SONOLUMINESCENCE AS AN INDICATOR
OF CELL MEMBRANE DISRUPTION
BY ACOUSTIC CAVITATION

A Thesis
Presented to
The Academic Faculty

by

Stephen Andrew Cochran

In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Bioengineering

Georgia Institute of Technology
November 2000
SONOLUMINESCENCE AS AN INDICATOR
OF CELL MEMBRANE DISRUPTION
BY ACOUSTIC CAVITATION

Approved: __________________

Mark R. Prausnitz, Chairman

William D. Hunt

Feng B. Dong

Date Approved 11/20/00
DEDICATION

To the creator, God of the universe, personal, and unchanging,

to my parents and sisters, and

to my wife.
ACKNOWLEDGEMENTS

There are many whom I would like to acknowledge and so I will inevitably leave someone off of this list but let me begin by thanking Hector Guzmán, Thomas Lewis, Robyn Schlicher, Esi Gharney-Tagoe, Paul Canatella and Steve Woodard for technical assistance and helpful discussions. I also thank many of the others in lab who helped to make my stay there an enjoyable one. I would like to especially thank my thesis advisor, Dr. Mark Prausnitz, who found funding for me and brought me into his lab. He was always available for quick questions and strongly encouraged me to think independently. In times when I was confused about which direction to take, he was there to guide me in which experiments to conduct or which analysis method was better. I really appreciate all that he did for me. The time that I spent here enriched me not only professionally but personally as well.

I also thank the following organizations for their support of this work: the National Science Foundation, the Whitaker Foundation and the National Institutes of Health (through a sub-contract from EKOS Corporation).
TABLE OF CONTENTS

DEDICATION .............................................................................................................. ii
ACKNOWLEDGEMENTS ............................................................................................ iii
LISTS OF TABLES ........................................................................................................ vi
LISTS OF FIGURES ....................................................................................................... vii
SUMMARY .................................................................................................................... viii

CHAPTER I – INTRODUCTION ................................................................. 1

1.1 Introduction ............................................................................................................. 1
1.2 Objectives ................................................................................................................. 3
1.3 Overview of the Thesis ............................................................................................ 3

CHAPTER II – BACKGROUND AND THEORY ........................................... 4

2.1 Introduction and Overview ..................................................................................... 4
2.2 Acoustic Waves ...................................................................................................... 4
2.3 Cavitation and Sonoluminescence .......................................................................... 5
2.4 Drug Delivery using Acoustic Cavitation ............................................................... 7
2.5 Relevance of Work ................................................................................................. 8

CHAPTER III – MATERIALS AND METHODS ........................................... 9

3.1 Introduction and Overview ..................................................................................... 9
3.2 Ultrasound apparatus ............................................................................................ 11
3.3 Verification of ultrasound apparatus ..................................................................... 12
3.4 Preparing Cell Samples ........................................................................................ 12
3.5 Flow Cytometry Assay ........................................................................................ 15
3.6 Data Analysis ........................................................................................................ 16
3.7 Analysis of Three Populations ............................................................................. 17
3.8 Sonoluminescence Analysis ................................................................................ 20

CHAPTER IV – RESULTS AND DISCUSSION ......................................... 22

4.1 Introduction ............................................................................................................ 22
4.2 Heterogeneous molecular uptake ........................................................................ 22
4.3 Effects of ultrasound parameters on bioeffects
  4.3.1 Effects of intensity on bioeffects
  4.3.2 Effects of total exposure time on bioeffects
  4.3.3 Effects of pulse length on bioeffects
  4.4 Correlation of sonoluminescence with bioeffects
  4.5 Discussion

CHAPTER V - CONCLUSIONS AND RECOMMENDATIONS

  5.1 Conclusions
  5.2 Recommendations

APPENDICES

  Appendix A - Figure 6 graphs shown in greater detail
  Appendix B - Figure 6 graphs shown in greater detail

REFERENCES
LISTS OF TABLES

Table 1. Ultrasound transducer specifications........................................9
LISTS OF FIGURES

Figure 1. Diagram of apparatus......................................................... 9
Figure 2. Results of apparatus validation experiments.......................... 13
Figure 3. Chemical structure of the calcein molecule........................... 15
Figure 4. Series of representative histograms...................................... 18
Figure 5. Typical light intensity waveforms........................................ 21
Figure 6. Cell viability and distribution of cells.................................... 25
Figure 7. Data from Figure 6 are re-plotted.......................................... 26
Figure 8. Uptake and cell viability shown as functions of sonoluminescence... 30
Figure 9. Cell viability correlations with other sonoluminescent parameters... 32
SUMMARY

Ultrasound has been shown to transiently disrupt cell membranes and thereby load drugs and genes into viable cells. In this study, we quantified the number of calcein molecules delivered and the loss of viability in prostate cancer cells exposed to 24 kHz ultrasound over a range of different pulse lengths (1-100 ms), total exposure times (0.1-10 s) and pressures (1.0-9.8 atm). Bioeffects were observed to increase with pulse length, total exposure time and pressure. Using this large data set, we established a correlation between bioeffects and the amount of light produced by sonoluminescence. These results support a cavitation-based mechanism for bioeffects and suggest a means to control ultrasound’s effects on cells using sonoluminescence-based feedback.
CHAPTER I

