SIZE AND LIFETIME OF TRANSIENT CELL MEMBRANES DISRUPTIONS CREATED BY ACOUSTIC CAVITATION

A Thesis
Presented to
The Academic Faculty

By
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Chemical Engineering
Size and Lifetime of Transient Cell Membrane Disruptions
Created by Acoustic Cavitation

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Date Approved by Chairman: 1/15/00
For my brother, Jacob Mark, who walked away from a million dollars to gain something of far greater value. Thanks for always being my friend.
ACKNOWLEDGEMENTS

This work would not have been possible without the assistance and support of many people:

My thesis advisor, Mark Prausnitz, who believed in me when I couldn't believe in myself. Thank you.

Keyhan Keyhani and Jin Liu who began this work.

The Prausnitz lab group for many hours of discussions, friendship and support; especially Hector Guzman for always answering my questions, Stephen Cochran for being my “undergraduate,” Esi Gharvey-Tagoe for listening, Shilpa Kaushik for always finding the solution, Paul Canatella for helping me learn the ropes, Devin McAllister for understanding, Matt Black for taking over, Derek Atkinson for making me laugh and Ellen Jett for being my friend.

John Shields and Mark Farmer at the University of Georgia Center for Advanced Ultrastructural Research, who taught me how to fix, fracture, and image cells; you guys made the 180 miles a day worth it.

Andrew Lyon and Stephen Quirk in the Chemistry Department at the Georgia Institute of Technology for assistance with the dextran purification and dynamic light scatter.

Steve Woodard for assistance with flow cytometry and confocal microscopy; and for always asking how my day is going.

Anna Chromiak for always being there when there was a crisis.

Bob Karaffa for assistance with flow cytometry and never losing his patience with me.

Chhaya Agrawal, who always knows what to say

Kerry and Debbie for listening to my defense 5 times in a row

Jacob, Ashley and Destiny; Kim and the Gerks; Kristi and the Atkinsons; Ian Harris, Sherri Coley, Thea Ellingson and Rita ‘Mom’ Rhodes: my large and extended family who help me through the rough spots
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SUMMARY

Medicine and biotechnology can benefit from the enhanced transport of molecules into cells. Acoustic cavitation created by low frequency ultrasound is a promising method that can achieve this goal. Cavitation is believed to increase uptake of molecules by creating transient disruptions in cell membranes, thus allowing the membrane to become permeable to drugs, proteins, and DNA.

The goal of this work is to determine the size and lifetime of transient disruptions created in cell membranes by acoustic cavitation. Low frequency (24 kHz) ultrasound was applied to cell suspensions to induce cavitation. Effective experimental conditions that cause large uptake and high viability were determined based on previous studies, in addition to experiments performed in this study to optimize acoustic pressure, location in the sound field, and cell concentration. Using this information, acoustic pressures were found that created moderate (just above the threshold of cavitation) and strong cavitation and thus, moderate and strong effects on cells.

The lifetime of disruptions was shown to be approximately 1 minute. Cells were first sonicated at moderate and strong cavitation conditions and subsequently exposed to either calcein (623 Da) or bovine serum albumin (67 kDa) a set amount of time after sonication was stopped. For up to about a minute after sonication, uptake of both
molecules was observed to be within one order of magnitude of samples sonicated in the presence of these molecules at the same conditions.

A study of disruption size determined that molecules at least as large as 28 nm can be transported into cells using acoustic cavitation. Cells were sonicated in the presence of five fluorescent compounds representing a radius size range (0.6 nm-28 nm): calcein, bovine serum albumin, and 3 FITC dextrans. Sonication was performed at moderate and strong cavitation as well as the maximum cavitation condition allowed by the apparatus. Light scatter data determined the effective size of the dextrans and showed that the molecules remained intact after sonication. Confocal microscopy images indicate that all five molecules were delivered into the cytosol.

Using freeze fracture and transmission electron microscopy (TEM), cells exposed to sonication and then fixed less than 1 minute after sonication exhibited a characteristic structure which could be interpreted to show holes or tears in the membrane. These membrane structures measured a few microns in size and were at high density (~10-15 per cell). These structures were not present in control samples (unsonicated) or in samples fixed 1 hour after sonication. In these experiments, samples of very high cell density were sonicated at moderate cavitation conditions, fixed by plunge freezing into liquid propane, fractured and viewed using TEM.
CHAPTER 1

1.1 Introduction

The cell membrane acts as a defense against external and harmful contaminants, but it also serves as a barrier to drugs or other molecules that can be helpful to the cell or the body. Many laboratory applications useful for biotechnology and possible medical treatments, such as gene therapy and targeted drug delivery, are hindered by an inability to transport molecules efficiently and easily into cells.

For biotechnology, traditional methods for gene transfection are specific to cell type, inefficient, or difficult to perform, making the processes inconsistent and expensive. In common medical applications, drug efficacy is lost due to rapid clearance from the gastrointestinal tract and/or blood stream. Protein molecules, including DNA, may degrade too rapidly or have too little uptake in the body to be of therapeutic use. High dosages of drug are often required and side effects, especially for serious illness such as cancer, are often severe. These problems frequently occur due to an inability to target the drug to a specified active site in the cell, or particular region of the body.

Acoustic cavitation produced by non-thermal low-frequency ultrasound can make cells permeable to drugs by a mechanism believed to involve the creation of transient disruptions in the cell membrane. These disruptions allow the transport of compounds,
including drugs, proteins, and DNA, across the cell membrane and into the cell in a noninvasive, targeted manner that is rapid, controlled and possibly applicable to a broad range of cell types.

Studies have shown acoustic cavitation to significantly enhance delivery of molecules across cell membranes; Liu et al. (1) have utilized low intensity, non-thermal ultrasound to disrupt red blood cell membranes and cause release of hemoglobin. Fechheimer et al. (2) and Wyber (3) have shown ultrasound to transfect live cells with DNA.

It is believed that cavitation is the mechanism by which ultrasound increases permeability of cell membranes. However, this hypothesis has not been fully confirmed. The existence and characteristics of disruptions created in cell membranes by acoustic cavitation are also uncertain. Establishing the existence of the disruptions, determining the maximum size molecule that can be delivered and finding the lifetime of the disruptions will aid in interpreting possible mechanisms and identifying suitable applications for this method of breaching cellular barriers.
1.2 Objectives

This work examines the biological effects of acoustically-induced cavitation. The governing hypothesis is that acoustic cavitation transiently disrupts cell membranes and thereby (a) increases uptake of molecules and/or (b) causes cell death. The overall goal of this research is to determine the size and lifetime of these membrane disruptions. The specific objectives are to:

1. Identify experimental conditions that cause large uptake and maintain high viability, by (a) quantifying the amount of fluorescent marker molecule (calcein) transported into the cytoplasm of cells and (b) finding the percent of cells that remain viable as a function of: pressure, location in the sound field and cell concentration.

2. Determine the effect of molecular size on uptake into the cell during moderate and strong cavitation using five model fluorescent compounds: (a) calcein (molecular weight = 0.623 kDa, radius = 0.6 nm), (b) bovine serum albumin (67 kDa, 3.5 nm), (c) FITC-dextran (150 kD, 8.9 nm), (d) FITC-dextran (500 kDa, 15 nm), (e) FITC-dextran, (2000 kDa, 28 nm).

3. Determine the lifetime of transient disruptions in cell membranes during moderate and strong cavitation by exposing cells to two model fluorescent compounds (calcein and bovine serum albumin) at different times after exposure to acoustic cavitation.
4. Image changes in cell membrane structure caused by acoustic cavitation using freeze fracture transmission electron microscopy.

In these experiments, cells were exposed to 24 kHz ultrasound at 10% duty cycle with 0.1 second pulse length and total exposure time of 2 seconds. Experiments are assayed using flow cytometry to quantify intracellular uptake of molecules and loss of cellular viability due to by sonication of a model prostate cancer line (DU 145).
1.3 Overview of the Thesis

In chapter 1, the purpose of this work and the objectives of the thesis are presented. Chapter 2 gives an overview of cavitation induced by low frequency ultrasound and its applications to targeted delivery of molecules. In chapter 3, the ultrasound system utilized is described and the experiments that were performed are defined. Chapter 4 shows and discusses the results and gives conclusions. Chapter 5 makes recommendations and suggests future work.
CHAPTER 2

2.1 Introduction and overview

Drug delivery applications can benefit from noninvasive technologies capable of targeting treatment in the body. One such method is the use of acoustic cavitation to transiently permeabilize cells and tissue to allow transport through previously impenetrable barriers. The creation of acoustic cavitation is discussed, its effects on the body are described and drug delivery applications are given. The relevance and importance of this work is also considered.

