EFSUMB Newsletter

European Federation of Societies for Ultrasound in Medicine and Biology

# Ultrasound and Microbubbles Assisted-gene Transfer: what is next?

The goal of gene therapy is to introduce a therapeutic nucleic acid material to cure genetic deficiencies and a large number of acute diseases. Exciting results from recent clinical trials demonstrate without doubt the promise of gene therapy. Despite the high gene transfer efficiency of viral vectors, there are still some drawbacks in their use due to their immunogenicity and mutagenesis features. Therefore, there is still room for non-viral methods to be developed since they are safer. However, gene delivery by non-viral methods is still a major challenge nowadays. The major limiting factor remains the lack of a suitable and efficient vector for gene delivery.

The challenge is to deliver the nucleic acid to the right intracellular compartment. Many efforts have been undertaken to identify the cellular barriers that have to be passed for this issue (**Fig. 1**). First, the nucleic acid has to be protected from nucleases in the extracellular compartment. Then the plasma membrane has to be crossed. There are two ways to enter in the cells; either a direct transfer in the cytosol through destabilization of the plasma membrane or via endocytosis process. This latter is the main way when chemicals delivery systems are used as carriers. Once internalized, nucleic acids particles end up inside endosomes where they must escape to reach the cytosol, where mRNA and siRNA or oligonucleotides can be translated and find their targets, respectively. In the case of plasmid DNA (pDNA), it must be imported into the nucleus where the expression machinery takes place. The size of pDNA limits its cytosolic motion and passive diffusion through pores of nuclear envelope. (**Fig. 1**).

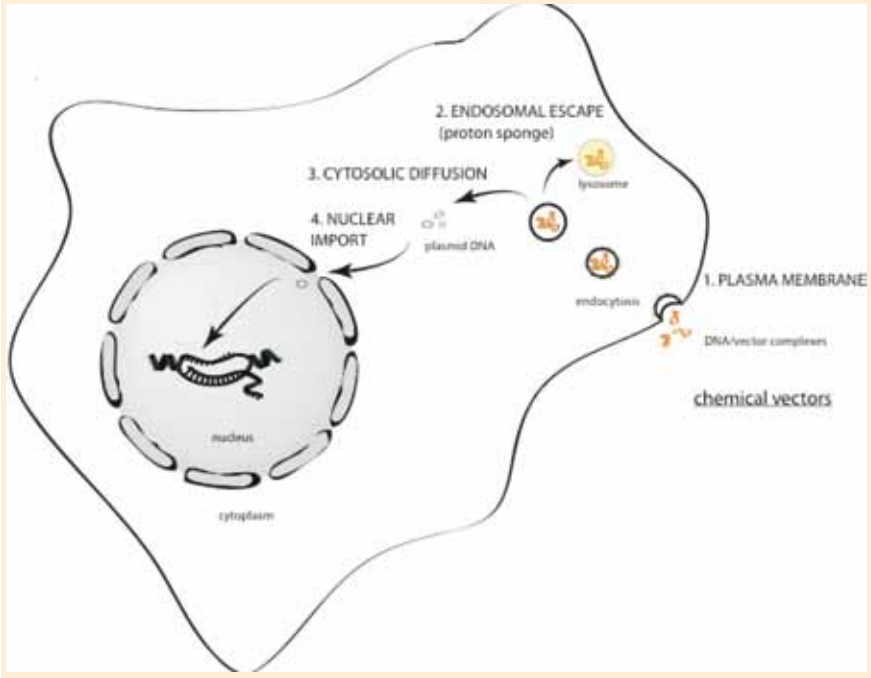


**Fig. 1** Main barriers for gene transfer by chemical vector: The first barrier is the plasma membrane. The main route of the DNA / vector complexes is the endocytosis, therefore the endosomal escape is the second barrier otherwise complexes would reach the lysosomes and can be degraded. Then, to be expressed the plasmid DNA has to be imported into the nucleus, this requires a cytosolic diffusion. The main barrier is the nuclear envelope which is highly selective since it does not allow the passive entry of molecules having a diameter size more than ~9 nm. Such molecules are able to be imported by active diffusion which involves the activity of nuclear import machinery via the recognition of specific nuclear localization signal (NLS).

