AN INVESTIGATION ON THE USE OF MICRO-ULTRASONIC-TRANSUDCERS FOR ANTICANCER DRUGS CYTOTOXICITY ENHANCEMENT

by

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ABSTRACT

Prototype ultrasonic transducers with resonance frequencies at 4 and 1MHz were constructed using lead zirconate titanate (PZT) ceramics. Their output ultrasonic powers were measured using an electronic ultrasound power meter. The power coefficients of the 4 and 1 MHz transducers were found to be 1.98 and 1.85 respectively, close to the theoretical value of 2. The theoretical transmitted ultrasonic power through a polystyrene plate was 88% whereas the experimental values were 86% for 1MHz and 71% for 4MHz ultrasound. Overall, the measured characteristics of the selected transducers were in good agreement with theoretical values. The output ultrasonic powers were also found to be consistent over four months.

Tone-burst ultrasound (32 Watt/cm², 20% Duty Cycle, 50msec burst period) was found to significantly (p<0.01) enhance cytotoxicity when used in combination with 20μM doxorubicin compared to doxorubicin-alone or ultrasound-alone controls. With 30 seconds exposure, immediate cytotoxicity was enhanced by 70% compare to doxorubicin-only control. The long-term cytotoxicity was enhanced by 83% compare to ultrasound-only control. The spatial-peak-temporal-peak ultrasonic power density threshold for cytotoxicity enhancement at 4MHz was 30W/cm², which translates to minimal actuation amplitude of 112nm. A Micro-Ultrasonic-Transducer (MUT) must satisfy that minimal actuation amplitude in order to enhance cytotoxicity at 4MHz and be useful for sonodynamic therapy.

Tone-burst ultrasound was also found to significantly enhance (p<0.05) cytotoxicity of micellar paclitaxel. The most significant enhancement (53%) was observed with micelles formed with 150μM PDLLA-MePEG diblock copolymers and loaded with 0.1% paclitaxel. The use of ultrasound to enhance cytotoxicity of micellar paclitaxel was found to be another promising application of implantable MUTs.
# TABLE OF CONTENTS

ABSTRACT .......................................................................................................................... ii  
TABLE OF CONTENTS ......................................................................................................... iii  
LIST OF TABLES .................................................................................................................... vi  
LIST OF FIGURES ................................................................................................................ vii  
LIST OF SYMBOLS AND ABBREVIATIONS ........................................................................ x  
ACKNOWLEDGEMENTS ....................................................................................................... xiv  

## CHAPTER 1: BACKGROUND ......................................................................................... 1  
1.1 Introduction ................................................................................................................... 1  
1.1.1 Ultrasound and Acoustic Cavitation ....................................................................... 1  
1.1.2 Micro-Ultrasound-Transducers ............................................................................. 4  
1.1.3 Output Power Density of an Ultrasonic Transducer ............................................. 7  
1.2 Research Rationale and Objectives ........................................................................... 8  
1.3 References ..................................................................................................................... 13  

## CHAPTER 2: DEVELOPMENT OF PROTOTYPE ULTRASONIC TRANSDUCERS AND THEIR PERFORMANCE EVALUATION ......................................................... 15  
2.1 Introduction .................................................................................................................. 15  
2.2 Materials ..................................................................................................................... 17  
2.2.1 Ultrasound Transducers ...................................................................................... 17  
2.3 Experimental Protocol .............................................................................................. 18  
2.4 Experimental Results ................................................................................................. 19  
2.5 Theoretical Calculation on Equivalent Circuits of Transducers ................................ 21  
2.6 Discussion ................................................................................................................... 22  
2.7 References .................................................................................................................. 39  

## CHAPTER 3: AN INVESTIGATION ON THE SONODYNAMIC EFFECT OF DOXORUBICIN AND THE POWER DENSITY REQUIREMENT OF A MICRO-ULTRASONIC-TRANSDUCER FOR SONODYNAMIC THERAPY ....................... 41  
3.1 Introduction .................................................................................................................. 41  
3.1.1 Sonodynamic Therapy ......................................................................................... 41  
3.1.2 MUTs for Sonodynamic Therapy ......................................................................... 43  
3.2 Materials ..................................................................................................................... 44  
3.2.1 Chemicals ............................................................................................................. 44
APPENDIX 3: ADDITIONAL INFORMATION ON THE MTS AND LDH-RELEASE ASSAY .......................................................................................................................... 100

APPENDIX 4: CELL PASSAGING PROTOCOL ................................................................................................................................. 102

APPENDIX 5: PREPARATION OF PC3 MEDIA ............................................................................................................................. 104

APPENDIX 6: WATER-BATH DRAWING ................................................................................................................................. 106

APPENDIX 7: PROCEDURES FOR FITTING DATA FROM FIGURE 3.10 TO EQUATION 3.4 ........................................................................................................... 107

APPENDIX 8: DOSE-EFFECT RESPONSE OF DOXORUBICIN BASED ON DRUG-RECEPTOR HYPOTHESIS ................................................................................. 108
LIST OF TABLES

Table 2.1: A few examples of the variety of ultrasonic transducers and parameters used in the literature ................................................................. 25

Table 2.2: Established techniques for measuring ultrasound intensity .................. 26

Table 2.3: Theoretical values of equivalent electrical circuits elements for the 4MHz and 1MHz transducers ......................................................... 26

Table 3.1: Values of $\alpha$ and corresponding $R^2$ from fitting the data from Figure 3.10 to Equation 3.4 .............................................................................. 60
LIST OF FIGURES

Figure 1.1: A conventional ultrasonic transducer make out of piezoelectric ceramic operated at the fundamental thickness-mode resonance frequency ............... 10

Figure 1.2: Basic design and operation of a pMUT ........................................ 11

Figure 1.3: Basic design and operation of a cMUT ........................................ 12

Figure 2.1: Schematic diagram of the prototype ultrasonic transducer ............... 27

Figure 2.2: A) A 1MHz 25% duty cycle tone-burst sinusoidal waveform with burst period of 50msec displayed over 200msec. B) Enlarged view of the same waveform for the first 6μsec clearly display the 1MHz sinusoidal nature of the wave ................................................................. 28

Figure 2.3 Diagram of the ultrasonic power measurement experimental setup ........... 29

Figure 2.4: Acoustic power output of a 2.00mm thick PZT over a range of actuation frequencies ................................................................. 30

Figure 2.5: Acoustic power density output of a typical 4MHz transducer ............... 31

Figure 2.6: Acoustic power density output of a typical 1MHz transducer ............... 32

Figure 2.7: Effect of the intervening polystyrene plate on the transmitted intensity of 4MHz ultrasound ................................................................. 33

Figure 2.8: Effect of the intervening polystyrene plate on the transmitted intensity of 1MHz ultrasound ................................................................. 34

Figure 2.9: A typical screen capture of the digitalizing oscilloscope ....................... 35

Figure 2.10: Electro-acoustic conversion efficiency of a typical 4MHz transducer...... 36
Figure 2.11: Comparison of tone-burst wave acoustic power density to that of continuous wave................................................................. 37

Figure 2.12: Equivalent electrical circuits for a lossless transducer operating at resonance frequency................................................................. 38

Figure 3.1: Structure of doxorubicin.......................................................... 61

Figure 3.2: Ultrasound exposure setup.......................................................... 62

Figure 3.3: Calibration curve of the MTS assay using known number of PC3 cells........ 63

Figure 3.4: Calibration curve of the LDH-release assay using known number of PC3 cells........................................................................................................... 64

Figure 3.5: Long-term cytotoxicity of doxorubicin on the PC3 cells at different concentrations........................................................................................................... 65

Figure 3.6: Long-term cytotoxicity due to ultrasound on PC3 in suspension and in adherent monolayer exposed under same ultrasonic conditions........ 66

Figure 3.7: Comparison of the result from the MTS assay and the LDH-release assay using PC3 cells........................................................................................................... 67

Figure 3.8: Immediate cytotoxicity in PC3 cells using: ultrasound alone, ultrasound in combination of 2μM doxorubicin and ultrasound in combination of 20μM doxorubicin........................................................................................................... 68

Figure 3.9: Long-term cytotoxicity in PC3 cells using: ultrasound alone, ultrasound in combination of 2μM doxorubicin and ultrasound in combination of 20μM doxorubicin........................................................................................................... 69

Figure 3.10: Cytotoxicity enhancement in PC3 cells using ultrasound in combination of 2μM doxorubicin over a range of ultrasonic power densities............... 70
Figure 3.11: Values of α from table 3.1 plotted against the corresponding power densities.

Figure 3.12: Cytotoxicity in PC3 cells using 4MHz and 1MHz ultrasound in combination with 2μM doxorubicin.

Figure 3.13: Immediate effect of 1MHz 4.8 W/cm² ultrasound in combination with 2μM doxorubicin: A) Before exposure; B) After 1min ultrasonic exposure.

Figure 4.1: Structure of paclitaxel.

Figure 4.2: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers alone and in combination with tone-burst ultrasound.

Figure 4.3: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG polymeric micelle loaded with 2.5% paclitaxel alone and in combination with tone-burst ultrasound.

Figure 4.4: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers loaded with 0.5% paclitaxel alone and in combination with tone-burst ultrasound.

Figure 4.5: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers loaded with 0.1% paclitaxel alone and in combination with tone-burst ultrasound.

Figure 4.6: Cytotoxicity in PC3 cells induced by 300μM and 150μM PDLLA-MePEG diblock copolymers loaded with 0.5% paclitaxel in combination with different duration of tone-burst ultrasound.

Figure 4.7: The five possible mechanisms for the ultrasonic enhancement of micellar paclitaxel cytotoxicity.
LIST OF SYMBOLS AND ABBREVIATIONS

\( \times g \) Times relative gravity

A Area

\( A_{492} \) Absorbance at 492 nm

AC Alternating current

C Concentration

cm Centimetre

cMUT Capacitive Micro-Ultrasonic-Transducer

\( C_0 \) Capacitance in equivalent circuit

\( c_t \) Compressional wave velocity of the ultrasound

dB Decibel

DC Direct current

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

Dox Doxorubicin, also known as Adriamycin

E Effect (defined as cytotoxicity in this study)

\( EC_{50} \) The concentration required to achieve 50% effect of the drug

EDTA Ethylenediaminetetraacetic acid
\( e_{ii} \)  \hspace{1cm} \text{Piezoelectric stress coefficient} \\

\( E_{\text{max}} \)  \hspace{1cm} \text{Maximal effect} \\

\( F \)  \hspace{1cm} \text{Frequency} \\

\( f_l \)  \hspace{1cm} \text{Fundamental thickness mode vibration frequency} \\

\( g \)  \hspace{1cm} \text{Gram} \\

\( \text{hr} \)  \hspace{1cm} \text{Hour} \\

\( I \)  \hspace{1cm} \text{Ultrasonic intensity, also known as power density} \\

\( I_{\text{SATA}} \)  \hspace{1cm} \text{Spatial-average-temporal-average intensity} \\

\( I_{\text{SATP}} \)  \hspace{1cm} \text{Spatial-average-temporal-peak intensity} \\

\( I_{\text{SPTA}} \)  \hspace{1cm} \text{Spatial-peak-temporal-average intensity} \\

\( I_{\text{SPTP}} \)  \hspace{1cm} \text{Spatial-peak-temporal-peak intensity} \\

\( J \)  \hspace{1cm} \text{Joule} \\

\( K_{\text{eac}} \)  \hspace{1cm} \text{Electro-acoustic conversion efficiency of the transducer} \\

