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## **Sonoporation: mechanistic insights and ongoing challenges for gene transfer**

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### **Abstract**

Microbubbles first developed as ultrasound contrast agents have been used to assist ultrasound for cellular drugs and gene delivery. Their oscillation behavior during ultrasound exposure leads to transient membrane permeability of surrounding cells, facilitating targeted local delivery. The increased cell uptake of extracellular compounds by ultrasound in the presence of microbubbles is attributed to a phenomenon called sonoporation. In this review, we summarize current state of the art concerning microbubble-cell interactions and cellular effects leading to sonoporation and its application for gene delivery. Optimization of sonoporation protocol and composition of microbubbles for gene delivery are discussed. We also outline ongoing challenges for clinical applications.

**Key words:** ultrasound; microbubbles; physical gene delivery method; gene therapy



## 1. Introduction

The principle of gene therapy is to introduce gene or nucleic acids into cells to cure genetic deficiencies. The success of gene therapy obtained with the use of viral vectors demonstrates unambiguously the feasibility of this innovative therapy [1-3]. To date, viral vectors remain the best vehicles to introduce genes into cells. Nevertheless, there are still drawbacks inherent to the use of viral molecule observed in gene therapy clinical trials raising serious safety concerns [4, 5]. In addition, size limitation capacity, cell targeting and manufacturing issues are still difficult to handle despite tremendous progresses made on viral vector bioengineering. Therefore, there is still some room for the development of alternative approaches of high safety, low immunogenicity and easy manufacture. This last decade, many efforts have been done to search for non-viral options. The goal is to design synthetic gene delivery systems that incorporate viral-like features to transfect efficiently cells [6-8]. Among non-viral systems, chemical vectors are the most widely used. These vectors have to face on several extracellular and cellular barriers to reach efficiently the target cells. One of the main challenges is the lack of selectivity towards target tissues explaining their narrow therapeutic index. Lack of specificity causes high toxicity which hampers the efficacy at the target site. For that, designing an efficient targeted delivery strategy is of importance to further improve the delivery systems while reducing side effects. To target chemical vectors, it is possible to couple them with ligands specific to receptors present at the cellular surface of target cells. A second option is to use an externally applied trigger to control the gene delivery in the targeted area. These two strategies are not mutually exclusive and could be combined. Physical trigger can be used either alone or combined with chemical or viral vectors to improve the targeting and/or gene expression efficiency. There are several physical methods

starting from hydrodynamic injection to more sophisticated systems such as electroporation-based on electric fields or ultrasound-mediated delivery.

## **2. Ultrasound as physical method for delivery**

Ultrasound can be used for imaging (ultrasonography) and for physical therapy (pulsed ultrasound mode) [9, 10]. These last years, therapeutic applications of ultrasound have gained new interests as a result of its exploitation for drug or gene delivery. Depending on the energy delivered by ultrasound, two types of effects can be produced either thermal or non-thermal each of them having their own application. High ultrasound intensities produce heating due to the absorption of acoustic energy by tissues; this property is employed in high-intensity focused ultrasound (HIFU) surgery or ultrasound-based physiotherapy. "World Federation for Ultrasound in Medicine and Biology Temperature" has stated that an elevation of 1.5°C is considered safe while an elevation of 4-5°C during 5 minutes could be dangerous [11]. At low ultrasound intensities, cavitation and mechanical streaming are the main non-thermal effects obtained. These effects can induce some benefits as tissue healing or ultrasound-mediated delivery. Inertial cavitation is the process of formation, oscillation and collapse of gaseous bubbles driven by an acoustic field [24]. The presence of preformed microbubbles in the environment allows reducing the threshold of energy needed for cavitation. Used as external trigger, ultrasound permits to spatiotemporally control the release of drug encapsulated in microbubbles or in their surrounding in a non-invasive manner [12-18].

### 3. Microbubbles and sonoporation

Microbubbles are gas-filled particles consisting of a gas core encapsulated by a stabilizing shell. They have been first developed as ultrasound contrast agents to differentiate blood and their surroundings under ultrasound due to their low acoustic impedance difference. When microbubbles are driven by ultrasound at a frequency close to their resonance frequency, they oscillate and produce sound [19, 20]. These oscillations lead to an increased permeability of surrounding cells allowing a targeted local drug delivery. The increased cellular uptake has been attributed to the formation of transient pores in the cell membrane facilitating trans-membrane transport of drugs into the cell [21-24]. This transient permeabilization of a cell membrane is called sonoporation.