2.2 Acoustic waves

Acoustic cavitation is bubble activity induced by the application of sound waves to a medium. Acoustic waves are three dimensional energy waves that spread out spherically from a point source and displace particles in a medium. They are longitudinal and particles are displaced parallel to the direction of the motion of the wave. Over the course of an acoustic cycle, there exists compression, i.e., high-pressure regions of positive displacement, during which the particles move ‘forward;’ and rarefaction, i.e., low-pressure regions of negative displacement, during which time the particles move ‘backward’ (4).
Some important parameters of an acoustic wave are the frequency, intensity/pressure, exposure time and duty cycle.

**Definitions:**

- **Frequency:** the number of wavelengths in a given time (Hz).

- **Acoustic intensity:** the rate at which energy in the wave crosses a unit area perpendicular to the direction of propagation (W/cm²).

- **Incident pressure:** a measure of the strength of the ultrasonic wave, defined as the pressure level existing in the absence of cavitation (primarily at the driving frequency).

- **Exposure time:** the ultrasound pulse duration multiplied by the number of pulses applied.

- **Duty cycle:** the fraction of time that the ultrasound is ‘on’ during pulse application.

An acoustic wave becomes “ultrasonic” when its frequency is greater than 20 kHz which is above the range of human hearing; thus ‘ultra’ sound. The application of an acoustic wave to a medium is known as sonication. The wave is often applied in a single burst of a given time duration or in timed ‘pulses’ (on/off cycles of the sound wave) to a transducer. At the driving frequency, the transducer resonates (creating sound). During cavitation, the acoustic energy frequency can be shifted from the driving frequency to a broad range of varying frequencies, due to bubble activity (5).
2.3 Creation of Cavitation

An acoustic wave stimulates cavitation by (6):

i) creating nuclei/nucleation sites for bubble formation

ii) tearing the liquid of the media (allowing bubble formation)

iii) creating bounded volume cavities (e.g. bubbles)

iv) causing the expanding/contracting/oscillating of bubbles

Acoustic waves can affect a bubble at any point in its evolution. Bubble evolution consists of three steps (5):

i) inception, during which bubble configurations are developed;

ii) activity, which can include oscillation, translation and collective motion of the bubbles;

iii) effects or dynamics, which include shocks, jetting, shattering, and sonochemistry.

When an acoustic wave passes through liquid, the acoustic pressure in the liquid is equal to the sum of the static and oscillation pressures created by the sound wave. If the acoustic pressure amplitude is greater than the static pressure, the liquid pressure is negative during a portion of each acoustic cycle and the liquid is in tension. The tensile strength of the liquid is the tension that a liquid can support without breaking and thus causing bubbles to evolve, an effect known as cavitating. Most cavitation occurs from the presence of dissolved gas in the medium, which is pulled out of solution in the
presence of ultrasound. The point at which the liquid begins cavitating is the threshold of cavitation. The threshold varies by the properties of the liquid and the conditions to which the liquid is subjected.

Cavitation can refer to either bubble inception, activity or dynamics. The relative amplitude of the bubble motion categorizes bubble activity: low, medium, or high. The parameters that determine bubble dynamics include liquid and gas properties of the medium that can cause attenuation of the wave; thermodynamic variables; and acoustic variables, such as pressure, frequency, waveform, duty cycle, pulse shape and width.

Cavitation can be described in two ways, as stable (noninertial) or transient (inertial) cavitation. Stable cavitation causes the bubbles to oscillate in size, but not to implode. Stable cavitation is associated with higher frequency and lower intensity sound waves than transient cavitation. Transient cavitation is created by high acoustic pressures and causes bubbles to violently implode after a few wave cycles. Transient cavitation shows high bubble motion and a great deal of bubble dynamics. It is a great energy concentrator, increasing the magnitude of the temperature, pressure and energy density in the upon bubble implosion (5).

2.4 Mechanisms of drug delivery

According to Tachibana (7), the use of non-thermal ultrasound as a delivery system can be classified into three major categories. Acoustic cavitation energy can:
i) help agents cross the boundaries of tissues,

ii) make membranes permeable to molecules, and

iii) change the chemical properties of the drug.

Ultrasound may increase permeability via several possible mechanisms: heating (acoustic thermal effect), radiation pressure, acoustic microstreaming, or cavitation. Studies have indicated cavitation to be the primary mechanism by which ultrasound transiently permeabilizes cells and tissues (8,9). During the low-pressure (negative) portion of the sound wave, dissolved gas and vaporized liquid in the medium form gas bubbles. These bubbles are affected by the sound wave, creating acoustic cavitation, and this stimulated behavior by the bubbles is believed to be the cause of biophysical effects observed from ultrasound, such as membrane disruption.

The bubbles shrink and grow in size, oscillating in response to the subsequent high- and low-pressure portions of the wave (stable cavitation) (4,5). It is possible that jetting, a radial motion from low to high amplitudes, shape oscillations and/or single and multiple bubble effects create the membrane disruptions. Jetting occurs when the presence of a surface near a bubble undergoing implosion during transient cavitation causes a non-symmetric collapse. The hydrodynamic effect of fluid rushing inward from the radial direction creates asymmetric flow, distorting the bubble and causing it to “jet” at very high speeds toward the existing surface (5). This makes the bubble like a bullet,
allowing it to punch holes in the surface, an effect that can be visually observed as metal pitting or underwater explosions.

Translation effects also allow the bubble to move, usually in the direction of the projected acoustic wave. Shape oscillations cause a bubble to 'pulse,' the pulsation of the bubble creates a pressure field, attracting particles like red blood cells and may be a factor in moving the particles through membranes (5). Multiple bubble effects cause the bubbles to be attracted to each other. During rarefaction, the bubbles flow toward each other, forcing fluid from between them. Compression then shrinks the bubbles, drawing the fluid back, however, the net effect is that of attraction.

Research suggests biological acoustic effects, including membrane disruption, to increase at lower frequency ranges (10). Increased cell membrane permeability has also been shown to increase with incident ultrasound pressure; however, cell viability may be compromised (11). At higher frequencies, cavitation is believed to be more 'gentle;' however, fewer effects on the cells are observed and it is more difficult for large molecules to access the cell when the disruptions are less severe. Higher cell viability may be possible, which is important for targeted delivery when the goal is treatment and not destruction.
2.4 Drug delivery using acoustic cavitation

The cell membrane acts as a barrier to external contaminants and works to regulate the cell's function by transporting nutrients into the cell and waste out. The cell membrane consists of a lipid bilayer containing covalently linked proteins embedded throughout. It is a selective filter that controls transport, receives and initiates a response to external stimuli and allows communication between cells. It also acts as a barrier against drugs or other molecules that can be helpful to the body. Acoustic cavitation induced by ultrasound can create transient disruptions in cell membranes that allow the transport of compounds, including drugs and large proteins such as DNA and insulin, across the cell membrane and into the cell.

When placed in a sound field, cells can show alterations in motility and be stimulated to synthesize and secrete proteins (12). These changes are associated with increased cell permeability that has been shown to occur as an effect of low intensity, non-thermal ultrasound (1). The changes in the membrane structure can range from deadly, lysing of the cell, to subtle and reversible permeability, allowing transport into the cell to occur (13,14). It is believed that acoustic cavitation is responsible for these bioeffects, e.g. increased permeability, that occur during sonication.

Many studies have shown ultrasound to significantly enhance molecular delivery to the cells, through skin and tissues and into organs (9,15,16,17). Liu et al (1), have
shown effects to cells utilizing low intensity, non-thermal ultrasound to disrupt cell membranes and lyse cells. Fechheimer et al. (2) and Bao et al. (13) have shown ultrasound to be a methodology for transfecting live mammalian cells with DNA. Studies by Loverock et al (18) have shown ultrasound to significantly increase the cytotoxicity of adriamycin to mammalian cells, an effect believed to be caused by increased cell permeability after exposure to ultrasound.

2.6 Benefits

Acoustic cavitation is a simple, economical and reproducible method for delivery of molecules, proteins and drugs through cellular membranes and skin. It is a mechanical method, and as such, is applicable to a wide range of cell types and tissues (3). It is a relatively inexpensive, rapid technique that can be targeted externally, and non-invasively, to the body.

2.7 Relevance of presented work

Since it is believed that cavitation is the reason for the ultrasonically induced increased permeability of the skin and cellular membranes, it is a goal of researchers to understand the mechanisms that control the production and amount of bubbles during sonication. It is also important to understand the nature of the biological disruptions that are produced and the limitations that exist. This work studies the time for uptake after sonication, the range of particle sizes that can be taken into the cell and the physical
nature of the disruptions via imaging. Determination of these factors will aid in the
design of practical drug delivery systems.
CHAPTER 3

3.1 Introduction and Overview

To determine the effects of acoustic cavitation on cells, cavitation was induced in cell suspensions using an ultrasound generation and application system. The ultrasound system consisted of the following components:

i) sound wave generation system,

ii) exposure chamber and cell sample container, and

iii) monitoring system.

