Sonoporation

Monica M. Forbes[1] and William D. O'Brien, Jr.[1,2]

[1]Department of Bioengineering and [2]Department of Electrical and Computer Engineering
University of Illinois at Urbana-Champaign, 405 N. Mathews, Urbana, IL 61801 USA

Abstract: Sonoporation utilizes the interaction of ultrasound (US) with ultrasound contrast agents (UCAs) to temporally permeabilize the membrane of cells allowing for the uptake of DNA and drugs. This membrane alteration is transient, leaving the compounds trapped inside the cell after US exposure. Small compounds, macromolecules, DNA, and other therapeutic compounds have been delivered into cells using US with UCAs. US with UCAs can also deliver protein and DNA into tissues. Low- and high-frequency US treatment of cells in the presence of plasmid DNA has been shown to cause cell transfection *in vitro* and *in vivo*. Thus, sonoporation has great possibilities in both targeted drug delivery and gene therapy. Little is known about the mechanism of sonoporation both physically and biologically, and until the mechanism(s) is(are) identified, optimal sonoporation effect will be left to trial and error. The presence of UCAs is necessary to induce a significant sonoporation event. This UCA requirement has led to the identification of various bubble-associated phenomena (shear stress, microjetting, inertial cavitation, etc.) as possible mechanism(s). However, these data provide only circumstantial, not direct, evidence about sonoporation mechanisms. Sonoporation has significant advantages for therapy, notably the ability for its spatial and temporal control, and thus a better understanding of its mechanism(s) could hasten its clinical acceptance.

Key words: Sonoporation, ultrasound contrast agent, inertial cavitation, thresholds.

A. Introduction

A significant problem in cancer therapy is the compromised quality of life experienced by the patient due to the side effects of the therapeutic compounds. Delivery of molecular medicine to solid tumors is often inefficient and as a result, the patient's healthy cells and tissues are subject to the toxic effects of the drugs. Thus, it is important to develop approaches that deliver drugs to the appropriate cells within the patient in a way that is specific, efficient, and safe. One such method involves the use of ultrasound (US) to enhance cell permeabilization. With this method it is possible, by using US and contrast microbubbles, to deliver therapeutic compounds noninvasively into specific target cells.

Lack of site specificity is a major obstacle for gene and drug delivery. The fundamental clinical goal is to develop approaches that deliver therapeutic material to the appropriate cells in the patient in a way that is specific, efficient, and safe. The necessary step of all forms of genetic manipulation is transfection, the uptake and expression of foreign DNA by the cell. Currently, transfection techniques can be divided into two categories: viral and nonviral.

Viral vectors have been shown to be efficient in transfection, but have drawbacks such as lack of site specificity, potential for new mutations, and severe immunological reactions [1]. The two major viral vectors are retroviruses and adenoviruses. In the other transfection option, nonviral transfection, DNA is naked, packaged in liposomes, conjugated to protein, or formed into artificial chromosomes. Presently, nonviral techniques include electroporation, particle bombardment, and lipofection. Electroporation involves the transfer of DNA through membrane pores formed in high-voltage electric fields. This process allows for some spatial targeting, but requires possibly invasive electrode placement. Particle bombardment uses high-speed DNA-coated projectiles to mechanically introduce the DNA into the cells. It allows for accurate placement of gene delivery, but is limited to surface applications. In lipofection, liposomes encapsulate the negatively charged DNA and facilitate transfer of the gene through the cell membrane. Lipofection has high transfection rates with minimal cellular toxicity, but does not allow control of spatial or temporal specificity. Table 1 outlines the various transfection techniques and rates.

Table 1. Transfection methods and their properties.