#### 3.1. Microbubble-cell interactions

Microbubble interactions with cells under ultrasound are the key step for the sonoporation process. To date, five types of interactions have been described (Figure 1). Microbubble oscillations near a plasma membrane could lead to a cell “massage”, the “push and pull” phenomena [25, 26] and microbubble jetting through the plasma membrane [27]. This latter is less likely to be the dominant mechanism as shown by *in silico* studies, *in vitro* by high-speed optical observations, and *in cellulo* experiments [28]. A microbubble attached to a cell membrane, could also create enough shear to rupture the membrane due to the fluid streaming around the oscillating bubbles. Recently, we have observed a new event: a penetration of microbubble into a cell during sonoporation process under a specific ultrasound setting [29]. The tracking of fluorescent-labeled microbubbles inside cells after sonoporation has proved this phenomenon. Figure 2 shows two microbubbles (black circle) entering into a cell during ultrasound stimulation, one microbubble was pushed

toward the cell deforming the plasma membrane before its cellular penetration. While entering, the microbubble underwent shrinking. This phenomenon took place 6.2 seconds after ultrasound stimulation and 850 ms later, microbubble penetrated inside the cell. This event is slow, compared to the other phenomena described above. Indeed, jetting occurs in the millisecond time scale. Fluorescent microbubbles were observed inside the cells after sonoporation proving their penetration (figure 2C, black arrows). However, it is hard to state if the microbubble has only fused with the plasma membrane or if the structure of the microbubble was preserved after entry. Nevertheless, these results reinforce the strategy on using microbubbles as drug or gene carriers.

We have also tracked the early interactions between cells and microbubbles at the beginning of the ultrasound stimulation [29]. In this study, we have used several phospholipid-based microbubbles (SonoVue®, Micromarker™, Definity®). Before ultrasound stimulation, microbubbles were randomly distributed all around cells. When ultrasound was turned on, microbubbles immediately interacted with each other forming small clusters (Figure 3) as described by Kotopoulos *et al.* [30]. These clusters could be observed approximately 15 ms after ultrasound application. Interestingly, every cluster present in the field seemed to be attracted to cells and was found at the vicinity of their plasma membrane several seconds after ultrasound stimulation independently of the ultrasound field direction. It is worth noticing that we did not observe such behavior for hard-shelled microbubbles like Quantison™ with the same ultrasound settings. Quantison™ microbubbles interacted also to each other by clustering but they moved only in the direction of the ultrasound field. Since sonoporation is based on microbubble and cell interactions, these observations indicate the importance of microbubble features (composition and acoustic activity). It

also arises the question if microbubbles are attracted to specific areas of the plasma which could be worth to be determined.

### *3.2. Cellular effects of ultrasound application*

Pore formation has been reported to be essential for sonoporation-mediated delivery [23, 31, 32]. The presence of 100 nm-sized cell membrane pores was observed by electron microscopy and confirmed by flow cytometry with the use of 75 nm-sized fluorescent nanospheres. Using an electro-diffusion model, Zhou and colleagues have evaluated the pore size at  $110\pm 40$  nm during sonoporation process in the presence of Definity® microbubbles [22]. Concomitant to pore formation, a transient intracellular calcium entrance has been observed during ultrasound application in absence or in presence of microbubbles [21, 33-39]. This intracellular calcium entrance has been supposed to promote the pore closure [21] and to induce the endocytosis and exocytosis processes [40, 41]. The pore closure time has been estimated at 5 seconds after the ultrasound stimulation by flow cytometry and patch clamp experiments [22, 23]. Several studies have reported the pore formation after sonoporation, however, it is still not known if these pores are directly responsible of the drug entry. In addition, a production of hydrogen peroxide ( $H_2O_2$ ) was detected after sonoporation and it seems to be important for the calcium entrance hence for the pore formation [42]. Some reports have suggested that this  $H_2O_2$  production could be related to the cavitation phenomenon [38, 43, 44].

Another cellular effect observed during sonoporation was an hyperpolarization state of cells that can occur in absence [38] or presence of microbubbles [21, 45, 46]. This hyperpolarization is directly attributed to the opening of the  $BK_{Ca}$  channels [45, 46]. It is related to a mechanical stress of the plasma membrane similar to that obtained when applying a mechanical pressure on the membrane using a glass



probe [47]. Both hyperpolarization and calcium signaling could increase macromolecules uptake. Besides these phenomena, we have observed an outward transport of small intracellular molecules likely due to membrane destabilization [24]. A transient release of small molecules such as enhanced green fluorescent protein (eGFP) was observed from the cytosol of HeLa cells stably expressing eGFP gene while preserving cell viability. These results reinforce the hypothesis of transient pore formation induced by sonoporation.

### 3.3. Cargo size and cellular localization

Sonoporation has been used to deliver drugs *in vitro* [48, 49] and *in vivo* [50]. The presence of microbubbles is a requirement for an efficient delivery. The delivery mechanism by sonoporation appeared to be dependent on the size of the molecules to transfer. The cell membrane permeabilization and viability are highly dependent on the ultrasound parameters used during sonoporation [51]. Following sonoporation, fluorescent labeled molecules have a different localization profile depending on their molecular size [39]. Dextran with small size as 3 kDa has a diffuse localization in the cytoplasm and in the nucleus whilst 70 kDa dextran was only found in the cytoplasm. For dextrans with size over 150 kDa, they were observed as patchy structures localised in the cytoplasm suggesting either aggregates in the cytosol or localisation inside endosomes (Figure 4). After sonoporation, a similar localization was observed for plasmid DNA of 7.5 kb which has an estimated size of approximately 4.95 MDa. Figure 5 shows the localization of fluorescent-labeled plasmid DNA 10, 30 and 45 minutes post sonoporation. Ten minutes after sonoporation, plasmid DNA was still found at the plasma membrane. It went closer to the nucleus from 30 to 45 minutes. These observations suggest a slow plasmid DNA trafficking inside the cells. Taking

into account that naked plasmid DNA injected inside the cytosol does not diffuse at all [52]. It is likely that these fluorescent spots corresponded to plasmid DNA located inside endosomes. Overall, these data indicate that pore formation is not the unique mechanism occurring during sonoporation and that endocytosis might also be involved in the uptake [39]. However, it is still not clear if the type of mechanism(s) involved could be both dependent on the microbubble chemical composition and on the type of tissue insonified.

