ON THE ACOUSITC RESPONSE OF ULTRASOUND AND
INDUCED CELL DEATH

By

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Fatimah Alsaiari

Abstract

On the acoustic response of ultrasound and microbubble induced cell death

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Ultrasonically-stimulated microbubbles can enhance cell membrane permeability and decrease cell viability where the underlying acoustic mechanism has been associated with both non-inertial and inertial cavitation. In this study, breast cancer cells (MDA-MB-231) were exposed to 0.5MHz ultrasound pulses of 16 μ s duration at varying peak negative pressures (PNP: 218kPa, 335kPa and 908kPa) and pulse repetition period (PRP 10ms and 100ms) in the presence of Definity microbubbles (3.3% v/v). The acoustic response of microbubbles was measured using passive cavitation detection with 2.25MHz transducer, and characterized by their frequency a cavitation dose (CD). Results show that the number of non-viable cells and integrated cavitation dose (ICD) significantly increases with PNP, whereas no significant differences were found between 10ms and 100ms PRPs. In this study, no correlation was found between (ICD) and cell non-viability.

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Dedication

To my husband, Rahed Alshamasy, and our children

To my mother, brother and sisters, especially “Sharifah Alsaiari”

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LIST OF SYMBOLS AND ABBREVIATIONS

PNP	Peak negative pressure
PCF	Pulse center frequency
CD	Cavitation dose
US	Ultrasound
MB	Microbubbles
RMS	Root mean square
RA	Receiver amplifier
TD	Time domain signal
ICD	Integrated cavitation dose
UCAs	Ultrasound contrast agents
PRP	Pulse Repetition Period
USMB	Ultrasound and Microbubbles
HR	Harmonic
ULTR-HR	Ultra-harmonic

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Chapter 1 Introduction

1.1 Background

Clinically, microbubbles (MB) are utilized in ultrasound (US) imaging as a contrast agent to enhance detection of blood circulation [1]. Experimentally, ultrasound and microbubbles (USMB) have potential applications in enhancing targeted drug delivery of chemotherapeutic drugs and genetic materials [2] and in improving the effectiveness of radiotherapy in cancerous tissues [3,4]. Microbubbles exposed to an ultrasound field can undergo stable linear and non-linear oscillations, or disruption depending on the ultrasound exposure conditions [3]. The interaction of USMB with cells has been shown to kill cells and enhance plasma membrane permeabilization allowing cell-impermeable molecules to enter a cell, a phenomenon referred to as sonoporation [5,6]. The biological mechanism of sonoporation appears to be associated with membrane disruption and enhanced endocytosis mediated by USMB [7]. This dissertation investigates the effect of USMB on cell death and the associated acoustic response under varying ultrasound exposure conditions.

1.2 Ultrasound physics

Ultrasound is a pressure wave with frequencies above 20 kHz. An ultrasound wave propagates through tissue through longitudinal oscillation of the particles of the medium about their equilibrium position in the direction of propagation [8]. As a wave propagates through a medium, energy is lost due to absorption and scattering; attenuation is a term used to account for scattering and absorption of a wave. Scattering is a process during which reflected acoustic waves change direction in regards to the incident acoustic wave. The scattering of ultrasound is due to the difference in the acoustic impedance within a medium [9,10]. The acoustic impedance of a medium is given by:

$$Z = \rho c \quad (1.1)$$

where ρ is the density of the medium [kg/m^3] and c is the speed of sound [m/s].

Absorption is a process during which acoustic wave energy is converted to thermal energy. At high intensity ultrasound conditions, the absorption of ultrasound energy interacts with tissue and cause tissue coagulation; this is referred to as thermal bio-effects [11]. HIFU has been used to treat soft cancer tissue [12]. HIFU causes tissue temperatures to rise in a particular focal area while the surrounding tissue remains unheated. *In vivo* studies have shown that a 1.7MHz frequency pulse with an intensity of 1500W/cm^2 and time exposure of 1- 2 sec, cause the temperature to exceed 56°C . This causes coagulative necrosis with a margin of 6-10 cells between live and dead cells at the focal region [12, 13]. In addition, ultrasound can cause bioeffects through non-thermal mechanisms such as cavitation, which describes the interaction between acoustic wave and gas bubbles or nucleus [14, 15, 16].

1.3 Ultrasound and Microbubbles

1.3.1 Microbubble Agent

Microbubbles are small in size, less than 10 μm in diameter making them smaller than red blood cells. The structure of microbubbles consists of a gas core and an outer shell. The outer shell of microbubbles is composed of a variety of biocompatible materials such as proteins, lipids or polymers, ranging approximating 10-20 nm in thickness. The core of microbubbles comprise of gas of high molecular weight such as perfluorocarbon, sulphur hexafluoride, and perfluoropentane [17, 18].