	Spatial Targeting	Temporal Targeting	Average Transfection Rate
Adenovirus	No	No	50% [53]
Retrovirus	No	No	1-15% [54]-[55]
Electroporation	Yes, but limited	Yes	13-36% [56]
Particle Bombardment	Yes, but limited	Yes	3.96% [57]
Lipofection	No	No	9-20% [57]-[58]

A transfer method that could spatially and temporally target the DNA would be a significant improvement over the current transfection methods. Sonoporation provides such advantages. US is used to permeabilize the membrane of cells allowing for the uptake of DNA and other molecules and can be focused on almost any location in the body [2]. Sonoporation combines the capability of enhancing gene transfer with the possibility of restricting this effect to the desired area and the desired time; thus allowing for spatial and temporal specificity without the side effects of other transfection agents.

Sonoporation alters the permeability of cell membranes in a transient fashion [3], leaving the compounds trapped inside the cell after US exposure. Small compounds [4]-[6], macromolecules [7]-[11], and other therapeutic compounds [6], [12]-[14] have been delivered into cells using US. US can also deliver protein [15]-[16] and DNA [17]-[20] into tissues. Low- and high-frequency US treatment of cells in the presence of plasmid DNA has been shown to cause mammalian cell transfection *in vitro* [9], [21]-[23] and *in vivo* [8], [19], [24]-[25]. Thus, sonoporation has great possibilities in both targeted drug delivery and gene therapy.

Little is known about the mechanism of sonoporation both physically and biologically. Tachibana et al. [26] and Meheir-Humbert et al. [27] have shown that pores form in a cell membrane following US exposure. It has been suggested that the pores are the means by which DNA and drugs can enter the cell, however the biological structure of the pores is unknown. Cellular and molecular damage of human RBCs does occur due to US [28], however, the role this damage plays in pore formation is unknown. It has been shown that the membrane permeability change in sonoporation is transient [3]-[4], [9], [25], and the recovery rate does not vary significantly with US parameters or the maximum amplitude of the transmembrane current [29]. Additionally, hyperpolarization of the cell membrane occurs in the presence of US and UCA, due most likely to activation of channels sensitive to mechanical stresses and nonspecific ion channels [30]. However, this hyperpolarization does not explain the presence of the pores in the membrane.

The presence of UCAs is necessary to induce a significant sonoporation event [9]-[10], [22]. This UCA requirement has led to the identification of IC as the probable mechanism. Studies supporting such a mechanism include work by Koch et al. [31] that found acoustic pressure levels of 0.3 MPa, at 2 MHz, and 200-mg/mL Levovist caused significant sonoporation. This acoustic pressure was in the range of that for IC, cited as 0.4 MPa for 2 MHz. Greenleaf et al. [10] found shards of fluorescently tagged Albunex® embedded in the cell membranes of sonicated cells, concluding that destruction of the microbubbles was the mechanism of the transfection process. Transfection due to sonoporation has been shown to increase for exposures above the cavitation collapse threshold, using the hydrogen peroxide sonochemical-production test for IC activity [9]. Sonoporation also displayed a strong dependence on peak rarefactional (negative) pressure amplitude, just as IC did [32]-[33]. However, the data cited above provide only circumstantial, not direct, evidence that collapse cavitation is the sonoporation mechanism.

B. UCAs and their Responses to Ultrasound

Microbubbles undergo complex behaviors in the presence of US. As acoustic waves are incident on the UCA, it grows and shrinks due to the time-varying pressure of the wave. The three categories of behavior that UCAs can undergo in a pressure field are linear oscillation, nonlinear oscillation, and bubble collapse. These behaviors are dependent on US frequency and peak rarefactional pressure. Literature has shown that bubble collapse pressure thresholds increase with increasing frequency [34]-[37]. Studies have shown that pulse duration (PD) does not appear to have an impact on the collapse threshold of UCA [37]-[38], whereas others have shown a weak effect [39]-[40].

At low-level acoustic pressure amplitudes, the bubble may undergo linear oscillation. During linear oscillation microstreaming can occur. Microstreaming is formed by the oscillation of the air-liquid interface at the surface of a bubble. This eddying motion is generated in the adjoining liquid and may cause shearing motions, which could affect membranes. Theoretical and experimental studies have shown that microstreaming near a cell boundary can adversely affect a cell membrane. The critical stress (in terms of viscous stress) for hemolysis is well defined [41]-[42]. Both groups found a threshold, with Rooney reporting a value of 150 Pa and Williams et al. 560 Pa for a 20-kHz frequency. Microstreaming produced by a vibrating a Mason horn demonstrated a threshold shear stress for sonoporation (≈ 12±4 Pa at 21.4 kHz) [43]. Thus, microstreaming could play a role in sonoporation.

