The Role of Inertial Cavitation of Ultrasound Constrast Agents in Producing Sonoporation

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Abstract—**The objective of this project is to elucidate the relationship between ultrasound contrast agents (UCA) and sonoporation by varying the peak rarefactional pressure (P_r) in a threshold type study. The results of sonoporation in the presence of Optison™ or Definity® are directly compared to the collapse thresholds of the respective contrast agent to uncover the role inertial cavitation plays in sonoporation. Chinese Hamster Ovarian (CHO) cells were grown as a monolayer in a 96-microwell plate. Each well was filled with an exposure medium consisting of 0.05 mL Fluorescein isothiocyanate-dextran (FITC-dextran), 8.8 µL Optison™ or 0.57 µL Definity®, and Phosphate Buffered Saline. CHO cells were exposed for 30 s to pulsed ultrasound at 3 MHz center frequency, 5-cycle pulse duration, and 10-Hz pulse repetition frequency. P_r was varied over a range from 10 kPa to 3.5 MPa. Flow cytometery was used to determine the percentage of positively labeled cells. Over the P_r range applied, the sonoporated cells in the presence of Optison™ increased from 0.63% to 10.21%, with a maximum occurring at 2.4 MPa. Above 2.4 MPa, a significant drop in sonoporation activity was observed. Sonoporation in the presence of Definity® presented the same trend, with sonoporated cells increasing from 5.26% to 26.39%, with a maximum occurring 172 kPa. Above 172 kPa, a drop in sonoporation activity was observed. These results illustrate that sonoporation is not due inertial cavitation of the UCA. Instead the evidence directly suggests that the sonoporation effect was caused by linear and/or nonlinear oscillation of the UCA, as these responses occur at lower P_r where sonoporation activity was present. Moreover, at higher pressures, the UCAs are rapidly collapsing and as such, are likely not present for enough time to significantly oscillate, thus minimal sonoporation activity was observed.**

Keywords-sonoporation, ultrasound contrst agent, inertial cavitaion, thresholds)

I. 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, healthy cells and tissues are subject to toxic effects of the drugs. Thus, it is vital to develop approaches that deliver drugs only to the appropriate cells within the patient in a specific, efficient, and safe manner. One such method, termed sonoporation, involves the use of ultrasound (US) to enhance cell permeabilization. With this method, it is possible, using US and contrast microbubbles, to noninvasively deliver therapeutic compounds into specific target cells.

Sonoporation alters the permeability of cell membranes in a transient fashion, leaving the compounds trapped inside the cell once US exposure is complete. Small compounds, macromolecules, and other therapeutic compounds have successfully been delivered into cells using US *in vitro* and *in vivo*. Thus, sonoporation has great possibilities in both targeted gene and drug delivery.

Little is known about the mechanism of sonoporation both physically and biologically. Tachibana et al. [1] and Meheir-Humbert et al. [2] have shown that large pores form in a cell membrane following US exposure. Cellular and molecular damage to human red blood cells occurs as a result of US exposure [3], though, the role this damage plays in pore formation is unknown. It has been shown that the enhanced membrane permeability in sonoporation is transient and the recovery rate does not vary significantly with US parameters [4]. Additionally, hyperpolarization of the cell membrane occurs in the presence of US and ultrasound contrast agent (UCA), most likely due to the activation of channels sensitive to mechanical stresses and nonspecific ion channels [5].

The presence of UCA is necessary to induce a significant sonoporation event. This UCA requirement has led to the identification of inertial cavitation (IC), which is the rapid collapse of a bubble, as the probable sonoporation mechanism, theorized by several studies [6-8]. However, the data provided in the literature is only circumstantial, not direct evidence that collapse cavitation is the sonoporation mechanism. UCAs have a complex dynamic behavior in an ultrasonic field. The major behaviors are linear oscillation, nonlinear oscillation, and IC. Determining whether oscillation or IC of UCAs is involved in producing sonoporation is essential for determining the physical phenomenon responsible for this biological effect.