#### **4. Optimization of gene delivery**

The number of publications relative to the use of sonoporation for gene delivery gives evidence of the potentiality of this method. Ultrasound-enhanced gene delivery has been successfully demonstrated both *in vitro* and *in vivo* [53-55]. The efficacy is dependent on acoustic parameters, the presence of microbubbles and the local concentration of plasmid DNA. Commercial microbubbles as well as customized bubbles lipid-or polymer-shelled have been used in those studies. In most cases, plasmid DNA was not complexed with microbubbles rendering it prone to degradation. As a consequence, the level of gene expression is still not as much as expected even though it is sometimes 1 or 2 orders of magnitude higher than the level obtained with plasmid DNA alone [53, 54]. Note that it is rather difficult to make comparisons between different reported studies because of the ultrasound set-up and acoustic conditions diversity used by each group.

Ultrasound-enhanced gene transfer has been successful, both with reporter and therapeutic genes *in vitro* [43, 56, 57] and *in vivo* [58-61]. Biological effects and high spatial targeting of transgene expression were achieved but again not as high as that obtained with electroporation for instance. Still, the specific targeting of the gene expression observed upon local or systemic administration in addition to low

toxicity demonstrates the potentiality of this delivery system [62, 63]. Our recent data showed remarkably that it is possible to get an efficient and sustained gene transfer up to 100 days in Achilles tendons by BR14 lipid shelled MB as shown in figure 6 [58, 59]. Optimized gene transfer was obtained with 1 MHz US frequency, 200 kPa and 40% duty cycle in the presence of 10 µg plasmid DNA and BR 14 microbubbles. The level of gene transfer was 130-fold more than that obtained with naked plasmid DNA. The level of gene expression obtained here was as good as with adenoviral vectors in tendons highlighting the potential of this system [64, 65].

In addition to acoustic parameters and microbubbles, sonoporation protocols could also be optimized to achieve a good gene transfer. After optimizing acoustic parameters and set-up to transfect HeLa cells, we have assessed if the number of ultrasound applications could improve the gene transfer efficiency since microbubbles interacted with cells and vanished in few seconds. Optimal ultrasound parameters found were 1 MHz frequency, 40% duty cycle, 10 kHz pulse repetition frequency, 150 kPa during 60 seconds in presence of 2.5 µg of plasmid DNA encoding the GFP and 5 µl of Micromarker microbubbles. Gene transfer efficiency was evaluated by flow cytometry 48 hours after sonoporation on HeLa cells. As shown in figure 7, when the ultrasound stimulation was made sequentially in more than one step (the total amount of microbubbles and plasmid DNA being equally dispatched in two or three sequential injections), the gene transfer efficiency was enhanced. Here, the ultrasound stimulation total time was preserved (1×60 sec, 2×30 sec or 3×20 sec). A two-times insonation enhanced by 2.2-fold the gene transfer efficiency (16.2% vs 7.3% for single exposure sonoporation) and a three sequential insonation improved the efficiency to reach 19.9% of GFP expressing cells. This effect could be explained by an improvement of the number of stimulated cells due to

a new supply of activable microbubbles. It is worth noticing that the mean fluorescence intensity of transfected cells was not significantly different between all sonoporation conditions validating this hypothesis.

## **5. Microbubbles features for efficient gene delivery**

While the range of size (mean diameter  $>1\mu\text{m}$ ) of microbubbles clinically used is appropriate for imaging, they are nevertheless too big and present a polydispersity unsuitable for therapeutic applications [66] (for extensive reviews see [18, 67-69]). Under ultrasound exposure, lipids are compliant to dilatation and compression and this renders gas-filled liposomes more echogenic. The development of acoustically active liposomes has been optimized (for a review [69]) but efforts have been mainly focused to get liposomes bubbles with a high acoustic response. These microbubbles are made of classical phospholipids without any specific feature. Recently, more complex molecular architecture of liposomes based microbubble have been described: bubbles liposomes that consist of PEG-modified liposomes that encapsulate perfluorocarbon gas enclosed in PEG-lipid micelles [70-72] and a hybrid particle made with microbubble loaded with liposomes that are made of thousands of small unilamellar biotinylated liposomes attached *via* avidin molecule to biotinylated microbubble [73].

To achieve improvements in the sonoporation method, the development of new microbubbles able to reach specifically the target and locally deliver the nucleic acid is also needed (figure 8). Targeting microbubbles with antibodies that can recognize a specific antigen present on a cell membrane has allowed microbubble binding even under flow [74]. For example, microbubbles targeted to the P-selectin and to the single chain VEGF were used to analyze the endothelium inflammation

[75] and to investigate tumor angiogenesis [76], respectively. These microbubbles mainly used in molecular imaging were only based on the acoustic properties and are not able to bind or carry nucleic acids.