INTRODUCTION

1.1 Introduction

One of the critical elements of drug therapy is effective and targeted delivery of drugs into cells and tissue (Park 1997; Langer 1998). The lipid bilayer cell membrane poses the primary barrier to transport of molecules into cells (e.g., gene therapy (Rolland 1998)), into tissues (e.g., targeted chemotherapy of tumors (Mir and Orlowski 1999)) and across tissues (e.g., transdermal drug delivery (Bronaugh and Maibach 1999)). Some approaches to increase drug transport have used chemical methods, whereby physicochemical properties of drug molecules are modified to promote their diffusion across membranes or to facilitate binding to receptors expressed, for example, preferentially on cancer cells (Silverman 1992). Another approach to enhancing and targeting drug delivery involves using ultrasound to transiently disrupt cell membranes (Fechheimer et al. 1987; Mitragotri et al. 1995; Liu et al. 1998). The advantage of this method is that as a physical, rather than chemical, approach, the enhancement is likely to be broadly applicable (i.e., can be applied to many cell types and many drugs, without modifying drug structure) and can be readily targeted, based on the established ability of ultrasound to be focused almost anywhere in the body (Kremkau 1998).
A number of studies have shown that ultrasound can disrupt cell membranes in a reversible manner and thereby load viable cells with molecules of interest. These studies have demonstrated delivery of small molecules, such as drugs (Saad et al. 1992; Mitragotri et al. 1995), as well as DNA for transfection (Fechheimer et al. 1987; Bao et al. 1997; Wyber et al. 1997). This "proof-of-principle" that ultrasound can be used for drug and gene delivery motivates our study, in which we strive to better elucidate ultrasound's mechanism of action and develop methods to predict and control ultrasound's effects on cells.

In this study, we sought to bridge the gap between simply seeing the bioeffects and being able to control them. Our approach to controlling ultrasound's biological effects is based on achieving feedback in the form of real-time physical measurements. Using a similar approach, Liu et al. (1998) correlated observed bioeffects (red blood cell disruption) with measured features of the acoustic frequency spectrum known to be associated with cavitation, i.e., subharmonic frequency (f/2). This correlation could be used to determine the effects a given exposure of ultrasound had on cells based only on a relatively simple measurement of the acoustic spectrum. Using a related approach, Wyber et al. (1997) correlated ultrasound's bioeffects (cell viability) with an iodine-based measurement of cavitation. Although assay of this technique is more time intensive and unlikely to be used in vivo, it further validates the idea of using physical measures of cavitation as correlates for ultrasound's bioeffects. In this study, we seek to correlate observed bioeffects (molecular uptake and cell viability) with another physical phenomenon associated with cavitation: sonoluminescence.
1.2 Objectives

The objectives of this study were threefold:

1) study the effects of different ultrasound pressure, total exposure time, and pulse length on molecular uptake and cell viability,

2) establish a relationship between ultrasound’s effect on cell viability and molecular uptake with a known acoustic cavitation phenomenon, sonoluminescence and

3) identify regions of high molecular uptake and high viability.

1.3 Overview of the Thesis

This chapter provides an introduction to the thesis. Chapter 2 provides the background and theories behind acoustic cavitation as well as the relevance of this study. In chapter 3, the materials and methods that were used in this study are described in detail. Results and discussion then follow in chapter 4. Lastly, in chapter 5, conclusions and recommendations for future studies are provided.
CHAPTER II

BACKGROUND AND THEORY

2.1 Introduction and overview

The cell membrane poses a difficult barrier across which to deliver drugs into cells or tissues for targeted drug therapy. There is a need for new and innovative technology that is able to transiently disrupt the cell membrane in a controllable fashion. Ultrasound-induced cavitation has been shown to disrupt cell membranes and provides an attractive solution if its effects can be controlled. It is believed that there exist methods to control these effects by monitoring physical measures of acoustic cavitation. In this study, we seek to find a correlation between sonoluminescence, which is closely associated with acoustic cavitation, and molecular uptake and cell viability. In order to build a foundation for this study, some background must be given on acoustics, acoustic cavitation and sonoluminescence.

2.2 Acoustic Waves

Acoustic waves are mechanical energy waves that create changes in the density of the medium as they travel through it. These waves are considered longitudinal waves because particles are displaced parallel or anti-parallel to the plane of motion. Over the cycle of an acoustic wave there exists a compression stage, where the particles are
pressed together, and a rarefaction stage, where the particles are spread apart. A more
detailed explanation of the physical phenomenon that occurs with the passage of an
acoustic wave can be found, for example, in Pierce (1989).

Acoustic waves can be classified according to the frequency at which these
compressions and rarefactions occur. In this study, the waves oscillate at a frequency of
24 kHz and are therefore considered to be ultrasonic, i.e. above the normal threshold of
human hearing, 20 kHz (Pierce 1989). There are other ways to describe acoustic waves
and some of the terms used to characterize them are: frequency, pulse length, incident
pressure, total exposure time, and duty cycle.

Terms Defined

- Frequency – number of cycles in a given time period.
- Pulse length - duration of one pulse of acoustic energy.
- Total exposure time - the pulse length multiplied by the total number of pulses
during an exposure.
- Incident pressure – the peak pressure of an acoustic wave in the absence of
acoustic cavitation.
- Duty cycle - the ratio of the time that the transducer generates acoustic waves to
the total duration of the exposure.