\( \text{kHz} \)  \hspace{1cm} \text{Kilo-hertz} \\

\( K^T \)  \hspace{1cm} \text{Relative dielectric constant} \\

\( \text{L} \)  \hspace{1cm} \text{Litre} \\

\( l \)  \hspace{1cm} \text{Thickness} \\

\( \text{LDH} \)  \hspace{1cm} \text{Lactate dehydrogenase}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>MEMS</td>
<td>Micro-electro-mechanical systems</td>
</tr>
<tr>
<td>MHz</td>
<td>Mega-hertz</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mm</td>
<td>Millimetre</td>
</tr>
<tr>
<td>mM</td>
<td>Milimolar</td>
</tr>
<tr>
<td>MRayl</td>
<td>Mega-Rayleid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance Imaging</td>
</tr>
<tr>
<td>msec</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt</td>
</tr>
<tr>
<td>MUT</td>
<td>Micro-Ultrasonic-Transducer</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>PC3</td>
<td>Human prostate cancer cells</td>
</tr>
<tr>
<td>PDLLA-MePEG</td>
<td>Poly(D,L-lactide)-block-methoxypolyethylene glycol</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>pMUT</td>
<td>Piezoelectric Micro-Ultrasonic-Transducer</td>
</tr>
<tr>
<td>PTX</td>
<td>Paclitaxel</td>
</tr>
<tr>
<td>PZT</td>
<td>Lead zirconate titanate</td>
</tr>
<tr>
<td>RCF</td>
<td>Relative centrifugal force</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>$R_m$</td>
<td>Resistance in equivalent circuit</td>
</tr>
<tr>
<td>SDT</td>
<td>Sonodynamic Therapy</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>$u$</td>
<td>Velocity of a particle in the medium or surface of the transducer</td>
</tr>
<tr>
<td>$V_{pp}$</td>
<td>Peak-to-peak voltage</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>$Z$</td>
<td>Acoustic impedance</td>
</tr>
<tr>
<td>$\varepsilon_0$</td>
<td>Relative permittivity of free space</td>
</tr>
<tr>
<td>$\mu l$</td>
<td>Microlitre</td>
</tr>
<tr>
<td>$\mu M$</td>
<td>Micromolar</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohms</td>
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</table>
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Finally, I would like to dedicate this thesis to my parents, who has left behind their own successes and friendships to bring me here and allow me to pursue better opportunities and higher aspirations.
CHAPTER 1

BACKGROUND

1.1 Introduction

Today, ultrasound is best known for its medical imaging applications such as those utilized in obstetricians’ clinics. Physiotherapists also use ultrasound regularly to treat muscle and tendon ailments. The use of ultrasound for cancer therapy is not widely known, although its history can be traced back to as early as 1933 [1]. Two biological effects of ultrasound in biological tissues are used to treat cancer: 1) thermal and 2) non-thermal. Thermal ablation of tumours using ultrasound is already being utilized in some clinics [2]. The use of non-thermal ultrasonic effect for cancer treatment, however, is still in the developmental stage. This approach is best illustrated by sonodynamic therapy, which will be discussed in detail in Chapter 3. The physical phenomenon behind sonodynamic therapy is believed to be acoustic cavitation [3]. Therefore a brief review of acoustic cavitation is present below.

1.1.1 Ultrasound and Acoustic Cavitation

Ultrasound is defined as acoustic wave with frequency above normal human hearing at 20kHz. Acoustic wave is simply the vibration of molecules and atoms in an organized manner. Acoustics cavitation is a physical phenomenon in which cavities are created in the liquid by
a large negative (i.e. rarefational) pressure, either produced intentionally, such as by ultrasound, or unintentionally, such as by the movement of a ship's propellers. The cavities that are produced can disappear during the subsequent compression or remain to grow and oscillate for several cycles [4]. When these cavities finally collapse, they give out enormous amounts of energy in the form of intense pressure and temperature for a very brief moment [5]. Using theoretical equations developed by Noltingk, Neppiras and Flynn, the collapse of a cavity containing nitrogen in water at 1 atm ambient pressure and room temperature (20°C) will achieve maximum temperature and pressure of 4,200K and 975atm respectively [4]. As a comparison, please note that the temperature found on the surface of the sun is approximately 5,800K and the pressure at the bottom of Mariana Trench, the deepest ocean trench on earth (11km below sea level), is approximately 1000atm. In fact, the condition in a collapsing cavity is so extreme that light is released in a process called sonoluminescence and physicists have proposed that nuclear fusion can possibly be attained [6]. The production of this high pressure over such a short time (μsec) in the cavity can produce shockwaves that physically disrupt the biological membrane [7]. Furthermore, the extreme temperature can produce hydrogen and hydroxyl free radicals by pyrolysis (or thermal decomposition) of water molecules [8]. Free radicals are molecules that have unpaired electrons on the outer most electronic shell. They are highly reactive because the unpaired electron is very unstable and seeks to return to the stable configuration by combining
with an electron of opposite spin from other molecules. The most common day-to-day reaction where free radicals can be found is combustion. In the laboratory and in the upper atmosphere, free radicals are also produced by the bombardment of ionizing radiation. These free radicals would have biological consequences including DNA degradation, enzyme inactivation, and lipid peroxidation [9].

The occurrence of cavitation depends on a variety of parameters including temperature, hydrostatic pressure, impurity in the solution as well as frequency and intensity of the applied ultrasound [4]. Please note that ultrasonic intensity (with unit of W/cm$^2$) is also called power density and these two terms will be used interchangeably depending on the context. With all parameters set as constant, there is a threshold power density that the applied ultrasound must achieve before cavitation occurs. This is because cavities will only be formed when the applied power density (force acting over a distance over a given time) is strong enough to overcome the attractive forces between the liquid molecules.

There are numerous ways to detect the onset of cavitations. They include:

1) Detection of the free radicals produced from the cavitation by using spin tripping molecules and Electron Spin Resonance Spectrometer [9];

2) Detection of the free radicals by use of colorimetric assay such as iodine [10];

3) Detection of the light produced by sonoluminescence [11];
4) Measurement of broadband and sub-harmonic noises using hydrophone [12];

5) Capturing the image of the collapsing cavities using high-speed camera [13].

1.1.2 Micro-Ultrasonic-Transducers

Most ultrasonic transducers today use piezoelectric ceramic as their active elements. In recent years, researchers have used micromachining techniques to fabricate ultrasonic transducers that have active acoustic element with the size of a few microns. Proposed applications for these Micro-Ultrasonic-Transducers (MUTs) include medical ultrasonography [14], acoustic anemometry [15], aerial ranging [16], etc. In medical ultrasonography in particular, MUTs have many advantages over conventional ultrasonic transducers such as greater precision in fabrication and better acoustic matching [17]. In spite of the many MUTs research around the world, no one has looked into the possibility of using MUTs for sonodynamic therapy.

There are currently two approaches in fabricating MUTs. The first approach requires the deposit of a piezoelectric thin film on a micromachined diaphragm and the resultant transducers are called Piezoelectric Micro-Ultrasonic-Transducers (pMUTs). Piezoelectric materials change their physical dimension when an electric field is applied across them. The dimension change occurs in both the direction along the electric field as well as in directions perpendicular to the electric field.
In a conventional ultrasonic transducer, only the piezoelectric effect along the electric field is typically used to generate ultrasound. For power ultrasound applications, such as sonochemistry and sonodynamic therapy, the objective is to obtain the maximal ultrasonic intensity. In such cases, the transducer is usually operated in a continuous wave fashion at the fundamental thickness-mode resonance frequency. At that frequency, a standing compressional wave is set up within the thickness of the transducer with the largest strain and therefore the least stress on its two faces [18]. Figure 1.1 illustrates a conventional transducer operated at the fundamental thickness-mode resonance frequency.

Since acoustic wave has a constant compressional velocity in a given material, the fundamental thickness-mode resonance frequency is related to its thickness and given by:

\[ f_t = \frac{c_t}{2l} \quad (1.1) \]

where \( f_t \) is the fundamental thickness-mode resonance frequency, \( c_t \) is the compressional acoustic wave velocity in the ceramic and \( l \) is the thickness of the ceramic.

In a pMUT, however, the deposited piezoelectric thin film typically has a thickness of a few microns. The thickness-mode resonance frequency of such a thin film will be in the order of a few Giga-Hertz. To allow the pMUT to operate at the Mega-Hertz frequency more typical in
medical applications, the piezoelectric effect perpendicular to the applied electric field is employed instead [17]. The micromachined diaphragm, on which the thin piezoelectric film rests, is made to buckle. When an electric field is applied across the piezoelectric film, the film will experience dimension changes in the directions perpendicular to the electric field. The change in area in the piezoelectric film will force the buckled diaphragm to flatten or further deform, generating the necessary motions to produce the ultrasound. Therefore, the resonance frequency of the pMUT will depend on the dimension and the material properties of the diaphragm. Figure 1.2 illustrates the operation of a pMUT. For the detail fabrication process of a pMUT, the readers are suggested to refer to published papers of Muralt et al. [19] and Wang et al.[20].

The second way to fabricate MUTs is to utilize the electrostatic force between parallel plates with opposite charges. The resultant transducers are called Capacitive Micro-Ultrasonic-Transducers (cMUTs). In general, a bottom electrode is first laid down, and then a thin membrane is suspended on the bottom electrode with a cavity in between. On top of the thin membrane is the top electrode. When an alternating voltage is applied across the two electrodes, the electrostatic force between them will attract and then repel them from each other, thereby creating the necessary motions to generate the ultrasound. The resonance frequency of a cMUT will also depend on the dimension and the material properties of the membrane. The operation of a cMUT is illustrated in Figure 1.3.
A crucial step in the fabrication of cMUT is the incorporation of a precisely controlled cavity between the bottom electrode and the membrane. This cavity needs to be deep enough to allow the free movement of the membrane but not so deep so as to overly weaken the electrostatic effect. Methods that are used to incorporate this cavity include: 1) the use of sacrificial layer that is etched by chemical [21, 22] and 2) a wafer bonding process [15].

1.1.3 Output Power Density of an Ultrasonic Transducer

As explained in the previous section, cavitation, and therefore sonodynamic phenomena, occurs only after a certain acoustic power density threshold has been reached. Therefore, the most important design criterion for a MUT intended for sonodynamic therapy is the output acoustic power density.

The power density of an acoustic wave is defined as “the instantaneous power flowing through a unit area perpendicular to the direction of propagation of the wave as one elemental volume of the fluid acts on a neighbouring element” [23]. Given that we can write:

\[
\text{Power Density} = \frac{\text{power}}{\text{area}} = \frac{\text{work}}{\text{area} \times \text{time}} = \frac{\text{force} \times \text{distance}}{\text{area} \times \text{time}} = \text{pressure} \times \text{velocity} \quad (1.2)
\]

We also define the acoustic impedance \(Z\) of a material as the relationship between the pressure
and velocity that a particle in the medium feels:

\[ Z = \frac{\text{pressure}}{\text{velocity}} \]  

(1.3)

Combining Equation 1.2 and Equation 1.3, we can write

\[ I = Zu^2 \]  

(1.4)

where \( I \) is the power density of the acoustic wave and \( u \) is the velocity of a particle in the medium. Now let us consider what happens at the interface between the transducer face and the liquid at a power density below the threshold for cavitation. The velocity of a liquid particle that is adjacent to the transducer face must be the same as the transducer face; otherwise we will have a void formation, which we know is not possible under the threshold power density. Therefore the power emitted by an ultrasonic transducer is determined by the velocity of its emitting face’s movement.