Some studies have reported the use of cationic microbubbles to directly complex plasmid DNA [77-81]. They consist of gas-made cationic liposomes made with neutral classical phospholipids (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine ; DMPC or 1,2-distearoyl-*sn*-glycero-3-phosphocholine ; DSPC) and cationic lipids as (1,2-distearoyl-3-trimethylammoniumpropane). Two main strategies have been proposed to produce microbubbles able to bind nucleic acids: (i) cationic lipids-based microbubble allowing electrostatic interactions between the nucleic acid and microbubbles, (ii) compaction of the nucleic acid using polymers or liposomes linked to the microbubble by biotin-streptavidin interaction [82]. Despite a good acoustic response of these microbubbles, the level of gene transfer obtained in those studies was rather low likely due to different limitations as a large bubble size and the intracellular fate of delivered plasmid DNA. If in addition to pore formation, endocytosis could be also involved in sonoporation, the compartmentalization of therapeutic molecules, and most importantly genes, may affect their efficiency and should be taken into consideration. Therefore, it is necessary to rethink on microbubble composition and to pay more attention on the intracellular fate of both bubbles and their payload.

## **6. Conclusion**

The non-invasiveness of sonoporation renders it superior to other physical methods as electroporation. The combination of the ultrasound with targeted gas microbubbles as gene carriers holds great promise by offering a double targeting

controlling both gene release and gene transfer location [12-18]. Nevertheless, there are some challenges that have to be tackled to improve its efficiency. It is important to combine a judicious choice of microbubbles composition and plasmid DNA composition to deliver gene in a specific cell type. So far, cationic microbubbles did not reach the expectations in terms of gene delivery efficiency. This is not surprising because if plasmid DNA is internalized during sonoporation, it has to overcome the same cellular barriers to those encountered by chemical vectors [83]. Therefore, plasmid DNA must escape from endosomes and be routed inside the nucleus. This could be tackled by fine tuning microbubble composition and reducing their size.

Most importantly, we need to improve our knowledge concerning microbubble-cell interactions and how their impact on the gene delivery to fully exploit this method in a safe and efficient way. Since a mechanical stress is induced during ultrasound stimulation, it is of importance to evaluate carefully the impact of this stress on cell metabolism. This would allow us to establish a rational design of sonoporation protocols and to propose sonoporation for possible clinical applications.

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Figures

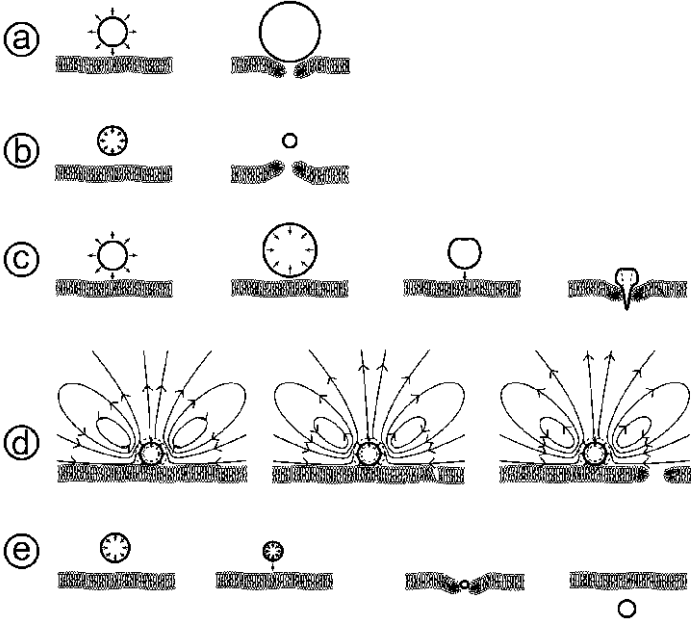


Figure 1. Five mechanisms of a cell membrane pore formation by an oscillating microbubble under ultrasound. (A) and (B) : push and pull, (C) : jetting, (D) : streaming and (E) : microbubble translation. Figure adapted from [29].

