

Sonoporation: Underlying Mechanisms and Applications in Cellular Regulation

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Abstract

Ultrasound combined with microbubble-mediated sonoporation has been applied to enhance drug or gene intracellular delivery. Sonoporation leads to the formation of openings in the cell membrane, triggered by ultrasound-mediated oscillations and destruction of microbubbles. Multiple mechanisms are involved in the occurrence of sonoporation, including ultrasonic parameters, microbubbles size, and the distance of microbubbles to cells. Recent advances are beginning to extend applications through the assistance of contrast agents, which allow ultrasound to connect directly to cellular functions such as gene expression, cellular apoptosis, differentiation, and even epigenetic reprogramming. In this review, we summarize the current state of the art concerning microbubble–cell interactions and sonoporation effects leading to cellular functions.

Keywords

Microbubble, multidisciplinary, reprogramming, sonoporation, ultrasound.

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Introduction

Ultrasound is widely used in clinical practice due to its advantages of being non-invasive, non-radioactive, convenient, and cost-effective [1–5]. The acoustic impedances of various tissues and organs of the human body are different, which in turn causes sound waves to generate specific echoes during the propagation in human tissues [6]. On the other hand, the safety characteristic of ultrasound application, its ability to focus on specific sites, and the potential to achieve a great diversity of therapies enrich the clinical treatment strategy [7–10] (Figure 1). For the past decades, ultrasound had shown good compatibility in combination with different technologies for disease therapy [11–15], especially ultrasound-mediated drug delivery. The key to the drug delivery system is delivering therapeutic molecules to the target area across endothelial barriers and plasma membrane to tissues or cells. Efficient aggregation at specific sites obviously reduces side effects to normal tissues. [16]. Ultrasound combined with gas-filled microbubbles is well known for its non-virus, non-invasive strategy to enhance intracellular gene or drug delivery [17–21]. By mixing cells with microbubbles, numerous temporary pores occur in the cell membrane

after ultrasound exposure, which is called sonoporation [22]. The capture of the phenomenon of sonopores for the first time on the cell membrane by a scanning electron micrograph in 1999 has aroused people's wide concern [23]. Improvement of cell membrane permeability and increase in the cellular uptake of impenetrable molecules from the extracellular matrix by sonoporation have a positive stimulation for cell growth and development [24]. Therefore, ultrasound has been utilized not only for diagnosis but also as a therapeutic modality [25].

However, many of the biophysical mechanisms still remain unknown. Technological innovation of ultrasound medicine has driven the development of the research in ultrasonic biophysics, which provides the necessary basic theory for the development of both diagnosis and treatment in modern ultrasound field. In this review, we state the present status of the sonoporation-mediated drug delivery system, the mechanisms of sonoporation production, and the relationship between ultrasound parameters and microbubbles. Next, we summarize the utilization of ultrasound-mediated sonoporation leading to cell endocytosis, production of reactive oxygen species (ROS), and life cycle. Finally, we discuss the role of



Figure 1 Application of ultrasonic therapy technique.

ultrasound-mediated sonoporation in an unexplored field for cellular fate regulation.

Definition and parameters of sonoporation

Definition of Sonoporation

Ultrasound energy can induce temporary disruptions in the cell membrane. This manifestation of morphological changes is called sonoporation, which is generally considered as the basic principle of the ultrasound-mediated drug delivery system. The interaction of ultrasound energy with tissues or cells *in vivo* first induces opening endothelial junctions, and secondly reversible perforation on the cell membrane [26], stimulating cellular uptake and endocytosis [27] (**Figure 2**). Microbubbles usually serve as cavitation nuclei to amplify the sonoporation effects. These microbubbles are 2~8 μm in size with a biocompatible shell (lipid, protein, or polymer) [28]. The underlying mechanism of sonopore formation and cellular uptake of impermeable molecules are the activities of microbubbles on the membrane driven by ultrasound [29, 30]. Oscillating microbubbles would go through contraction and expansion and collapse with the action changing of negative acoustic pressure [31, 32]. The proximity of microbubbles to the cell surface set the surrounding liquid into motion, create shearing force to form microstreaming around the microbubbles [33, 34]. Collapsed microbubbles generate shock wave and micro-jet in the fluid (**Figure 3**).

The deformation and collapse of microbubbles are related to the acoustic energy field, specifically to the ultrasound parameters.