In chemical based-methods, several strategies have been developed to bypass these limitations. Protection and stabilization of nucleic acids in extracellular medium have been achieved by nucleic acid condensation or encapsulation by chemical vectors. For the endosomal escape, strategies that exploit the proton-sponge effect enabling endosomal membrane destabilization have been proposed [1]. The nuclear importation of nucleic acid has also been improved by addition of devices bearing nuclear localization signal which can be put either on the chemical vector or the nucleic acid. Despite of all of these tremendous efforts, the efficiency of these chemical based-strategies is still far from that of the viral vectors.

In parallel, physical based methods for gene delivery have been developed. Electric, magnetic, light or ultrasound fields have been exploited as physical trigger. Among them, electrotransfer is one of the most used and efficient method but its invasiveness still hampers its wide application.

Twenty-five years ago, an alternative method for drug delivery based on ultrasound stimulation was proposed [2]. This method was proven to be more effective when coupled with gaseous microbubbles [3]. These micron-sized structures

**EFSUMB**

Lynne Rudd
36 Portland Place, London
W1B 1LS, United Kingdom
Tel: +44 (0) 20 7099 7140
Fax: +44 (0) 20 7436 7934
Email: efsumb@efsumb.org

containing gas encapsulated by elastic shell have allowed the improvement of ultrasound imaging. It has been found that microbubbles oscillations under ultrasound stimulation resulted in an increased permeability of surrounding cells. The increased uptake by ultrasound has been attributed to the formation of transient pores on the plasma membrane with a phenomenon called sonoporation which is amplified when microbubbles are present.

Several studies have been conducted these last years to delineate mechanisms involved in sonoporation [4, 5]. However, it is still ill-known how exactly cells that are subjected to ultrasound and microbubbles internalize extracellular compounds, and which cellular responses ultrasound and microbubble evoke. It was also suggested recently that besides transient pore formation, endocytosis mechanism might also be involved in the uptake during ultrasound-mediated drug / gene delivery [6, 7]. The mechanisms of sonoporation are summarized in **Fig. 2**. These results open a new research area. Indeed, the type of mechanism(s) involved in the delivery could be both dependent on the microbubble chemical composition, the type of drug to deliver and on the type of insonified tissue or cells. Improving the knowledge on both extracellular and intracellular fates of microbubbles and their cargo will be crucial to clearly specify limitations of this method. (**Fig. 2**)

Ultrasound enables control of both the drug release by activating the microbubbles and the delivery location by positioning the ultrasound probe in a specific area on the skin. The combination of the ultrasound trigger effect with targeted gas microbubbles as drug or gene carrier holds a great promise by offering a targeting method controlling both pDNA release and the location gene expression. The non-invasiveness of this system renders it superior to other physical methods as electroporation. Still, some challenges must be overcome to ensure its efficiency. Ultrasound and microbubbles assisted-gene delivery have been reported to be efficient in different tissue types including cardiac, endothelium, liver, kidney, tumour, skeletal muscles, bones and tendons (for a review see [8]). The best result is obtained with 1 MHz of frequency, the other optimal acoustic parameters clearly being dependent on the tissue type. In most studies reported so far, the microbubbles type used are those that have been developed as ultrasound contrast agents. In those reports, experiments have been done by separately injecting microbubbles and pDNA, mostly after local injection. These bubbles are not able to load and to complex nucleic acids. Liposomes bubbles, specifically designed for gene transfer, seem to be effective on a variety of tissue either or not in a targeted form [9].

For systemic injection, the pDNA must be complexed with or loaded in the microbubble without affecting the acoustic properties of the latter. This is still challenging even though the production of microbubbles carrying genetic materials via viral particles, PEI or complexed cationic lipids [10, 11] have been produced. However, the gene transfer levels still have to be improved.

It is obvious that designing an efficient microbubble for ultrasound gene delivery is the next step to further improve this method. Ideally, this microbubble should be able to interact specifically with its cell target, carry the gene to deliver and be optimized for nucleic acid delivery to permit its specific expression. Before developing new bubbles, it would be worthwhile to pinpoint all requirements by clearly delineating the DNA / microbubble intracellular routing. We have shown that under a specific ultrasound exposure microbubbles could enter into cells [12]. It is important to note that at these settings an efficient gene delivery was achieved suggesting that microbubble entry and gene transfer could be linked.

For most genetic deficiencies, treating the patient via intravenous administration could be more advantageous for gene therapy. In this case, the nucleic acids must cross the endothelium barrier and reach target tissues without being degraded. Some studies have also shown that ultrasound used at a specific regime was able to permeabilize endothelial barriers [13, 14]. For this particular issue, it would be of interest to establish specific ultrasound settings that could act first on the vascular barrier and then controlling the microbubble activation leading to gene transfer.