The samples were assayed using flow cytometry, spectrofluorimetry, fluorescence microscopy, confocal microscopy, and transmission electron microscopy. Quantification of cellular uptake and viability utilized WinMDI 2.8 (Windows Multiple Document Interface, Joseph Trotter, Scripps Research Institute, La Jolla, CA), Microsoft Excel version 5.1 spreadsheets and a statistical software package, MiX3.1 (Ichthus Data Systems, Hamilton, Ontario, Canada).

3.2 Apparatus

The generation system, shown in figure 3.1, controlled the frequency, intensity, duty cycle, and pulse length. Total exposure time was controlled manually, by turning
Figure 3.1 Ultrasound generation system
Figure 3.2 Schematic of exposure chamber and transducer
the system on/off. A function generator (DS345 SRI, Stanford Research Systems, Sunnyvale, CA) was used to generate sine waves at 24 kHz. The output of the function generator was sent to an ultrasonic amplifier (Macro-Tech 2400, Crown Technology) to control the amplitude of the signal, which was then sent to a matching transformer (MT-56R, Krohn-Hite Corporation) which drove the transducer located in the exposure chamber, as shown in figure 3.2.

The exposure chamber consisted of two pieces of 2" OD PVC (poly-vinyl chloride) pipe with a lead zirconate titanate transducer of 24 kHz resonance (Channel Industries, Santa Barbara, CA) sandwiched between them (Transducer Specifications are given in table 3.1). The exposure chamber was filled with deionized water, which was then degassed for 3 hours using a vacuum chamber at ~760 mm Hg (Nalgene, Rochester, NY), and pump (KNF Laboratory). The degassed water has few cavitation nuclei and serves as a bath through which acoustic waves can travel to the sample container with minimized cavitation occurring in the bath. The sample containers were Sedi-Pet transfer pipets (SAMCO 241) with stems cut to 2 cm in length.

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The monitoring system was composed of an oscilloscope (54603B, Hewlett Packard) and a spectrum analyzer (SR670, Stanford Research Systems). The wave amplitude set in the function generator was sent as a voltage signal to the transducer and read in the oscilloscope as the applied voltage. This voltage measurement was converted to incident pressure (atm), using a correlation developed by Liu et al (19) for this apparatus.

\[ P[\text{atm}] = V[\text{volts}] \times 0.0089 \]

*equation 3.1*

Where \( P \) is the incident pressure and \( V \) is the voltage applied to the transducer.

A hydrophone (Bruel and Kjaer) was inserted into the bottom of the exposure chamber; its signal was sent to the spectrum analyzer, which yielded an acoustic spectrum from 0-100 kHz. Features of the spectrum are believed to be associated with the amount of cavitation produced during ultrasound application (1,19). Acoustic spectra were collected for use in other studies and not analyzed in this thesis.

### 3.3 Preparing Suspensions

Du 145 prostate cells (American Type Culture Collection, Rockville, MD) were used in all experiments. This cell line was used as a model mammalian cell because it has been well characterized in this laboratory by previous experiments and is readily available. The cells were grown as a monolayer in 5% \( \text{CO}_2 \) and 37°C environment (model 3110, Forma Scientific incubator).
Complete growth media used:

90% RPMI-1640 (Sigma and Cellgro)

10% fetal bovine serum, heat inactivated (Cellgro)

10 ml antibiotic, Penicillin/Streptomycin or AB/AM (Cellgro)

The cells were then harvested during the exponential growth phase using trypsin (2.5%, without Ca\(^{2+}\)/Mg\(^{2+}\), Cellgro) diluted 10 X in Dulbecco’s phosphate buffered saline (PBS) (Cellgro).

3.3.1 Cell Harvest Protocol

To harvest a 150 cm\(^2\) flask (approximately 10,000,000 cells):

1.) Growth media over cell monolayer was decanted and disposed of as biohazardous.

2.) Monolayer was washed with 5-10 ml phosphate buffered saline (Cellgro) without Ca\(^{2+}\)/Mg\(^{2+}\) for 1-2 minutes and then decanted.

3.) 5 ml of trypsin were added to the flask. The cap was replaced upon the flask. 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 removed from the incubator. When held up to a strong light, the monolayer can be seen to slide off the flask wall. The flask was tapped gently to ensure that all cells are suspended in the trypsin.
5.) 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 haemocytometer (Hauser Scientific Partnership) and then diluted to a final concentration; for most uptake and viability experiments, the concentration was either 500,000 or 1,000,000 cells/ml.

The experimental design for most uptake and viability experiments is given (Fig 3.3). Cells are exposed to ultrasound in the presence of fluorescent molecules, washed to remove any excess external fluorescence and then assayed for uptake and viability using flow cytometry. There are four possible outcomes:

1) Live cell with no uptake (unaffected)
2) Live cell with uptake (desired)
3) Intact dead cell
4) Non-intact dead cell debris

In certain experiments (lifetime of disruptions) the fluorescent molecule is added after sonication and then assayed the same way as standard uptake samples.
Molecule
Viability stain

- Molecule
- Viability stain

Viable
Non-permeabilized

Viable
Permeabilized

Non-viable
Intact

Non-viable
Not intact
3.4 Fluorescent molecules

Fluorescein-based fluorescent molecules were introduced into the cell suspensions at varying times in the study and are described in more detail in the next section. The molecules were over a broad range of sizes (MW = 0.623 – 2000 kDa, r = 0.6 - 28 nm) for experiments to determine the size of cavitation-induced disruptions. The small molecules, calcein and bovine serum albumin, were selected for comparison to previous and on-going work by other researchers. The fluorescein isothiocyanate (FITC)-dextrans were selected because they represent a range of sizes, with the highest molecular weight used in this study as the maximum size fluorescent dextran commercially available.

3.4.1 Addition of fluorescent molecules

For experiments on cell density, molecular uptake and cell viability which used calcein (Molecular Probes lot #2532-5), fluorescent molecules were added to the cell suspension prior to sonication in sufficient quantity to make the final calcein concentration of the suspension 10 μM.

For lifetime of disruption experiments using calcein (Molecular Probes lot #2532-5) or bovine serum albumin (Molecular Probes lot #6581-1), after sonication, individual samples were added to a solution containing fluorescent molecule to make the final fluorescent concentration of the suspension 10 μM.
For size of disruption experiments using calcein, bovine serum albumin or FITC-dextrans, fluorescent molecules were added to cell suspensions prior to sonication. The final concentration varied by molecule.

The larger FITC dextrans have a much higher fluorescent intensity than the other molecules due to many FITC molecules bound to each dextran molecule. This made assaying the samples with flow cytometry difficult. It was therefore decided to maintain constant fluorescence intensity instead of constant dextran concentration, which allowed the standard settings of the flow cytometer and customary calibration standards to be unchanged.

3.5 Sonication

Ultrasound experiments were performed at room temperature. Heating due to cavitation effects was previously determined to be negligible (1) using this apparatus and similar protocols.

3.5.1 Sample Preparation

For samples assayed using flow cytometry:

1.2 ml of cell suspension was introduced to the sample container (Sedi-Pet transfer pipet, 2 cm stem) using a 22 gauge needle (Becton Dickinson) and 3 cc syringe (Becton Dickinson). A meniscus was left at the top of the pipet stem and
a 1/16" metal rod was inserted 0.5 mm into the stem. Overflow of solution was allowed to ensure no air bubbles were trapped in the sample.

For freeze fracture samples:

0.35 ml of a highly concentrated cell suspension (2.0 x 10^7-2.4 x 10^7 cells/ml) was introduced to a PADL-PET (SAMCO 422) sample container, stem cut to 5 cm, using a 22 gauge needle and 3 cc syringe (Becton Dickinson). A meniscus was left at the top of the pipet stem and a 3/16" metal rod was inserted 0.5 mm into the stem. Overflow of solution was allowed to ensure no air bubbles were trapped in the sample.

Sample containers were never re-used and were disposed of as biohazardous after each experiment was performed.

The sample was positioned in the cylindrical water bath (300 ml degassed deionized water) so that the center of the sample was in the radial and axial center of the transducer (unless otherwise noted).