1.3.2 Microbubble Physics

The response of microbubbles in an ultrasound field depend on (1) the MB characteristics (size, gas and shell) and environment, and (2) on the ultrasound exposure parameters such as peak negative pressure (PNP) and pulse center frequency (PCF) [19]. Microbubbles in an ultrasound field can undergo stable oscillation – linear and non-linear – or disruption [20]. The oscillation of MBs is maximized at their resonant frequency [21,22]. The resonant frequency for free gas bubbles, assuming a linear and an undamped system, is:

$$f_r = \frac{1}{2\pi a} \sqrt{\frac{3\gamma_c}{\rho} P_o} \quad (1.2)$$

where a is the initial radius of the bubble, $\gamma_c = \gamma_p / \gamma_v$ (where γ_p is the heat capacity at constant pressure and γ_v at constant volume), ρ is the density of the surrounding medium, and P_o is the static pressure at the bubble surface [23].

As acoustic pressure increases, the microbubbles shift towards nonlinear oscillation [24]. Microbubbles expand during negative pressures and contract during positive pressures. Non-inertial cavitation occurs during low pressure and microbubbles oscillate both linearly and non-linearly about their equilibrium radius [25]. Inertial cavitation occurs at high acoustic pressures where microbubbles undergo large expansion followed by a violent collapse [26]. The microbubbles experiencing inertial cavitation generally reach a radius two times larger than their initial diameter [17, 18]. The microbubbles undergo inertial cavitation (IC) depending on shell and gas properties. It has been shown that the thick shell has a high threshold compared with thin shell microbubbles. For example, Optison microbubbles are destroyed at 0.13MPa while Biospheres are destroyed at 0.23MPa [27]. Furthermore, it has been shown that the threshold increases as center frequency increases in both thin shell microbubbles and thick shell microbubbles. Microbubbles that undergo linear oscillation emit signal frequencies equal to that of driving frequency (fundamental frequency). Bubbles that undergo non linear oscillation emit signal frequencies in the harmonics ($2f$, $3f$, $4f$ etc), subharmonic($f/2$, $f/3$ etc) and ultraharmonic ($3f/2$, $5f/2$,etc) [27]. Figure 1 illustrates the occurrence of harmonic, ultra harmonic and sub harmonic signals after transmitting a pulse at 20 kHz [28].

Figure 1.1: The acoustic emission spectrum for bubbles using the passive cavitation detection measurement. The figure includes fundamental signals, 2nd Harmonic, Ultra-Harmonic, 2nd Ultra-Harmonic and Sub-Harmonic [28].

1.4. Sonoporation

Sonoporation has recently been studied as new modality for cancer treatment. Ultrasound and microbubbles have the ability to enhance cellular uptake of molecules to target areas. Several investigations have already examined the effect of varying ultrasound parameters on cells and microbubbles, however there is limited understanding on the correlation between microbubble acoustic response during sonoporation and cell viability. Studies have reported on the impact of different ultrasound parameters on cell membranes [29,30,31]. Sonoporation has been used for cancer treatment but it remains a challenge to achieve consistent and controlled delivery of drugs to the interior of tumor. Furthermore, the acoustic dynamic of sonoporation remains unclear. Sonoporation is dependent on ultrasound exposure parameters including: frequency, acoustic pressure, pulse repetition period and pulse duration. One investigation has indicated that an optimal ultrasound condition for sonoporating endothelial cells is 30 s [32]. Generally, as

acoustic pressure and pulse repetition frequency increase, both cell permeability and cell death increases [33].

Figure 1.2: Schematic diagram of ultrasound and microbubble induced sonoporation by cell membrane disruption [33].

Figure 1.3: The acoustical mechanisms associated with ultrasonically-stimulated microbubbles include microstreaming induced by oscillating microbubbles, shock waves due to non-stable cavitation, and micro-jet formation due to collapsing microbubbles. A: drug molecules; B: stable cavitation of microbubbles; C: Microstreaming; D: non stable cavitation of microbubbles produce shock waves; E: fluid microjet; F: cell membrane disruption; G: non ruptured cell membrane with increase permeability due to USMB; H: cell membrane damage due to shock waves and microstreaming; I: extravascular tissue; J: microbubble loaded with drug molecules on its surface; K: microbubbles loaded with drug molecules in lipophilic phase; L: micelle loaded with drug molecule lipophilic phase; M: liposome with aqueous interior; N: targeted microbubble [20].