Microbubble deformation and various ultrasound parameters

Ultrasound parameters include acoustic pressure, frequency, acoustic intensity, exposure time, pulse duration, duty cycle, cavitation index (CI), mechanical index (MI), pulse repetition frequency (PRF), and others. Most studies generally indicated that acoustic pressure has a positive relationship with sonoporation-mediated delivery rate, but the opposite with cell viability [35–39]. The cavitation in fluid environment is more like a random event, which can be reduced when enough microbubbles exist simultaneously. Therefore, the concentration of microbubbles would be regarded as an optimization index for the production of sonopores and transfection efficiency [39–41]. Parameters related to cavitation, such as acoustic intensity [42–44], exposure time [39, 45], duty cycle [46], pulse duration [47], MI [48], and PRF [49] have similar trends in *in vitro* experiments. The opening degree of sonopores is related to the energy provided by ultrasound. The energy absorbed by the microbubbles is positively correlated with cavitation effect, which leads to more obvious sonopore formation. However, when the acoustic energy is too high, irreversible damage will be caused to the cells. This threshold depends on the tolerance and sensitivity of the cells to ultrasound. In an *in vivo* environment with complex conditions, the ultrasound-mediated sonoporation follows a similar rule. Shapiro et al. [39] optimized the sonoporation-based gene delivery parameters *in vivo*, including acoustic pressure, microbubble concentration, plasmid dosage, and irradiation time. Green fluorescent signal from the luciferase gene in the treatment group

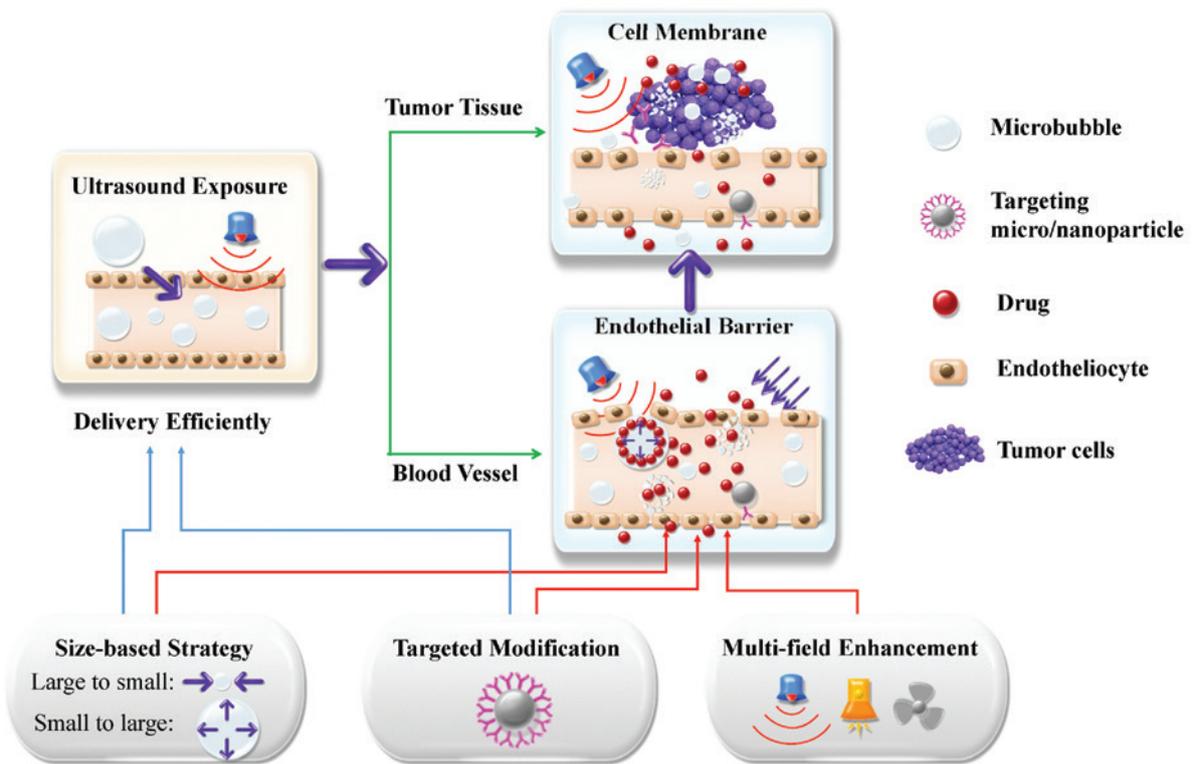


Figure 2 Overview of the delivery routes enhanced by sonoporation.