2.3 Cavitation and Sonoluminescence

Cavitation involves the creation of gas bubbles in a liquid medium and is a
phenomenon that occurs in many places (Munson et al., 1994). For example, people who
design propellers for boats have to understand how the propeller will cut through the
water and if it will induce regions of cavitation. In other applications, people who design artificial heart valves have to consider the pressures their valve generates in blood flow and if it will induce cavitation as well. The term, cavitation, has been used to describe the creation as well as the dynamics of bubbles. Cavitation occurs when liquid that was at high pressure enters a region of low pressure, resulting in the “tearing” apart of the liquid and formation of a cavity or bubble. When this bubble reenters a region of high pressure, it can result in the violent collapse of the bubble. This violent phenomenon can lead to pitting of metal propellers and heart valves.

Cavitation that is acoustically driven has been named acoustic cavitation. Acoustic cavitation harnesses the mechanical energy inherent in acoustic waves to generate, grow, and subsequently collapse bubbles in liquid media. The size of the bubbles generated in liquid media is inversely related to the driving frequency. One of the interesting aspects of acoustic cavitation is that acoustic waves can be focused and thereby target areas where cavitation activity would be useful. Focusing could play an important role in situations where only a small region of the body should be affected while leaving other tissues unharmed.

Acoustic cavitation is believed to be the mechanism by which cell membranes are ruptured by ultrasound. The violent bubble collapse that occurs during cavitation creates extremely high local temperatures (thousands of degrees Kelvin) and pressures (hundreds to thousands of atmospheres), high-velocity jets of fluid, and free radicals (Leighton 1994, Apfel 1997). Light can also be generated as a result of the extremely high temperatures and pressures, a phenomenon called sonoluminescence. We believe that
these phenomena directly or indirectly lead to cell membrane disruption. It is important to point out that the resonant bubbles created in water at 24 kHz have been shown to be ~150 microns (Leighton, 1994), which is approximately 10 times the size of the cells we utilized. During our experiments, we measured changes in molecular uptake and cell viability induced by a broad range of ultrasound conditions. We also measured the amount of sonoluminescence light generated. Our hypothesis is that since cavitation is believed to cause the observed bioeffects, then a measure of cavitation – i.e., sonoluminescence – should correlate with these bioeffects over the many ultrasound conditions examined.

2.4 Drug Delivery Using Acoustic Cavitation

Given the violent nature of acoustic cavitation, it can be deduced that there are several ways that acoustic cavitation could reversibly or irreversibly damage cell membranes. A cell in close proximity to one of these violent collapses could be damaged in a variety of ways. Some cells might avoid damage, others might be reversibly damaged and still others might be fatally damaged. As will be explained in the Results section, this range of damage is often what is seen among cells exposed to ultrasound.

In this study, we do not attempt to identify which of the physical events caused by cavitation leads to the bioeffects that we see. Instead, we are attempting to correlate the observed bioeffects with quantitative measurements of acoustic cavitation. With a quantitative measurement of acoustic cavitation, its effects can be monitored and thereby controlled independent of acoustic parameters. In this study, we monitored two effects of acoustic cavitation; its ability to kill cells and its ability to deliver a model drug, calcein,
to living cells. Depending on the acoustic conditions utilized, these occurrences are observed in varying proportions.

2.5 Relevance of Work

A critical problem in pharmaceutical research is the desire to deliver molecules into cells more efficiently. Acoustic cavitation is the mechanism that we propose to use to transiently disrupt cell membranes and thereby accomplish this goal. Ultrasound-induced cavitation is especially attractive because of its ability to target local regions in the body. For example, tumors could be targeted and made more permeable to chemotherapeutic agents utilizing ultrasound. In this study, we sought to increase the utility of ultrasound-induced cavitation by developing a method to control the effects caused by acoustic cavitation. To that end, we monitored one of the physical measures of acoustic cavitation, sonoluminescence. By recording the sonoluminescent light output as we monitored bioeffects and by finding a relationship between the two, we achieved a feedback mechanism with which to monitor and control molecular uptake and cell viability.
CHAPTER III

MATERIALS AND METHODS

3.1 Introduction and Overview

To determine the effects of acoustic cavitation on molecular uptake and cell viability, and to correlate those effects with sonoluminescence, cavitation was induced in cell suspensions using a cylindrical piezoelectric transducer (Table 1) and sonoluminescent light output was measured using a photo-multiplier tube (PMT) (Figure 1). The exposure chamber was similar to that described by Liu et al. (1998) with the addition of a lens and PMT (R5600U Series, Hamamatsu, Bridgewater, NJ) attached to the bottom of the chamber. The exposure chamber was filled with 200 mL deionized water that was degassed for 3 hr at -650 mmHg gage pressure using a vacuum chamber (Nalgene, Rochester, NY) and pump (KNF Neuberger, Trenton, NJ). Degassing the water bath removes nucleation bubble sites and therefore limits the majority of cavitation, and hence sonoluminescent light output, to within the cell sample.