24 kHz pulsed ultrasound (0.1 s pulse length, 10% duty cycle for 20 pulses, 2 s total on time) was applied. All ultrasonic parameters were kept constant in all experiments with the exception of amplitude of the sine wave, which was controlled by the function generator. This allowed us to vary incident pressure applied to the sample.
Table 3.2 Ultrasound Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency</td>
<td>24 kHz</td>
</tr>
<tr>
<td>Pulse length</td>
<td>0.1 s</td>
</tr>
<tr>
<td>Duty cycle</td>
<td>10%</td>
</tr>
<tr>
<td>Total on time</td>
<td>2 s</td>
</tr>
</tbody>
</table>

After ultrasound application, the cells remained at room temperature for approximately 10 minutes to allow cell recovery. Samples were washed 3 times by spinning down (735 x g, 3 min, Eppendorf 5415 C, Brinkman) and resuspending the cell pellet in Dulbecco's phosphate-buffered saline (Cellgro).

3.5.2 Sample Assay

To assess viability and uptake of molecules, 5 μl of 10 mg/ml of propidium iodide (Molecular Probes), a red fluorescent cell viability stain, and 10^5/ml green fluorescent latex microspheres (d = 2.5 μm, Molecular Probes, 488/515, lot #5881-2, 100% relative intensity) were added to the final cell suspensions. Non-viable cells were stained red by the propidium iodide. The microspheres were used as an internal volumetric standard to determine the cell concentration after ultrasonic application, as described below (20).

3.5.3 Flow Cytometry

A FACScan flow cytometer (Becton Dickinson) using Cellquest (BD Software Corporation) software was utilized. At least 10,000 cells were collected from each sample. The samples were excited using a 488 nm argon laser for all fluorescent uptake.
compounds. The fluorescence of propidium iodide (525/25 nm bandpass filter for emission collection) was used to distinguish between viable and nonviable cells; nonviable cells were high in propidium iodide (red) fluorescence.

Viable cells were analyzed to determine the mean fluorescence intensity of the fluorescent molecules (e.g. calcein). All were fluorescein based (green, 530/30 nm bandpass filter for emission collection): calcein or FITC-labeled BSA or FITC-labeled dextran. The mean fluorescence intensity was converted to an average number of molecules per cell using green fluorescent calibration beads (Flow Cytometry Standards Corporation, Quantum 25, d = 7.2 μm, lot #B00136) with a known fluorescence intensity that can be quantified as a number of free fluorescein molecules in solution or "Molecules of Equivalent Standard Fluorochromes" (MESF) (21).

To compare the uptake molecules to MESF values, the fluorescence of the marker molecule must be converted to a measurement in terms of free fluorescein. A spectrofluorimeter (Photon Technology International) was used to determine the ratio of fluorescence of free tracer molecule to free fluorescein (sigma lot #2532-5), or intensity of the marker relative to the intensity of fluorescein. The intensity ratios (R) were determined to be (using 3 replicates):

i) Calcein (Molecular Probes lot #2532-5): 0.93 ± 0.1
ii) Bovine serum albumin (Molecular Probes lot #6581-1): 0.97 ± 0.2
iii) FITC dextran (150 kDa, Sigma lot #77H5086): 1.01 ± 0.2
Calcein, bovine serum albumin and FITC-dextrans of high molecular weights cannot cross the intact cellular membrane, thus any fluorescence in the controls should be due to autofluorescence and surface binding of the compounds. The fluorescence of a sonicated sample is found by subtracting its fluorescence from that of the control sample. For viability calculations, the control is designated as 100% viable. The ratio of the number of viable cells after sonication to the number of cells in the control(s) is represented as % viability.

Intracellular and extracellular concentrations of fluorescent compounds are related as the % external concentration. This was calculated by dividing final intracellular concentration, determined using the measured molecules/cell and a cell volume of $5.6 \times 10^3 \ \mu m^3 / cell$, by the known initial external concentration.

3.5.4 Analyzing data

After readings were taken using the flow cytometer, data were analyzed in multiple steps:

1) WinMDI was used to determine fluorescent events, which were represented as a histogram of uptake in a logarithmic scale and a number of events for viability. In flow cytometry, different types of lasers and arrangements of lasers are used. In forward scatter, the laser shines directly through the sample and is an indication of size. Side scatter uses a laser at a 45-degree angle and is a measure of internal complexity. The
histograms show the amount of fluorescence (e.g. calcein) on the x-axis and the number of events (e.g. cells) with that particular fluorescence value. Histograms that show molecular uptake exhibit two (or possibly more) populations. In Figure 3.4, (a) is a representative density plot of side scatter vs. forward scatter of the control, a representation of the data that allows the cells to be identified as region R1 (drawn to define events that are cells); (b) shows the fluorescence of propidium iodide (PI) vs. forward scatter, a representation of the data that shows the amount viable and nonviable cells; region R2 is drawn to define live cells, region R3 is drawn to define dead cells; (c) shows calcein vs. forward scatter, a representation of the data that allows the MESF microspheres to be identified; region R4 is drawn to define the fluorescent microspheres, (d) is a typical control histogram; (e) and (f) show sonicated samples at two pressures; the multiple populations can be observed.

The data collected from WinMDI software were:

i) fluorescent histogram raw data
ii) number of live cells (R2),
iii) number of dead cells (R3),
iv) number of fluorescent microspheres (R4),
v) MESF fluorescent peak values.

2) To put the data in a format useable by the statistical software package, the histogram data from WinMDI was imported to an Excel spreadsheet using Ldata, a numerical conversion formula.
Figure 3.4 Data Analysis using WinMDI 2.8
3) The histogram data was imported to the statistical software package, MiX3.1. Comparison of sonicated samples to control samples shows multiple peaks in the sonicated sample. The peaks were defined as the low uptake population and the high uptake population. The package was used to analyze the histogram data as two normal distributions, a function that cannot be performed in WinMDI. Assuming that the sample is 100% cells and giving the entire sample a value of 100%, the proportion each population represents is found via MiX3.1. The mean, standard error of the mean and sigma (standard deviation) for each proportion is found and correlated to the fluorescence using Excel spreadsheets and the data from WinMDI.

The data collected from this software were:

i) % cells per population (assuming 100% cells total),

ii) mean fluorescent intensity per population,

iii) the standard error of the mean

iv) the sigma of fluorescent intensity per population.

4) Collected data were entered on Excel spreadsheets, which calculated

i) uptake (as the number of molecules per cell),

ii) viability (as a percentage of the control), and

iii) % equilibrium for each population.
The histogram of the control sample shows a single peak in the first decade \((10^1)\) of fluorescence (Figure 3.4 (d)). Histograms of sonicated samples show multiple peaks of fluorescence that change in size and shape due to: pressure, type of molecule or time. Events present in the first decade of fluorescence are defined as a low uptake population, all higher events (if present) are defined as a high uptake population for the purposes of this study. It was initially hypothesized that all events indicated in the sonicated samples could be defined by a bimodal distribution: low uptake population or high uptake population. However, the presence of events between these populations is seen to occur. Additionally, some samples do not have a high uptake population, but include a significant number of events at a higher fluorescence than the control.

All events in the first peak are defined as the low uptake population, all events of higher fluorescence are defined as the high uptake population for this work. If the first population is greater than 97\% (by proportion) of the histogram, it is reported as no uptake, i.e., the high uptake population is insignificant.

3.6 PERFORMED EXPERIMENTS

3.6.1 Optimizing conditions

Early work with this apparatus and cell line were performed previously by Dr. K. Keyhani (11). However, some further optimization was required.
3.6.1.1 Sample Chamber

The sample chamber used in previous experiments was a 3.5ml tube (cut from a 15 ml centrifuge tube, Fisherbrand, 05-539-12), filled to overflowing and capped with a rubber stopper.

It is likely that the rubber stopper absorbed some of the sound, causing less cavitation to occur and lowering the effectiveness of the system. The new chambers, 1.2 ml transfer pipets (used in this study) have thinner walls and do not utilize a rubber stopper. More cavitation, and thus more effects, is expected to occur using this system. Furthermore, the previous sample chambers required more than twice the volume of cell suspension, causing waste to occur.

3.6.1.2 Cell density

Cell suspensions over a range of cell densities, from 500,000 cells/ml to 3,000,000 cells/ml, containing 10 μM calcein were sonicated at 5.3 atm. Viability and uptake of molecules were assayed using flow cytometry the same day.

3.6.1.3 Sample location

Cell suspensions (1,000,000 cells/ml) containing 10 μM calcein were sonicated at 5.3 atm. The samples were positioned in the radial center of the transducer, but at a range of axial positions. Based on the axial center of the transducer = 0, the range was ± 1.25 cm. Viability and uptake were assayed using flow cytometry the same day.
3.6.1.4 Ultrasound Pressure

To determine the uptake of compounds and the viability of the cells after exposure to a range of cavitation conditions created in the ultrasound system, the standard cell protocol was used. After resuspending cells to a known concentration, calcein was added to the suspension to a final concentration of 10 μM. The samples were exposed to ultrasound at a range of pressures (1.8 atm – 8.9 atm, where 0 atm = control) and then assayed using flow cytometry the same day.