Cavitation has been associated with sonoporation-mediated delivery of drugs and genetic materials [18]. Stable oscillation of microbubbles has been shown to generate microstreaming within the surrounding fluid and induce shear stress on nearby biological membranes [47]. In addition, microbubbles undergoing inertial cavitation can generate shockwaves and fluid microjets and induce bioeffects on biological cells [7]. Furthermore, microbubbles can be loaded with molecules and targeted to a diseased site, and subsequently releasing the drug molecules by disrupting the microbubbles acoustically (Figure 1.3).

1.5 Microbubble Acoustic Response

1.5.1 Microbubble Characterization

Microbubbles can be characterized acoustically using active and passive detection methods. Active cavitation detector uses a pulse-echo technique, which transmits and receives ultrasound pulses using the same transducer [34]. In passive cavitation detection (PCD), one transducer emits the pressure wave while the other transducer is set only in receive mode, which listens to the signal emissions from microbubble passively [35].

1.5.2 Cavitation Dose

Cavitation in this study refers to the acoustic activity of micorbubbles exposed to an ultrasound field. Cavitation dose is important in characterizing microbubble acoustic response; it quantifies the amount of energy emitted by USMB [36]. Integrated cavitation dose quantifies the cavitation activity of microbubbles when exposed to ultrasound pulses. The integrated cavitation dose has been related to various bio-effects induced by ultrasound and microbubbles [37]. One of the specific aims in this project is to measure the integrated cavitation doses. The frequency spectrum of the signal emitted by microbubbles is calculated using:

$$S(f, \tau) = \int_{-\infty}^{+\infty} s(t, \tau) e^{-j2\pi ft} . dt \quad (1.3)$$

where $s(t, \tau)$ is the time domain signal and $S(f, \tau)$ is the frequency spectrum of $s(t)$; t refers to the fast time and τ refers to the slow time of the ultrasound exposure pulse sequence.

The cavitation dose (CD) was calculated using Equation 1.4 over a certain frequency range at each of the slow time-points τ :

$$CD(\tau) = \int_{f_n+\Delta f}^{f_n-\Delta f} |S_i(f, \tau)| df \quad (1.4)$$

The integrated cavitation dose over the exposure duration was calculated using;

$$ICD = \int_0^{IT} CD(\tau) d\tau \quad (1.5)$$

Where IT is the insonation time of the ultrasound pulse sequence.

1.6 Thesis Outline

This project aims to study USMB-induced cell death through the characterization of microbubble acoustic response. In this study, the effect on cell viability was associated to harmonic cavitation doses in an effort to better understand USMB induced cell death. This study is guided by the hypothesis that the integrated cavitation dose is correlated with cell death when treated with USMB. The specific objectives of this investigation are:

- 1- To determine the effects of peak negative pressure and pulse repetition period on cell viability and acoustic response of microbubbles measured by PCD (Passive Cavitation Detection)
- 2- To investigate the relationship between cell viability and inertial cavitation dose.

Chapter 2 Materials and Methods

2.0 Introduction

Cells and microbubbles in suspension were exposed to varying ultrasound exposure conditions. During ultrasound exposure, the microbubble acoustic response was measured using a passive cavitation detector (PCD). Following ultrasound exposure cell viability was measured using flow cytometry.

2.1 Cell model

A human adenocarcinoma breast cell line (MDA-MB-231- ATCC, MD, USA) was used in this project. MDA cells were grown in RPMI media with 5% streptomycin antibiotic and 10% fetal bovine serum. The cells were grown as a monolayer in 175cm tissue culture flasks with humidity of 5% CO₂ at 37 °C. The cells were exposed to USMB (ultrasound and microbubbles) at a concentration of 1×10^6 cells / mL at a volume of 1 mL. Cells were suspended in RPMI cell culturing media at room temperature during the experiment.

2.2 Ultrasound exposure system

The system consists of an arbitrary wave generator (AWG610, Tektronix, USA), power amplifier (AG series Amplifier, T&C power conversion, Inc., USA) and AlazarDso acquisition system. Two transducers attached to micropositioning systems were used in this project: Transmitting transducer was 0.5 MHz (IL0508HP, Valpey Fisher Inc, Hopkinton, MA) with a diameter of 25.4 mm, a focal length of 4.3 cm and a beamwidth of 9.2 mm; receiving transducer was 2.25MHz (IL0208HP, Valpey Fisher Inc, Hopkinton, MA) with a diameter of 25.4 mm and bandwidth of 30% at -6dB. The passive cavitation detection transducer was positioned at 90° in relation to the transmitting transducer. The ultrasound apparatus held the sample submerged in degassed water inside a tank with dimensions 50 x 30 x 20 cm³. A schematic diagram of the ultrasound apparatus is shown in Figure 2.1. The sample was placed at the intersection of the focal zones of the transducers in a cylindrical treatment chamber with 8mm internal diameter. Focusing was done using a micropositioning system with a needle to receive the pulse-echo from the needle.