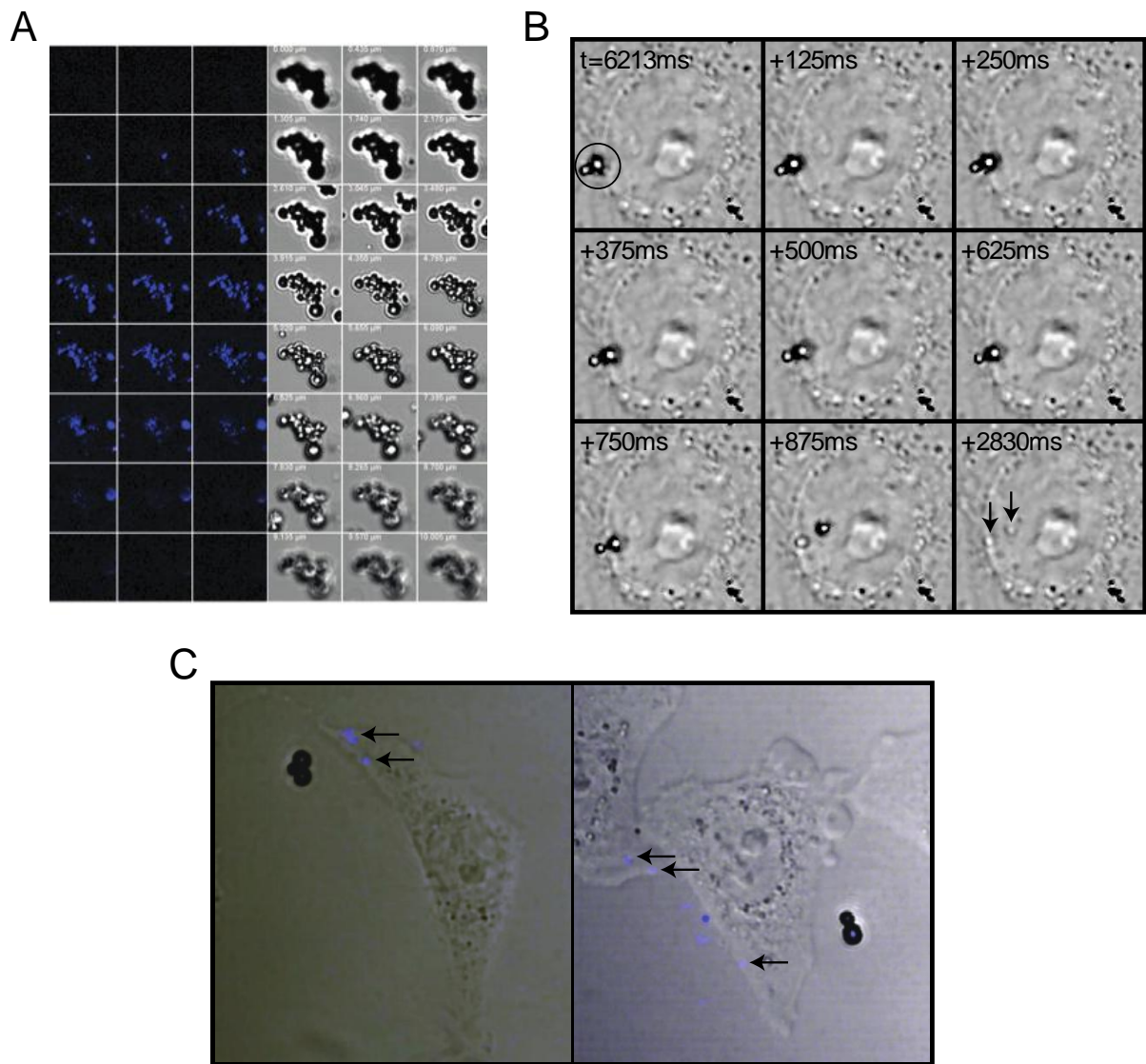


Figure 2. Microbubble entry under sonoporation. (A) Z-stack of fluorescent-labeled microbubbles observed by confocal microscopy. (B) High-speed imaging of two microbubbles (circle) close to a cell under 6.6MHz pulsed ultrasound. The microbubbles were seen to push the plasma membrane and entered (frame +875ms) in the cell until their disappearance (arrows). (C) Fluorescence images of HeLa cells after sonoporation, the fluorescence observed was due to DiD-labeled microbubbles entered in a cell (arrows). Intact microbubbles were still in the media after sonoporation.