Most UCAs are gas filled, encapsulated microbubbles designed to increase acoustic reflectivity. As acoustic waves are incident on the UCA, it grows and shrinks due to the time-varying pressure of the wave. The behavior of the UCA is dependent on US frequency and peak rarefactional pressure. At low-level acoustic pressure amplitudes, linear oscillation of the UCA occurs. These oscillations lead to local steady flows that are termed microstreaming. Theoretical and experimental studies have shown that microstreaming near a cell boundary can adversely affect a cell membrane. The critical stress for

hemolysis is well defined [9, 10]. Additionally, microstreaming from a vibrating Mason horn demonstrated a threshold for enhanced membrane permeability (12±4 Pa at 21.4 kHz) [11]. Thus, microstreaming due to linear oscillation of UCAs could play a role in sonoporation.

At higher pressure amplitudes, UCAs exhibit nonlinear oscillation. During these conditions, the UCA slowly expands to several times its initial radius during rarefaction and is followed by a rapid contraction, but not collapse, during compression. Nonlinear oscillation produces microstreaming, as well as the potential for liquid jets. Liquid jets are formed as a result of the asymmetric behavior of the UCA near a surface, such as a cell. Liquid jets provide increased transport of heat and gas by streaming and have the capacity to puncture the cell membrane, producing openings that could allow for the transport of extracellular material into the cell.

As the pressure amplitude is increased further, the maximum-to-initial diameter ratio reaches 2, a common criterion for UCA collapse. This collapse is termed 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. The violent collapse of the bubble during IC produces many mechanical effects and chemical agents that could cause bioeffects. Some of the consequences of collapse are mechanical shock waves, a bubble temperature that may reach thousands of degrees Kelvin (4,300-5,000 K), and free radical production (FRP). However, it has been shown that FRP is not required for transfection [12].

Sonoporation is a promising drug delivery and gene therapy technique, limited only by a lack of understanding regarding the biophysical mechanism that causes the cell membrane permeability change. The objective of this project is to elucidate the relationship between UCAs and sonoporation, specifically determining the role IC plays in sonoporation.

II. Materials and Methods

A. Cell Culture

Chinese Hamster Ovarian (CHO) cells (American Type Culture Collection (ATCC), Manassas, VA) were cultured in F-12K Medium (ATCC, Manassas, VA) with 10% v/v fetal bovine serum (ATCC, Manassas, VA), 1% Penicillin/Streptomycin (Sigma-Aldrich, St. Louis, MO), and 0.1% Fungizone (Invitrogen, Carlsbad, CA). The cells were propagated as a monolayer in 75 cm^3 tissue culture flasks at 37˚C and a humidified atmosphere of 5% CO_2.

B. Ultrasound Contrast Agents

Optison™ (Amersham Health Inc., Princeton, NJ) contains perflouropropane and is stabilized by a human serum albumin shell, with a mean diameter between 2 and 4.5 µm. The concentration of Optison™ is 5 to $8x10^8$ mL^{-1} gas bodies.

Definity® (Bristol-Myers Squibb, North Billerica, MA) contains perflouropropane and is stabilized by a phospholipid shell, with a maximum of $120x10^8$ mL^{-1} gas bodies with a diameter ranging from 1.1 to 3.3 µm.

C. Ultrasound Exposure Vessels and Cell Preparation

The sample vessel was a flat bottomed 96-well cell culture microplate (BD Falcon, San Jose, CA) constructed from medical-grade polystyrene. Each well holds 0.37 mL, with a diameter of 6.4 mm, 4.25 times the -6 dB focal beamwidth of the 3.15-MHz transducer. The open face of the microplate was covered by plastic cling wrap, forming a barrier between the external water bath and internal cell solution, as well as an acoustic window for the US to pass into the well unperturbed.

For preparation of an experiment, $0.3x10^6$ CHO cells in 0.37 mL of growth medium were added to each well. The vessel was incubated overnight to allow the seeded cells to settle to the bottom of each well, thus forming the monolayer. On the day of the experiment, the growth medium was removed and the monolayer rinsed twice with Phosphate Buffered Saline (PBS) to remove any dead cells and debris.