3.6.2 Lifetime of disruptions

In this study, two fluorescent compounds (calcein and FITC-BSA) were introduced into cell suspensions after sonication and in set time increments up to 120 seconds. The sonication protocol was followed at two pressures selected using the uptake/viability curve data to determine a midrange pressure (just after threshold of cavitation is reached) and a high pressure (low viability and high uptake). The voltages were 600 V and 800 V, correlating with pressures of 5.34 atm and 7.10 atm.

Samples were sonicated without the marker, then added to microcentrifuge tubes (1.5 ml, Eppendorf, 23398) containing a known concentration of marker, making the final concentration in the sample 10 μM for both molecules. After the last pulse of sonication was applied, the function generator was turned off and a timer starter simultaneously. The sample was removed from the water bath, added to the marker solution and mixed by
pipeting up and down with the sample container (a transfer pipet) for five seconds. The
time increments used: no sonication (control), 0 (marker present during sonication), 15,
30, 45, 60, 90, and 120 s after the final sonication pulse was applied.

The samples, including control, remained at room temperature for 15 minutes
after addition to the marker, then placed on ice, washed and assayed the same day using
flow cytometry.

3.6.3 Size of disruptions

Five fluorescent compounds of a varying molecular weight distribution were
utilized. Calcein, bovine serum albumin, and FITC-dextrans of 150 kDa, 500 kDa and
2,000 kDa were studied at three pressures: 5.3 atm, 7.1 atm and 8.9 atm. The
approximate radii of the dextrans were calculated using an empirical relation by Oliver et
al. (21):

\[
r \text{ [Angstroms]} = 0.488 \times M^{0.437}
\]

*equation 3.2*

Where \( r \) is the radius of the molecule and \( M \) is the molecular weight in daltons. Dynamic
light scatter measurements were made to confirm this calculation (see below).

The approximate radii were found to be:

i) Calcein (Molecular weight = 0.623 kDa): \( r = 0.6 \text{ nm} \) (26)

ii) Bovine Serum Albumin (67 kDa): 3.5 nm (27)
iii) FITC-dextran (150 kDa): 8.9 nm
iv) FITC-dextran (500 kDa): 15 nm
v) FITC-dextran, (2000 kDa): 28 nm

In order to keep the fluorescence from overwhelming the calibration standards, dextran concentrations were selected to keep FITC concentration approximately constant. Since larger dextrans have more FITC per dextran molecule, the molar dextran concentration was lower for dextrans of greater molecular weight. This allowed the same fluorescent calibration beads to be used for all samples, and also reduced the amount of free fluorescein likely to be found in the dextran samples.

The final concentrations used were:

i) Calcein (Molecular Probes lot #2532-5): 10 μM
ii) Bovine serum albumin (Molecular Probes lot # 6581-1): 10 μM
iii) FITC dextran (150 kDa, Sigma lot #77H5086): 10 μM
iv) FITC dextran (500 kDa, Sigma lot #97H5084): 0.8 μM
v) FITC dextran (2000 kDa, Sigma lot #68H5079): 0.1 μM

The markers were added to cell solutions prior to sonication. To ensure that no cross contamination occurred while loading the samples, a new syringe and needle were used for each molecule solution. The samples were then sonicated and assayed using flow cytometry. Fluorescent and confocal microscopy were also used to determine that uptake of the dextrans had occurred (described below).
3.6.3.1 Dynamic Light Scatter

Since dextrans are sold with a size distribution, the size range of the dextrans was determined using light scatter. Light scatter measurements were made using a DynaPro Dynamic Light Scatter Instrument (Protein Solutions Incorporated, Charlottesville, VA), accurate for detecting samples with radii in the range of 1 nm - 1μm. Data correlation was done using Dynamics Graphical Size Analysis Software (Protein Solutions, Inc.) to interpret the correlatives. Light scatter measurements were also made after sonicating a dextran solution to determine whether the molecule size was affected during the course of the experiment.

3.6.5 Fluorescent and Confocal Microscopies

Fluorescent microscopy (IX70, Olympus Microscope Corporation, Osaka, Japan) and confocal microscopy (Carl Zeiss Incorporation, Thornwood, NY) were used to visualize uptake. Confocal microscopy was utilized for better resolution and because specific areas of uptake can be viewed. Confocal microscopy uses a “pinpoint” of light that blocks the fluorescence above and below the sample. This allows an internal image of the cell to be taken in optical sections. Images can be taken in the z-direction from the top of the cell to the bottom. Cells tested for uptake of all five molecules were imaged in this way.

Confocal Magnification: 40 X objective with various numerical apertures.
3.6.6 Freeze fracture and transmission electron microscopy (TEM)

Samples of extremely high cell density (10,000,000-12,000,000 cells/sample) were exposed to ultrasound, then fixed by plunge freezing and then fractured. In these experiments, smaller sample volumes were utilized and cells were suspended in either full growth media (supplemented RPMI-1640) or PBS. No fluorescent molecules were added. An image of cavitation effects upon cell membranes was desired and the presence of molecules may have additional effects. Furthermore, the presence of additional molecules could make interpreting images more difficult.

Sample preparation in PBS reduces particles in samples, but offers little protection against freeze damage. The protein component of the supplemented RPMI-1640 acts as antifreeze and was used to reduce cellular damage caused by freeze fixation.

High cell density and small sample volumes were used to maximize the number of cells/volume. The sample size for freeze fracture is extremely small (less than 1 µL), but the magnification of the microscope is very high (up to 250,000X), which makes finding cells in the sample difficult. It is therefore desirable to maximize cell density and decrease volume size.

The sample containers, PADL-PET transfer pipets, contained 0.35 ml of cell suspension. After sonicating at 5.34 atm and standard conditions, a small amount of sample (less than 1 µL) was loaded onto a gold planchet (Electron Microscopy Sciences
The planchet with sample was plunged into a copper reservoir of liquefied propane suspended in liquid nitrogen and frozen.

Three types of sample were studied:

1) those fixed less than 1 minute after sonication,
2) those fixed 1 hour after sonication, and
3) controls (no sonication).

Samples were stored in liquid nitrogen and fractured using a Balzers 360 (Bal-Tec, Arlington Heights, Illinois) freeze fracture device. After fracture, samples were coated with platinum for 10 minutes to create metal replicants, which were then washed in glycerol, distilled water and chlorine bleach. The metal replicants were picked up on copper mesh grids (Electron Microscopy Sciences (EMS), Fort Washington, Pennsylvania) and viewed using transmission electron microscopy. A JEOL 100 CX TEM (JEOL Limited, Akishima, Japan, JEOL USA, Deerborn, MS) was used because of its capacity for high resolution of thin sections (the replicants), magnification used was 100,000–250,000X. Photographic negatives of the cell images were taken.
CHAPTER 4

4.1 Introduction

24 kHz ultrasound was used to create acoustic cavitation. Experimental conditions were developed from previous work (1,11) and further optimized in this study by determining the effect of cell density and sample location upon uptake and viability. Correlation of pressure with the amount of uptake and % viability was conducted and from this information, two operating pressures were identified: one that created moderate cavitation (just above the threshold) and one that created strong cavitation. These cavitation conditions were utilized to conduct studies of the lifetime of acoustically-induced disruptions using two fluorescent marker molecules. Moderate, strong and the maximum cavitation conditions allowed by the apparatus were utilized in conjunction with five fluorescent marker molecules to determine the size of the disruptions. The fluorescent dextran molecules were sized using dynamic light scatter (DLS) and the determined values compared to those calculated from an empirical relation. Finally, microscopy was performed to visualize the uptake of molecules into the cell and to show the effect of ultrasound upon the cell membrane.

4.2 Distribution of Uptake and Viability

To quantify the amount of uptake and the viability of sonicated samples, flow cytometry and several software packages are used.
Results: Figure 4.1 shows typical histograms (WinMDI 2.8) of events vs. fluorescence intensity using calcein as the marker molecule. The x-axis is the amount of fluorescence (divided into 1023 bins) and the y-axis represents the number of events/bin (cells). Figure 4.1 (a) is the positive control sample, a single peak of fluorescence; (b) is a histogram of uptake at a moderate pressure, the bimodal distribution is clearly shown as 2 peaks: population 1 is low uptake and population 2 is high uptake; (c) is a histogram of uptake at high pressure, it can be seen that more events are present in population 2 (high uptake), (d) shows a low pressure sample, no uptake is observed.