1.2 Research Rationale and Objectives

Given the fact that an ultrasonic transducer, either conventional or MUT, intended for
sonodynamic therapy must meet the requirement of the power density threshold, the objectives of the research laid out in the following chapters are:

1) To develop prototype ultrasonic transducers as a reliable source of ultrasound;

2) To investigate the sonodynamic effect of doxorubicin and the minimal power density required to effect sonodynamic enhancement of doxorubicin cytotoxicity

3) To investigate a new application for therapeutic ultrasound: the targeted cytotoxicity enhancement of micellar paclitaxel.
Figure 1.1: A conventional ultrasonic transducer made out of piezoelectric ceramic operated at the fundamental thickness-mode resonance frequency.
Figure 1.2: Basic design and operation of a pMUT
Figure 1.3: Basic design and operation of a cMUT
1.3 References


CHAPTER 2

DEVELOPMENT OF PROTOTYPE ULTRASONIC TRANSDUCERS AND THEIR PERFORMANCE EVALUATION

2.1 Introduction

Recently, there has been much interest in the use of ultrasound in a wide range of therapeutic applications including the enhancement of transfection for gene therapy [1-3], the facilitation of drug delivery [4,5] and the potentiation of drug efficacy [6-8]. The ultrasonic transducers used in these experiments differ to a large degree, ranging from custom-built devices to off-the-shelf therapeutic transducers. The ultrasonic parameters (such as frequency, acoustic power density, exposure time, waveform, etc.) tested also vary extensively. Table 2.1 illustrates this variety in the ultrasonic transducers and parameters by summarizing a small portion of articles from the literature.

The custom-built approach was adopted for this study because of the flexibility it offers and the possibility of scaling down to dimensions close to that of a MUT. Many months were spent simply to test out different transducer designs by trial-and-error. In the end, a design similar to Umemura et al. [8] was selected.

Another challenge that came with using custom-built transducers was to ensure their...
performance reliability. It was found that transducers made using the same procedures could have vastly different performance. Further, a working transducer could fail catastrophically (due to breakage of lead wire and overheating) or slowly over time (due to fatigue of the insulation and aging of the piezoelectric ceramic). Therefore a method to screen newly made transducers and monitor transducer performance over time was needed. Since the acoustic power density of the ultrasound is the major factor for any ultrasound-mediated therapy, it was taken as the performance marker. There are four main approaches to measure acoustic power density level and they are summarized in Table 2.2. Each of those approaches was attempted but a commercial ultrasound meter based on the acoustic force balance approach was chosen for the rest of this study because of the ease of operation and reliability. All subsequent mention of acoustic power densities are in reference to spatial-average-time-average intensity ($I_{SATA}$) unless noted otherwise.

At its resonance frequency, an ultrasonic transducer could be represented by a simple equivalent circuit consisting of a capacitor in parallel to a resistor$[9]$. These parameters were calculated for the custom-built transducers to aid in the analysis of their experimental performance.
2.2 Materials

2.2.1 Ultrasound Transducers

Lead zirconate titanate ceramics (PZT) (dimension: 25.00mm×25.00mm ×0.50mm and 5.00mm×5.00mm×2.00mm; material: 841) were purchased from APC International Ltd. (Mackeyville, PA, USA). The larger ceramics were then diced into 5.00mm×5.00mm pieces by hand using a diamond cutter. They were then adhered to aluminums foils or aluminums sheet (thickness: 0.032 in) using silver conductive epoxy (MG Chemicals, Surrey, BC, Canada). The aluminums sheets were pre-cut into 0.625in diameter pieces before the PZT attachment. After the silver epoxy was allowed to cure overnight, a lead wire was soldered onto the backside of the PZT. The PZT with lead wire and aluminums front plate were then adhered to aluminums pipes (diameter: 0.625in, wall thickness: 0.070in, length: 2.0cm) using silver epoxy and allowed to cure overnight. The material properties of the PZT used are available in Appendix 1.

The main purpose of the aluminums front plate, either foil or sheet, and the aluminums pipe was to serve as water insulated casing to prevent shorting of opposite faces of the PZT. They also served as heat sink to remove heat generated from the PZT during high power operation. The aluminums front plate also served as electrical ground during actuation. Figure 2.1 shows a schematic diagram of the prototype ultrasonic transducer.

The transducers were always actuated at their thickness-mode resonance frequencies of
4MHz (for 0.50mm thick PZT) or 1MHz (for 2.00mm thick PZT). Electrical signal from an arbitrary waveform generator (Model: 33220A, Agilent Inc., Palo Alto, CA, USA) was amplified by a 50dB broadband power amplifier (Model: 240L, Electronic Navigation Industries, Inc., Rochester, NY, USA) before being applied to the transducers. The voltage applied to the PZT was monitored by a 10X attenuation passive voltage probe. The current through the PZT was measured by an AC/DC current probe (Model: TCP202, Tektronix Inc., Beaverton, OR, US). Both signals were displayed on a digitizing oscilloscope (Model: TDS 420, Tektronix Inc.). The acoustic power output of the each transducer was measured using an UPM-DT-10 electronic ultrasound power meter from Ohmic Instruments Co. (Easton, MD, USA). Both continuous sinusoidal wave and tone-burst wave signals were used to actuate the transducers. A 25% duty cycle tone-burst waveform is illustrated in Figure 2.2. The 25% duty cycle tone-burst wave lessened the effect of overheating and was used for experiments in subsequent chapters.

2.3 Experimental Protocol

The performances of the ultrasonic transducers were evaluated by measuring their acoustic power output using an electronic ultrasound meter. The ultrasound meter was an electronic balance specially designed to measure the force generated by an acoustic wave. It reported the spatial-average-time-average power of the ultrasound and that value was divided by the active
area of the ultrasonic transducer to obtain the spatial-average-time-average intensity ($I_{SATA}$).

Each ultrasonic transducer, pointing toward the reflective cone of the digital ultrasound meter, was secured in place by a test tube holder and a stand. The water-bath of the electronic ultrasound meter was filled with degassed water. Continuous sinusoidal signals (1 or 4MHz) at six different voltages were used to actuate the transducer. The acoustic power measurements were conducted at least five different times over six weeks and with over twelve samples to ensure the long term reliability of the ultrasonic transducers. The performances of the ultrasonic transducers were also monitored continuously between actual experiments.

The attenuation of the ultrasonic intensity by the degassed water from the transducer to the 48-wells cells culture plate over a distance of approximately 5mm was assumed to be insignificant. The attenuation and reflection of the ultrasonic intensity by the 2mm polystyrene cells culture plate bottom was determined experimentally by placing a piece of polystyrene with equivalent thickness between the ultrasonic transducer and the reflective cone of the ultrasound meter during the acoustic power measurement. Figure 2.3 shows the experimental setup. Photos of the setup are available in Appendix 2.

2.4 Experimental Results

The output acoustic power of the transducers peaked at the thickness mode vibration
resonance frequency as calculated from the material properties (Figure 2.4). As illustrated in Figure 2.5 and 2.6, the output acoustic power density of the transducers were found to be approximately proportional to the square of the input voltage (power coefficient is 1.98 for 4MHz and 1.85 for 1MHz).

The relationships of the acoustic power density measured with and without the intervening polystyrene plate are shown in Figure 2.7 and 2.8. The slopes of the regression lines were the percentages of the acoustic power density transmitted and were found to be 71% for 4MHz and 86% for 1MHz ultrasound. This decrease in acoustic power density due to the cells culture plate bottom was taken into consideration for the calculation of the applied acoustic power density in subsequent experiments.

A typical screen capture of the digitizing oscilloscope showing the instantaneous voltage, current and input electrical power is shown in Figure 2.9. When the input electrical power was plotted against the output acoustic power, a linear relationship was found (Figure 2.10). The slope of the linear regression line is the electro-acoustic conversion efficiency of the transducer and was found to be 7.2% for a typical 4MHz transducer. The acoustic power density of a 25% tone-burst waveform was approximately a quarter of a continuous waveform as expected (Figure 2.11).
2.5 Theoretical Calculation on Equivalent Circuits of Transducers

When it is operating at the resonance frequency, a lossless ultrasonic transducer can be represented by an equivalent electrical circuit consisting of a capacitor in parallel with a resistor (Figure 2.12). The values of the resistor ($R_m$) and the capacitor ($C_0$) are given by Equations 2.1 and 2.2.

\[
R_m = \frac{l^2 Z}{4e_{ii}^2 A} \quad (2.1)
\]

where $Z$ is the acoustic impedance of water ($1.5 \times 10^6$ secN/m$^3$); $l$, $e_{ii}$ and $A$ are the thickness, piezoelectric stress coefficient and the area of the PZT respectively.

\[
C_0 = \frac{K^T \varepsilon_0 A}{l} \quad (2.2)
\]

where $\varepsilon_0$ is the relative permittivity of free space ($8.854 \times 10^{-12}$ F/m); $l$, $K^T$ and $A$ are the thickness, relative dielectric constant and the area of the PZT respectively. For a complete derivation of these equations, please reference to Christensen [9]. Given the PZT material properties in Appendix 1, the $R_m$ and the $C_0$ calculated for the transducers are summarized in Table 2.3.
2.6 Discussion

The performances (output acoustic power density) of selected transducers were found to be reliable over an extended period (Figure 2.5 and 2.6). From the definition of power and Ohm’s law, the acoustic power output of a transducer is expected to fit the relationship:

\[
I_{\text{SATA}} = K_{\text{eac}} \frac{V_{pp}^2}{8R_mA} \quad (2.3)
\]

where \(I_{\text{SATA}}\) is the spatial-average-time-average intensity, \(R_m\) is the resistance value in the equivalent circuits, \(V_{pp}\) is the applied peak-to-peak voltage, \(A\) is the transducer area and \(K_{\text{eac}}\) is the electro-acoustic conversion efficiency of the transducer.

The power coefficients in the regression fit of Figure 2.5 and 2.6 are 1.98 and 1.85 respectively, which are very close to the expected value of 2. A possible reason for the slightly lower power coefficient of the 1MHz transducer is the fact that low level cavitation might be occurring at the ultrasonic intensities being tested. The cavitation could deflect ultrasound wave and therefore decrease the measured acoustic power density.

The linear factor in the regression fit of Figure 2.5 is 0.0044, which should equal to the combination of \(K_{\text{eac}}\) and \(\frac{1}{8R_mA}\) in Equation 2.3. Given that the electro-acoustic conversion
efficiency ($K_{eo}$) of a 4MHz transducer is 7.2% (Figure 2.10) and the transducer area is 0.25cm$^2$, the corresponding $R_m$ value calculated is 8.2$\Omega$ which is in the same magnitude but larger than the theoretical value of 10.5$\Omega$ (Table 2.3). A possible reason for the larger experimental value is the fact that the theoretical value assumed the transducer is air-backed. In reality, the solder spot on the backside of the transducer is of considerable size compared to the total transducer area and therefore adds to the acoustic impedance in Equation 2.1 and increases the value of equivalent resistance.

The lowering in the transmitted ultrasonic intensity through the intervening polystyrene plate is due to the reflection of the ultrasonic wave at the interface between the polystyrene and the water as well as the attenuation through the polystyrene. The reflection of the ultrasonic wave is a phenomenon due to the acoustic impedances mismatch of the different materials (water vs. polystyrene). Assuming the incident ultrasonic wave is exactly normal to the interface, the percentage of transmitted intensity is given by:

$$\frac{\text{Transmitted ultrasonic intensity}}{\text{Incident ultrasonic intensity}} = 1 - \left(\frac{Z_2 - Z_1}{Z_2 + Z_1}\right)^2$$  \hspace{1cm} (2.4)

as derived by Christensen [20], where $Z_1$ and $Z_2$ are the acoustic impedances of the two materials.
Note that the ultrasound is reflected twice because there are two water-polystyrene interfaces (ignoring the effect of reverberation). Given the acoustic impedance of polystyrene is 2.5MRayl [21] and that of water is 1.5MRayl [20], the theoretical percentage of transmitted intensity is 88% which is close to the experimental value for 1MHz ultrasound wave at 86% (Figure 2.8). The percentage of transmitted intensity is decreased further to 71% for 4MHz ultrasound wave (Figure 2.7) because the second effect, the attenuation within the polystyrene, is expected to be more severe for higher frequency wave.

