imaging duration but with sufficient temporal resolution to track microbubble motion. For this work we used immortalised cervical cancer cells (HeLa cells). 1.6×10^6 cells were seeded into an OptiCell[™] and incubated for 24 hours. Following incubation, low concentrations of DiD lipophilic fluorescent probe (Vybrant[™] Molecular Probes) tagged lipid shelled microbubbles, Definity[®], SonoVue[®] or MicroMarker[™] (Bracco) were injected into the OptiCell[™]. Concentrations of 1 bubble to 1 cell were aimed for. The OptiCell[™] was then placed in a water bath cell-side up, ensuring the floating bubbles were in contact with the cells. Two ultrasound frequencies were evaluated: 1.0 MHz and 6.6 MHz with duty cycles of 40% and 50%, respectively. The MI for both frequencies was ≤ 0.2 and acoustic intensity (I_{SPTA}) was $\ll 1 \text{ W/cm}^2$. Imaging was performed using a high-magnification, high-numerical aperture (NA) setup using a $60\times/0.90\text{-NA}$ objective or using a confocal fluorescence microscope with a $40\times/1.30\text{-NA}$ objective. Following sonication, a range of phenomena was observed, including bubbles hammering back and forth into cells [11], to complete bubble translation into a cell. To validate if the microbubble had truly translated into the cell, 3D confocal fluorescence imaging was performed. Results showed that the lipid bonded fluorescent probe was then within the cell. To exclude the chance of endocytosis, experiments were re-done with endocytosis inhibitors where no change in localised microbubble translation was seen.

In conclusion, to date there are several non-exclusive phenomena that can be directly related to this increased uptake [12]: *Push and pull*, where the microbubble near, or in contact with the cell, stretches the bilayer membrane opening pores. *Jetting*, when a bubble collapses asymmetrically, making a needle-like point towards the cell. *Streaming*, where the fluid flow around the bubble near a cell is strong enough to shear the membrane apart. *Translation*, where a microbubble with a therapeutic load can be forced inside a cell. *Inertial cavitation*, where the violent formation

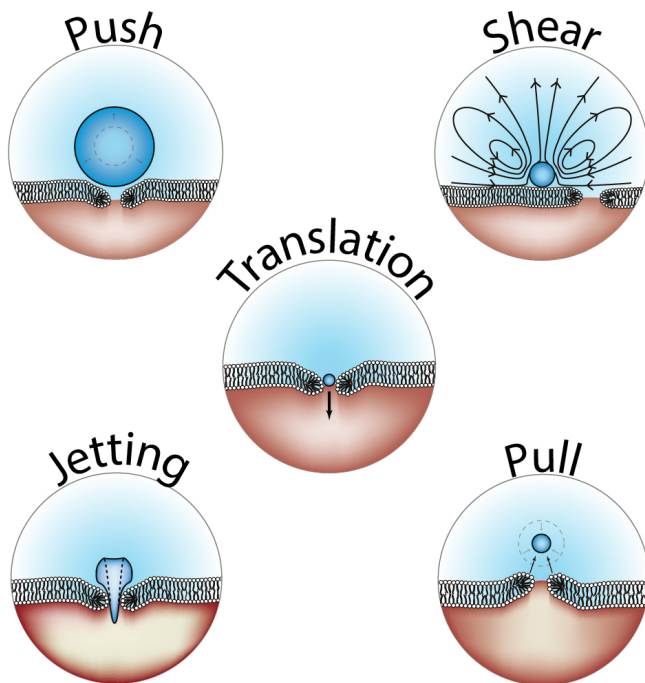


Fig. 2. Illustration depicting five of the sonoporation mechanisms.

157 and destruction of bubbles generates shock-waves damaging
 158 everything in the local area. It is important to understand
 159 that all these phenomena are non-exclusive, and each and
 160 all have advantages and disadvantages. In addition, when
 161 sonoporation will occur most effectively when exciting the
 162 microbubbles at their resonance frequency. Figure 2 illustrates
 163 five of the sonoporation mechanisms that induce transient cell
 164 membrane porosity. In addition to these phenomena there are
 165 several controversial yet thought-provoking theories regarding
 166 the acoustic activity of the cell membranes themselves [13].