At higher pressure amplitudes, UCAs undergo nonlinear oscillation. During these conditions, the UCA slowly expands to several times its initial radius during rarefaction, which is followed by a rapid contraction during compression, but not collapse. Nonlinear oscillation can produce microstreaming and liquid jets. Possible cellular effects of microstreaming have been discussed. Liquid jets are formed as a result of the nonlinear waves produced near a surface, such as a cell. These high-velocity jets provide increased transport of heat and gas by streaming and have the capacity to puncture the membrane of cells, producing openings that could allow for transport of extracellular material into the cell [44].

As the pressure amplitude is increased, the maximum-to-initial diameter ratio reaches 2. This value of 2 has been used as a common criterion for microbubble collapse [39], [45]-[46]. This collapse is termed inertial cavitation (IC) because the UCA motion is dominated by the inertia of the liquid. For a shelled UCA, this violent collapse causes the shell to fragment, releasing the encapsulated gas that then possibly rebounds; rebounds can occur when a microbubble ruptures and generates daughter/free bubbles that grow and collapse. The shell fragments and then dissolves, as does the microbubble. The violent collapse of the bubble during IC produces many mechanical and chemical agents that could cause bioeffects. Mechanical shock waves can be produced. During collapse, the temperature of the bubble may reach thousands of degrees Kelvin (4,300-5,000 K), an increase capable of inducing thermal injury [47]-[48]. Free radical production (FRP), caused by dissociation of water vapor during the contracted part of the cycle, can mediate chemical changes. However, it has been shown that FRP is not required for transfection [49].

Fig 1 presents the numerous responses of UCA to US

and the possible bioeffects, thus emphasizing the critical junction of IC versus oscillation of UCA in determining the mechanism for sonoporation.

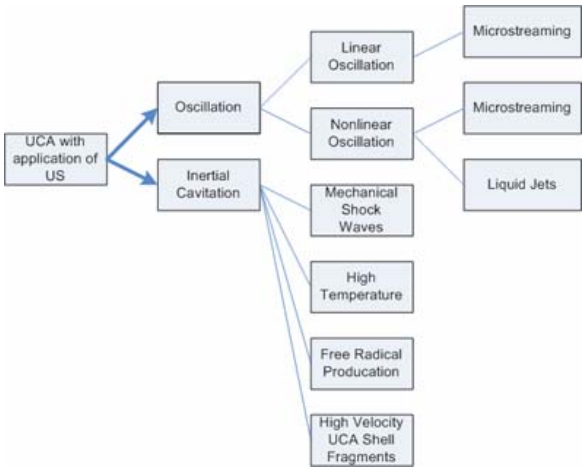

Fig 1. The response of UCA to US, the resulting behaviors and bioeffects.

UCAs for US imaging were proposed in 1968 [50] and a search for a deliberately designed UCA started in the 1980s. Most UCAs are gas filled, encapsulated microbubbles that are injected into the venous system to act as a red blood cell tracer. By increasing acoustic reflectivity, UCAs enhance echo amplitudes to improve sensitivity in deep tissues or in otherwise invisible small vessels. In the presence of an US field, UCAs are nonlinear resonators that, under certain conditions, can change size, cavitate, fragment, or be moved. Well beyond their original intended applications, UCAs have potential in therapy, drug delivery, and the location of targeted sites.

The UCA structure is typically a sphere about 1-10 μm in diameter, containing a gas core and encapsulated by a thin elastic shell approximately 10-200 nm thick. One major difference between air bubbles and UCAs is the effect of the shell, which constrains and raises the resonant frequency. The makeup of the shell also determines the rigidity of the UCA, which in turn affects the collapse threshold.