Table 1. Ultrasound transducer specifications

<table>
<thead>
<tr>
<th>Materials</th>
<th>Lead Zirconate Titanate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outer Diameter</td>
<td>2.0 inches</td>
</tr>
<tr>
<td>Wall Thickness</td>
<td>0.125 inches</td>
</tr>
<tr>
<td>Length</td>
<td>1.0 inches</td>
</tr>
<tr>
<td>Piezoelectric Constant</td>
<td>-107 x 10^{12} m/V</td>
</tr>
</tbody>
</table>
Figure 1. Diagram of apparatus, which applies ultrasound to cell suspensions and simultaneously measures sonoluminescence light production. A cell suspension contained in a plastic sample chamber is placed within a water bath containing a cylindrical transducer that is controlled by a function generator and amplifier to produce ultrasound. At the base of the exposure chamber is a photo-multiplier tube (PMT) which is used to measure light produced by sonoluminescence.
3.2 Ultrasound Apparatus

The ultrasound generation system consisted of a function generator (DS345 SRI, Stanford Research Systems, Sunnyvale, CA), ultrasonic amplifier (Macro-Tech 2400, Crown Audio, Elkhart, IN), matching transformer (MT-56R, Krohn-Hite, Avon, MA), and transducer (24kHz, Channel Industries, Santa Barbara, CA). This system controlled the frequency, duty cycle, incident pressure, and pulse length. The total exposure time was controlled manually by turning the system on and off.

The data acquisition system was composed of a digital oscilloscope (54603B, Hewlett Packard, Palo Alto, CA) equipped with a HPIB interface module (54650A) allowing the voltage applied to the transducer and the voltage produced by the PMT to be downloaded to a PC and stored for analysis. The voltage applied to the transducer was measured and converted to an incident pressure using the method of Liu et al (1998). This approach determines the voltage-pressure correlation under conditions where there is no cavitation and then linearly extrapolates this correlation out to higher pressures. This approximation will be an overestimate of the actual pressure but provides a better measure of the total acoustic input. According to this analysis, the following correlation was established: \( P = 0.0083 \, V \), where \( P \) is incident pressure (atm) and \( V \) is voltage (V) applied to the transducer. To be sure this correlation was developed in the absence of any cavitation, the acoustic spectrum was monitored using a FFT spectrum analyzer (SR 670, Stanford Research Systems) and incident pressure measurements were only used if there was no peak present at the subharmonic frequency \( (f/2) \).
3.3 Verification of Ultrasound Apparatus

In order to verify that the light we gathered during the ultrasound exposure was coming from the cell sample and not from the water bath, we performed a series of experiments. The first set of experiments performed included setting up the apparatus exactly like we would for any other experiment, the only difference being that the water was not degassed and there was no cell sample. We then ran the apparatus through the different ultrasound conditions and recorded the light output, as shown in Figure 2.

The second set of experiments was similar to the first except this time we degassed the water bath thereby reducing the number of cavitation nucleation sites (again no cell sample used). Again we tested the range of different ultrasound conditions and from these two sets of experiments, we concluded that the light generated was indeed coming from the cell sample and not from the water bath itself.

3.4 Preparing Cell Samples

DU 145 prostate cancer cells (American Type Culture Collection, Rockville, MD) were grown and harvested using protocols described below.

Cell Harvest Protocol

Harvesting a 150 cm² flask (approximately 12 million cells):

1) Growth media over the cell monolayer was removed using a pipette.

2) Monolayer was washed with 15 mL PBS (Cellgro) without Ca²⁺ / Mg²⁺ for 1-2 minutes and then removed using a pipette.
Figure 2. Results of apparatus validation experiments showing the ability to distinguish the light output from the sample and the light output from the water bath. The integrated light output from a sample was five times or more the light output generated from the degassed water bath without a sample.
3) 5 mL of trypsin were then added to the flask and the flask’s cap was replaced and tightened down. The flask was gently shaken to ensure full coverage of the monolayer by the trypsin. The flask was then placed in the incubator (Model 3110, Forma Scientific, 37°C) for 2 minutes.

4) The flask was then removed from the incubator. The trypsin was known to have successfully dislodged the cells when the monolayer could be seen to slide off the flask wall. The flask was tapped gently to ensure that all cells were suspended in the trypsin. 30 mL of full growth media were added to the cell suspension to deactivate the trypsin.

After harvest, the cells were spun down (1000 x g, Model BS-15R, Beckman centrifuge) and resuspended in growth media. The cell concentration was determined using a cell counter (Coulter Multisizer II) and then diluted to a final concentration of 750,000 cells/mL. The sample chambers (Sedi-Pet transfer pipette, 241, SAMCO, San Fernando, CA; stems cut to 2 cm in length) each contained 1.2 mL of a well-mixed suspension of 750,000 cells/mL in a 10 μM solution of calcein (623 Da, r = 0.6 nm, Molecular Probes, Eugene, OR). Calcein (Figure 3) was used as a model drug because it is of similar size to many drugs in common use and is unable to readily penetrate the cell membrane. The suspension was introduced into the chambers using a 22-gauge needle and 3 mL syringe (Becton Dickinson, Franklin Lakes, NJ). A meniscus was left at the top of the pipette stem and a 1/16” stainless steel rod was inserted 2 mm into the stem. Overflow of solution was allowed to ensure no visible air bubbles were trapped in the sample.
The sample was positioned in the cylindrical water bath exposure chamber so that the center of the sample was in the radial and axial center of the transducer. Pulsed ultrasound at 24 kHz and 10% duty cycle was applied to the cell sample using combinations of 3 pulse lengths (1-100 ms), 3 total exposure times (0.1-10 s), and 10 different pressures (1.0-9.8 atm). After ultrasound application, the cells remained at room temperature for approximately 5 min to allow cell recovery. Cell samples were then washed 3 times by centrifugation (735 x g, 3 min, Eppendorf 5415 C, Brinkmann Instruments, Westbury, NY), resuspended in Dulbecco’s phosphate-buffered saline (Cellgro, Herndon, VA), and kept on ice until analysis the same day.