167 IV. PRE-CLINICAL WORK

168 Pre-clinical mouse work was performed to evaluate the
 169 effect of sonoporation in combination with a chemotherapeutic
 170 on an orthotopic pancreatic ductal carcinoma (PDAC) model
 171 [14].

172 The orthotopic xenograft model was developed by injecting
 173 1×10^6 bioluminescent human PDAC cells (MIA PaCa-2^{luc})
 174 into the head of the pancreas of immunodeficient (NOD-scid
 175 IL2 γ^{null}) mice. The bioluminescent cells allowed for 2D full
 176 body imaging helping visualise the spread of the tumour cells.
 177 The 3D development of the primary tumour was measured
 178 using high-resolution 3D ultrasound (MS250 probe and Vevo
 179 2100, VisualSonics Inc, Ontario, Canada).

180 Mice were imaged and treated once weekly using Gemzar
 181 (Eli Lilly and Company, Indianapolis, IN) the current
 182 best chemotherapeutic for treating PDAC, or Gemzar in
 183 combination with ultrasound and SonoVue[®] microbubbles.
 184 Acoustic conditions remained the same as in the *in-vitro* work,
 185 where bubbles were forced into the cells, *i.e.*, 1-MHz, 40

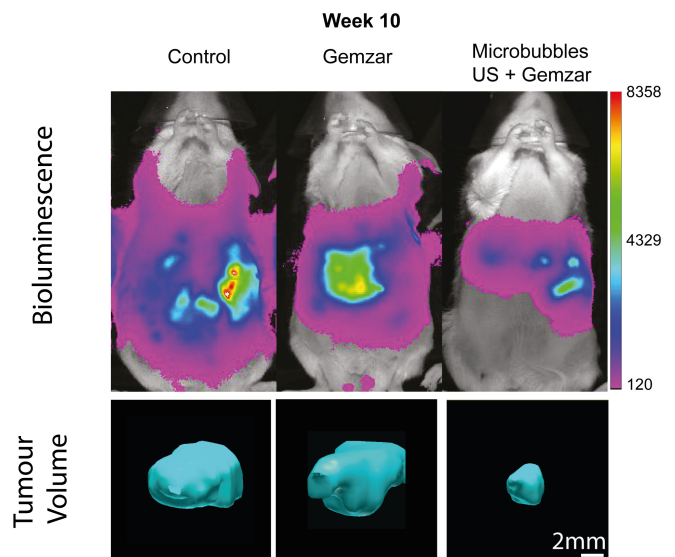


Fig. 3. Bioluminescence imaging and tumour volume of mice with orthotopic pancreatic cancer treated with Gemzar alone or Gemzar with microbubbles and ultrasound.

186 cycles, MI=0.2, with a 40% duty cycle. Treatment was started
 187 3 weeks after xenografting.

188 After only two treatments, *i.e.*, 5 weeks after xenografting,
 189 a statistical significant difference in tumour volume was seen
 190 between the sonoporated group and the chemotherapy alone
 191 group. Figure 3 shows the bioluminescent images and tumour
 192 volumes 10 weeks after xenografting (after six treatments).
 193 By week 10, the primary tumour in the sonoporation group
 194 was nearly 4 times smaller than the control and chemotherapy
 195 alone tumour. In addition the metastatic spread was limited
 196 to the liver in the sonoporation group whereas the Gemzar
 197 and control group showed whole body metastatic spread.
 198 In conclusion, translating the identical acoustic conditions
 199 seen to force microbubbles into cells *in vitro* resulted in
 200 primary tumour inhibition and delayed metastatic spread when
 201 combining a chemotherapeutic.

202 V. CLINICAL TRIALS

203 In many cases pre-clinical work does not translate well into
 204 the clinic. In our work here we perform, to our knowledge,
 205 the world first clinical trial on sonoporation [15].