The exposure medium added to each well contained Fluorescein isothiocyanate-dextran (FITC-dextran) (FD500S, Sigma-Aldrich Co., St. Louis, MO), with an average molecular weight (MW) of 500,000 Daltons. The FITC-dextran is normally unable to cross the cell membrane, and thus used as the marker for change in cell membrane permeability. A volume of 0.05-mL FITC-dextran solution (25 mg/mL in PBS), and 8.80-µL Optison™ or 0.57µL Definity® were added to each well, with PBS filling the remainder of well volume. The plate was then sealed with plastic cling wrap. Any wells containing air bubbles were excluded from the experiment.

The vessel was placed in a room temperature, degassed water bath with the plastic cling wrap located near the transducer (Fig 1). Thus, the monolayer was on the back window of the chamber, allowing the UCAs to rise to the monolayer due to buoyancy and be pushed toward the monolayer by the radiation force. Each sample was either exposed or sham exposed (US turned off). Immediately following exposure, each sample was washed with PBS to remove any FITC-dextran in solution and placed on ice, to prevent pinocytosis of residual FITC-dextran.

D. Ultrasound Exposure

US was produced by a 3.15-MHz f/3 single-element focused transducer (diameter 0.75 in) (Valpey Fisher, Hopkinton, MA). The -6 dB beamwidth of the transducer was 1.5 mm. Sinusoidal tone bursts were generated by a pulser-receiver (Ritec RAM5000, Warwick, RI) for a pulse duration (PD) of 5 cycles, pulse repetition frequency (PRF) of 10 Hz, and exposure duration of 30 s. The peak rarefactional pressure (P_r) was varied over a range from 0.12 MPa to 3.5 MPa, and five replicates were performed at each P_r value. This P_r range encompassed the threshold for Optison™ and Definity® collapse, 0.83 MPa and 0.8 MPa, respectively [13, 14].

The transmit pressure waveforms were calibrated at the field's focus for each exposure condition. Calibrations were

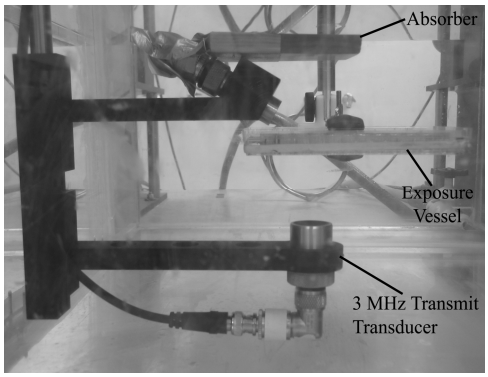

Figure 1. The Experimental Setup

routinely performed according to well-established calibration techniques using an NPL-calibrated PVDF bilaminar shielded membrane hydrophone (diameter of active element: 0.5 mm, Marconi 699/1/00001/100; GEC Marconi Ltd., Great Baddow UK). The hydrophone was located in the same position that the exposure vessel was located during experiments. Additionally, an absorber was placed above the microplate to prevent reflection from the water-air interface at the top of tank from interfering with the exposure conditions.

E. Post-exposure Analysis

Following exposure, the vessel was removed from the water bath and the monolayer trypsinized from the chamber. Each cell suspension was transferred to a microcentrifuge tube and immediately washed twice with 1-mL cold PBS to avoid pinocytosis. To assess cell viability, 1-µL propidium iodide (PI) (Sigma-Aldrich Co., St. Louis, MO) was added. Samples were analyzed using flow cytometry (Beckman Coulter, Inc. Epics XL-MCL, Fullerton, CA). Results are expressed in percentages of positively labeled cells, using the software program Summit v3.1 (Cytomation Inc., Fort Collins, CO). The percent of positive cells is relative to whole cells only, as debris from cells was ignored. FITC-dextran does not bind to the cell membrane, so fluorescence of a cell indicates internalized material (*i.e.*, sonoporation).