3.5 Flow Cytometry Assay

The samples were assayed using flow cytometry to quantify cell viability and the number of calcein molecules taken up by each cell. To assess viability, we added to each cell sample 5 μL of 10 mg/mL propidium iodide (Molecular Probes) to stain non-viable
cells. We also added $10^5$/mL green fluorescent latex microspheres (d = 2.5 μm, Molecular Probes, 488/515, lot #5881-2), which were used as an internal volumetric standard to determine the cell concentration after ultrasound application, as described by Prausnitz et al. (1994).

A FACScan flow cytometer using Cellquest software (BD Immunocytometry Systems, San Jose, CA) was utilized to measure green calcein fluorescence associated with molecular uptake and red propidium iodide fluorescence associated with non-viable cells. At least 10,000 viable cells were collected from each sample. The samples were excited using a 488 nm argon laser. The fluorescence of propidium iodide (675/20 nm bandpass filter for emission collection) was used to distinguish between viable and non-viable cells. Viable cells were analyzed to determine their mean fluorescence intensity of calcein (530/30 nm bandpass filter). Fluorescence intensity was then converted to an average number of calcein molecules per cell using calibration beads (Quantum 25, Flow Cytometry Standards Corporation, Fishers, IN, d = 7.2 μm, lot #B00136) with known fluorescence intensity (Prausnitz et al. 1994).

3.6 Data Analysis

As described previously (Guzmán et al. submitted), the fluorescence intensity of a population of cells can be represented by a histogram (Figure 4). These histograms from flow cytometry data were initially analyzed with WinMDI 2.8 (Windows Multiple Documents Interface, Joseph Trotter, Scripps Research Institute, La Jolla, CA) to determine the fraction of cells that were viable and, among the viable cells, the number of molecules per cell. Then, molecular uptake data were imported to a Microsoft Excel
(version 9.0) spreadsheet using Ldata (Robinson and Kelly, 1998), a numerical conversion formula; reformatted; and finally imported to the statistical software package, MiX 3.1 (Ichthus Data Systems, Hamilton, Ontario, Canada) where histograms of molecular uptake were fitted as three normal distributions.

3.7 Analysis of Three Populations

As described in the Results section, histograms were best described as complex distributions containing up to three sub-populations. In order to determine the relative percentages of cells in the three sub-populations, we first had to decide how many populations were present. As shown in Figure 4 A, a single population sometimes adequately describes histograms. In other cases, two sub-populations are evident: one at the same position as the single population and another over a broad distribution at higher levels of fluorescence (Figure 4 B). Finally, three sub-populations are sometimes needed to describe the data, which include the previously mentioned two distributions, with a third population at even higher fluorescence (Figure 4 C-E).

Although one could visually identify when multiple sub-populations were present, we wanted a uniform, quantitative method to determine the number of sub-populations present. First, we classified control (no ultrasound) samples as having a single population and identified the value below, which the fluorescence of 97% of cells fell. This value was considered the maximum fluorescence of control (i.e., the “cut-off”). Excluding the brightest 3% of “events” in flow cytometry is often done to discard artifacts (Canatella et al., submitted).
Figure 4. (Caption on next page)
Figure 4. Series of representative histograms showing calcein uptake by viable cells at increasing ultrasound pressures. (A) A control population of cells shows weak background fluorescence. (B) Cells exposed to ultrasound at 1.9 atm show a large sub-population with essentially no effect and smaller group of cells with higher fluorescence, indicating uptake of calcein. (C-E) Ultrasound applied at increasing pressure (2.9, 3.9 and 4.9 atm) causes increasingly more cells to take up calcein molecules. Because these highly non-Gaussian distributions cannot be characterized by a single average, we separated each population into nominal uptake (NUP), low uptake (LUP) and high uptake (HUP) sub-populations by fitting three Gaussian distributions, as illustrated in (C) (Guzmán et al. submitted). Ultrasound exposures were performed for 2 sec total exposure time and 10 ms pulse length.
For samples exposed to ultrasound, we determined the fraction of cells above the "cut-off" fluorescence. If this value was less than 5%, then that sample was classified as one population (i.e., no different from control); if it was greater than 5% but less than 15% then it was classified as two populations (unless we could see from the uptake distribution that it was clearly three populations); and lastly if cells with fluorescence above the "cut-off" represented 15% or more of the cells then we classified that sample as having three populations. The selection of 5% and 15% criteria is somewhat arbitrary. In the absence of a more rigorous way to determine the number of sub-populations, we selected these criteria because they classified the samples in a manner consistent with our visual classification. Once we had classified all of the sample histograms, then we could properly analyze them with the MiX 3.1 software, which cannot determine how many sub-populations are present but will find the distribution among a known number of sub-populations.