The effect of the following ultrasound exposure parameters on viability and microbubble response was investigated at varied pulse repetition periods (PRP) of 10ms and 100ms over different pressures (218,335 and 908kPa) The acoustic pressure was measured in the absence of cell exposure chamber using a needle hydrophone (Precision Acoustics Ltd, Dorchester, and Dorset, UK), 8 cycles tone-burst pulse (pulse duration of 16µs) and a total insonation time of 30s. The trigger delay in the acquisition system was set to 70 µs. The acoustic emission from MBs acquired using the passive cavitation detection was analyzed using FFT spectral analysis (MATLAB) and cavitation dose calculations [38].

2.3 Cavitation dose

The Power Spectral Density (PSD) was calculated in MATLAB to understand the strength of a signal distribution in the frequency domain (FFT). This was implemented in MATLAB using equation 2.1. Spectrograms were used to analyze the microbubbles' response throughout the entire treatment time.

$$S(f, \tau) = 20 \log \text{FFT} [(s(t, \tau))] \quad (2.1)$$

Where t is the fast time and τ is the slow time.

The cavitation dose was calculated using Equation 2:

$$CD(\tau) = \sum_{f_n - \Delta f'}^{f_n + \Delta f'} |S(f, \tau)| \Delta f \quad (2.2)$$

Where $S(f, \tau)$ is the FFT of $s(t)$, $\Delta f = 0.012\text{MHz}$ is the step size in frequency domain, $\Delta f' = 0.1\text{MHz}$, and $f_n = 1.00, 1.50$ and 2.00 MHz at the harmonic frequencies and $1.25, 1.75$ and 2.25 MHz at the ultra-harmonic frequencies. The CD as a function of τ represents the MB signal at each ultrasound pulse within the sequence.

The calculation of integrated cavitation dose was performed using the ICD equation below:

$$ICD = \sum_1^{\tau_i} CD \cdot \Delta \tau \quad (2.3)$$

Where τ_i is 3000 and $\Delta \tau = 10\text{ms}$ for the 10 ms PRP, and τ_i is 300 and $\Delta \tau = 100\text{ms}$ for the 100ms PRP [45].

2.4 Microbubble contrast agent

Definity Microbubbles (Lantheus Medical Imaging, North Billerica, MA0186, USA), a clinically approved ultrasound contrast agent, were used. The microbubbles comprised of a phospholipid shell encapsulating gaseous octafluoropropane (C_3F_8) with a mean diameter of 1.1-3.3 μ m to a maximum diameter of 20 μ m according to the manufacturer. Definity microbubbles had an initial concentration of 1.2×10^{10} bubbles/mL, which was activated by shaking with Vial-Mix (Lantheus Medical Imaging Inc) for 45 seconds. The microbubbles concentration used in this experiment per sample was 33 μ L/mL. Microbubbles were diluted with PBS at 1:1 and 1:4 ratios, respectively.

2.5 Treatment procedure

Cell samples were placed in the chamber and treated with ultrasound pulses in the presence of microbubbles. Microbubbles were added just prior to ultrasound exposure. During ultrasound treatment, the cells were uniformly distributed using gentle magnetic stirring. The chamber was washed between exposures using PBS.

2.6 Cell viability

Following treatment with ultrasound and microbubbles, cell viability was measured using flow cytometry (BD FACSCalibur; San Jose, CA, USA) and propidium iodide (PI) staining. Cells unstained with PI were referred to as viable. Cells stained with PI were considered non-viable; PI stain cells whose membrane integrity has been compromised. Dilute Propidium Iodide (PI), 5 μ L/samples, was made by 1:4 dilutions with PBS.

Figure 2.1: The schematic diagram of the ultrasound exposure system. Microbubbles and cells were exposed in a cylindrical chamber with an internal diameter of 8mm and a height of 90 mm; 30 mm was covered with acoustic membrane. Microbubbles were exposed to an ultrasound field using a 0.5MHz transducer and emissions were received using 2.25MHz transducer. Harmonic and ultra-harmonic frequencies were detected.