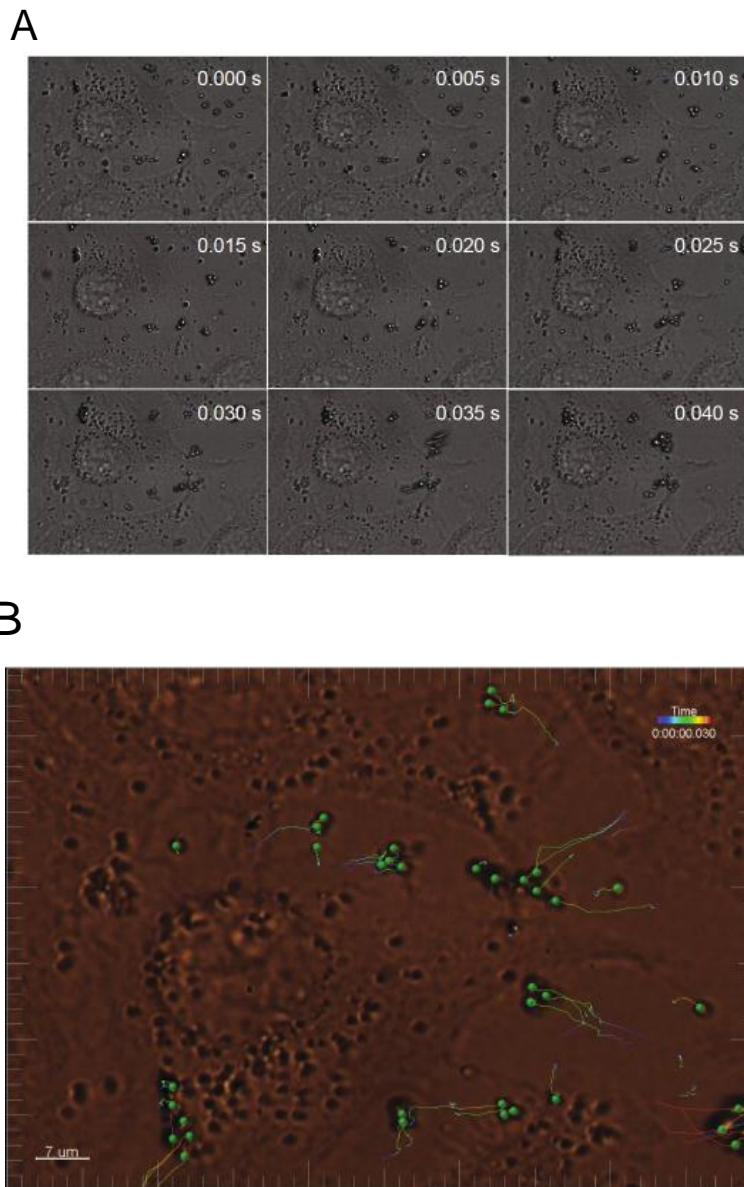


Figure 3. Microbubble-cell interaction during sonoporation. Observation of microbubble behavior during sonoporation by high-speed imaging (A). Microbubbles were randomly distributed before ultrasound (0s). After 15 ms of ultrasound stimulation, clusters of microbubbles could be observed around the cell membrane and all them were found in contact with plasma membrane after 40 ms suggesting a specific attraction between microbubbles and cells. Movements of microbubbles were analyzed using a tracking software (B). Microbubbles are represented by green circles, the line next to them corresponds to the analysis of their displacement during 30 ms of ultrasound.

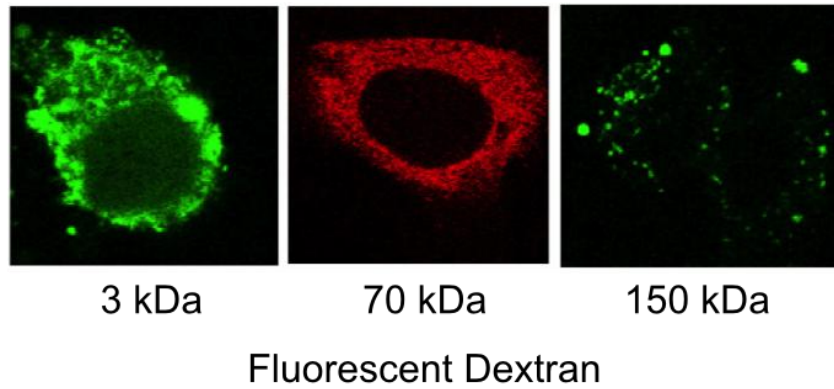


Figure 4. Influence of the cargo size on its cellular localization after sonoporation. Observation of the dextran of different molecular size (3, 70 and 150 kDa) in HeLa cells after sonoporation.

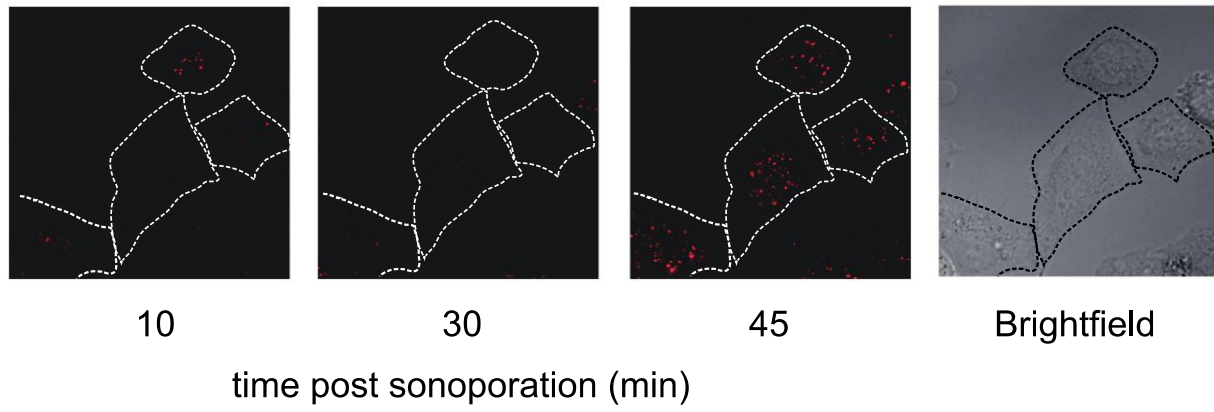


Figure 5. Plasmid DNA intracellular trafficking after sonoporation. Fluorescent-labelled pDNA (Cy3) was followed in HeLa cells after sonoporation during 45 minutes under confocal microscopy (dot lines correspond to cell boundaries).