3.8 Sonoluminescence Analysis

Light output gathered by the digital oscilloscope (Hewlett Packard, 54603B) was downloaded to a computer (Ginstar Computer, Norcross, GA) for analysis. Light output generated during all ultrasound pulses applied to a given cell sample were combined to yield an average light-vs.-time trace for each sample. Although the light data had different shapes, in Figure 5 we have displayed some representative waveforms.
Figure 5. Typical light intensity waveforms showing that for the same conditions the light output waveform varied. We sought to determine if ultrasound's effects on cells could be correlated with integrated light output (i.e., total area under the curve), light output above a threshold (i.e., area under the curve above a threshold level) or peak light output (i.e., light intensity at its peak value).
CHAPTER IV

RESULTS AND DISCUSSION

4.1 Introduction

This study examined the effects of pressure, pulse length, and total exposure time on calcein uptake and cell viability, and sought to correlate those effects with sonoluminescence. Prostate cancer cells were exposed to 24 kHz ultrasound and then analyzed using flow cytometry to determine the number of calcein molecules taken up into the cytosol and the loss of cell viability.

4.2 Heterogeneous molecular uptake

The number of calcein molecules taken up by cells showed significant heterogeneity. Figure 4 displayed a series of uptake histograms in which acoustic pressure was varied, while pulse length and total exposure time were held constant. Each histogram shows the distribution of molecular uptake among a population of 10,000 viable cells. The control sample (Figure 4 A) shows a single peak with weak background fluorescence probably due to autofluorescence, membrane-bound calcein, and optical and electrical noise. Histograms of sonicated samples (Figures 4B-E) show broad distributions of fluorescence, which indicates heterogeneous amounts of calcein uptake within single populations of cells. Although the distribution shape depended on pressure,
pulse length, and total exposure time (see below), the existence of a low-fluorescence peak, a high-fluorescence peak and a broad valley in between was seen in most samples (e.g., Figure 4).

As described previously (Guzmán et al. submitted), to facilitate analysis we have divided each uptake histogram into three sub-populations: nominal uptake population (NUP), which corresponds to the low-fluorescence peak interpreted as indistinguishable from controls; low uptake population (LUP), which corresponds to the broad valley between the peaks interpreted as cells with varying amounts of uptake; and high uptake population (HUP), which corresponds to the high-fluorescence peak interpreted as cells with the maximum possible uptake (i.e., achieved equilibrium with extracellular environment).

Although the relative sizes of these sub-populations depended strongly on ultrasound conditions, the number of molecules taken up within each sub-population remained the same order of magnitude. Graphically, this means that the heights of the peaks changed, but their positions did not vary much. For all of the ultrasound conditions examined in this study, the average number of molecules taken up by NUP cells was $3.53 \times 10^4 \pm 4.08 \times 10^4$, LUP cells was $2.55 \times 10^6 \pm 4.74 \times 10^5$, HUP cells was $8.70 \times 10^6 \pm 8.77 \times 10^5$ (average ± standard error of the mean). Based on a cell volume of $2 \times 10^{-9}$ ml per cell (data not shown), the average calcein concentration within HUP cells was 7 μM. Because calcein was supplied extracellularly at 10 μM, this supports the interpretation that HUP cell uptake approaches equilibrium.
4.3 Effects of acoustic parameters on molecular uptake and cell viability

The effects of pressure, pulse length, and total exposure time on molecular uptake and cell viability are shown in Figure 6. The overall height of each bar represents cell viability, while the three stripes within each bar represents the fraction of cells in NUP (gray), LUP (white) and HUP (black). Each graph shows the effect of pressure, while each row corresponds to a different pulse length and each column to a different total exposure time.

4.3.1 Effects of pressure on bioeffects

These graphs show that as pressure increases, cell viability generally decreases. Similarly, as pressure increases, NUP cells also decrease in number. However, the fraction of LUP and HUP cells (i.e., cells with significant levels of uptake) does not change much as a function of pressure, above an apparent threshold of approximately 1.0 atm. Except when cell viability is extremely low, the sum of LUP and HUP cells in each graph is relatively constant.

4.3.2 Effects of total exposure time on bioeffects

Figure 7 replots the data shown in Figure 6 to better display the effects of total exposure time and pulse length. In each graph, pressure is held constant and the effects of three different pulse lengths are examined at three different total exposure times. Scanning across each graph shows that as total exposure time increases, cell viability generally decreases.
Figure 6. Cell viability and distribution of cells among NUP (gray), LUP (white) and HUP (black) sub-populations following exposure to ultrasound as a function of pressure, total exposure time and pulse length. The height of each bar represents total cell viability, whereas the stripes show the distribution of cells among the three sub-populations. Each column shows results at the same total exposure time (TET) and each row is at the same pulse length. Data represent averages of at least three replicates with standard errors of the mean shown. (Graphs can be seen in greater detail in Appendix)
Figure 7. (Caption on next page)
**Figure 7.** Data from Figure 6 are re-plotted to better show dependence on total exposure time and pulse length. Each graph provides results at a different pressure. Within each graph, three total exposure times (TET) are considered, each at three different pulse lengths. (Graphs can be seen in greater detail in Appendix)
4.3.3 Effects of pulse length on bioeffects

Looking within each triplet of bars indicates that pulses of 1 ms duration often cause weaker effects than those of 10 ms or 100 ms duration.