Chapter 3 Results

3.0 Introduction

This project investigated the response of both MDA-Mb-231 cells and Definity microbubbles when exposed to varying acoustic pressures (0-908kPa) and pulse repetition period (10ms and 100ms), with 16 μ s pulse duration and a microbubble concentration of (3.3% v/v) for an insonation time of 30s. Cell viability results are shown in section 3.1, the acoustic characteristics of microbubbles in section 3.2 and the relationship between cell viability and integrated cavitation dose in section 3.3.

3.1 Non-viable cells

Figure 3.1 illustrates the effects of acoustic pressure and pulse repetition period on cell viability. The viability of a cell when exposed to ultrasound and microbubbles is influenced by both peak negative pressure and pulse repetition period. The percentage of non-viable cells significantly increases with peak negative pressure and only slightly decreases with pulse repetition period. In terms of peak negative pressure, there were statistical differences between the control (0 kPa, 2.3% cell death), and all the pressures (218 kPa, 335 kPa, 908 kPa) for both 10ms and 100ms. Cell death at 218 kPa (14% at 10ms PRP, and 11% at 100ms PRP) and 335 kPa (10ms= 16%, 100ms= 14%) are similar with no significant differences found between these pressures. The

maximum amount of cell death observed for pulse repetition periods of both 10ms and 100ms was found at 908 kPa with 26% and 22%, which was also statistically higher than both 218 kPa and 355 kPa treatments. In addition, the cell viability was not statistically different between the pulse repetition periods of 10ms and 100ms.

Figure 3.1: Non-viable cell of MDA-MB-231 cells exposed to a 0.5 MHz center frequency at varying acoustic pressures (218 kPa, 335 kPa, 908 kPa) with a 10ms (black bars) and 100ms (grey bars) PRP and total treatment time of 30s in 3.3% Definity volume concentration. The percentages of Non-viable cells are shown with respect to different pressures at center frequencies of 0.5MHz.

3.2 Acoustic characteristics of Microbubbles

The effect of acoustic pressure (PNP) on acoustic MB behaviors is shown in Figures 3.2-to-3.5. The acoustic behavior of a MB exposed to 8 cycles at 0.5 MHz frequency pulse at acoustic pressures (218kPa, 335kPa, 908kPa) for 30 s depends on the applied PNP. Spectrograms associated with the acoustic response time domain signal and the FFT of the MB at 10ms and

100ms pulse repetition period are presented in Figure 3.2 and 3.3 respectively, and the FFT spectrograms are shown in Figure 3.4 and 3.5, respectively.

3.2.1 Effect of peak negative pressure

The time domain signals received with the 2.25 MHz transducer and their corresponding frequency spectra are shown in Figure 3.2 at 10ms pulse repetition period. As the pressure of the transmitted wave was increased, the signal from the MBs increased as shown in Figure 3.2 (A, B and C). As seen in Figure 3.2 (A), the amplitude of the time domain signal is 1, and as pressure increases up to 908kPa the amplitude increases to 1.5. At 218kPa, the MBs experience weak non-linear oscillation emitting the fundamental (f), harmonic signals ($2f$, $3f$, $4f$ and $5f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$ and $4.5f$) as shown in Figure 3.2(D). At 335kPa, the MBs strong non-linear oscillation with an emission of the fundamental (f), harmonic signals ($2f$, $3f$, $4f$, $5f$ and $6f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$ and $4.5f$) shown in Figure 3.2(E). At 908kPa, the MBs undergo irregular compression emitting the fundamental (f), harmonic signals ($2f$, $3f$, $4f$, $5f$ and $6f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$, $4.5f$) shown in Figure 3.2(F). Also, in Figure 3.2 (C) the emission spectrum from the MB appears to broaden at higher pressures indicating increased frequency emissions associated with non-linearity. In comparing the FFT graphs of the different pressures it is also notable that the amplitude of the harmonic signals increases on average, as the pressure increases (Figure 3.2 D, E, F). For example, the amplitude of the second harmonics ($2f$) increases from ~ 350 at 218kPa to ~ 620 at 335kPa. The second harmonics increases further when the pressure goes up to 908kPa (~ 700). At the higher pressures a higher amplitude harmonic and sub-harmonic signals are generated.

218kPa

335kPa

908kPa

Figure 3.2: The acoustic behavior of Definity microbubbles at varying acoustic pressure at 10ms PRP. The first column represents Time Domain Signal of the echo received, where the x-axis represents the time y-axis represents the amplitude. The second column represents the corresponding FFT of the time domain signal, where x-axis represents the frequency of the waves emitted from the microbubble and the y-axis represents the amplitude of the wave.