Discussion: Multiple peaks of uptake occur in sonicated samples. The reason for the existence of multiple peaks (i.e., low and high uptake) and what they represent is not fully understood at this time.

Conclusion: Sonicated samples show multiple peaks of uptake that are analyzed separately and represented as the low uptake population and high uptake population.

4.3 Effect of cell density

To determine the effect of cell density on uptake and viability, cell density was varied from $0.5 \times 10^6$ cells/ml to $3 \times 10^6$ cells/ml. Using calcein to test for uptake, sonication at 5.34 atm was performed for 2 s total on time with 0.1 s pulse length and 10 % duty cycle.
Results: Figure 4.2 (a) shows the curve of % viability with cell density. Each bar represents the overall viability (as subtracted from the control), the bottom part (light gray) represents the low uptake population and the upper part (dark gray) represents the high uptake population. At greater cell density, more cells were found in the high uptake population and higher overall cell viability was observed. The performance of a student’s $t$-Test (two tailed, critical value $t_{0.95}, P > 0.05$) (28) shows a significant differences to exist in the % viability for the following (results of tests are shown in the Appendices):

- Total cell population: 0.5 M cells/ml and 1 M cells/ml samples
  - 0.5 M cells/ml and 3 M cells/ml samples

- Low uptake population: no significant differences

- High uptake population: 0.5 M cells/ml and 1 M cells/ml samples
  - 1 M cells/ml and 2 M cells/ml samples
  - 1 M cells/ml and 3 M cells/ml samples

In figure 4.2 (b), the curve for uptake vs. cell density shows the number of molecules/cell in the high uptake population to be on the order of $1.3 \times 10^7$ molecules/cell. Little change in the amount of uptake occurs in the high uptake population as cell density increases, but the number of molecules in the low uptake population shows a modest decrease.

Discussion: The results show that cell density has little effect on uptake or viability.

This study was suggested because cavitation effects may be limited by an available number of cavitation nuclei (23); once the nuclei are used up; no more effects will be seen. This suggests that only a limited number of cells can be affected during sonication,
and the higher the cell density, a smaller percentage of cells will show effects. It is also possible that highly dense solutions of cells create a “shielding” effect and protect each other from the effects of sonication. However, our data did not suggest this phenomena occurred, since increased cell density did not dramatically reduce acoustic effects.

Conclusion: Cell density has little effect, but at higher densities slightly reduced cavitation effects (lower uptake and higher viability) may occur.

4.4 Effect of varying axial sample location

To determine the effect of axial position upon uptake and viability, samples were positioned in the radial center of the transducer, with axial position varied. The axial center of the transducer is denoted as the 0 position. The center of the sample was positioned over the range of the transducer, -1.25 cm (bottom of transducer) to +1.25 cm (top of transducer). Cell density was 2,000,000 cells/ml. Using calcein to test for uptake, sonication at 5.34 atm was performed for 2 s total on time with 0.1 s pulse length and 10 % duty cycle.

Results: For a well mixed sample, the position of lowest viability (less than 60 %) occurs when the center of the sample is located in the axial and radial center of the transducer, as shown in figure 4.3 (a).
In figure 4.3 (b), the maximum uptake observed (9.0 x 10^5 molecules/cell) at position equal to 0 cm (axial center of the transducer). At locations varying from this position, lower uptake is observed.

Discussion: When the center of the sample is in the axial and radial center of the transducer, it is more likely to be in the focal point of the sound wave. Furthermore, in this position, most of the sample is located within the transducer, allowing more cells to see the cavitation effect. Note that less uptake and higher viability are observed in this experiment than in other experiments in this work. The water bath was degassed for a shorter period of time than in other experiments. This probably caused fewer cavitation effects to occur in the sample due to cavitation in the bath.

Conclusion: For maximum cavitation effect, the center of the sample should be positioned in the axial and radial center of the transducer.

4.5 Effect of varying pressure

To determine the effect of pressure upon uptake and viability, sonication of samples containing calcein was performed from 0.9 to 8.9 atm for 2 s total on time with 0.1 s pulse length and 10 % duty cycle. Cell density was 1,000,000 cells/ml. The center of the sample was positioned in the axial and radial center of the transducer.
Results: In figure 4.4(a) the curve of % viability vs. pressure is shown. Viability is affected by sonication, dropping immediately from the control (100%) to 65%, after which the overall viability decreases with pressure, reaching a minimum at the strongest cavitation condition tested. The fraction of viable cells in the high uptake population increases with pressure.

Over the pressure range, little uptake occurs until a minimum pressure threshold is reached, as shown in figure 4.4 (b). High uptake, on the order of $1.2 \times 10^7$ molecules/cell is not achieved until the pressure is 3.6 atm. After this point, the high uptake population reaches a maximum value that remains unchanged as pressure increases. The low uptake population remains relatively constant. A "transition point" where some uptake occurs, but is not necessarily the high uptake population occurs at 3.6 atm ($5.00 \times 10^6$ molecules/cell).

Discussion: No uptake is observed until a threshold, probably correlating with the threshold of cavitation for this system, is achieved. At the higher pressure, there is more energy input to the system and more cavitation is likely to occur. Therefore, at the higher pressures, more cavitational effects are generated and uptake is increased while viability decreases. The "transition point" at 3.6 atm may be the result of a third, "medium uptake" population. Viability is affected by sonication, and decreases rapidly after the threshold of cavitation is reached at 3.6 atm, where more cells are affected and the percent cells in the high uptake region increases.
Conclusion: No uptake is seen until the threshold of 3.6 atm is reached. Above that, the high uptake population increases with pressure until reaching a plateau; after which, little change occurs. The results suggest a limit in the number of molecules that can be taken into the cell. From this data, two pressures, 5.3 and 7.1 atm (moderate and strong cavitation) were selected for use in future experiments.

4.6 Lifetime of disruptions: calcein and bovine serum albumin

To determine the lifetime of disruptions created by acoustic cavitation at 24 kHz, samples were sonicated for 2 s total on time with 0.1 s pulse length and 10% duty cycle at two pressures: 5.3 atm and 7.1 atm. Cell density was 1,000,000 cells/ml and samples were centered in the transducer. Individual samples were added to a calcein solution a known amount of time (from 0-120 s in 15 s increments) after ultrasound application was stopped. The 0 s result contained fluorescent molecules at the time of sonication.

Results: In figure 4.5, representative histograms show the change in the uptake populations with time after sonication at 7.10 atm. (a) shows a typical control (no sonication); (b) shows a standard uptake sample: calcein is present at the time of sonication; (c) calcein added 15 s after sonication; (d) calcein added 30 s after sonication; (e) calcein added 45 s after sonication; (f) calcein added 60 s after sonication; (g) calcein added 90 s after sonication; (h) calcein added 120 s after sonication. Note that the size of the second peak decreases with time, becoming nearly equal at 60 s and 90 s, and becomes insignificant at 120 s.
Figure 4.5 (b) calcein and figure 4.6 (a) bovine serum albumin, show overall % viability as a function of time. The high uptake population decreases with time until it is no longer present, and overall viability remains constant and in agreement with previous data (e.g. Figure 4.4 (a)).

Figure 4.5 (c), calcein and figure 4.6 (b) bovine serum albumin show molecules of uptake as a function of time. The greatest amount of uptake for both pressures ($1.2 \times 10^7$ molecules/cell) and both molecules occurs at 0 s (when cells are in the presence of fluorescent molecules during sonication), and sharply decreases with time, reaching a minimum non-zero value at 60 s ($2.0 \times 10^6$ molecules/cell) and going to 0 by 120 s for calcein. The bovine serum albumin samples reach a minimum non-zero value at 45 s (on the order of $2.0 \times 10^6$ molecules/cell) and go to 0 uptake by 60 s. For both molecules, the higher-pressure condition, 7.1 atm, shows more uptake in the high uptake population with time than 5.3 atm.

Discussion: Uptake into cells that are in the presence of marker molecules and drugs has been observed in many experiments and in the literature (9,11,13,17); however, the length of time available for uptake to occur after sonication has not before been examined.

The results show that an extended disruption lifetime exists after sonication, enabling the uptake of molecules for a relatively long period of time. This is potentially important for many drug treatment therapies, especially those involving sensitive molecules, such as DNA that could be damaged in the presence of ultrasound. The results also suggest that
the mechanism of uptake is at least largely diffusion; uptake after sonication occurs without any gradient but that of concentration.

Using the method of data analysis (described in Methods section), negligible uptake is considered to occur when the low uptake population is 97 % (or greater) of total cells. Data showing this result are reported as 0 uptake. The decrease in the high uptake population reported may be the result of a "medium uptake" population that is neither low nor high uptake, but is reported as part of the high uptake population for the purposes of this study.