Cells exposed to ultrasound are known to elicit a variety of biological response that can be deleterious or with therapeutic potential [15]. Recently, Furusawa and colleagues have shown that ultrasound could induce host DNA modifications up to double breaks [16]. One can ask if it is possible to fine tune the setting to act on the accessibility of the DNA structure, thus allowing more efficient transgene integrati-

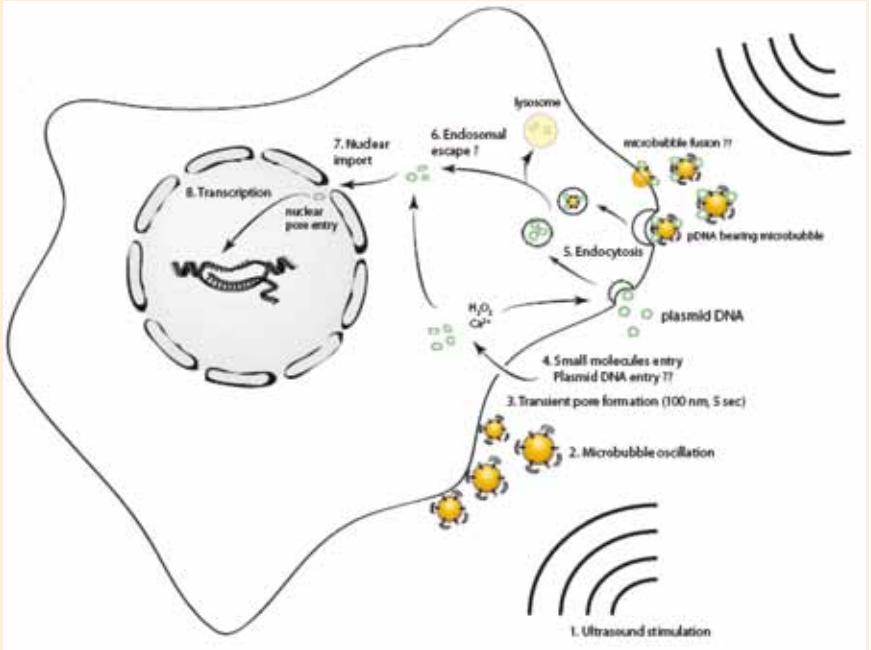


**Fig. 2** Gene delivery by sonoporation: When activated by ultrasound waves, microbubbles oscillate and are able to create transient pores in the plasma membrane. Through these pores can transit small molecules like calcium and hydrogen peroxide that are known to induce the endocytosis process. So far, it has not been proven if pDNA could be transferred inside these pores. During sonoporation, pDNA has been found to enter via endocytosis process. Therefore, it has to face the same barriers as those pointed on during gene transfer by chemical delivery systems. Microbubbles bearing / loading pDNA must have specific attributes that would permit pDNA delivery inside the cytosol.

on. This is not as idealistic as it sounds since ultrasound stimulation has been shown to induce an over-expression of some genes, especially in musculoskeletal tissues which can sense external stimuli. Acquiring more knowledge on the cellular effects in terms of molecular signaling will be worthwhile to take advantage of these phenomena for gene delivery purposes.

Chantal Pichon, Anthony Delalande
Center of Molecular Biophysics, University of Orléans and CNRS, Orléans, France