The three most commonly studied UCAs in the realm of sonoporation have been Albunex®, Optison™, and Definity®. Albunex® contains air and is stabilized by a human albumin shell. Albunex® contains a maximum of $7x10^8$ mL^{-1} gas bodies and a diameter ranging from 1 to 15 μm. Optison™ contains perflouropropane, is stabilized by a human serum albumin shell, with a diameter between 2 to 4.5 μm. The concentration of Optison™ is 5 to $8x10^8$ mL^{-1} gas bodies. Optison™ seems to show much greater transfection enhancements than Albunex®. Definity® contains perflouropropane, the same gas as Optison™. However, Definity® is stabilized by a phospholipid shell and has a maximum of $120x10^8$ mL^{-1} gas bodies with a diameter ranging from 1.1 to 3.3 μm. Definity® shows similar transfection results as Optison™ when using similar gas-body numbers.

C. Mechanism Identification

Elucidating the mechanisms of transient change in cell membrane permeability is crucial for the future use of sonoporation as a drug delivery or gene therapy method. Two forms of sonoporation occur, lethal and sublethal. In the lethal case, the cell is unable to repair the membrane permeability change and the cell subsequently lyses and disintegrates. In the sublethal form, molecules in the surrounding medium are able to pass in or out of the cell, followed by membrane sealing and cell survival. This allows foreign macromolecules to be trapped inside the cell. Determining the balance between lethal and sublethal forms of sonoporation can only occur after knowledge of the mechanism exists. The results of this study can be used to guide the setting of exposure conditions to obtain maximal sonoporation with minimal cell death.

In addition, with advanced understanding of the sonoporation mechanism, designing an exposure protocol to obtain a predefined transfection rate would be possible. Such a system is ideal for agents with adverse side effects, but requiring a minimum transfection for success. Such a treatment would only be possible with a thorough understanding of the mechanism. Thus, the future of clinical usage of sonoporation is dependent on a comprehension of the mechanisms that cause the transient cell membrane permeability.

D. Methodology

The objective of the study is to determine the relationship between sonoporation and the collapse threshold of the UCA. This comparison is intended to reveal the requirement for IC in producing sonoporation.

The cell line used is Chinese Hamster Ovarian Cells (CHO) (ATCC, Manassas, VA) and cultured in F-12K Medium (ATCC) with 10% fetal bovine serum (ATCC), at 37˚C and in a humidified atmosphere of 5% CO_2 in air. This cell line is commonly used in sonoporation studies. The cells are grown as a monolayer.

For this study, Optison™ (Amersham Health Inc., Princeton, NJ) was the UCA used.

The sample vessel used for this study was a 96-well cell culture microplate (Corning Lowell, MA) constructed from medical grade polystyrene. Each well is flat bottomed with a diameter of 6.4 mm, 4.25 times the beam width (1.5 mm) of the 2.82-MHz transducers. Each well is 10-mm deep and the top will be made from plastic cling wrap, forming an acoustic window.

For preparation of an experiment, CHO cells were harvested with trypsin and pipetted into a clean, sterilized exposure vessel. Approximately 0.3 million cells in 0.36 mL of growth medium were added to each well. The vessel was placed in an incubator overnight to form a monolayer. On the day of the experiment, the monolayer was gently rinsed with PBS to remove unattached gas bodies and dead cells. The chamber was then filled with exposure medium.

The exposure medium contained Fluorescein isothiocyanate-dextran (FITC-dextran) (FD500S, Sigma-

Aldrich Co., St. Louis, MO), with an average molecular weight of 500,000 daltons. These molecules are normally unable to cross the cell membrane, and thus were used as the marker for cell membrane permeability change. If FITC is detected inside a cell, then that cell is considered to have undergone sonoporation. Dextran was chosen because it can be conjugated to a variety of fluorophores and it has a range of molecular weight from 3000-2,000,000 daltons. These different sizes can be used to determine the size-exclusion limit of the membranes permeability. A volume of 0.065-mL FITC-dextran solution, which was 25 mg/mL in PBS, was added to the chamber.