Given the agreement between the experimental and theoretical values discussed above, it is concluded that the selected ultrasonic transducers are suitable to be used as prototypes to investigate the therapeutic applications of ultrasound.
Table 2.1: A few examples of the variety of ultrasonic transducers, frequency, acoustic power density, cells and drugs used in the literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Type of Transducer</th>
<th>Frequency</th>
<th>Acoustic power density</th>
<th>Cells/Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>[8]</td>
<td>Custom built (24mm diameter piezoelectric ceramic)</td>
<td>1.93 MHz</td>
<td>6 W/cm²</td>
<td>Sarcoma/Adriamycin</td>
</tr>
<tr>
<td>[10]</td>
<td>Commercial in-vitro ultrasound device (Soniرون 1000, Richmar, Inola, Okla, US)</td>
<td>1 MHz</td>
<td>0.5 or 1. W/cm²</td>
<td>In vivo/Plasmid DNA</td>
</tr>
<tr>
<td>[11]</td>
<td>Therapeutic ultrasound machine (Mark 3, EMS Limited, Oxford, UK)</td>
<td>1 MHz</td>
<td>0.5 to 1 W/cm²</td>
<td>Mouse myoblasts/Plasmid DNA</td>
</tr>
<tr>
<td>[4]</td>
<td>Sonicator (VCX 400, sonics and materials)</td>
<td>20 kHz</td>
<td>1.6 to 14 W/cm²</td>
<td>Not applicable</td>
</tr>
<tr>
<td>[12]</td>
<td>Commercial Lithotripter Piezolith 3000 (Richard Wolf GmbH, Knittlingen, Germany)</td>
<td>Pulsed-shock wave</td>
<td>28Mpa</td>
<td>HeLa/ Not applicable</td>
</tr>
<tr>
<td>[13]</td>
<td>Custom built (38mm diameter piezoelectric ceramic)</td>
<td>1.765 MHz</td>
<td>0.25 W/cm²</td>
<td>Chinese hamster CHO-UM/Adriamycin and diaziquone</td>
</tr>
<tr>
<td>[14]</td>
<td>Custom-built (ceramic sandwiched by metal resonator of appropriate lengths ~10cm)</td>
<td>20kHz, 57kHz, 76kHz, 93kHz</td>
<td>0.8 W/cm²</td>
<td>3T3 mouse cell/Calcein</td>
</tr>
<tr>
<td>[15]</td>
<td>Custom-built (25mm diameter piezoelectric ceramic)</td>
<td>2.6 MHz</td>
<td>2.3 W/cm²</td>
<td>Chinese hamster lung fibroblasts/Adriamycin</td>
</tr>
</tbody>
</table>
Table 2.2: Established techniques for measuring ultrasound intensity. For a discussion on the different type of ultrasonic intensity, please refer to Christensen [19].

<table>
<thead>
<tr>
<th>Ultrasound Intensity Measurement Technique</th>
<th>Type of Intensity Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrophone [16]</td>
<td>Spatial-peak-temporal-peak intensity (ISPTP) or spatial-average-temporal-peak intensity (ISATP) depending on size of probe</td>
</tr>
<tr>
<td>Calorimetry [17]</td>
<td>Spatial-peak-temporal-average intensity (ISPTA) or spatial-average-temporal-peak intensity (ISATA) depending on size of probe</td>
</tr>
<tr>
<td>Laser Interferometry [18]</td>
<td>ISPTP</td>
</tr>
<tr>
<td>Acoustic Force Balance [19]</td>
<td>ISATA</td>
</tr>
</tbody>
</table>

Table 2.3: Theoretical values of equivalent electrical circuit’s elements for the 4MHz and 1MHz transducers

<table>
<thead>
<tr>
<th>Transducer</th>
<th>$R_m$</th>
<th>$C_\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4MHz</td>
<td>10.5Ω</td>
<td>0.598nF</td>
</tr>
<tr>
<td>1MHz</td>
<td>168Ω</td>
<td>0.149nF</td>
</tr>
</tbody>
</table>
Figure 2.1: Schematic diagram of the prototype ultrasonic transducer
Figure 2.2: A) A 1MHz 25% duty cycle tone-burst sinusoidal waveform with burst period of 50msec displayed over 200msec. B) Enlarged view of the same waveform for the first 6μsec clearly displays the 1MHz sinusoidal nature of the wave.
Figure 2.3 Diagram of the ultrasonic power measurement experimental setup
Figure 2.4: Acoustic power output of a 2.00mm thick PZT over a range of actuation frequencies. The PZT was actuated by 276Vpp continuous sine wave. The thickness mode vibration resonance frequency calculated from the material properties (available in Appendix 1) is 1MHz, which is where the peak output is located.
Figure 2.5: Acoustic power density output of a typical 4MHz transducer. Each data point consists of 23 samples taken over four months. Voltages reported are peak-to-peak values. Data expressed as mean ± 1 standard deviation. Regression analysis: \( y = 0.0044x^{1.98} \) \( R^2 = 1.00 \)
Figure 2.6: Acoustic power density output of a typical 1MHz transducer. Each data point consists of 12 samples taken over one month. Voltages reported are peak-to-peak values. Data expressed as mean ± 1 standard deviation. Regression analysis: \( y = 0.00022x^{1.85} \)  \( R^2 = 1.00 \)
Figure 2.7: Effect of the intervening polystyrene plate on the transmitted intensity of 4MHz ultrasound. The slope of the regression line represents the percentage of transmitted acoustic power density. Data expressed as mean ± 1 standard deviation. Regression analysis: $y = 0.71x$ $R^2 = 1.00$
Figure 2.8: Effect of the intervening polystyrene plate on the transmitted intensity of 1MHz ultrasound. The slope of the regression line represents the percentage of transmitted acoustic power density. Data expressed as mean ± 1 standard deviation. Regression analysis: $y = 0.86x$, $R^2 = 1.00$
Figure 2.9: A typical screen capture of the digitalizing oscilloscope. Channel one (top waveform) shows the current through PZT. Channel two (bottom waveform) shows the 10X attenuated voltage on the PZT. Channel Math One (middle waveform) shows the calculated input electrical power on the PZT.
Figure 2.10: Electro-acoustic conversion efficiency of a typical 4MHz transducer. Output acoustic power was measured without the intervening polystyrene plate. Data expressed as mean ± 1 standard deviation. Regression analysis: \( y = 0.072x \quad R^2 = 1.00 \)
Figure 2.11: Comparison of tone-burst wave acoustic power density to that of continuous wave. The tone-burst wave has 25% duty cycle. Data expressed as mean ± 1 standard deviation. Regression analysis: $y = 0.27x$  \( R^2 = 1.00 \)
Figure 2.12: Equivalent electrical circuits for a lossless transducer operating at resonance frequency
2.7 References


CHAPTER 3

AN INVESTIGATION ON THE SONODYNAMIC EFFECT OF DOXORUBICIN AND THE POWER DENSITY REQUIREMENT OF A MICRO-ULTRASONIC-TRANSDUCER FOR SONODYNAMIC THERAPY

3.1 Introduction

3.1.1 Sonodynamic Therapy

Sonodynamic therapy is a cancer treatment modality using ultrasound in which the efficacy of a molecular cancer drug is enhanced by acoustic cavitation[1]. It has been investigated as a method for targeted-drug delivery and enhancement because of the ability of ultrasound to focus precisely on a volume in the body. The mechanism of sonodynamic therapy is not well defined and is probably a combination of several effects due to acoustic cavitation including:

1) Chemical activation of the molecular drugs from reaction with free radicals produced by the cavitation [2]

2) Transient pore formation in cell membranes due to the physical disruption caused by shockwave and acoustic streaming from the cavitation. Molecules that could not transverse the cell membrane previously could then enter the cells efficiently [3]; this process is termed sonoporation.

3) Induction of cellular apoptosis due to membrane damage [3].

A version of this chapter will be submitted for publication.
4) Direct cell lysis [1]

Doxorubicin, a common chemotherapeutic agent, is one of the many molecular drugs that are used in sonodynamic therapy. Other drugs used in sonodynamic therapy include cyclophosphamide, daunomycin, diaziquone and 5-fluorouracil [1]. Doxorubicin has a molecular weight of 579.98 g/mol [4] and a octanol:water partition coefficient of 0.52 [5]. With a pKa of 7.6 due to the amine group, doxorubicin acts as a weak base and would be ionized in acidic solution [6]. The charge on the ionized doxorubicin will prevent its efficient penetration through the cell membrane and is thought to be a reason for the “physiological” resistance observed in vivo [6]. Doxorubicin also contains a quinone structure (Figure 2.1) and can generate highly reactive chemical species that would damage membrane and cytoplasmic proteins and DNA [7]. This inherent cytotoxicity could be further enhanced by free radical produced from acoustic cavitation and is demonstrated by Umemura et al [8]. Other mechanisms of action for doxorubicin such as inhibition of topoisomerase II and simulation of apoptosis could also be further enhanced by increased cellular uptake due to sonoporation. This mode of enhancement is supported by evidence from Saad and Hahn 1989[9]. General synergistic effect of ultrasound and doxorubicin were reported by numerous investigators including: Loverock et al. [10], Harrison et al [11] and Yu et al. [12].
3.1.2 MUTs for Sonodynamic Therapy

An often cited advantage of using ultrasound for cancer therapy is the non-invasiveness and spatial specificity [13]. The ability to localize the treatment volume brings about another challenge, namely "how to find and target that volume of interest". The most developed clinical protocol of ultrasonic cancer therapy calls for the use of a magnetic resonance imaging system to guide the treatment [7]. However, such an approach has obvious drawbacks including increased complexity and cost. This challenge would be applicable to the development of clinically relevant sonodynamic therapy as well.

An alternative to the MRI-guided approach is to implant the ultrasonic transducer directly in the vicinity of the volume of interest. The first requirement for such an ultrasonic transducer is to be reasonably sized so that implantation is acceptable. Recent advances in micro-fabrication technology have made MUTs a reality [14-16]. There are many advantages in using MUTs for sonodynamic therapy over the use of extra-corporeal application of ultrasound with conventional transducers. Some of these advantages include:

1) No need to re-align and re-focus the ultrasound for every treatment

2) Reduced unnecessary ultrasound exposure in normal tissues

3) Possible integration with controlled-release mechanism for the cancer drug
4) Fully automatic operation where sonication is carried out at specific times and for specific duration.

While MUTs have been successfully fabricated for medical imaging applications, no one has yet designed an MUT specially intended for sonodynamic therapy. One of the most important parameters in sonodynamic therapy is the acoustic power density because acoustic cavitation is known to occur only above certain acoustic intensity thresholds [17]. In this study, the acoustic intensity threshold necessary for sonodynamic enhancement of doxorubicin cytotoxicity is investigated. The result of this study will be a crucial design criterion for a MUT intended for sonodynamic therapy and will pave the way for its eventual realization.

3.2 Materials

3.2.1 Chemicals

Doxorubicin was purchased from Sigma-Aldrich Corporation (Oakville, ON, Canada) and made up to 1mM concentration stock solution in DMSO and stored at -20°C. Trypsin-EDTA (0.25%) was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). Chemicals used for the cell culture media preparation are outlined in section 2.2.2.