**References**

1 Midoux P, Pichon C, Yaouanc JJ, Jaffres PA. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. British journal of pharmacology, 157 (2), 166–178 (2009).
2 Fechheimer M, Boylan JF, Parker S, Sisken JE, Patel GL, Zimmer SG. Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading. Proc Natl Acad Sci U S A, 84 (23), 8463–8467 (1987).
3 Bao S, Thrall BD, Miller DL. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. Ultrasound Med Biol, 23 (6), 953–959 (1997).
4 Mehier-Humbert S, Bettinger T, Yan F, Guy RH. Plasma membrane poration induced by ultrasound exposure: implication for drug delivery. J Control Release, 104 (1), 213–222 (2005).
5 Duvshani-Eshet M, Machluf M. Therapeutic ultrasound optimization for gene delivery: a key factor achieving nuclear DNA localization. J Control Release, 108 (2–3), 513–528 (2005).
6 Paula DM, Valero-Lapchik VB, Paredes-Gamero EJ, Han SW. Therapeutic ultrasound promotes plasmid DNA uptake by clathrin-mediated endocytosis. J Gene Med, 13 (7–8), 392–401 (2011).
7 Meijering BD, Juffermans LJ, van Wamel A et al. Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation. Circ Res, 104 (5), 679–687 (2009).
8 Delalande A, Postema M, Mignet N, Midoux P, Pichon C. Ultrasound-assisted gene delivery: recent advances and ongoing challenges. Therapeutic delivery, in press (2012).
9 Suzuki R, Oda Y, Utoguchi N, Maruyama K. Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. J Control Release, 149 (1), 36–41 (2011).
10 Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. Ultrasound Med Biol, 29 (12), 1759–1767 (2003).
11 Nomikou N, Tiwari P, Trehan T, Gulati K, McHale AP. Studies on neutral, cationic and biotinylated cationic microbubbles in enhancing ultrasound-mediated gene delivery in vitro and in vivo. Acta Biomater, 8 (3), 1273–1280 (2012).
12 Delalande A, Kotopoulis S, Rovers T, Pichon C, Postema M. Sonoporation at a low mechanical index. Bubble science, Engineering and Technology, 3 (1), 3–11 (2011).
13 Yang FY, Lin YS, Kang KH, Chao TK. Reversible blood-brain barrier disruption by repeated transcranial focused ultrasound allows enhanced extravasation. J Control Release, 150 (1), 111–116 (2011).
14 Xia CY, Liu YH, Wang P, Xue YX. Low-Frequency Ultrasound Irradiation Increases Blood-Tumor Barrier Permeability by Transcellular Pathway in a Rat Glioma Model. J Mol Neurosci, (2012).
15 Mitragotri S. Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. Nat Rev Drug Discov, 4 (3), 255–260 (2005).
16 Furusawa Y, Fujiwara Y, Campbell P et al. DNA double-strand breaks induced by cavitational mechanical effects of ultrasound in cancer cell lines. PLoS One, 7 (1), e29012 (2012).

# Shear Wave Elastography

In the last years, liver elastography became an important target for many research groups, which tried to establish the real value of this new method, especially for the evaluation of diffuse hepatopathies.

Liver biopsy was considered as the "gold standard" for liver fibrosis assessment and the first elastographic method, transient elastography, was compared to the morphologic evaluation (liver biopsy). Later studies, evaluating new elastographic methods, tried to demonstrate their non-inferiority as compared to this validated method.

In the last years we observed a dramatic decrease in the number of liver biopsies performed for liver fibrosis assessment in chronic hepatitis, as a result of increasing use of the non-invasive modalities, especially in Europe. Biological tests (direct or indirect) and elastographic methods for liver stiffness evaluation became more popular and are used for prognosis assessment, for decision regarding treatment and for the follow-up of patients with chronic hepatopathies.

Tissue elasticity can be described qualitatively by evaluating the relative displacements caused by static or dynamic deformations. Quantitative techniques estimate the deformation rate, which indirectly characterize environmental stiffness.

The first elastographic method used in clinical practice was transient elastography (TE). Now, after more than 10 years experience with this method, several meta-analyses showed its real value. The weak points of this method are: It is a quite blind method (no real time ultrasound use); it is not feasible in patients with ascites and in approximately 15–20 % of patients, reliable measurements cannot be obtained.

More recently, shear wave elastography (SWE) was developed. Quantitative elastography techniques provide high resolution information about tissue elasticity in a region of interest. To obtain a quantitative elastographic assessment, shear waves are generated into the tissue, then tracked and measured. Complex equipment is used to generate them and to make high-resolution measurements of their propagation velocity. Several techniques can be used to generate the shear waves:

## Acoustic Radiation Force Impulse (ARFI) Elastography

The acoustic radiation force causes tissue displacement centered on a region of interest. These displacements propagate through the tissue in the form of shear waves and an ultrasound system is used to follow the shear waves' propagation. The time scale of tissue's response is much slower than that of the ultrasonic wave propagation. ARFI elastography is integrated into an Acuson S2000 ultrasound machine, from Siemens.

**Principle of ARFI Elastography:** A short duration, high-intensity acoustic "push pulse" is transmitted by the transducer, followed by a series of diagnostic intensity pulses, which are used to track the tissue displacement caused by the push pulse. The tissue response to the radiation force is observed using conventional B-mode imaging pulses, and it is possible to measure and display the quantitative shear-wave velocity (meters/second – m/s) from the ARFI generated displacements. This velocity is proportional to the square root of the tissue's elasticity modulus. Because the shear wave velocity depends on tissue stiffness, it is possible to