4.4 Correlation of sonoluminescence with bioeffects

Although the data presented in Figures 6 and 7 characterize the effects of ultrasound parameters on cells, we wanted to determine if there was a single measurable parameter that would correlate with uptake and viability for all of the ultrasound conditions tested. The parameter we selected was sonoluminescence, or the light generated by cavitation. Our hypothesis was that since cavitation is believed to cause the observed effects on cells, then a measure of cavitation – i.e., sonoluminescence – might correlate with these bioeffects over the broad range of ultrasound conditions used in this study.

To test this hypothesis, we have replotted the uptake and viability data not as a function of ultrasound parameters, as shown in Figures 6 and 7, but instead as a function of total integrated light output produced by sonoluminescence as measured using a photomultiplier tube mounted at the base of the ultrasound exposure chamber. Figure 8 shows that molecular uptake, viability and the fraction of cells in NUP, LUP, and HUP all correlate with sonoluminescence. The many graphs shown in Figures 6 and 7 can be reduced to the set of single curves shown in Figure 8. As mentioned above, molecular uptake in each of the sub-populations shows scatter, but is generally of the same order of magnitude within each sub-population (Figure 8 A). Figures 8 B – 8 E indicate an apparent threshold at an integrated light output near $10^{-2}$ Vxsec, above which, cell
viability drops steeply. However, at lower levels of integrated light output, viability remains high and a significant fraction of cells (e.g., 20 - 30 %) are in the LUP or HUP sub-populations.

Effects on cells are seen to correlate with total integrated light output, which is defined as the average integrated light output during a pulse (e.g., the integral of one of the curves shown in Figure 5) multiplied by the total number of pulses applied during the given exposure. We also looked for correlations with other features of the measured sonoluminescence, but none correlated as well as integrated light output. For example, we tried to correlate bioeffects with the peak level of light output (see Figure 5), since the creation of bioeffects may be a catastrophic event that only requires achieving some peak level of cavitation, even for just an instant. However, the data were extremely scattered using this approach (Figure 9 A), suggesting that this interpretation was incorrect. Similarly, correlation of peak light output multiplied by the number of pulses (i.e., the number of times the peak was achieved) also yielded a poor correlation (Figure 9 B).

Another approach was to correlate bioeffects with sonoluminescence only above a threshold, since weak cavitation that created low levels of sonoluminescence might not produce bioeffects. However, correlation of bioeffects with light output integrated above a number of different thresholds (see Figure 5) yielded progressively better correlations as the threshold was lowered (Figure 9 C), but the best correlation remained when the threshold was zero (i.e., total integrated light output shown in Figure 8). This suggests that any cavitation capable of generating sonoluminescence was sufficiently “strong” to induce effects on cells.
Figure 8. (Caption on next page)
Figure 8. Uptake and cell viability shown as functions of sonoluminescence light output. (A) Calcein molecules per cell in each of the three sub-populations are relatively independent of light output. (B) Cell viability decreases sharply above a threshold level of light output (e.g., $1 \times 10^{-2}$ Vxsec). (C – E) Percent cells in NUP, LUP and HUP is relatively constant and then decreases sharply above a threshold level of light output (e.g., $1 \times 10^{-2}$ Vxsec). All of the data from Figures 6 and 7 collected over a broad range of ultrasound conditions are re-plotted here and shown to correlate well with sonoluminescence light output.
Figure 9. Cell viability correlations with other sonoluminescent parameters show a more scattered relationship. (A) Correlation with peak light output, (B) Correlation with peak light output times the number of pulses, (C) Correlation with integrated light output above a threshold of 10 mV.
4.5 Discussion

The most significant observation from this study is the overarching correlation between cavitation-induced sonoluminescence and ultrasonic bioeffects. We believe that this is the first time such a correlation has been demonstrated. In a related study, Liu et al. (1998) showed that disruption of red blood cells correlated with another measure of cavitation – the subharmonic pressure measured at one-half the applied frequency. Combined, these and other observations suggest that cavitation is the mechanism by which ultrasound disrupts cells.

The observed correlations also suggest possible applications for drug and gene delivery, where sonoluminescence or subharmonic pressure might be used to provide feedback about the cavitation and its associated bioeffects generated by a given ultrasound exposure. For example, a tissue in the body or solution of cells in the lab could be exposed to ultrasound while simultaneously monitoring sonoluminescent output. Once the total integrated light output reached a desired level (e.g., $10^3$ V·sec for the system used here), sonication could be stopped. In this way, a constant dose of cavitation (as measured by sonoluminescence), rather than a constant dose of ultrasound, can be applied and thereby potentially achieve more reproducible bioeffects.