The time domain signals received with the 2.25 MHz transducer and their corresponding FFT spectra are shown in Figure 3.3 at 100ms pulse repetition period. As the pressure of the transmitted wave was increased, the echo signal was also increased as shown in Figure 3.3(A, B, C). At 218kPa, the MBs also experience weak non-linear oscillation emitting the fundamental (f), harmonic signals ($2f$, $3f$, $4f$ and $5f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$ and $4.5f$) seen in Figure 3.3(D). At 335kPa, the MBs strong non-linear oscillation with an emission of the fundamental (f), harmonic signals ($2f$, $3f$, $4f$, $5f$ and $6f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$ and $4.5f$) shown in Figure 3.3(E). Similar to 335kPa, MBs exposed to 908kPa also exhibit strong non-linear oscillation emitting the fundamental (f), harmonic signals ($2f$, $3f$, $4f$, $5f$ and $6f$) and ultra-harmonic signals ($1.5f$, $2.5f$, $3.5f$ and $4.5f$) shown in Figure 3.3(F). It is important to note that though the harmonic signals remain the same as those received from the tests with a 10ms PRP, the amplitudes of the signals not including the fundamental signal are lower with 100ms. In comparing the FFT graphs of the different pressures the amplitude of the harmonic signals seem to decrease as the pressure increases (Figure 3.3 D, E, F). When looked at the first harmonic ($2f$) at 218kPa the amplitude appears to be at ~ 380 , drops slightly at 335kPa and then down to ~ 310 at 908kPa. It is evident that higher pressures produce more harmonic and sub-harmonic signals.

218kPa

335kPa

908kPa

Figure 3.3: The acoustic behavior of Definity microbubbles at varying acoustic pressure at 100ms PRP. The first column represents Time Domain Signal of the echo received, where the x-axis represents the time y-axis represents the amplitude. The second column represents the corresponding FFT of the time domain signal, where x-axis represents the frequency of the waves emitted from the microbubble and y-axis represents the amplitude of the wave.

The spectrogram in Figure 3.4 present the time dynamics of the MBs acoustic behavior exposed to a pulse with a pulse repetition period of 10ms. Figure 3.4 (A, B, C) show 30s of the time dynamic while Figure 3.4 (D, E, F) magnify the spectrogram to 5s in order to clearly observe the signal. The non-linear oscillation of microbubbles appears to increase as the pressure of the pulse increased from 218kPa to 908kPa. The time dynamic results suggest that MBs experience strong non-linear oscillation within the first few seconds at each pressure and then undergo a loss of signal (Fig. 3.4). At 218kPa, the loss of signal from the MB appears to occur approximately between 4-5s with clear harmonic signals suggesting weakly oscillating microbubbles (Fig. 3.4 A, D). At 335kPa, the spectrograms show additional harmonics as well as a clean MB signal up to 1s after the start of the signal (Fig. 3.4 B, E). The shorter oscillation signal compared to 218 kPa implies that the MB experienced stronger non-linear oscillation. At 908kPa, the MB signal was lost within milliseconds during exposure (Fig. 3.4 C, D). The signal recorded on the spectrograms from Fig. 3.4 F was slightly cluttered in comparison to the other pressure spectrograms. The results show that as the pressure of the pulse increases, the amount of time for the loss of MB signal decreases.

Figure 3.4: The spectrograms of acoustic behavior of Definity microbubbles at varying acoustic pressure received with 2.25MHz at 10ms PRP of 30s total treatment times. The x-axis represent frequency and y-axis represent treatment times. Figure 3.4 (A, B, C) show 30s of the time dynamic while Figure 3.4 (D, E, F) magnify the spectrogram to 5s in order to clearly observe the signal. (A) and (D) at PNP=218kPa;(B) and (E) at PNP=335kPa; (C) and (F) at PNP=908kPa.

The spectrogram in Figure 3.5 present the time dynamics of the MBs acoustic behavior exposed to a pulse with a pulse repetition period of 100ms, received with a 2.25 MHz transducer. Figure 3.5 (A, B, C) show 30s of the time dynamic while Figure 3.5 (D, E, F) magnify the spectrogram to 5s in order to clearly observe the signal. The non-linear behavior of microbubbles appears to increase as the pressure of the pulse in increased from 218kPa to 908kPa. The time dynamic

results suggest that the MBs experience strong non-linear oscillation within the first few seconds at each pressure (Fig. 3.5). At 218kPa, the intensity of the signal is low and the signal appears to dissipate at ~ 10 s during the exposure. The harmonics from the microbubble suggest that undergo weak non-linear oscillation (Fig. 3.5 A, D). At 335kPa, the spectrograms show additional harmonics as well as a signal up to 25s into the exposure and lost thereafter (Fig. 3.5 B, E). Based on the harmonics and the increased cluttering of the signal the MB seems to undergo strong non-linear oscillation. At 908kPa, the signal from the MB did not appear to be lost during the entire 30s time frame indicating continued oscillation from the MB (Fig. 3.5 C, F).