Conclusion: Uptake of calcein occurs during and up to 90 seconds after sonication. Uptake of bovine serum albumin occurs during and up to 45 seconds after sonication. The degree of uptake decreases over time. Post-sonication uptake suggests transport by diffusion through long-lived membrane disruptions.

4.7 Determination of disruption size

To determine the maximum size of disruptions created by acoustic cavitation, samples were sonicated for 2 s total on time with 0.1 s pulse length and 10 % duty cycle at two pressures: 5.3 atm and 7.1 atm, using five fluorescent molecules. Different concentrations of molecules were used and the uptake was normalized using the apparent % equilibrium (% of initial concentration taken into cell). Radii of molecules were
calculated using an empirical relation (23); light scatter analysis was used measure the radii as validation.

Results: Dynamic light scatter analysis (DLS) (using 3 replicates):

Table 4.1 Comparison of dynamic light scatter to calculated values

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Calculated Radius (nm)</th>
<th>DLS Radius (nm)</th>
<th>DLS Radius (nm)</th>
<th>% Difference</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$P = 0 \text{ atm}$</td>
<td>$P = 8.9 \text{ atm}$</td>
<td>$P = 0 \text{ atm}$</td>
<td>$P = 8.9 \text{ atm}$</td>
</tr>
<tr>
<td>150 kDa</td>
<td>8.92</td>
<td>8.17 ± 0.11</td>
<td>8.72 ± 0.26</td>
<td>8.41 ± 1.20</td>
<td>-7.58 ± 0.249</td>
</tr>
<tr>
<td>500 kDa</td>
<td>14.6</td>
<td>13.5 ± 0.39</td>
<td>15.3 ± 0.45</td>
<td>13.5 ± 2.54</td>
<td>-8.67 ± 1.15</td>
</tr>
<tr>
<td>2000 kDa</td>
<td>27.7</td>
<td>*26.5 ± 0.58</td>
<td>28.5 ± 0.52</td>
<td>5.14 ± 1.81</td>
<td>-4.40 ± 1.33</td>
</tr>
</tbody>
</table>

*shows significant difference from the empirical value

The light scatter data shows good agreement with the values calculated using the empirical relation. It is assumed that no damage occurs to the molecules when exposed to cavitation. The use of a student’s $t$-Test (two tailed, critical value $t_{0.95}$, $P > 0.05$) (28) shows no significant difference between non-sonicated ($P = 0 \text{ atm}$) and sonicated ($P = 8.9 \text{ atm}$) samples. All samples but the non-sonicated 2000 kDa dextran show no significant difference from the empirical (calculated) values. Data from the $t$-Test application is given in the Appendices.

Figure 4.7 (a) is the curve of % viability as a function of radius. The number of cells in the high uptake population decreases with molecule size. Overall viability shows little effect with radius and is consistent with previous data for the cavitation conditions studied.

Figure 4.7 (b) shows molecular uptake as a function of molecule radius. It was possible to achieve uptake of all five molecules. Little change with pressure is observed.
Discussion: Uptake is reported in the literature for many types of molecules. However, the maximum size of a particle that can be taken in the cell has not been determined. Since molecules are believed to be taken into the cell through disruptions created by cavitation bubbles of varying size (24), the size and shape of the disruptions may also vary. The cavitation effect is mediated by the amount of pressure applied, as well as the frequency of the sound wave; therefore, differing ultrasound conditions may create different types and sizes of disruptions. The size of the molecule that can be taken into the cell may be mediated by the conditions of the applied ultrasound.

The number of molecules that can be taken into a cell can reach a maximum, most likely based upon available volume of the cell. Although the dextrans were seen to be reasonably monodisperse using dynamic light scatter, they do represent a range of sizes and the uptake may represent only the smallest molecules of the sample, which are limited in number.

Conclusion: Molecules of at least 28 nm radius (i.e. 2,000,000 Da) can be taken up by sonicated cells, indicating that membrane disruptions are at least that large.

4.8 Imaging using confocal microscopy

To obtain images of control cells and sonicated cell, samples were sonicated for 2 s total on time with 0.1 s pulse length and 10 % duty cycle at 7.10 atm, using five
fluorescent molecules. The samples were stained with a nuclear dye and propidium iodide, then highly concentrated and fixed on microscope slides.

Results: Figure 4.8 shows confocal images of cells at 40X magnification and various numerical apertures. Images were obtained using 3 lasers: blue (nuclear stain), red (propidium iodide, non viable cells), and green (uptake of fluorescent molecules). In uptake samples, all channels but the green were turned off in order to show the location of the uptake molecule in the cell. In some cases, dark areas, such as in figure 4.8 (e), show an area that appears to be excluded from uptake (e.g. the nucleus of the cell). In figure 4.8: (a) The control sample represented as blue stained nuclei; (b) calcein is present throughout the entire cell, including the nucleus; (c) bovine serum albumin is present throughout the cell, but does not appear as concentrated as the calcein; (d) 150 kDa dextran is present within the cytosol, but excluded from the nucleus of the cell; (e) 500 kDa dextran is present in the cytosol, but not the nucleus of the cell; (f) 2000 kDa dextran is present in the cytosol, but appears to be locally concentrated in particular regions of the cell. Confocal images show that all five molecules are transported internally to the cell.

Discussion: To strengthen the molecular uptake data, discussed above (section 4.7) confocal microscopy was used to show that the fluorescence assayed using flow cytometry was the result of actual molecules internal to the cell and not surface bound. This microscopy takes images through sections of the cell and shows the internal
composition. The entire sample was used for microscopy in this study and no samples were assayed for uptake with flow cytometry. This microscopy does not have high enough resolution to show individual molecules and quantification of the actual size or amount of molecular uptake was not performed for these data.

Conclusion: All five molecules are taken into the cell and are present throughout the cytosol.

4.9 Freeze Fracture and imaging with transmission electron microscopy

To obtain images of cell membranes using freeze fracture, samples were sonicated for 2 s total on time with 0.1 s pulse length and 10% duty cycle at one pressure, 5.34 atm, then fixed by plunge freezing, fractured, and imaged using TEM. Three types of samples were studied: a) control with no sonication; b) frozen less than 1 minute after sonication; c) frozen 1 hour after sonication. No fluorescent molecules were added to the samples.

Results: Figure 4.9 shows TEM images of freeze fractured cells. In (a) and (b), control samples show an intact cell membrane. The view shown contains a single round cell that fills most of the figure. In (c) and (e), samples frozen less than 1 minute after sonication show “disruptions.” A close up of the disruptions shown in (c) is shown in (d). Pictures (f) and (g) show samples frozen 1 hour after sonication, possible remnants of the disruption structures remain. A control cell is shown in (h) and (i), the presence of
surface proteins is observed as the darker region, the lighter region is probably the hydrophobic region of the cell membrane, exposed during fracture of the cell. In samples frozen less than 1 minute after sonicating, the presence of structures in the cell membrane are observed. When focusing into these structures, regions similar to the control sample image of the hydrophilic part of the cell membrane are observed. Samples frozen 1 hour after sonicating do not show these structures, and appear “smoother” than the control samples. Observations were made over 18 hours (3 days x 6 hours/day) of microscope time. Over the course of the microscope time, 10 control cells with structures that could be interpreted as the surface of the cell membrane were observed, 15-20 cells fixed less than 1 minute after sonication were observed to contain similar structures to those shown in (c), (d), and (e), and 10-15 cells fixed 1 hours after sonication were observed to be similar in appearance to those shown in (f) and (g).

Discussion: Little is known about the actual physical effects of cavitation to the cell. Our studies concerning the lifetime of the disruptions have indicated that they are no longer present or no longer available for uptake after approximately 1 minute. Therefore, the cells must be fixed rapidly. Freezing is a rapid method, but ice damage often occurs. This method is useful for imaging, but is not often used for quantifiable results. The images are difficult to interpret and due to the nature of the fracture method, membranes are often “split apart” at the hydrophobic region of the membrane, giving a less useful picture of the cell membrane, or through the cytosol of the cell, showing internal structure, but not the cell membrane.
Conclusion: Comparison to micrographs of freeze fractured cell membranes in the literature (25) suggests that the images showing the difference between the outside of the cell membrane (hydrophilic region) and the inside (hydrophobic region) to have an accurate interpretation. The observed structures appear only in samples sonicated for less than 1 minute and may represent the physical effect of acoustic cavitation upon the cell membrane.
Control sample: described as 100% viable with no uptake. 100% of cells are in the first population.

Histogram of sonication at moderate pressure showing 2 populations. Region 1 is defined as the low uptake population and region 2 is defined as the high uptake population. MiXa describes the proportion of population 1 to be 78% and the proportion of population 2 to be 22%.