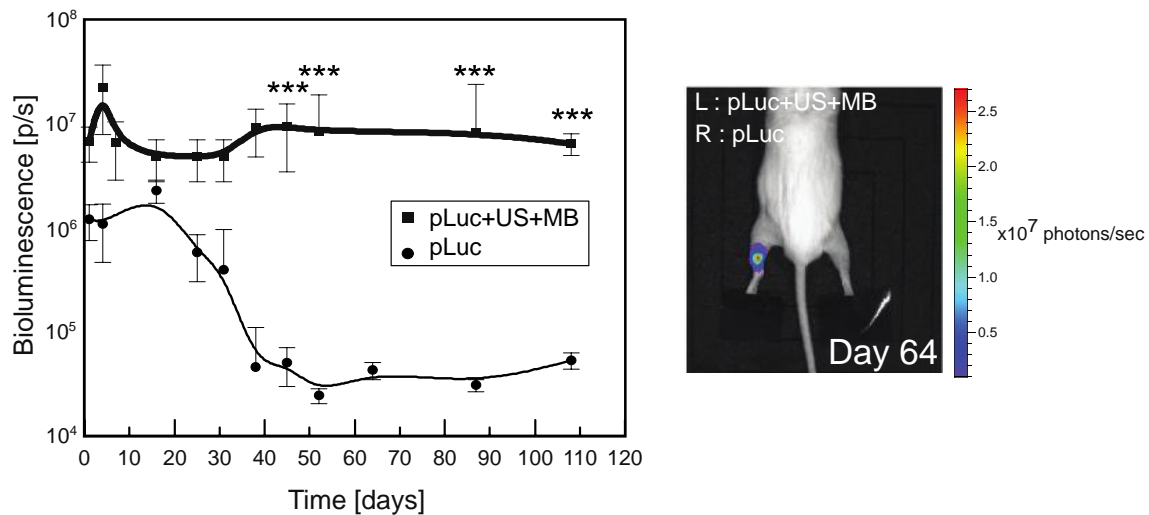


Figure 6. Kinetic of luciferase gene expression after sonoporation in mouse Achilles tendons. Mice Achilles tendons were injected either with 10  $\mu$ g pLuc only or 10  $\mu$ g pLuc mixed or not with  $5 \times 10^5$  of microbubbles (MB) followed by ultrasound (US) exposure at 1 MHz, 200 kPa, 40% duty cycle during 10 min. Bioluminescence signals (expressed in p/s: photons per second) that correspond to the luciferase activity were measured after luciferin injection in the tendon area. Luciferase gene expression of mice groups injected with either pLuc only (circle), pLuc mixed with MB and exposed to US (square). Values are means $\pm$ SEM. Data are representative of two experiments with five mice per group. (\*\*\*: p-value  $\leq$  0.001). Right panel: representative CCD image showing luciferase expression in tendons injected with pLuc mixed with MB and US exposed (left tendon) or pLuc only (right tendon). Adapted from [58].