III. Results

CHO cells exposed to US in the presence of the UCA, Optison™, were observed, by means of FITC-dextran internalization, to have undergone sonoporation. A threshold-type study examining sonoporation activity as a function of P_r was performed. Over the P_r range 10 kPa to 3.5 MPa, the live sonoporated cells in the presence of Optison™ increased from 0.63% to 10.21%, with a maximum occurring at 2.4 MPa (Fig 2). Above 2.4 MPa, a significant drop in sonoporation activity was observed. Sonoporation in the presence of Definity® presented the same trend (Fig 3). The live sonoporated cells increased from 5.25% to 26.39%, with a maximum occurring at 172 kPa. Above 172 kPa a drop in sonoporation activity was observed. The error was calculated using standard error of measurement for the 5 samples at each P_r. The sonoporation in the presence of Definity® was consistently higher that the sonoporation in the presence of Optison™.

The collapse data of Optison™, obtained by Ammi et al. [13], is also plotted on Fig 2 to show the relationship between sonoporation and IC. The percentage of observed bubbles that underwent collapse at each P_r is displayed. The minimum IC threshold for Optison™ was defined as the 5% occurrence rate using logistic regression analysis. At these exposure conditions, the IC threshold for Optison™ is 0.83 MPa. At this threshold pressure, sonoporation had already reached over 50% relative to the maximum sonoporation activity. At P_r>2.4 MPa a significant drop in sonoporation activity was observed. This decrease corresponds to the pressure where greater than 95% of the Optison™ was collapsing.

The percentage of nonviable cells at each P_r is plotted in Fig 4. For the range of P_r examined, 120 kPa to 3.5 MPa, the nonviable cells varied between 0.74% and 3.9%, for Optison™ samples, and 5.5% to 8.0% for Definity® samples, with no distinct pattern emerging with respect to P_r. This emphasizes that sonoporation is not immediately lethal to the cells and that cell death is not related to the activity of the UCA, nor is cell death a contributor for the drop in sonoporation seen above 2.4 MPa for Optison™ and 172 kPa for Definity®.

IV. Discussion

Previously conducted research provides only circumstantial, not direct, evidence regarding the role of IC in the sonoporation mechanism. The experimental observations reported herein provide evidence that sonoporation is not

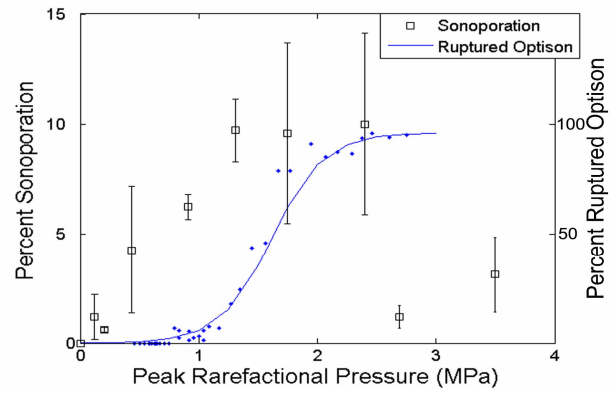

Figure 2. Sonoporation of CHO cells exposed at 3.15 MHz, 5 cycles, 10 Hz and for 30 s cycles, 10 Hz and for 30 s compared to the occurance of ruptured Optison™. The collapse threshold for Optison™ occurs at 0.83 MPa.

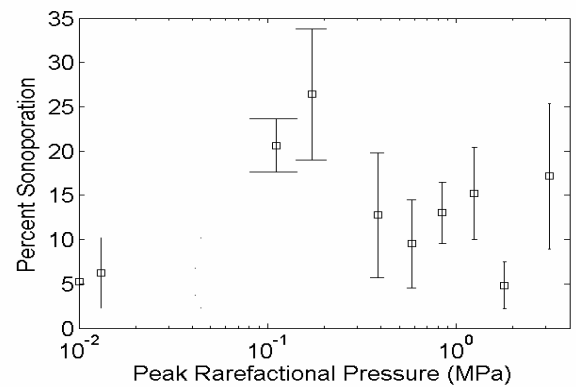

Figure 3. Sonoporation of CHO cells exposed at 3.15 MHz, 5 cycles, 10 Hz and for 30 s cycles, 10 Hz and for 30 s in the presence of Definity®. The collapse threshold for Definity® occurs at 0.8 MPa.