Histogram of sonication at high pressure showing 2 populations. Region 1 is defined as the high uptake population and region 2 is defined as the low uptake population. The proportions are: 36% and 64%, respectively.

Histogram depicting a sonicated sample at low pressure. The proportions are 98% and 2%, respectively. This result is reported as no uptake.

Figure 4.1 Representative Histograms from WinMDI 2.8
Figure 4.2 (a) The % viability is shown as a function of cell density at 5.3 atm.
Figure 4.2 (b) The uptake of calcein is shown as a function of cell density at 5.3 atm
Figure 4.3 (a) The % viability as a function of axial position is shown at 5.3 atm
Figure 4.3 (b) The uptake of calcein is shown as a function of axial sample position.
Figure 4.4 (a) The % viability is shown as a function of pressure.
Figure 4.4 (b) The uptake of calcein is shown as a function of pressure.
Figure 4.5 (a) Histograms depicting calcein uptake as a function of time after sonication ($P=7.1$ atm)

(i) control (ii) 0 s (iii) 15 s (iv) 30 s (v) 45 s (vi) 60 s (vii) 90 s (viii) 120 s
Figure 4.5 (b) % viability is shown as a function of time, the top graph is at 5 atm and the bottom is at 7 atm.
Figure 4.5(c) The uptake of calcein is shown as a function of time after sonication.
Figure 4.6 (a) % viability as a function of time for bovine serum albumin, the top graph is at 5 atm and the bottom is at 7 atm.
Figure 4.6 (b) The uptake of bovine serum albumin is shown as a function of time after sonication.
Figure 4.7 (a) % viability is shown as a function of molecular radius.
(i) 5 atm (ii) 7 atm (iii) 9 atm
Figure 4.7 (b) The apparent % equilibrium is shown as a function of increasing molecular radii.
Figure 4.8 Confocal images of DU 145 prostate cancer cells.
(a) control, (b) calcein, (c) bovine serum albumin (d) 150 kDa dextran (e) 500 kDa dextran (f) 2000 kDa dextran. Samples showing uptake were sonicated at 24 kHz and 7.1 atm for 2 seconds total on time.
Figure 4.9 TEM images of DU 145 prostate cancer cells

(a) TEM of DU 145, magnification = 4,000 X
A control cell (unsonicated) is shown as a round object with a rough surface.

(b) TEM of DU 145, magnification = 4,000 X
A control cell is shown. This cell is less clearly defined than (a), but displays a similarly rough surface.
(c) TEM of DU 145, Magnification = 4,000X
Cell sonicated at 5.3 atm and then fixed less than 1 minute after sonicating. Structures that appear to be disruptions in the cell membrane are observed.

(d) TEM of DU 145. Close-up of (c) Magnification = 8,000X
A closer view of the structures shows what might be structures internal to the cell membrane.

(e) TEM of DU 145
Magnification = 4,000X
Cell sonicated at 5.3 atm and fixed less than 1 minute after sonication. The image is less detailed than (c) but shows similar structures.
(f) TEM of DU 145 Magnification = 4,000X
Cell sonicated at 5.3 atm and fixed 1 hour after sonication. The surface appears smoother than the control and structures that appear to be “tears” in the cell membrane are observed.

(g) TEM of DU 145 Magnification = 4,000X
Cell sonicated at 5.3 atm and fixed one hour after sonication. Cell surface is very smooth with a single structure similar to those in (f).
(h) TEM of DU 145 Magnification = 4,000X
A control cell that has been fractured (in part) along the hydrophobic part of the cell membrane. The darker portion is the surface of the cell and the lighter portion is the hydrophobic part of the membrane.

(i) TEM of DU 145 Magnification = 10,000X
Close-up of (h) showing the surface of the cell and structures internal to the cell membrane.
CHAPTER 5

5.1 Recommendations

Due to the nature of the circular transducer, this system cannot use a higher frequency and cannot be focused. Neither the amount of oxygen in the water bath nor temperature is measured or controlled, two variables that may have effects upon cavitation. The existing apparatus and protocols provide useful data, but in an improved system, with better control of variables and capacity for focused application and higher frequencies, would make more accurate measurements and allow a greater variety of experiments to be performed.

Although some acoustic spectra were collected during this work, they were not analyzed in this study. Comparison of spectra to data presented here may be of value for studies that quantify acoustic cavitation.

5.2 Future work

Using the conditions and apparatus in this study, the following work would help to clarify the presented data.

In order to determine if intracellular uptake is induced by cellular mechanisms (e.g. pinocytosis), it is recommended that red blood cell ghosts or liposomes (non-living
cellular membranes) be sonicated in the presence of fluorescent molecules and assayed using flow cytometry.

A more complete time study with molecules larger than bovine serum albumin is suggested to show if disruption lifetime is molecule (size) dependent.

A study of long-term viability and function after sonication would better determine cell viability. Since viability is quantified the same day, it is unknown whether cavitation affects cells' ability to function normally. This can be performed, for example, by plating sonicated samples and determining viability over time.

The 2000 kDa dextran molecule is the largest commercially available fluorescent dextran, however, molecules larger than 28 nm should be used to determine the maximum size molecule that can be taken into the cell.

The effect of sonication upon delivered molecules needs to be considered for any gene therapy applications. Inert molecules, e.g. dextrans, were shown to have relatively unchanged radii. Plasmids or oligonucleotides must be undamaged to be effective. This can be performed by delivery of DNA with fluorescent expression.
A quantification (size and number per cell) of acoustically-induced disruptions should be performed with a different method for cell fixation. Additionally, the exact nature of disruptions created by cavitation remains to be fully determined.

It is possible that the use of a simple membrane structure, (e.g. liposomes) would make the imaging disruptions easier, but may not show the effect to live cells.
REFERENCES


11. K. Keyhani, in progress.


25  http://utk-biogw.bio.utk.edu/Bio2


## APPENDIX I

**Typical Data Sheet for Experiments, generated using Excel (Microsoft)**

### Date: 

Experiment #

- water degassed:3h

### Description:

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<th>Calibration</th>
<th>Vpp (V)</th>
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</table>
APPENDIX II

Protocol for Plunge Freezing DU 145 cell suspensions

1. After harvest, combine cell pellets from multiple flasks and resuspend as one highly dense solution: 1 ml RPMI-1640 or PBS/ per l flask cells, mix well
2. Load cells into 0.35 ml sample volume (SAMCO PADL-PET) chamber
3. Sonicate using standard ultrasound parameters
4. After sonication, transfer cell suspension to microcentrifuge tube and load 5-10 μL of cell suspension into a 10 μL pipet (fine tip)
5. Load solid gold planchette (< 1 μL of sample/planchette): DO NOT OVERLOAD
6. Using fine tip forceps, quickly plunge planchette containing sample into liquid propane bath
7. Quickly place frozen sample into labeled microcentrifuge tube and store in liquid nitrogen.

Important: Once the sample is frozen, it must not be allowed to thaw. It is necessary to work quickly to keep samples in liquid nitrogen until they are fractured (Balzer 360 fracture device)

Perform in chemical hood:
To liquify propane, modify gas propane container outlet to allow a stream of gas to be release, cool the liquid propane reservoir in liquid nitrogen (approximately 5 minutes), slowly add liquid propane gas to the reservoir, it will cool and liquefy with time. Leave the reservoir suspended in the liquid nitrogen for the duration of the experiment. When finished, remove the reservoir from the liquid nitrogen and allow the propane to vaporize. Dispose of the liquid nitrogen.
APPENDIX III

EXAMPLE OF STATISTICAL SOFTWARE FOR STUDENT'S $t$-TEST

EasyStat®

Student's t-Test

© Vitaliy Pozharov and Olympus Research, 1998

This program calculates Student's $t$ value, compare it with a critical value $t_{0.95}$ (two tailed) and makes the decision if the differences between means is by chance only (if $P>0.05$) or is statistically significant (if $P<=0.05$) at the 95% level of significance. Enter data for both series and press <Submit> button. Have fun!

More complicated programs available from the author. Please send requests or comments to: vitaliy@xmission.com

Enter data for the first series here (N, SD or SEM):

N1 : 3
Mean1 (average) : 30.7
SD1 : 4.04
SEM1 : 2.333

Enter data for the second series here (N, SD or SEM):

N2 : 3
Mean2 (average) : 41
SD2 : 1
SEM2 : .578

Results:

Degrees of freedom 4
Student's $t$-test value 4.287
Critical $t_{0.05}$ value 2.776

$P <$0.05
t-Tests of Cell Density

Total cell population % viability:

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High uptake population % viability:

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Dynamic light scatter (DLS) data of dextrans:

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