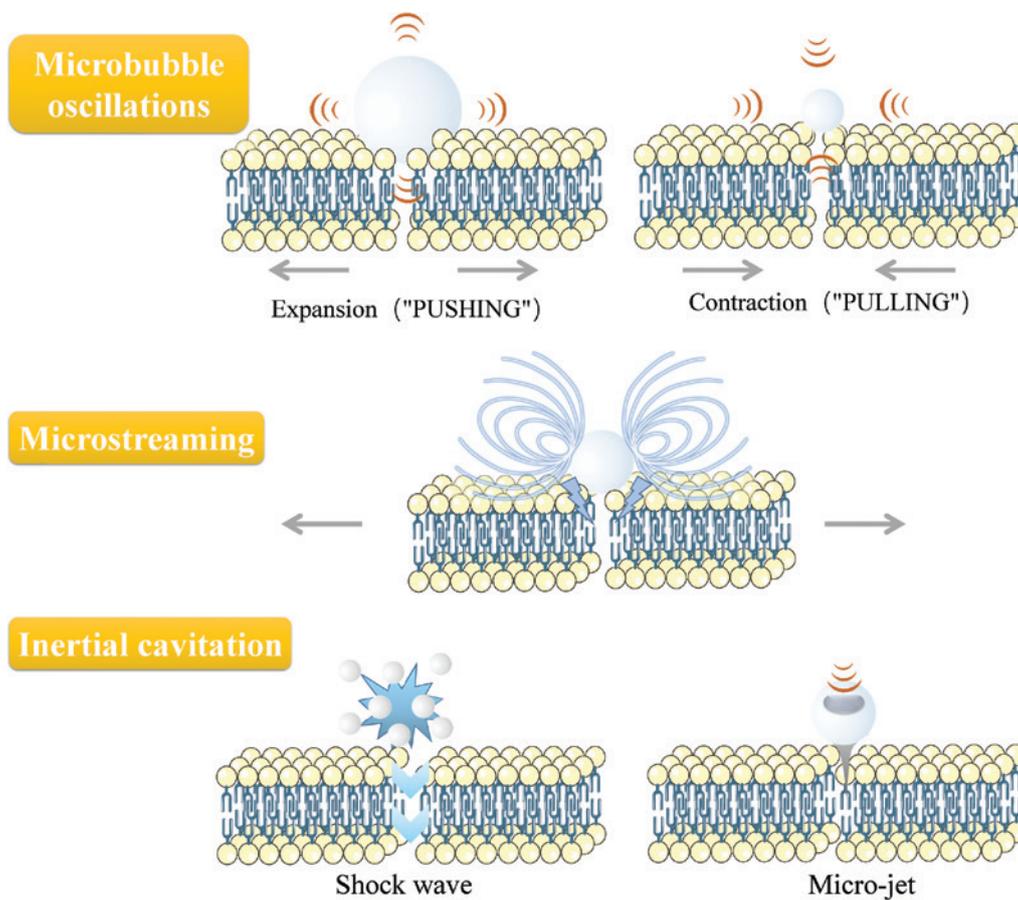


Figure 3 The generation of sonoporation.

was 100-fold higher than that in the control group, which indicated that *in vivo* delivery efficiency is positively correlated with acoustic pressure, microbubble and plasmid DNA (pDNA) dosage, and treatment time.

Microbubble size and distance from cell membrane

The distance between the microbubbles and the cells is another consideration [50]. Qin et al. [51] revealed the impact of bubble-cell interaction parameters. The energy produced by bubble-cavitation decreases as the distance between microbubbles and cells increases. The sonoporation phenomenon cannot be detected when the microbubble-to-cell distance is larger than the microbubble's diameter, and a larger microbubble can improve this situation, but it is easily prone to unpredictable motion when microbubbles' diameter is larger than 5.5 μm . Furthermore, the influences of different cell lines also have been studied. Shi et al. [52] showed that four cancer cell lines (breast, ovarian, liver, and thyroid) have their own sonoporation under ultrasound treatment. The conditions (acoustic intensity, concentration of microbubbles, and treatment time) for each cancer cell line were different. This suggests that cells from different tissue sources have different sensitivity and tolerance to ultrasonic stimulation. Jia et al. [53] compared Adriamycin-resistant breast cancer MCF-7/ADR cells and MCF-7 cells at the same ultrasound exposure conditions. They found that the cell viability of both MCF-7/ADR cells and MCF-7 cells decrease with a high-intensity acoustic field, and MCF-7/ADR cells were more sensitive to ultrasound exposure.