Using the proper dispensing system, 8.80 µL of Optison™ was added to the vessel. The concentration of Optison™ was equal to 2%. The remainder of the chamber was filled with PBS and the top was clamped with a sheet of plastic cling wrap, sealing the chamber. The exposure vessel was placed in a room temperature water bath with the plastic cling wrap located near the transducer (Fig 2). Thus the monolayer was on the back window of the chamber, allowing the contrast agent to rise to the monolayer due to gravity and the radiation force to push the contrast agent toward the monolayer. Samples were then either exposed or sham exposed (US turned off) one at a time, and immediately washed with PBS to remove any FITC-dextran in solution and placed on ice, to prevent pinocytosing of the FITC-dextran following exposure.

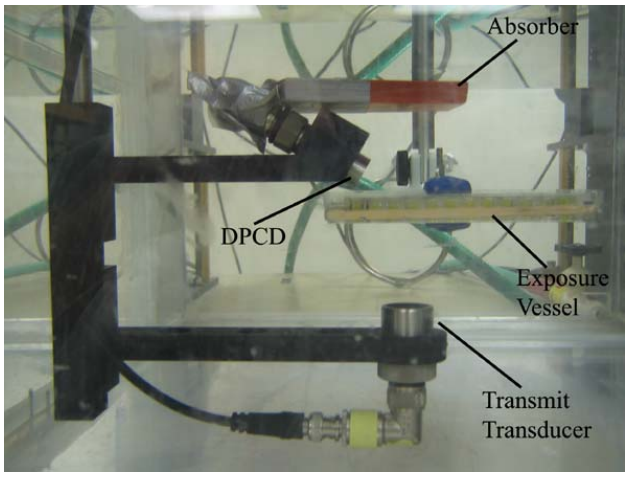

Fig 2. Experimental setup.

E. Results and Discussion

Logistic regression anaylsis [51] was used to analyze the dependence of ruptured microbubble occurrence rates on the peak rarefactional pressure, P_r (Fig 3), and the 5% occurrence rate was used to quantify the shell rupture (inertial cavitation) threshold; an automated algorithm applied to the PCD signals detected the number of IC events out of 128 data realizations. The experimental results show a frequency-dependent threshold for collapse of Optison™ ($p<0.0001$) but not a significant PD-dependent threshold [37].

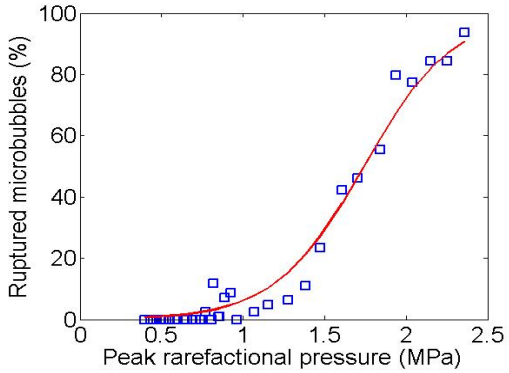

Fig 3. Squares represent the occurrence of ruptured microbubbles based on IC detection at 2.8 MHz for a 3-cycle PD. The line describes the logistic regression result.

Our early sonoporation results have been shown to be consistent with those of Miller and Quddus [52]. They used Human epidermoid carcinoma cells, while we used Chinese Hamster Ovarian Cells (CHO). Both cell lines grow in a monolayer morphology. Miller and Quddus exposed the cells using 3.5 MHz at a P_r of 0.84 MPa. The experiments in our lab were conducted at 2.82 MHz and 0.97 MPa P_r. With these differences in mind, the resulting sonoporation over 5 trials was 5.05±0.9% (Table 2). This compared well to the 7.7±1.0% found by Miller and Quddus. Fig 4 shows optical and fluorescence microscope images of CHO cells exposed and not exposed to US with 2% Optison™. The fluorescence microscope results were confirmed with flow cytometeric analysis.