CytoTox 96® Non-Radioactive Cytotoxicity Assay (referred to as the lactate dehydrogenase (LDH)-release assay subsequently) was purchased from Promega Corporation (Madison, WI,
USA). It was prepared and stored as instructed by the manufacturer in Technical Bulletin No. 163.

CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (referred to as the MTS assay subsequently) was purchased from Promega Corporation (Madison, WI, USA). It was prepared and stored as instructed by the manufacturer in Technical Bulletin No. 169. More details about the MTS and LDH-Release assays are available in Appendix 3.

3.2.2 Cell Culture

Adherent human prostate cancer cells (PC3) were kindly provided by Dr. Helen Burt's laboratory at the Faculty of Pharmaceutical Science at The University of British Columbia. They were grown as monolayer in T-75 flask (Corning Inc., Corning, NY, USA) and passaged weekly. The protocol for cell passaging is available in Appendix 4.

The PC3 media contained F-12 Nutrient Mixture, 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin, all purchased from Invitrogen Canada Inc. It also contained 1.176g of sodium bicarbonate and was filter-sterilized and stored at 2-4°C. The protocol for preparing the PC3 media is available in Appendix 5.
3.2.3 Ultrasound Transducers

Ultrasonic transducers were made and their performances were evaluated as described in Chapter 2.

3.2.4 Ultrasound Exposure Setup

A custom-made polymethyl-methacrylate (PMMA) water-bath was constructed for the experiments outlined in this chapter. A drawing of this water-bath is available in Appendix 6. During the experiments, an ultrasonic transducer was fixed at the bottom center of the water-bath and pointed directly upward. The 48-wells cell culture plate was placed above the ultrasonic transducer. Water was used to couple the transmission of the ultrasound and was degassed by boiling before being added to the water-bath. Figure 3.2 summarizes the ultrasound exposure setup.

The attenuation of the acoustic power density by the degassed water from the transducer to the 48-wells cells culture plate over a distance of approximately 5mm was assumed to be insignificant. The attenuation and reflection of the acoustic power density by the 2mm polystyrene cells culture plate bottom was determined to be 71% for 4MHz and 86% for 1MHz ultrasound. This decrease in acoustic power density due to the cells culture plate bottom is taken into consideration for the calculation of the applied acoustic power density in subsequent experiments.
3.3 Experimental Protocol

3.3.1 Calibration of Cytotoxicity Assay

Two commercially available assays- the MTS assay and the LDH-release assay, were used to measure cytotoxicity of doxorubicin and ultrasound. The MTS assay measured the long-term (3 days) cytotoxicity which includes proliferation inhibition while the LDH-release measured the immediate cytotoxicity. In order to find out the valid parameters for conducting the experiments (i.e. number of cells to be seeded per well, number of days for incubation, minimal detectable number of cells, etc.) a number of calibration experiments were carried out with these two assays.

For the MTS calibration experiment, the cell concentration of a stock of harvested PC3 was determined by counting a 50μl aliquot with a haemocytometer. Specific numbers of cells were seeded in a 48-wells cell culture plate and were allowed to equilibrate for 1 day. Then MTS solution was added to PC3 media at 10μl/100μl media and the mixture was added to each well at 200μl per well. After incubation of the specified time, the solution in each well was transferred to a 96-wells plate and the absorbance of each well were read with the Multiskan ELISA Plate reader at 492nm.

For the LDH-release experiment the cells density of a harvested stock PC3 was determined as previously described. Specified numbers of cells were seeded in 48-wells cell culture plate
with 400μl PC3 media. Solution of 10X lysis buffer was added at 50μl per well and the plate was
shaken at speed 6 for 1min and then incubated for 1.2hr. 50μl content from each well was then
transferred to a 96-wells plate and 50μl LDH substrate was added to each well. The 96-wells
plate was protected from light and incubated for 30min and the absorbance of each well was read
with a Multiskan ELISA Plate Reader at 492nm.

3.3.2 Cytotoxicity of Doxorubicin

Using 48-wells cell culture plate, PC3 cells were seeded at 10,000 cells/well
(25,000cells/ml at 400μl per well). The cells were allowed to equilibrate and attach to bottom of
the wells for 1 day after seeding. PC3 media were mixed with doxorubicin to 16μM stock. Serial
dilution of the stock doxorubicin was added to corresponding well. The plate was incubated in
room temperature for 2hr to simulate experimental conditions after which the plate was
incubated at 37°C for 72hrs. Then cytotoxicity was determined by the MTS assay.

3.3.3 Cytotoxicity of Ultrasound

The cytotoxicity of ultrasound on PC3 in suspension was compared to the cytotoxicity of
ultrasound on PC3 in adherent monolayer. For the suspended PC3, 50,000 cells per well
(50,000cells per ml at 1ml per well) were seeded in 48-wells cell culture plate. Tone-burst
ultrasound (4MHz, 50msec repetition period, 25% duty cycle) was applied right after seeding. After sonication, an aliquot of 100μl from each well were taken and transferred to a new 98-wells plate. 1ml of new media was added to each well and the media was also changed on the next day. The plate was incubated for 72hrs and the MTS assay was used to assess the cytotoxicity.

For the PC3 monolayer, the cells were seeded at 10,000 cells per well (50,000 cells per ml at 200μl per well) in 48-wells cell culture plate. The cells were allowed to equilibrate and attach to the bottom of the wells for 24hrs. The 300μl media was added and tone-burst ultrasound (4MHz, 50msec repetition period, 25% duty cycle) was applied. The plate was incubated for 72hrs and the MTS assay was used to assess the cytotoxicity.

The cytotoxicity results of the MTS assay and the LDH-release assay was compared. PC3 cells were seeded under the same conditions as in section 2.3.2. After 24hr of equilibration and re-attachment to the well bottom, 50μl of 10x lysis buffer was added to each positive control well for the LDH-release assay. The specific well was exposure to 4MHz ultrasound for various times. The LDH-release assay was carried out immediately after ultrasound exposure and the MTS assay was done after 72 hrs of incubation at 37°C.
3.3.4 Cytotoxicity of Doxorubicin in Combination with Ultrasound Exposure

PC3 cells were seeded with the same procedures as outlined in section 3.3.2. After 1 day of equilibration and re-attachment to the well bottom, 400μl of fresh PC3 media or media plus 2μM doxorubicin were added to corresponding wells. Tone-burst ultrasound (4MHz, 50msec repetition period, 25% duty cycle) at various acoustic power densities was then applied to the specific wells for varies times. After the ultrasound exposure, the content of all wells was aspirated and replaced by fresh PC3 media. The LDH-release assay was used to measure the immediate cytotoxicity and the MTS assay was used to measure the long-term cytotoxicity (after 3 days of incubation).

3.4 Experimental Results

3.4.1 Calibration of Cytotoxicity Assay

From Figure 3.3 we can see that the MTS assay gives a fairly linear relationship between the absorbance at 492nm (A₄₉₂) and the number of seeded cells up to 20,000 cells. Starting at 40,000 cells and more significantly at 80,000 cells, the A₄₉₂ appears to deviate from the linear relationship and becomes less than expected. The longer incubation times gives a more sensitive response ratio because of the greater slope of the relationship but also exacerbates the non-linearity.
Figure 3.4 shows the $A_{492}$ in relation to the number of lysed cells. It is clear that the relationship is linear up to 20,000 cells. For this particular calibration the background $A_{492}$ is at 0.9589, which corresponds to minimal detectable number of cells to be about 1,250. For subsequent LDH-release experiments, it was observed that the background $A_{492}$ is much lower. The higher background $A_{492}$ during the calibration experiments might be due to some cells that were lysed during the cell passaging. However, this situation is avoided in subsequent LDH-release experiments because the content of each well was replaced with PC3 media before the LDH-release assay.

Cytotoxicity in all the subsequent experiments is defined as follow:

For MTS assay:

$$Cytotoxicity = 1 - \frac{\text{Normalized } A_{492} \text{ of treated well}}{\text{Normalized } A_{492} \text{ of no treatment control well}}$$  \hspace{1cm} (3.1)

in which, Normalized $A_{492} = A_{492}$ of the sample - $A_{492}$ of no cell control.

For the LDH-release assay:

$$Cytotoxicity = \frac{\text{Normalized } A_{492} \text{ of treated well}}{\text{Normalized } A_{492} \text{ of highest absorbance wells}}$$  \hspace{1cm} (3.2)
in which, Normalized $A_{492} = A_{492}$ of the sample - $A_{492}$ of intact-cells control. The normalized $A_{492}$ of highest absorbance wells is used for the definition of cytotoxicity in LDH-release experiments because it was found that the buffer lysed controls do not give 100% release of the LDH.

3.4.2 Cytotoxicity of Doxorubicin

The long term cytotoxicity of doxorubicin from 0.25µM to 20µM is summarized in Figure 3.5. The graph of the concentration-cytotoxicity relationship followed roughly a hyperbolic shape, i.e. the cytotoxicity levels off at high concentration, as predicted by the drug-receptor hypothesis [18].

3.4.3 Cytotoxicity of Ultrasound

Given the same ultrasound exposure condition, PC3 in suspension were found to be more prone to the cytotoxic effect of ultrasound compare to PC3 in adherent monolayer (Figure 3.6). This might be due to the fact that the spherical shape of the cells in suspension render them to more pressure variation than the flattened adherent cells. Furthermore, both arrangements showed increased cytotoxicity with increased exposure time as expected. Adherent monolayer of PC3 was selected to be the arrangement used for all subsequent experiments because the lower cytotoxicity allowed greater potential of enhancement when combined with doxorubicin.
Both the MTS and the LDH release assay showed increased cytotoxicity with increasing exposure time (Figure 3.7). The cytotoxicities reported by MTS assay were consistently less than the LDH-release results. One possible reason for this difference might be due to cells that were transiently permeabilized, therefore released some LDH, but recovered afterward and continued to proliferate. It also suggested that the major cytotoxic action of ultrasound (at the setting employed in this study) was immediately cell lysis. While there might also be some cytotoxicity from increased uptake of doxorubicin due to sonoporation and ultrasound induced apoptosis of cells, these effects were minimal.

3.4.4 Cytotoxicity of Doxorubicin in Combination with Ultrasound Exposure

Tone-burst ultrasound (32 Watt/cm\(^2\), 20% Duty Cycle, 50msec burst period) was found to significantly (p<0.01, student's t-test, unpaired, two tails, unequal variances) enhance cytotoxicity when used in combination with 20μM doxorubicin compare to doxorubicin-alone or ultrasound-alone controls (Figure 3.8 and 3.9). The greatest enhancement was found with 30 seconds exposure, where immediate cytotoxicity was enhanced by 70% compared to doxorubicin-only control and 57% compared to ultrasound-only control. The long-term cytotoxicity was enhanced by 26% compared to doxorubicin-only control and 83% compared to ultrasound-only control. To facilitate further analysis of the data, a new parameter, cytotoxicity
enhancement, is defined as:

\[ Cytotoxicity \text{ Enhancement} = CT_{\text{com}} - CT_{\text{Dox}} \quad (3.3) \]

Where \( CT_{\text{com}} \) is the Cytotoxicity of ultrasound in combination with doxorubicin and \( CT_{\text{Dox}} \) is the cytotoxicity of doxorubicin alone.

When ultrasound is used in combination with 2\( \mu \)M doxorubicin over a variety of ultrasonic power densities, cytotoxicity enhancement increases with applied ultrasonic energy density ( = ultrasonic power density \( \times \) duty cycle \( \% \) \( \times \) exposure time) as well as with ultrasonic power (Figure 3.10), which confirm the enhancement is not a thermal phenomenon.