A notable observation from this study is that there was a broad distribution of cells with different amounts of uptake characterized by two peaks and a broad valley in between. This type of distribution has been observed before using a similar protocol, but involving ultrasound applied at 500 kHz (Guzmán et al. submitted), which leads us to believe that this heterogeneity in uptake may be a general feature of ultrasound-disrupted
cells. It is not currently clear whether heterogeneity is due to cell-based differences in their response to ultrasound or physically-based differences due to non-uniformities in ultrasound-induced cavitation. In either scenario, one can imagine some cells being damaged to the point of cell death, some that are extensively disrupted but nevertheless survive (HUP), some with fewer disruptions (LUP), and some with essentially no disruptions at all (NUP). More work is needed to provide a fuller mechanistic interpretation of the observed heterogeneity and its implications for drug and gene delivery applications.

This study also presented data on the dependence of uptake and viability on ultrasound parameters. Generally, greater pressures and longer total exposure times yielded greater effects on the cells, which is similar to previous observations (Liu et al. 1998; Bao et al. 1997; Miller et al. 1996; Miller et al. 1999). This result is consistent with a cavitation-based mechanism, since cavitation generally increases in intensity with increasing pressure and increases in duration with longer exposure times (Leighton 1994).

We also observed how pulse length influenced bioeffects: 1 ms pulses had significantly less effect than 10 or 100 ms pulses. We hypothesize that 1 ms pulses, which generate only 24 acoustic cycles, may not be sufficiently long to nucleate, grow and collapse cavitation bubbles powerful enough to disrupt cell membranes. However, 10 ms pulses, which produce 240 cycles per pulse, may be long enough to generate sufficient inertial cavitation. Others have similarly observed a dependence on pulse length for disruption of red blood cells (Ciaravino et al. 1981; Kober et al. 1989; Liu et al.
1998). In contrast, Guzmán et al. (submitted) did not observe a dependence on pulse length for prostate cancer cells exposed to 500 kHz ultrasound for pulse lengths between 20 μs (10 cycles) and 60 ms (30,000 cycles). This apparent inconsistency may be explained by the presence of stabilized gas bubbles (i.e., Optison) in the work of Guzmán et al., which served as nucleation sites and thereby facilitated more rapid onset of cavitation (Flynn and Church 1988).
5.1 Conclusions

The main obstacle to delivering therapeutic drugs to cells is the cell membrane. In this study, we sought to overcome that barrier by using mechanical energy in the form of ultrasonic waves. The primary impetus of this study was to evaluate the effects of ultrasound parameters on cell viability and molecular uptake and to establish a relationship between acoustic cavitation and these effects. The acoustic cavitation phenomenon that we sought a relationship with is sonoluminescence, which is the burst of light that is given off when bubbles in solution collapse violently.

To meet our first objective of quantifying the effects of ultrasound parameters, we made the following observations:

1) As pressure increased, cell viability generally decreased above a threshold pressure. Also, at the same time that cell viability was decreasing, molecular uptake was often increasing, shown by the increasing percentages of cells with calcein uptake.

2) As total exposure time increased, we again observed increasing bioeffects, namely a decrease in cell viability and an increase in cell viability.
3) As pulse length increased from 1 ms to 10 ms, there was a notable difference in bioeffects at the higher total exposure times. There was not such a notable difference between 10 and 100 ms pulse lengths.

Regarding our second objective, we sought to establish a relationship between acoustic cavitation and bioeffects (cell viability and molecular uptake), and therefore we measured the amount of light generated during ultrasound exposures. We utilized several correlation techniques but the best relationship materialized when the integrated light output was multiplied by the number of pulses during an exposure, a measurement we called the total integrated light output. From this correlation, we accomplished the objective of establishing a relationship between bioeffects and a measurable gauge of cavitation, sonoluminescence.

The last objective was to identify regions of sonoluminescent light output where there was both high molecular uptake and high cell viability. This objective was accomplished by treating the nominal uptake population, low uptake population, and the high uptake population as a single population using a weighted average. With this representation we were able to locate a region where there was both high molecular uptake and high cell viability.

5.2 Recommendations

An interesting study might include observing not only the amount of light during ultrasound exposure but the location of the light generation as well. This might isolate whether the light is generated near cells and thereby further determine that acoustic cavitation is responsible for the bioeffects that are seen. This could be accomplished
through a camera device mounted on the bottom of the device. Another study might include disseminating the spectrum of light generated during routine ultrasound exposure. Perhaps the water bath and the cell sample emit spectrum that are different enough to be distinguished.

Further studies could also be done to see if the bioeffects that we observe would correlate with any other acoustic cavitation phenomenon, such as the generation of free radicals. This would be important in determining which measure of cavitation is primarily responsible for the bioeffects that we see. A free radical scavenger that fluoresces could be used to detect the presence of hydroxide radicals and through the analysis used in this thesis or a similar one perhaps a relationship would materialize.

One shortcoming of the device is that only the average light output during a total exposure can be observed instead of gathering the light output singly from each pulse. With a different data acquisition device, one might be able to gather each pulse and therefore get an even more accurate picture of the bioeffects.
APPENDIX A

FIGURE 6 GRAPHS SHOWN
IN GREATER DETAIL
100 ms TET, 1 ms pulse

100 ms TET, 10 ms pulse

100 ms TET, 100 ms pulse
2 s TET, 1 ms pulse

2 s TET, 10 ms pulse

2 s TET, 100 ms pulse
APPENDIX B

FIGURE 7 GRAPHS SHOWN IN GREATER DETAIL
REFERENCES