Additionally, a threshold-type study examining sonoporation activity as a function of P_r was performed. CHO cells in the presence of Optison™ were exposed to a 2.82 MHz transducer for 5-cycle PD, 10-Hz PRF, and exposure duration of 30 seconds. Sonoporation activity increases as P_r is increased, up to 2.5 MPa where a drop in sonoporation is observed (Fig. 5); the collapse thresholds of Optison™ are plotted on the same figure. Presented in this manner, the relationship between sonoporation and bubble collapse can be determined. Fig 5 clearly reveals that for P_r below the collapse threshold of 0.83 MPa, significant sonoporation is taking place.

Table 2. Comparison of our sonoporation results to results from Miller and Quddus [52].

	Cell	Freq	PD	P_r	Sonoporation
Miller and Quddus	A431	3.5 MHz	5 µs	0.84 MPa	7.7±1.0%
Our results	CHO	2.82 MHz	5 µs	0.97 MPa	5.05±0.9%

F. Conclusion

The previous research that has been conducted provide only circumstantial, not direct, evidence that collapse cavitation is the sonoporation mechanism. It is our view that we have shown evidence that inertial

cavitation is not directly involved in producing sonoporation. Rather, the evidence suggests directly that microstreaming is sufficient for sonoporation. And finally, above a peak rarefactional pressure of 2.4 MPa, a mechanism preventing sonoporation becomes apparent.

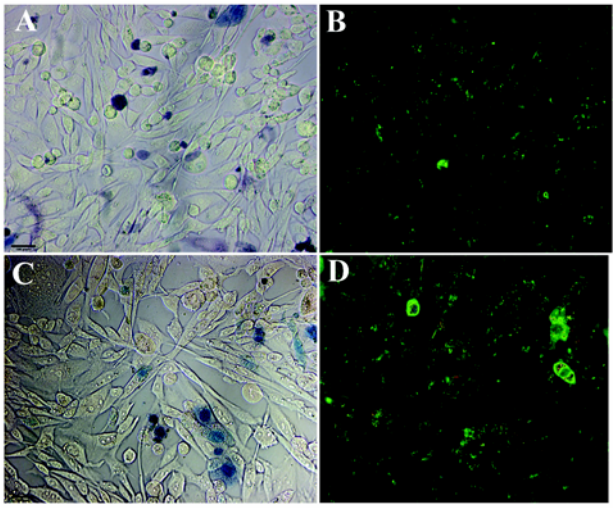

Fig 4. CHO cells in the presence of FITC-Dextran and Optison™. A) Phase Contrast Image with no US (7.9% nonviable cells). B) Fluorescence Image with no US (0.82% Fluorescent Cells). C) Phase Contrast Image with US applied at 2.8 MHz, 15 cycles, 0.97 MPa for 60s exposure (7.09% nonviable cells). D) Fluorescence Image with US applied at same settings as above (5.16% Fluorescent Cells).

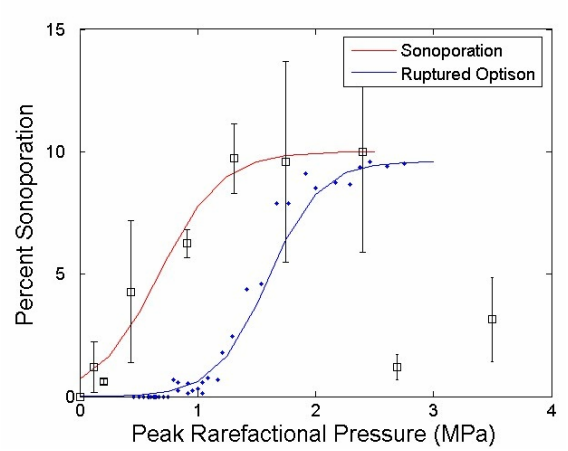

Fig 5. Sonoporation of CHO cell exposed at 2.82 MHz, 5 cycles, 10 Hz and for 30 s compared to the occurrence of ruptured Optison microbubbles. The collapse threshold for Optison occurs at 0.83 MPa.

G. Aknowledgements

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