In 2001, Guzman et al. [19] found that the ultrasound–mediated enhancement of molecular uptake could be fitted to following relationship:

\[ Cytotoxicity \text{ Enhancement} = 1 - e^{\alpha E^{2/3}} \quad (3.4) \]

where \( E \) is the ultrasonic energy density and \( \alpha \) is an adjustable parameter to take into account the difference in experimental conditions between each set of data. Adopting their approach, data from Figure 3.10 were fitted to Equation 3.4 and allowing \( \alpha \) to account for the differences in
ultrasonic power density. The procedure for doing the fit is outlined in Appendix 7. Table 3.1 summarizes the values of \( \alpha \) found at each power density. Plotting the value of \( \alpha \) to their corresponding ultrasonic power density, we obtain a straight line (Figure 3.11). When \( \alpha \) is zero, cytotoxicity enhancement is independent of the ultrasonic power density. In other words, threshold ultrasonic power density required for any cytotoxicity enhancement to be observable is the x-intercept of Figure 3.11 and it is found to be 15W/cm\(^2\).

The cytotoxicity of 1MHz ultrasound in combination with doxorubicin was much more pronounced than 4MHz ultrasound (Figure 3.12). The immediate action of cell lysis due to 1MHz ultrasound in combination with doxorubicin was also visible under optical microscope (Figure 3.13)

3.5 Discussion

The greatest cytotoxicity enhancement was observed with 30 seconds ultrasonic exposure while longer exposures resulted in less enhancement simply because cytotoxicity could not go above 100% by definition. The cytotoxicity enhancement of 20\(\mu\)M doxorubicin plus ultrasound over doxorubicin-only control dropped from 70% to 26% over the course of 3 days (difference in immediate and long-term cytotoxicity). This effect was consistent with the mechanism of doxorubicin (DNA intercalation and topoisomerase II inhibition: i.e. affecting cell proliferation),
which requires time to manifest itself. Similarly, the cytotoxicity enhancement of 20μM doxorubicin plus ultrasound over ultrasound-only control increased from 57% to 83% over the course of 3 days. This increase was consistent with the mechanism of ultrasonic cell killing (disruption of cell membrane) and its effect is immediate.

When deciding what concentration of drug should be used with ultrasound for sonodynamic therapy, one must remember that the cytotoxic effect of doxorubicin follows a hyperbolic shape as predicted by the Drug-Receptor Hypothesis (Figure 3.5). At low concentration, increasing the concentration of doxorubicin would increase the long-term cytotoxicity drastically. However, at high concentration, increasing the concentration of doxorubicin would yield only marginal effect. For example, using the data from Figure 3.5, the cytotoxicity of 20μM doxorubicin was 68%; if we wished to increase the cytotoxicity by 12%, we must double the concentration of doxorubicin to 40μM (detail of calculation in Appendix 8). But we could accomplish that same level of cytotoxicity enhancement (12%) by adding 15 seconds of ultrasound exposure in conjunction (Figure 3.9). Furthermore, our data suggested that to raise the cytotoxicity to 95% by doxorubicin alone would require a concentration of at least 171μM (see Appendix 8). On the other hand, a 60 seconds ultrasound exposure with 20μM would almost completely eradicate the PC3 (Figure 3.9). Therefore, sonodynamic therapy should be carried out with concentration from the levelling portion of the Dose-Response Curve (above the 50% Effective Concentration).
where its effect over drug-only treatment is most significant.

The \textit{a priori} assumption that the cytotoxicity enhancement was due to acoustic cavitation as opposed to thermal effect was confirmed by the following finding: 1) Cells in suspension were more susceptible to the cytotoxic action of ultrasound than adherent cells (Figure 3.6); 2) Cytotoxicity enhancement varied with ultrasonic energy density as well as ultrasonic power density (Figure 3.10); 3) Cytotoxic action of 1MHz ultrasound was more prominent than 4MHz ones (Figure 3.12).

The ultrasonic power density ($I_{SATA}$) threshold for the cytotoxicity enhancement at 4MHz was measured to be 15 W/cm$^2$. Since the ultrasonic power density threshold was proportional to actuation frequency raised to some power [17], any ultrasonic transducer that achieves this power density at a lower frequency would also be able to effect the cytotoxicity enhancement. Furthermore, the threshold ultrasonic power density discussed above is dictated by the physics behind the cavitation phenomenon and is not dependent on the nature and size of the ultrasonic transducer. Therefore a MUT that is intended for enhancing cytotoxicity in sonodynamic therapy must meet the same criterion.

In designing a MUT, ultrasonic power density is a very abstract idea. Therefore it is beneficial to relate it to some tangible quantities such as the velocity and the amplitude of the transducer. The procedure for doing so is outlined as follows:
Since this power density value is estimated using the acoustic force balance method, it is a spatial-average-temporal-average intensity (ISATA). Assuming that the ultrasound emitted from the transducer is a plane propagating wave, the intensity at any point with the same distance from the transducer will be the same (ISPTA = ISATA) and multiplying ISPTA by 2 to convert to ISPTP. Therefore, the spatial-peak-temporal-peak ultrasonic power density threshold for cytotoxicity enhancement at 4MHz is 30W/cm².

Furthermore, by rearranging Equation 1.4, we have:

\[ u = \sqrt{\frac{I_{SPTP}}{Z}} \]  \hspace{1cm} (3.5)

where \( u \) is the peak transducer face velocity and \( Z \) is the acoustic impedance of water (1.5×10⁶ secN/m³). Substituting the value of \( I_{SPTP} \) and \( Z \), we found that transducer face must move with a peak velocity of 0.447 m/s to produce that ultrasonic power density at 4MHz. For a transducer actuated by a sinusoidal waveform at a given frequency (\( f \)), the peak transducer face velocity (\( u \)) is also related to actuation amplitude (\( A \)) by:

\[ A = \frac{u}{f} \]  \hspace{1cm} (3.6)

therefore, for 4MHz ultrasonic transducer to effect any cytotoxicity enhancement, it must have
an actuation amplitude of at least 112nm.

Given the frequency and the required actuation amplitude the corresponding properties of the MUT (thickness, stiffness, area of the membrane and the amount of actuation force) can be decided upon.
Table 3.1: Values of $\alpha$ and corresponding $R^2$ from fitting data from Figure 3.10 to Equation 3.4.

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<th>Ultrasonic Power Density (W/cm$^2$)</th>
<th>Value of $\alpha$ from the regression fit to Equation 3.4</th>
<th>$R^2$ of the regression fit</th>
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Figure 3.1: Structure of doxorubicin
Figure 3.2: Ultrasound exposure setup.
Figure 3.3: Calibration curve of the MTS assay using known number of PC3 cells. Incubation time: — 30 min; — 1hr25min; — 2hr5min; — 4hr25min. Data expressed as mean ± 1 standard deviation.
Figure 3.4: Calibration curve of the LDH-release assay using known number of PC3 cells. Regression analysis of the portion from 2,500 to 20,000 shows excellent fit to a straight line ($R^2 = 1.00$). Data expressed as mean ± 1 standard deviation.
Figure 3.5: Long-term cytotoxicity of doxorubicin on the PC3 cells at different concentrations. Cytotoxicity was measured using the MTS assay. The PC3 cells were exposed to doxorubicin for 3hrs and then allowed to proliferate in fresh media for an additional 2-3 days. Data expressed as mean ± 1 standard deviation.
Figure 3.6: Long-term cytotoxicity due to ultrasound on PC3 in suspension (□) and in adherent monolayer (■) exposed under same ultrasonic conditions. Cytotoxicity was measured using the MTS assay. The PC3 cells were exposed to doxorubicin for 3hrs and then allowed to proliferate in fresh media for an additional 2-3 days Data expressed as mean ± 1 standard deviation.
Figure 3.7: Comparison of the result from the MTS assay () and the LDH-release assay () using PC3 cells. Acoustic power density used was 32Watt/cm². Data expressed as mean ± 1 standard deviation.
Figure 3.8: Immediate cytotoxicity in PC3 cells using: ultrasound alone (□), ultrasound in combination of 2μM doxorubicin (■) and ultrasound in combination of 20μM doxorubicin (■). The PC3 cells were exposed to doxorubicin for 3hrs and then had the cytotoxicity measured immediately using the LDH-release assay. Acoustic power density used was 32Watt/cm². Asterisk (*) indicates statistical significance (p<0.01) comparing to ultrasound alone and doxorubicin alone. Data expressed as mean ± 1 standard deviation. With 30sec ultrasound exposure and 20μM doxorubicin, cytotoxicity is increased by 70% compare to doxorubicin-only control and 57% compare to ultrasound-only control.
Figure 3.9: Long-term cytotoxicity in PC3 cells using: ultrasound alone (□), ultrasound in combination of 2μM doxorubicin (■) and ultrasound in combination of 20μM doxorubicin (□). The PC3 cells were exposed to doxorubicin for 3hrs and then allowed to proliferate for another 3 days before having the cytotoxicity measured using the MTS assay. Acoustic power density used was 32Watt/cm². Asterisk (*) indicates statistical significance (p<0.01) comparing to ultrasound alone and doxorubicin alone. Data expressed as mean ± 1 standard deviation. With 30sec ultrasound exposure and 20μM doxorubicin, cytotoxicity is increased by 26% compare to doxorubicin-only control and 83% compare to ultrasound-only control.
Figure 3.10: Cytotoxicity enhancement in PC3 cells using ultrasound in combination of 2μM doxorubicin over a range of ultrasonic power densities: 19W/cm² (●); 22W/cm² (○); 25W/cm² (▲); 32W/cm² (△); 36W/cm² (●); 40W/cm² (■). The PC3 cells were exposed to doxorubicin for 3hrs and then allowed to proliferate for another 3 days before having the cytotoxicity measured using the MTS assay. Data expressed as mean.
Figure 3.11: Values of $\alpha$ from table 3.1 plotted against the corresponding power densities. Regression analysis: $y = -4.79E-04x + 7.07E-3$ $R^2 = 0.86$
Figure 3.12: Cytotoxicity in PC3 cells using 4MHz (—in) and 1MHz (△—) ultrasound in combination with 2μM doxorubicin. 4MHz ultrasound was applied at 22W/cm² and 1MHz was applied at 4.8 W/cm². The PC3 cells were exposed to doxorubicin for 3hrs and then allowed to proliferate for another 3 days before having the cytotoxicity measured using the MTS assay.
Figure 3.13: Immediate effect of 1MHz 4.8 W/cm$^2$ ultrasound in combination with 2μM doxorubicin: A) Before exposure; B) After 1min ultrasonic exposure; Solid line circles PC3 cells that were not affected; Dashed line circles cells that were lysed or detached.
3.6 Reference


CHAPTER 4

TARGEDTED CYTOTOXICITY ENHANCEMENT OF MICELLAR PACLITAXEL USING TONE-BURST CONTINUEOUS ULTRASOUND

4.1 Introduction

Paclitaxel is a potent anticancer drug first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. Its anticancer activity arises from its action on the microtubule which leads to the disruption of both mitotic and non-mitotic events [1]. A major drawback for the use of Paclitaxel is its low water solubility, as evident from its structure (Figure 4.1). The reported aqueous solubility of paclitaxel is less than 0.25μg/ml [2] or 0.293 μM, given the molar weight of paclitaxel is 853.91g/mol [3]. The octanol:water partition coefficient of paclitaxel was found to be more than 99 comparing to doxorubicin at 0.52 [4]. Clinically, paclitaxel is used as a solution in Cremphor ® EL (polyethoxylated castor oil) which can cause serious side effects such as hypersensitivity, nephrotoxicity and neurotoxicity[5]. As a result, numerous alternative approaches to administer paclitaxel, including the use of nanospheres [6, 7], liposomes [8] and polymeric micelle [9] have been investigated.