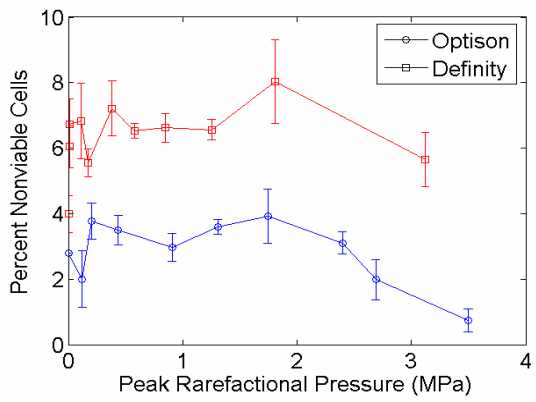

Figure 4. The percentage of nonviable cells immediately following exposure to 3.15 MHz, 5-cycle US for 30 s at a 10 Hz PRF in the presence of Optison™ or Definity®.

directly due to IC of UCAs. This is most apparent in comparing the collapse threshold of Optison™ and Definity® to the sonoporation response to Pr (Figs 2 & 3). The threshold for Optison™ collapse is at a P_r of 0.83 MPa, hence below this pressure fewer than 5% of the microbubbles are undergoing collapse. However, below this collapse threshold significant sonoporation is occurring. Furthermore, at the threshold for Optison™ collapse, sonoporation activity is half of the maximum observed activity. These results demonstrate that sonoporation is occurring while Optison™ is intact, thus IC is not the mechanism responsible for sonoporation.

Experimental results for Definity® have shown that the threshold for collapse is 0.8 MPa using the same exposure conditions applied in this study [14]. Below this threshold, sonoporation activity is dynamic, reaching a maximum and then declining. Thus, IC of Definity® is not the UCA behavior responsible for sonoporation. However, the determination of Definity™ collapse threshold is still being verified, so at this time these conclusions are preliminary for Definity®.

The exposure-dependent sonoporation activity between 120 kPa and 2.4 MPa for Optison™ and 10 kPa and 120 kPa for Definity® are P_r ranges that transition the UCAs' response from linear to nonlinear to inertial collapse (IC), suggests a mechanism that can likewise manifest itself exposure-dependent throughout this P_r range. It is likely that microstreaming is exposure-dependent between 10 kPa and 2.4 MPa. Liquid jets are an unlikely explanation for the sonoporation results presented in this paper due to the random nature of liquid jet formation.

At P_r>2.4 MPa, a drop in sonoporation activity of the Optison™ occurs. At these P_r values (2.7 and 3.5 MPa), greater than 95% of the Optison™ are collapsing quite rapidly. Due to their rapid collapse, the UCAs are not present to oscillate and contribute to microstreaming. Thus, at higher P_r microstreaming will be minimized. A similar drop in the sonoporation due to Definity® was observed. In addition, the drop in sonoporation does not have a corresponding increase in the percentage of nonviable cells. Thus, increased cell death is not a cause of this decreased sonoporation.

Microstreaming is influenced by the degree of oscillation of the UCA, which is influenced by the properties of the shell. Optison™ and Definity® have different shells, thus having different shell properties. These differences have the potential to manifest with respect to the quantity of sonoporation. For example, samples with Definity® displayed a maximum sonoporation of 26.39%, while samples with Optison® had a maximum of 10.21%. The degree of sonoporation could be directly related to the oscillating capacity of the UCA.

The evidence provided suggests directly that the sonoporation effect was caused by linear or nonlinear oscillation of the UCA. These responses occur at lower pressure amplitudes and could thus explain the presence of sonoporation at the lower pressure levels. Therefore, we conclude that IC is not the responsible mechanism for sonoporation and hypothesize that microstreaming due to microbubble oscillations is principally responsible.

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