Figure 3.5: The spectrograms of acoustic behavior of Definity microbubbles at varying acoustic pressure received with 2.25MHz at 100ms PRP of 30s total treatment times. The x-axis represent frequency and y-axis represent treatment times. Figure 3.5

(A, B, C) show 30s of the time dynamic while Figure 3.5 (D, E, F) magnify the spectrogram to 5s in order to clearly observe the signal. (A) and (D) at PNP=218kPa; (B) and (E) at PNP=335kPa; (C) and (F) at PNP=908kPa.

3.2.2 Integrated cavitation doses

Integrated cavitation doses of the received MB acoustic signal after exposed to 8 cycles, 0.5MHz center frequency pulsed wave at 10ms and 100ms PRPs are shown in Figure 3.6. The integrated cavitation dose (ICD) increases with acoustic pressure for all harmonic and ultra-harmonic frequency ranges. The ICD values at 10ms and 100ms PRP increased significantly among both harmonic (1MHz, 1.5MHz, 2MHz) and ultra-harmonic (1.25MHz, 1.75MHz and 2.25MHz) frequencies between 218kPa and 908kPa; the maximum ICD was observed at 908kPa. At 10ms PRP statistically significant differences were observed between 218kPa and 335kPa for all harmonic (1MHz, 1.5MHz, 2MHz) and the first ultra-harmonic (1.25MHz) frequencies. The general trend in ICD values within the harmonic frequencies shows an increase at the second harmonic (1.5MHz) and a decrease at the third harmonic (2MHz) relative to the first harmonic (1MHz) at all pressures. Relative to the first ultra-harmonic, the ICD values decrease at each subsequent ultra-harmonic frequencies for both 335kPa and 908kPa. At 100ms, statistically significant differences were observed between 218kPa and 335kPa at the second and third ultra-harmonic frequencies (1.75MHz, 2.25MHz). The general trend in ICD values within both the harmonic (1MHz, 1.5MHz, 2MHz) and ultra-harmonic (1.25MHz, 1.75MHz and 2.25MHz) frequencies follows a similar trend as the 10ms PRP treatment, however the ICD values are significantly lower for the 100ms PRP treatment.

Figure 3.6: Integrated cavitation dose of the harmonic and ultra-harmonic frequency at different acoustic pressures (218kPa, 335kPa, 908kPa) received with 2.25 MHz transducer at a PRP of 10ms and 100ms for 30s of treatment time.

3.3 The relationship between non-viable cells and ICD

Figure (3.7) shows the correlation of non viable cells and integrated cavitation dose for harmonic and ultra-harmonic exposed to a 30s, 8 cycles 0.5 MHz frequency pulse at different acoustic pressures (218kPa, 335kPa, 908kPa) and received with a 2.25MHz transducer. As the applied pressure was increased, the integrated cavitation dose and non-viable cells also increased. Results did not show any correlation between non-viable cells and ICD. For both PRPs (10ms and 100ms), exposure of the highest pressure (908kPa) resulted in the largest non-viable cell values

(~ 22% and ~26%) for all harmonic (1 MHz, 1.5 MHz, 2 MHz) and ultra-harmonic (1.25MHz, 1.75MHz, 2.25MHz) frequencies. However, the non-viable cell values obtained from the 10ms exposure were higher than that obtained from 100ms. The ICD values for each frequency window measured for both 10ms and 100ms were not equivalent, each with its own unique specific value. Although there appears to be a relationship between the ICD and cell non-viability, the correlation was not statistically significant.

Figure 3.7: The correlation of MDA-MB-231 breast cancer cells vs. integrated cavitation dose of harmonic (2f, 3f, 3f) and ultra-harmonic (2.5f, 3.5f, 4.5f) at different pressure (218kPa, 335kPa, and 908kPa), frequencies received with 2.25 MHz transducer exposed to an 8-cycle pulse sequence with a 10ms and 100ms PRP for a 30s treatment time (n=8).