Among the mentioned alternatives, polymeric micelle is a promising candidate because many of the important properties, such as the critical micelle concentration, micelle size and

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A version of this chapter will be submitted for publication.
drug loading capacity, can be modified by changing the structure of the polymers or the length of the block. In 1996, Zhang et al. reported a series of amphiphilic diblock copolymers that could be used to effectively deliver paclitaxel [10, 11]. Furthermore, due to their sizes (10nm-100nm) and non-ionic water-soluble shell, polymeric micelles can preferentially accumulate in tumours and escape uptake by phagocyte via the permeation and retention effect [12]. Therefore drug-loaded polymeric micelles can passively target tumour sites. Currently, researchers are investigating other methods to actively target the polymeric micelle to tumour sites and therefore further enhancing its selectivity [13].

Ultrasound has been found to enhance the delivery of liposome-encapsulated DNA plasmid [14, 15] and trigger the release of doxorubicin from polymeric micelles [16]. It is a promising tool for targeted-delivery of therapeutics because it could be applied by a conventional extra-corporeal device and be focused precisely into a small volume of the body. In addition, it has also been proposed that an implantable MUT could provide cost-effective and fully automatic targeted-drug enhancement (Chapter 3). This study investigates the possibility of targeted cytotoxicity enhancement of micellar paclitaxel using tone-burst continuous ultrasound.
4.2 Materials

4.2.1 Chemicals and Cell Culture

Paclitaxel and diblock copolymers of Poly(D,L-lactide)-block-methoxypolyethylene glycol (PDLLA-MePEG 2000-40:60) were kindly provided by Mr. John Jackson from Dr. Helen Burt’s laboratory at the Faculty of Pharmaceutical Science at The University of British Columbia. Each vial containing 50mg of materials (Paclitaxel and diblock copolymers) was dissolved in 1ml phosphate buffered saline and stored at 2-4°C until the experiments. All other materials used for the cell culture were the same as what has been described in section 3.2.1 and 3.2.2.

4.2.2 Ultrasound Transducers

Ultrasonic transducers were made and their performances were evaluated as described in Chapter 2.

4.2.3 Ultrasound Exposure Setup

The ultrasonic exposure setup is described in section 3.2.4.

4.3 Experimental Protocol

The PC3 were seeded at 10,000 cells per well (25,000 cells per ml at 400µl per well) in
48-wells cell culture plate. To avoid acoustic coupling, wells that are immediately adjacent to seeded wells are left empty. The cells were allowed to equilibrate and attach to the bottom of the wells for 2 days. Then micellar paclitaxel from the stock was made up to different concentrations with PC3 media and was added to the corresponding wells. Tone-burst ultrasound (4MHz, 50msec burst period, 50,000 burst count, 25% duty cycle) at 32Watt/cm² was applied for 15 sec for wells unless specified otherwise. After the ultrasound exposure, content of each well was replaced by fresh PC3 media. The plate was incubated at 37°C for 2 days and the MTS assay was used to assess the long-term cytotoxicity.

4.4 Experimental Results

The PDLLA-MePEG diblock copolymers are found to have negligible cytotoxicity at all concentrations tested for this experiment (Figure 4.2). At high concentration (600μM), PDLLA-MePEG diblock copolymers appear to counteract the cytotoxic action of tone-burst ultrasound (Figure 4.2).

Micelles that were loaded with 2.5% and 0.5% paclitaxel have consistent cytotoxicity despite the varying concentration of diblock copolymers (Figure 4.3 and 4.4). This result agreed with the fact that paclitaxel is very insoluble in water and paclitaxel in the system would be expected to partition between the solution (minority) and the micelle (majority). Given the high
concentration of paclitaxel in the micelle, the surrounding solution would be saturated with a constant concentration of free paclitaxel regardless of the concentration of diblock copolymers. That constant concentration of free paclitaxel contributes to the background cytotoxicity of 30% to 35% observed in Figure 4.3 and background cytotoxicity of 40% to 46% observed in Figure 4.4. Diblock copolymers loaded with 0.5% appeared to have greater cytotoxicity of those loaded with 2.5%. This effect was most likely due to experimental errors in preparing the samples; further experiments are needed to clarify this finding. However, both showed similar trends with the synergetic effect of ultrasound suppressed by high concentration of diblock. At and below 300μM diblock copolymers, ultrasound significantly enhanced the cytotoxicity compare to micellar paclitaxel only control (p<0.05, student’s t-test, unpaired, two tails, equal variances).

For micelles that were loaded with 0.1% (Figure 4.5), the background cytotoxicity decreased markedly at low concentrations 75 and 37.5μM. Cytotoxicity enhancement of the micelles with 0.1% due to tone-burst ultrasound appeared to be even greater than for 0.5% and 2.5% loaded micelles. With 150μM diblock copolymers, ultrasound increased the cytotoxicity from 28% to 69%.

The critical micellar concentration for PDLLA-MePEG diblock copolymers used was reported previously as 90μM and 23μM depending on the techniques of measurement [10]. In this study, we assumed that the micelle still formed at the lowest diblock copolymers
concentration used (37.5\textmu M). This assumption was supported by the fact that at the lowest concentration of copolymers, the cytotoxicity with 0.1% paclitaxel was lower than those at higher concentration (Figure 4.5). If the diblock copolymers existed as monomers at that concentration, we would expect the paclitaxel concentration, and therefore cytotoxicity, in the solution to increase compared to higher concentrations of diblock copolymers.

Finally, increasing the exposure time also increased the cytotoxicity as expected (Figure 4.7). Diblock copolymer at 150\textmu M and loaded with 0.5% paclitaxel with 60 second of tone-burst ultrasound gave 91% cytotoxicity, compare to 38% cytotoxicity of the micellar paclitaxel alone.

4.5 Discussion

Regardless of the amount of loaded paclitaxel, the highest cytotoxicity was always achieved at around 150 and 75\textmu M. At higher concentration, the diblock copolymers counteracted the effect of ultrasound, whereas at lower concentration the cytotoxicity enhancement was not as effective. Unlike the sonodynamic enhancement of doxorubicin, which appears to increase proportionally with the concentration of doxorubicin, this apparent optimal concentration for ultrasonic enhancement of micellar paclitaxel would be an important parameter for any therapeutic applications and should be explored further in future study. The suppression of ultrasonic cytotoxicity with high concentration of polymeric micelle has been reported recently
and thought to be caused by micelles deflecting the ultrasonic power [17]. Similar results were observed in this study.

The fact that diluting the copolymer concentration did not alter the background cytotoxicity of the 2.5% and 0.5% loaded micelles (Figure 4.3 and 4.4) suggested that the background cytotoxicity may be due to free paclitaxel in the solution. However, since the aqueous solubility of paclitaxel is so low (< 0.293 μM), the significant amount of background cytotoxicity (30-45%) may be due to the increase solubility of paclitaxel as it could associate with diblock unimer. Equilibrium dialysis can be used in the future to study the interaction between the diblock unimers and paclitaxel.

Of the four possible mechanisms underlying sonodynamic therapy outlined in section 3.1.1, all except one could have a potential role in increasing cytotoxicity of free paclitaxel in the solution. Paclitaxel is not expected to interact with ultrasound to form free radical because the lack of a quinone structure. However, because of the presence of micellar paclitaxel, there are two extra ways ultrasound could affect cytotoxicity enhancement:

1) The transient holes on the cell membrane produced by sonoporation [18] could allow micellar paclitaxel to enter the cell. Because the concentration of paclitaxel is much higher in the micelle than in the solution, such an event would dramatically increase the intracellular concentration of paclitaxel.
2) It is believed that in the absence of ultrasound, internalization of polymeric micelle mediated predominantly by endocytosis [12]. In the study of ultrasonic enhancement of delivery of DNA encapsulated in micelle, researchers suggested that acoustic cavitation could possibly disrupt endosomes containing the endocytosed micelle and release its content to the cytoplasm [14, 15]. The cytotoxicity of micellar paclitaxel could be enhanced in a similar way.

These different mechanisms are summarized in Figure 4.7. It has also been suggested that ultrasound could trigger the release of doxorubicin from polymeric micelles by disrupting the micelle [19]; however this mechanism is not expected to contribute to the increased cytotoxicity in our study because concentration of free paclitaxel in the solution was near or at saturation for our experiments.

Our study is not designed to distinguish the differences of all these possible mechanisms. In fact, all the mentioned mechanisms probably occur to some extent. With special design in future studies, some of these mechanisms could possibly be separated from the others; for example, the use of radioisotope-labelled paclitaxel could distinguish sonoporation from direct cell lysis or apoptosis induction. The use of fluorescent microscopy to study the distribution of fluorescently labelled paclitaxel could give clues about the extent of ultrasonic disruption of endosomes [20]. The key point of our study is that we have shown that tone-burst ultrasound is a viable method to
further increase cytotoxicity of micellar encapsulated drugs. With the careful consideration of the concentration of the polymeric micelle, this could be an additional application of an implantable MUT.
Figure 4.1: Structure of paclitaxel
Figure 4.2: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers alone (□) and in combination with tone-burst ultrasound (■). The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 2 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation Data expressed as mean ± 1 standard deviation (N=2).
Figure 4.3: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG polymeric micelle loaded with 2.5% paclitaxel alone (■) and in combination with tone-burst ultrasound (□). The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 2 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation (N=2). Asterisk (*) indicates statistical significance (p<0.01) using student’s t-test (two-tails, unequal variances) comparing to loaded micelle alone.
Figure 4.4: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers loaded with 0.5% paclitaxel alone (●) and in combination with tone-burst ultrasound (▲). The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 2 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation (N=2). Asterisk (**) indicates p<0.05 and (*) indicates p<0.01 using student’s t-test (two-tails, equal variances) comparing to loaded micelle alone.
Figure 4.5: Long-term cytotoxicity in PC3 cells induced by PDLLA-MePEG diblock copolymers loaded with 0.1% paclitaxel alone (□) and in combination with tone-burst ultrasound (■). The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 2 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation (N=2). Asterisk (*) indicates statistical significance (p<0.01) using student’s t-test (two-tails, unequal variances) comparing to loaded micelle alone.
Figure 4.6: Cytotoxicity in PC3 cells induced by 300μM (□) and 150μM (●) PDLLA-MePEG diblock copolymers loaded with 0.5% paclitaxel in combination with different duration of tone-burst ultrasound. The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 2 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation (N=1).
Figure 4.7: The five possible mechanisms for the ultrasonic enhancement of micellar paclitaxel cytotoxicity.
4.6 References


CHAPTER 5

CONCLUSION

5.1 Limitations and future work

In this study, prototype ultrasonic transducers were developed. Their ultrasonic intensity output performances were evaluated and a few were selected to be used for further experiments. Ultrasound was found to enhance the cytotoxicity of doxorubicin as well as micellar paclitaxel. The power density required for the cytotoxicity enhancement in vitro was found to be \(30\text{W/cm}^2\) (\(I_{\text{SPTP}}\)). Some points for improvement in future work are considered below:

In the ultrasound exposure setup of this study, the ultrasonic transducer was orientated upward as depicted in Figure 3.2. This setup was has been used by other researchers [1, 2]. The major advantages of this setup were the compatibility with commercial cell culturing plates and the ability to maintain sterility. However, the ultrasound wave was reflected at the surface of the liquid and potentially resulting in interference pattern with incoming wave or even a standing wave pattern. While this effect was somewhat ameliorated by the use of tone-burst ultrasound, the situation was still far from what would be expected in an \textit{in vivo} environment.