Although the basic structure and principles of sonoporation-based delivery have been established in previous work, the efficiency of ultrasound combined with microbubbles-mediated drug or gene delivery is far from meeting clinical requirements. In-depth study of relevant underlying mechanisms and corresponding adjustments are the key to solve this limitation. Many studies in the literature show that multiple factors and mechanisms work together to explain the generation of sonoporation, including cavitation activities and biological regulatory mechanisms [54–56]. In addition, a variety of biophysical effects after sonoporation treatment, including apoptosis, actin cytoskeleton changes, and mitochondrial permeability transition pore opening, may be used as potential cellular regulatory applications [57–59].

Bioeffects of sonoporation on cells

Changes in cytoskeleton dynamics

Transformation of F-actin represents the morphological changes in the cytoskeleton, which is related to cellular motility and morphology. FITC-phalloidin can bind to F-actin to show the arrangement of the cytoskeleton. Liufu et al. found that in comparison with the control group, the

sonoporation-treated group showed highlighted green fluorescent spots, which present a disorder of F-actin microfilaments [60]. The F-actin in cells aggregated and induced more dot-like structures, suggesting a complete disruption of actin microfilaments [57]. Experimental observations showed that the higher the acoustic pressure or closer the distance between the microbubble and the cell, the more obvious the pore formation occurred in the cell membrane, sometimes leading to cytoskeleton disassembly [61].

Drug delivery using sonopores or endocytosis

Sonopore formation triggers many motility mechanisms of the cellular membrane, including the movement of the cell membrane. The diameter of the sonopore has been determined (110 ± 40 nm) [62], and not all micromolecules pass through the pore pathway into the cell; endocytosis also plays an important role in ultrasound-mediated delivery. Several studies have indicated that endocytosis triggered by sonoporation may be involved in intracellular delivery of macromolecules. FITC-labeled dextran can enter the cell through sonopores (4.4 to 70 kDa) and endocytes (>155 kDa) after sonoporation. In the case of adenosine triphosphate (ATP) consumption, the delivery efficiency of the sonopore pathway decreased, and the endocytosis pathway was significantly limited, which was closely related to macropinocytosis and clathrin [63]. De et al. [64] showed that acoustic pressure is related to the approach of intracellular transport. FITC-dextran mainly enters the cell through pore formation at higher acoustic pressures, while endocytosis was more abundant at lower acoustic pressures, which was related to cytoskeletal deformation.

Production of ROS

Many studies have shown that ROS are products of the body's normal metabolism and play an important role in human disease and aging due to their strong oxidizability. However, the imbalance between antioxidants and oxidants may induce adverse effects. The shear stress from oscillated microbubble leads to the generation of superoxide and H_2O_2 . Jia et al. [65] assessed the degree of sonoporation and intracellular ROS changes and found that ROS production in sonopore-reversible cells increased and was positively correlated with the degree of sonoporation. Escoffre et al. also confirmed that ROS was produced during microbubble cavitation and that these reactive oxygen molecules are easily involved in cell membrane penetration and gene delivery [66].

Impact in cellular cycle function

In addition to cell membrane disruption, as a biophysical process, sonoporation induces some cellular responses, including regulation of cellular calcium ion signals [67], cell membrane potential changes [68], and cellular mechanical

events at the nuclear level [69]. The cell cycle is a periodic program that leads to DNA replication and cell division [70]. As the mechanical effects of ultrasound-driven sonoporation stimulate the physical vibration of cellular structures, Fan et al. [71] investigated the influence of the cell cycle phase on the regulation of HeLa cells and their cellular responses. Their research showed that the cell cytoskeleton structural change with cell cycle phases varies, and the instantaneous biophysical effect resulted in the fastest cytoskeleton disassembly. Synchronous with the stimulation of ultrasonic microbubble-mediated sonoporation trigger, they found that disruption of the α -tubulin microtubules at the position of the microbubble located accompanied propidium iodide being delivered into the cytoplasm through the microbubble site.

Regulation of cellular fate with ultrasound

Application of cell apoptosis

After undergoing sonoporation, the appearance of cellular disruption may lead to anti-proliferation effects, for instance, causing cell apoptosis through disrupting various cell signaling pathways. Zhong et al. [72] demonstrated that sonoporation induced HL-60 cells to apoptosis possibly through the mitochondrion, where Bcl-2 decreased while Bax increased over time. The opening mitochondrial membrane through the Bax pathway released pro-apoptotic molecules (cytochrome c) into the cytoplasm, inducing cell apoptosis. However, another research [73] using sonoporation increased the percentage of apoptosis in K562 cells, and it was found that these mitochondrial membrane-depolarized cells lead to mitochondrial dysfunction, which were significantly inhibited by cyclosporine-A but not the Bax-inhibiting peptide. They proved that cytochrome c was released from the mitochondrial permeability transition pore, the proteinaceous megapore consisting of cyclophilin D, but not the Bax pathway. The specific mechanism is contradictory, and the proof of more in-depth principles remains to be revealed.