Chapter 4 Discussion

4.0 Introduction

This study aimed at improving our understanding of the relationship between cells killed by USMB and microbubble acoustic response at different PNP and PRP using passive cavitation detection method and inertial cavitation dose. The study indicates that cell viability and acoustic response of MBs depend on ultrasound pressure and do not vary with PRPs of 10ms and 100ms.

4.1 Non-viable cells

It has been shown that ultrasound in the absence of microbubbles at exposure conditions similar to this study induce minimal or no effect on cell viability [36]. In the presence of microbubbles, higher PNPs have a more significant effect on cell death, which may be due to increased damage inflicted on the cell membrane by USMB [33, 39]. In addition, ultrasound and microbubbles has also been shown to cause stresses on the cell membrane increasing the incidence of apoptosis [31]. Whereas low ultrasound pressures have been shown to increase permeabilization of the membrane and allow large molecules to enter the cell and remain viable [41]. The integrated cavitation dose indicates that the MB response increases at higher acoustic pressures. Increasing the PRP from 10ms to 100ms reduces the number of ultrasound pulses sent to the sample within the treatment time. This may suggest that the increased rate of ultrasound pulses may be causing

increased levels of damage to the cell membrane or the lengthened gap between pulses may allow the cell additional time to recover from the exposure.

4.2 Acoustic characteristics of Microbubbles

The non-linear response of microbubbles increased with pressure; the amplitude of harmonics and ultra-harmonics signals emitted from the MB increased with pressure. Studies have shown similar results that change in PNP cause significant changes in MB behavior showing irregular oscillations [24,25,26]. The spectrogram in (Figure 3.4) present the time dynamics of the MBs acoustic behavior exposed to a pulse with a pulse repetition period of 10ms respectively at different pressures. This form of analysis can detect the loss of MBs from the solution as a result of MB disruption. As pressure was increased from 218kPa to 908kPa, the spectrogram showed subsequently stronger MB signals within 1s and progressively faster loss of MB signals, with the fastest loss at 908kPa (Figure 3.4 F). This suggests that higher PNPs cause stronger non-linear signals, apparent from the harmonic and ultra-harmonic signals, and the disruption of microbubbles at a higher rate. This observation is in accordance with past research papers that highlighted the dependence of MB rupture on PNP [23,24,25]. This information not only provides us with the behavior of microbubbles but also the stability of microbubbles at different pressures.

The integrated cavitation dose (Figures 3.8) at specific frequencies such as harmonic signals (2f, 3f, 4f,) and ultra-harmonic signals (2.5f, 3.5f, 4.5f), increased with pressure, which has been shown by previously published papers [43,44,45]. This may be due to non-linear microbubble oscillations or microbubble disruption. In addition, ICD also increased with PRP which may be due to the number of times MBs within the solution were exposed to US pulses within the 30 s

exposure time. At 10ms PRP, MBs are exposed to an US pulse 3000 times, whereas at 100ms the MBs are exposed to an US pulse 300 times.

4.3 Relationship of non-viable cells and integrated cavitation dose

Generally, as ICD increases the percentage of non-viable cells increases at all harmonic and ultra-harmonic frequencies. However, statistically there is no correlation between non-viable cells and ICD. More cells are killed with USMB at higher ICD doses. It is postulated that higher ICD would induce a higher level of stress onto cell membranes [46]. It is also important to note that the proximity of the MB is important for the effect to be realized. The energy emitted from the MB will only do damage to the membrane if it close enough to the surface [47].

4.4 Limitations of this study

This study has several limitations. The analysis of passive cavitation detection data did not take into account the bandwidth of the receiving transducer (2.25MHz). Cell viability was measured using Propidium iodide a few hours after the ultrasound exposure. Propidium iodide is a short-term analysis that only measures cell death. In addition, only two PRP and three PNP settings were used in this study.

Chapter 5 Conclusions and Future Work

5.0 Conclusions

The results show that the number of non-viable cells and ICD increases with PNP. The study suggests that there is no correlation between ICD and cell viability. In addition, cell viability appeared to be independent of PRP at the setting used in this study, although the ICD dose increased with PRP. A higher ICD was measured at 10ms PRP compared to 100ms PRP. Overall, no correlation was observed between ICD at a specific harmonic or ultra-harmonic frequencies and cell viability.

5.1 Future Work

Enhancing our understanding of the acoustic behaviors of MBs and the effect they have on cells is important for improving ultrasound and microbubble therapy. Further focus on the effect of PNP and the corresponding integrated cavitation dose on cell viability is valuable as it will allow for more controlled treatments during experiments. The effect of MB type and size is also important for quantifying MB response.

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