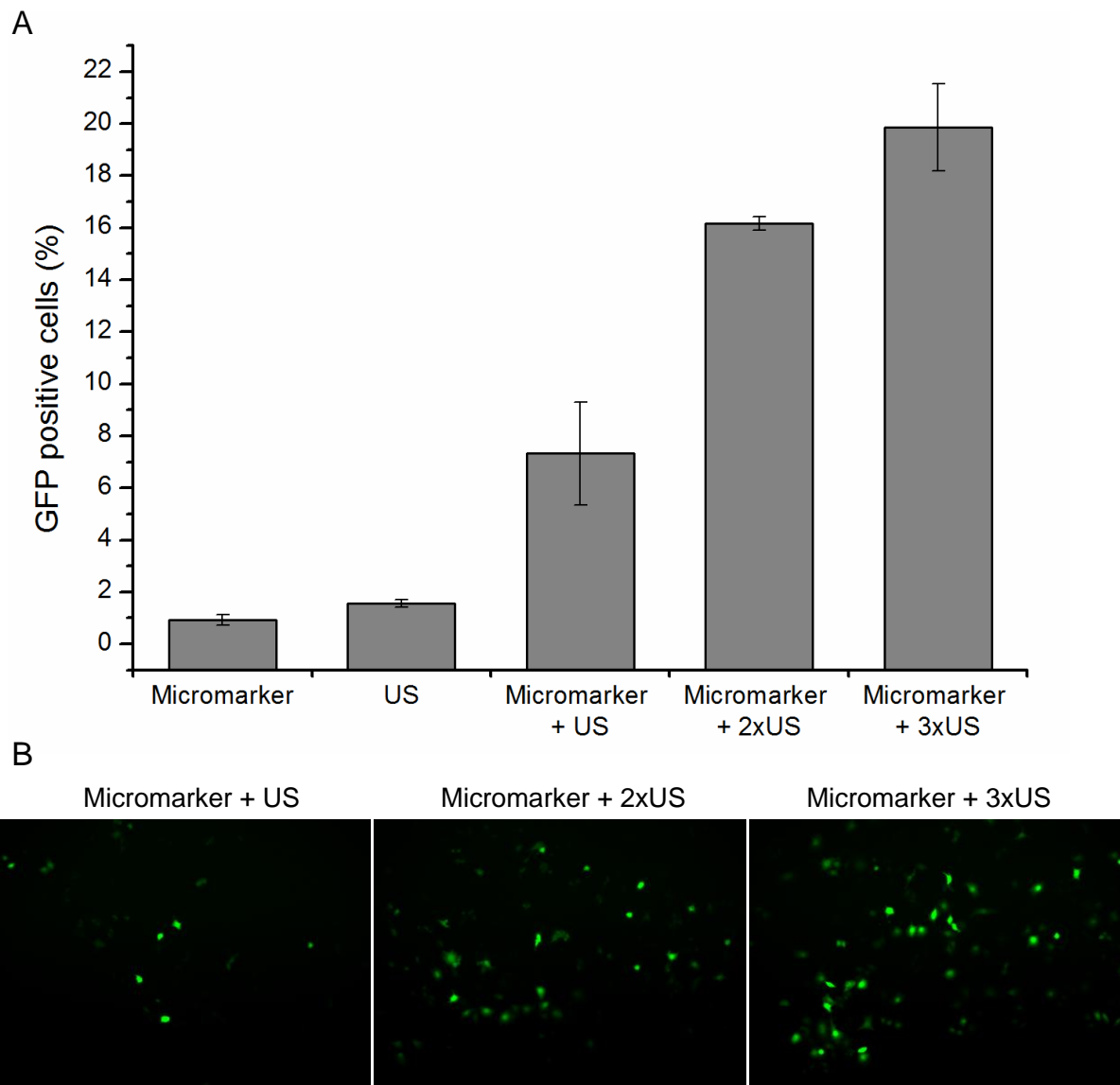


Figure 7. Gene transfer efficiency by sonoporation. Sonoporation was performed using 2.5  $\mu\text{g}$  of pGFP and Micromarker microbubbles using the following acoustic parameters: 1 MHz, 150 kPa, 40% duty cycle, 10 kHz pulse repetition frequency and 60 seconds total exposure time. (A) Percentage of transfected HeLa cells 48 hours after sonoporation. US: ultrasound, 2x: two-times insonation, 3x: three-times insonation. Data represents means  $\pm$  SD. (B) Representative fluorescence images of HeLa sonoporated cells in several conditions.

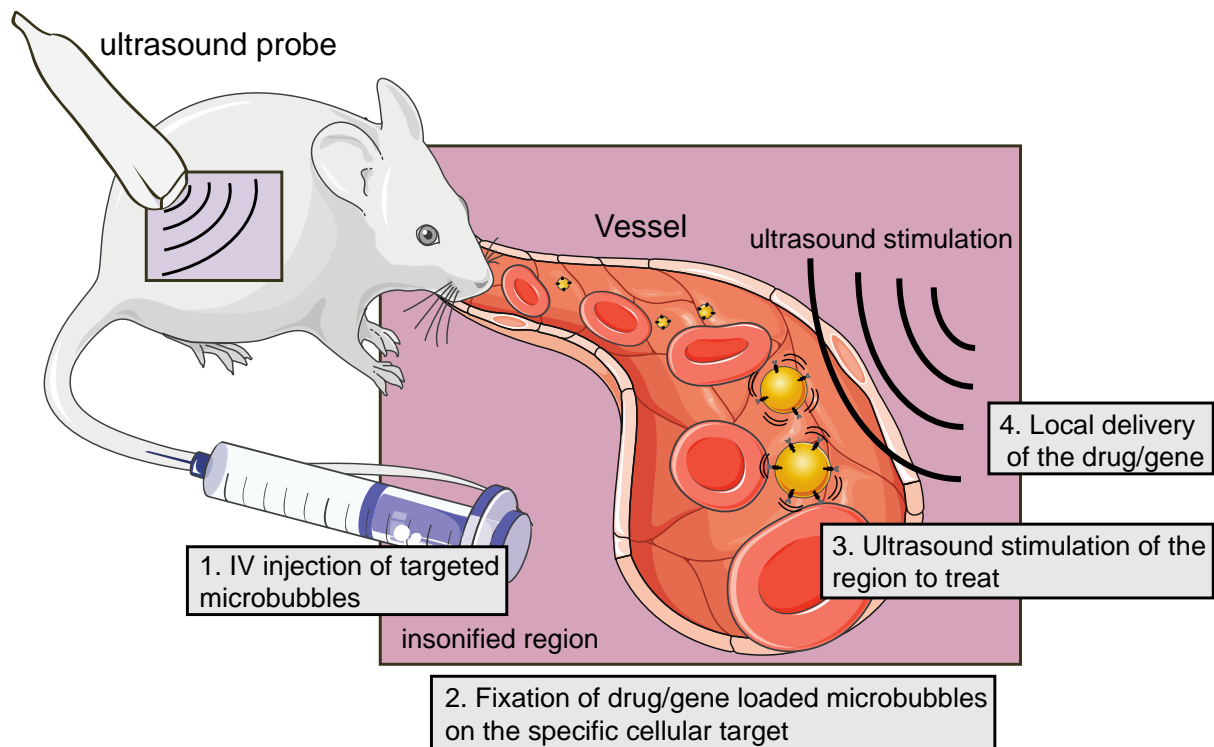


Figure 8. General overview of the sonoporation strategy for *in vivo* drug/gene delivery. The drug/gene loaded targeted microbubbles are injected in the systemic circulation. Targeted microbubbles can bind specifically to cells and ultrasound stimulation of the region of interest can activate these fixed microbubbles leading to the delivery of their payload through the plasma membrane or inside the cells depending on microbubbles composition.