Application of stem cell-differentiation

Stem cells can differentiate into many types of cells or tissues and have an important value in clinical transplantation treatment, disease model construction, and mechanism research. In order to successfully apply stem cells to tissue engineering and regenerative medicine, the behavior of stem cells, such as responses to biochemical and biophysical cues (including adhesion, proliferation, survival, and differentiation) must be precisely regulated. Some studies have investigated if ultrasound exposure promotes neural stem cell attachment and differentiation [74]. Lee et al. showed that ultrasound significantly enhances neural stem cell and neural progenitor cell differentiation and the utilization of growth factors [75]. Cancer stem cells (CSCs), which have

in common the ability to self-renew as normal stem cells, have the characteristics of stimulating tumor growth, recurrence, metastasis, and drug resistance. Differentiation of CSCs provides another therapeutic strategy that reverses the stemness of the CSC and forces it to lose its ability to self-renew [76].

Application of cellular reprogramming

In 2006, Takahashi and Yamanaka first transferred four transcription factors into mouse somatic cells through retroviral vectors and obtained induced pluripotent stem cells (iPSCs), which are very similar to embryonic stem cells (ESCs) in their morphology, as multipotent markers, and epigenetic status. The emergence of iPSCs has led to a breakthrough in the understanding of the extremely complex process of cell reprogramming [77]. The cellular signaling pathway and the pluripotency gene regulatory network together maintain the pluripotency of iPSCs. Lee et al. [78] employed biophysical stimuli such as ultrasound-mediated cellular permeation, which led to cell reprogramming, developing a non-invasive and gene/chemical-free method for multipotent cell generation from human dermal fibroblasts (HDFs). These multipotent cells exhibit a spheroid morphology, potential of differentiation, and pluripotency expression. They also found that the activation of the mitogen-activated protein kinase (MAPK) signaling pathways was closely related to cell reprogramming. Moreover, direct cell-to-cell connections facilitated reprogramming factors being released and transported into neighboring cells through ultrasound-induced sonoporation, causing a common expression of pluripotent markers and multi-lineage differentiation potential in the cell population.

Prospects and outlooks

Ultrasound is experiencing a transformation in terms of both clinical applications and technical developments. It is possible to improve the efficacy and minimize side effects by controlling sonoporation activities and designing biological regulatory strategies. Several pre-clinical studies have indicated the potential of novel ultrasound-responsive carriers to deliver multiple types of drugs including model drugs, anti-cancer drugs, therapeutic antibodies, genes, and nanoparticles, efficiently in various tumor models for immunotherapy [5], brain disease [79], pancreatic cancer [80], and bacterial infections [81].

Besides, one important research area encompasses the structure, morphology, and function of biological systems under ultrasound irradiation. The inherent advantage of ultrasound is that it applies basic physical stimulation to living tissue. The fields of molecular ultrasound and ultrasound genetics seek to link these physical forces with the functions of biomolecules and cells to achieve precise control of cells [82, 83]. In addition, genetic engineering editing allows biomolecules to interact with ultrasound. Ultrasound presents as both a waveform and a sound energy form. For example,

the thermal effect or mechanical effect of high-intensity focused ultrasound (HIFU) can be used to locally elevate tissue temperature and activate temperature-sensitive proteins and pathways [84, 85]. Although this field is still in its infancy, it has opened the door to precise ultrasound control. As an acoustic waveform, it can detect loading signals; as an energy form, it can change the medium and microenvironment for cell survival [86–88]. Although cavitation-induced sonoporation has the potential for drug and gene delivery, the cavitation events seem to be a random process, causing

a low consistent sonoporation outcome. The elucidation of all of the interactions between microbubbles and cells would help to improve the uniformity, efficiency, and safety of the sonoporation. Multidisciplinary technology integration should be advocated and promoted to address this limitation, including the machining device [89], biophysics [56], physics [90], and sonochemistry [91]. We believe that ultrasound will have more extensive research in the future, providing more options for targeted regulation and treatment protocol as well as revealing more biophysical mechanisms.

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