206 A total of five patients with PDAC were treated using
 207 Gemzar, SonoVue[®] and ultrasound. The chemotherapeutic
 208 was administered in accordance to the manufacturer's
 209 guidelines; infusion over 30 minutes. At the completion
 210 of the infusion, the concentration of the chemotherapeutic
 211 was maximal in the blood. At this point sonoporation was
 212 initiated. Ultrasound was generated using a clinical diagnostic
 213 scanner (GE LOGIQ 9 + 4C probe, GE Healthcare, Waukesha,
 214 WI), with optimised acoustic output conditions. The clinical
 215 diagnostic machine allowed for simultaneous imaging and
 216 treatment of the primary tumour. The output frequency was
 217 1.8MHz with a duty cycle of 1%. To compensate for the

218 reduced duty cycle the ultrasound treatment lasted 31.5 min.
219 SonoVue® were injected intravenously every 3.5 minutes
220 resulting in 9 doses of 0.5 mL each over 31.5 minutes.

221 To evaluate the effect of the treatment, we compared the
222 number of chemotherapy cycles our patients could undergo
223 versus 80 historical patients treated at the same hospital. The
224 healthier the patients are, the more treatment cycles they
225 can undergo. The historical group was able to receive 9 ± 6
226 treatment cycles. The patients treated with sonoporation were
227 able to undergo almost double the amount of treatment with
228 16 ± 7 cycles of chemotherapy. It is important to note that
229 several patients were removed from the study due to the
230 efficacy of the treatment. These patients would be able to go
231 substantially more cycles, hence the treated cycles number is
232 artificially low.

233 Maximum tumour diameters were measured using CT
234 imaging from independent radiologists. The primary tumour
235 growth was inhibited and in some cases, even reduced.

236 In conclusion, the clinical trials showed that there are
237 no immediate adverse side effects to sonoporation, and that
238 sonoporation can help increase the efficacy of the treatment.

239 VI. FUTURE WORK

240 Whilst sonoporation has shown success in the lab and in
241 the clinic, there is still a lot of room for improvement. To
242 date, sonoporation has primarily been performed by combining
243 existing materials and technologies aimed for other tasks, for
244 example the ultrasound contrast agents and clinical diagnostic
245 machines are designed for imaging not treatment. Both these
246 aspects need to be drastically improved.

247 A. Microbubbles

248 In the area of microbubbles, new agents need to be
249 developed that can carry a therapeutic load to the target region.
250 These bubbles need to be highly acoustically sensitive, and be
251 able to release their load on demand. Current concepts allow
252 loading inside the shell, or on the surface of the shell, hence
253 only small loads can be carried. New methods and concepts are
254 needed that allow for larger volumes of therapeutic loads and
255 specific release mechanisms. Such a method would only treat a
256 localised region, and would have minimal affect on metastasis,
257 for this reason, it may be ideal to combine sonoporation with
258 traditional chemotherapy, ensuring maximum affect on both
259 primary tumour and metastasis.

260 B. Ultrasound

261 Clinical diagnostic machines are highly specialised and have
262 inverse acoustic properties than those needed for sonoporation.
263 Sonoporation requires a large area to be treated, so a large
264 footprint probe, that can treat a whole tumour is necessary. In
265 addition, longer duty cycles are required to continuously excite
266 the microbubbles in the target region increasing the cellular
267 permeability.

VII. CONCLUSION

268 Although sonoporation was discovered only in 1997 and
269 clinical trials were first done from 2013, we have shown
270 that there is great potential in sonoporation. *In-vitro* results
271 have shown that microbubbles can be forced into cells,
272 whereas pre-clinical mice studies under identical conditions
273 have shown to reduce primary tumour burden and metastatic
274 development. Our clinical trial showed that combining
275 sonoporation and chemotherapy can help the patients to
276 undergo more chemotherapy cycles, presumably prolonging
277 survival.
278

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286

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