Beside ultrasonic intensity, acoustic cavitation is also influenced by the presence of impurities, temperature and hydrostatic pressure. These factors were not precisely controlled in this study and in fact, would be hard to maintain at conditions similar to those found in the
human body. Therefore, the real ultrasonic intensity required for sonodynamic enhancement of
drugs in vivo might be somewhat different from the 30W/cm² (ISPTP) that we found in this study.
The next step of the investigation should be carried out with in vivo experiments, which would
circumvent the two problems described above.

Furthermore, in this study, a commercial ultrasonic meter was used to measure the ISATA.
Simple mathematical relation was used to convert the ISATA to ISPTP, which is a more precise
measure of acoustic cavitation. However, factors such as the beam pattern of the transducer were
ignored, which could influence the ISPTP. For future study, a miniature hydrophone measuring the
ISPTP directly would be a better approach to estimate the ultrasonic intensity.

In the calculation of long-term cytotoxicity, the MTS assay was assumed to give a linear
relationship between the absorbance value and the number of viable cells. In reality, we observed
deviation from linearity starting at 40,000 cells (Figure 3.3) and that could contribute to the
experimental error. A more precise estimate of the viable cells could be achieved by using the
calibration curve to correlate each absorbance value to corresponding cell number [3]. This
approach was not used in this study because it would mean that a single incubation time was to
be strictly observed and flexibility of the experiment would be greatly limited. However, for
future experiments, these extra efforts should be considered in order to produce more accurate
data.
To account for the fact that the LDH-release assay consistently reported more cytotoxicity than the MTS assay under the same conditions (Figure 3.7), we hypothesized that some cells might release a small amount of LDH due to sonoporation but remain viable for subsequent proliferation. One can then argue that the LDH-release assay did not give an accurate account of the cytotoxicity. Therefore, for future study, we should consider using the MTS assay to measure the immediate cytotoxicity as well.

Finally, it is important to remember that prototype ultrasonic transducers with bulk piezoelectric ceramics were used in this study. While the size of the transducers is not expected to alter the power density threshold, experimental data with MUT would be necessary to convince the expected critics. The results from this study provide a concrete physical requirement for the design of MUT. It serves as an important first step for the realization of implantable MUT for sonodynamic and other therapeutic applications.
5.2 References


APPENDIX 1

MATERIAL PROPERTIES OF THE PZT USED

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APPENDIX 2

PHOTOS OF EXPERIMENTAL SETUP FOR MEASURING ULTRASONIC POWER
APPENDIX 3

ADDITIONAL INFORMATION ON THE MTS AND LDH-RELEASE ASSAY

Quantifying the number of viable cells underline the calculation of cytotoxicity and cytotoxicity enhancement in this study. There are many different ways to quantify the number of viable cells, the most common ones are:

1) Stain cell with Typhan Blue and count individual cells from a sample volume underneath a haemocytometer
2) Use of a Coulter Counter
3) Radioisotope-labelled Thymine uptake assay
4) MTT and MTS assays
5) LDH-release assay

There are advantages and disadvantages, such as the ease of use and accuracy, associated with each method. The MTS and LDH-release assays were used for this study because of their ease of use, accuracy and the materials were readily available.

MTS is a water-soluble tetrazolium compound and it is reduced into a formazan product by dehydrogenase enzymes found in viable cells [1]. The formazan product has a characteristic absorbance at around 490nm and could be quantified using any typical spectrometer. Therefore, after a given incubation time, the 490nm of solution is proportional to the number of viable cells. The accuracy of the MTS assay was found to be comparable to the Radioisotope-labelled Thymine uptake assay by Buttke et al. [2].

LDH is stable cytosolic enzyme found in normal viable cells. When the cell membrane is
disrupted or lysed, the LDHs are released into the surrounding solution and their activity can be measured by adding a tetrazolium salt (INT), which will be converted into a red formazan product. Again, the concentration of the formazan product is conveniently measured at 490nm by a spectrometer [3]. For more details regarding the MTS and the LDH-release assays, please refer to their respective technical bulletins from the manufacturer.

References


APPENDIX 4

CELL PASSAGING PROTOCOL

Materials:

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity/Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mL Beaker half filled with 10% bleach</td>
<td>5mL pipette tips</td>
</tr>
<tr>
<td>50mL centrifuge tubes (3)</td>
<td>Trypsin-EDTA (7mL)</td>
</tr>
<tr>
<td>Electric pipettor</td>
<td>PC3 media (20mL + volume for new plates)</td>
</tr>
<tr>
<td>Water and air incubators</td>
<td>Centrifuge</td>
</tr>
<tr>
<td>Invert microscope</td>
<td>Haemocytometer</td>
</tr>
<tr>
<td>Typhan Blue</td>
<td>Eppendorf pipettes (1mL and 200µL) and tips</td>
</tr>
<tr>
<td>New T-75 flask</td>
<td>PC3 in T-75 flask</td>
</tr>
<tr>
<td>Ethanol 70%</td>
<td></td>
</tr>
</tbody>
</table>

Methods:

Aseptic technique is to be followed at all times. Italic portion of the methods have to be done inside a biological safety cabinet

1. Warm PC3 media and trypsin to 37°C in water incubator
2. Wipe down Biological safety cabinet with 70% ethanol.
3. Inspect PC3 in T-75 flask under inverting microscope
4. Remove media from T-75 flask by decanting content into bleach
5. Rinse flask with 2ml trypsin, and decant into bleach.
6. Add 5ml of trypsin to the flask and return to air incubator at 37°C for 5 to 10 minutes to suspend the cells.
7. Decant the cell suspension in trypsin into a 50ml centrifuge tube.
8. Another 5ml of PC3 media was used to rinse out the flask and added to the same 50ml
9. Centrifuge the cell suspension was at 300×g RCF at 25°C for 5 minutes to softly pellet the cells.

10. After centrifugation, remove the supernatant aspiration and re-suspend the cell pellet in 4ml PC3 media.

11. Determine the cell concentration by adding 50μl of the cell suspension to 50μL of Typhan Blue and count on a haemocytometer under a microscope.

12. Re-seed new T-75 flask with 200,000 PC3 and approximately 15ml of PC3 media and allow cells to grow for 7 days until next passage. Change the PC3 media in the flask on the 4th day.

13. Seed new plates with remaining PC3 for experiment if necessary.
APPENDIX 5

PREPARATION OF PC3 MEDIA

Note: This protocol is adopted from Dr. Helen Burt’s lab at the Faculty of Pharmaceutical Science at The University of British Columbia

Materials:

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Code/Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-12 Nutrient Mixture</td>
<td>Invitrogen</td>
<td>21700-075</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Invitrogen</td>
<td>10437-028</td>
</tr>
<tr>
<td>Penicillin/Sterptomycin</td>
<td>Invitrogen</td>
<td>15140-122</td>
</tr>
<tr>
<td>Distilled and de-ionized water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoclaved bottle (1L or equivalent)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnetic stir bar and stir plate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Graduated cylinder (1000mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipettes (10 and 25mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottle top filter (0.22μm)</td>
<td>Sarstedt</td>
<td>83.1823.101</td>
</tr>
<tr>
<td>Bio-safety cabinet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate (Sigma S-5761)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium foil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beaker (2L)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Methods:

Aseptic technique is to be followed at all times. Only italic portions of the methods have to be done inside a biological safety cabinet. These instructions are for preparing 1L of media, adjust accordingly for a different volume.

1. Add 500mL of water and place a magnetic stir bar into a 2L beaker.
2. Empty one package of F-12 nutrient mixture into the beaker.
3. Rinse the package out with water and add the rinsing to the beaker.
4. Add 450mL of water to the beaker
5. Weigh out 1.176g sodium bicarbonate and add to beaker.
6. Cover the beaker with aluminium foil and mix thoroughly
7. *Inside the biological safety cabinet add the following*
- 111mL FBS
- 11ml antibiotics

8. Inside the biological safety cabinet, filter the media into a 1L bottle using the bottle top filter apparatus:
   - attach filter to bottle
   - connect vacuum line to filter
   - turn on vacuum
   - pour media into filter funnel and proceed
   - disconnect vacuum line from filter before removing filter from bottle

9. Label bottle with “PC3 Media”, date, initials of person that prepared the media.

10. Store media at 2-4°C
APPENDIX 6

WATER-BATH DRAWING

Centre holes diameters
1) 1.8 cm
2) 1.2 cm

Prototype transducer fits into the bottom centre hole and hold in place by securing the end piece to the water-bath using 4 cm long screws.
APPENDIX 7

PROCEDURES FOR FITTING DATA FROM FIGURE 3.10
TO EQUATION 3.4

Shortening Cytotoxicity Enhancement to C and rearranging Equation 3.4, we get:

\[ e^{-aE^{2/3}} = 1 - C \]  \hspace{1cm} (A.7.1)

Take natural logarithm of both sides:

\[ -\alpha E^{2/3} = \ln(1 - C) \]  \hspace{1cm} (A.7.2)

therefore, if we plot \( E^{2/3} \) with \( \ln(1-C) \) for the data set collected at each ultrasonic power density and fit the data for a straight line using Microsoft Excel, the slope of each of the straight line fit is the value of \( \alpha \) for the corresponding ultrasonic power density.
APPENDIX 8

DOSE-EFFECT RESPONSE OF DOXORUBICIN BASED ON DRUG-RECEPTOR HYPOTHESIS

The Drug-Receptor Hypothesis predicted that the dose-effect response of a drug to follow the relationship:

\[ E = \frac{E_{\text{max}} c}{c + EC_{50}} \]  
(A.8.1)

Where \( E \) is the Effect (defined as cytotoxicity in this study), \( E_{\text{max}} \) is the maximum effect, \( c \) is the concentration of the drug and \( EC_{50} \) is the concentration required to achieve 50% effect.

Using Lineweaver-Burk technique to analyze Equation A.8.1, we multiply the top and bottom of the fraction on the right by \( 1/c \) and obtain:

\[ E = \frac{E_{\text{max}}}{1 + \frac{EC_{50}}{c}} \]  
(A.8.2)

Rearranging Equation A.8.2 we get:

\[
\frac{1}{E_{\text{max}}} + \frac{EC_{50}}{E_{\text{max}} c} \frac{1}{E} = \frac{1}{E}
\]  
(A.8.3)

From Equation A.8.3, we know that if we plot \( 1/c \) against \( 1/E \), we should obtain a straight line with the y-intercept as \( 1/E_{\text{max}} \) and the slope as \( EC_{50}/E_{\text{max}} \). Using the result from Figure 3.5 (only the 4 to 20\( \mu \)M data were used because the uncertainty at the lower concentrations results in

108
Using regression data from Figure A.8.1 and Equation A.8.3, the values for $E_{\text{max}}$ and $EC_{50}$ were found to be 100% and 9.96μM respectively. Using these values to model the dose-effect response of doxorubicin, we obtain the Figure A.8.2 from which we can conclude that to achieve 80% cytotoxicity, 40μM doxorubicin is needed. In order to achieve 95% cytotoxicity, 171μM doxorubicin must be used.
Figure A.8.1 Lineweaver-Burk plot of dose-effect response of doxorubicin from Figure 3.5. Data expressed as mean. Regression analysis: $y = 9.96x + 1.00$  \( R^2 = 1.00 \)
Figure A.8.2 Model (◊) and experiment (■) data of dose-effect response of doxorubicin in PC3 cells. The PC3 cells were exposed to the treatment for 3 hrs and then allowed to proliferate in fresh media for an additional 3 days before cytotoxicity was measured using the MTS assay. Data expressed as mean ± 1 standard deviation.