SONOLUMINESCENCE AS AN INDICATOR OF CELL MEMBRANE DISRUPTION BY ACOUSTIC CAVITATION

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Abstract—Ultrasound (US) has been shown to transiently disrupt cell membranes and, thereby, facilitate the loading of drugs and genes into viable cells. Because these effects are believed to be mediated by cavitation, we hypothesized that measured levels of cavitation-induced sonoluminescence should correlate with levels of US bioeffects. We, therefore, quantified the number of calcein molecules delivered and the loss of viability in prostate cancer cells exposed to 24-kHz US over a range of different pulse lengths (1 to 100 ms), total exposure times (0.1 to 10 s) and pressures (1.0 to 9.8 atm). Consistent with previous observations, uptake increased and viability decreased with increasing pulse length, total exposure time and pressure. As a new observation, we established correlations between the amount of light produced by sonoluminescence and both molecular uptake and cell viability. These results support a cavitation-based mechanism for these bioeffects and suggest a means to control US effects on cells using sonoluminescence-based feedback. (E-mail: mark.prausnitz@che.gatech.edu) © 2001 World Federation for Ultrasound in Medicine & Biology.

Key Words: Ultrasound, Cavitation, Sonoluminescence, Drug delivery, Gene delivery, Feedback control, Cell viability, Molecular transport.

INTRODUCTION

One of the critical elements of medical 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., for gene therapy) (Rolland 1998), into tissues (e.g., for targeted chemotherapy of tumors) (Mir and Orlowski 1999) and across tissues (e.g., for 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 (US) 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 US to be focused almost anywhere in the body (Kremkau 1998). Limitations may include difficulty creating ultrasonic cavitation in vivo, where dissolved gas content is low and tissue structure may constrain bubble growth (Miller et al. 1996).

A number of studies have shown that US 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 and Hahn 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 US can be used for drug and gene delivery motivated our study, in which we strove to better elucidate the mechanism of action of US and develop methods to predict and control its effects on cells.

Our approach to controlling the biologic effects of US 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. This correlation could be used to determine the effects a given exposure of US had on cells based only on a relatively simple measurement of the acoustic spectrum. Using a related approach, Wyber et al. (1997) correlated US bioeffects (cell viability) with an iodine-based measurement of cavitation. Although the assay of the latter 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 US bioeffects. In this study, we seek to correlate observed bioeffects (molecular uptake and cell viability) with another physical phenomenon associated with cavitation; viz., sonoluminescence.

Sonoluminescence is a flash of light that occurs when cavitation bubbles collapse violently (Crum 1994; Leighton 1994). In addition to generating light, cavitation bubble collapse can create extremely high local temperatures and pressures, high-velocity jets of fluid and the generation of free radicals. We believe that these phenomena lead directly or indirectly to cell membrane disruption. During experiments, we measured changes in molecular uptake and cell viability induced by a broad range of US conditions and also measured the amount of sonoluminescent light generated. Our hypothesis is that, because cavitation is believed to cause the observed bioeffects, then a measure of cavitation (i.e., sonoluminescence) should correlate with these bioeffects over the many US conditions examined.

**MATERIALS AND METHODS**

*Ultrasound apparatus*

To determine the effects of acoustic cavitation on molecular uptake and cell viability, and to correlate those effects with sonoluminescence, US was applied to cell suspensions using a cylindrical piezoelectric transducer and sonoluminescent light output was measured using a photomultiplier tube (PMT) (Fig. 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 a water bath containing 200 mL deionized water that was degassed for 3 h using a vacuum chamber (Nalgene, Rochester, NY) and pump (KNF Neuberger, Trenton, NJ). Degassing the water bath removes bubble nucleation sites and, therefore, limits the occurrence of the majority of cavitation and, hence, sonoluminescent light output to within the cell sample.

The US 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 (24 kHz, Channel Industries, Santa Barbara, CA) (Fig. 1). This system controlled the frequency, duty cycle, incident pressure, pulse length and exposure time.

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 personal computer and stored for analysis. The voltage applied to the transducer was converted to an incident pressure using the empirical correlation, \( P = 0.0083 \times V \), where \( P \) is incident pressure (atm) and \( V \) is voltage applied to the transducer. This correlation was established by measuring pressure as a function of transducer voltage at subcavitation pressures and then extrapolating this relationship to higher pressures. In this case, pressure was measured using a calibrated hydrophone (Model 8103, Brüel and Kjær, Norcross, GA) placed in the axial and radial center of the transducer. Because the cylindrical transducer used here creates a heterogeneous acoustic environment, pressure variation was measured as a function of radial and axial position within the sample chamber volume containing cells. Radial variability in pressure across the width of the cell sample chamber (9 mm wide) was less than 10% and axial...
variability up and down the cell sample chamber height (21 mm tall) was less than 20%.

Preparing cell samples
DU 145 prostate cancer cells (American Type Culture Collection, Rockville, MD) were grown and harvested using standard protocols (Canatella et al. 2001). Briefly, cells were supplied with growth media (RPMI-1640 with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin; Cellgro, Herndon, VA) and grown to approximately 90% confluency on T-150 cell culture flasks (BD Falcon, Franklin Lakes, NJ). The cells were then harvested during their exponential growth phase using trypsin. The sample chambers (Sedi-Pet transfer pipette, No. 241, SAMCO, San Fernando, CA; stems cut to 2 cm in length) each contained 1.2 mL of a well-mixed suspension of 10^6 cells/mL in a 10-μM solution of calcein (Molecular Probes, Eugene, OR). This 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” (1.6 mm) 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 US at 24 kHz and 10% duty cycle was applied to the cell sample using combinations of 3 pulse lengths (1 to 100 ms), 3 total exposure times (0.1 to 10 s) and 10 pressures (1.0 to 9.8 atm). Negative controls were carried out in the same manner, except no voltage was applied to the transducer (i.e., “sham” exposure). After US 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 × g, 3 min, Eppendorf 5415 C, Brinkmann Instruments, Westbury, NY), resuspended in Dulbecco’s phosphate-buffered saline (Cellgro), and kept on ice until analysis the same day.

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 nonviable 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 US application (i.e., the product of the measured cell-to-bead ratio in each sample and the known bead concentration yields the cell concentration in each sample) (Prausnitz et al. 1994).

A FACSScan 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 nonviable cells. At least 10,000 viable cells were collected from each sample. Sample fluorescence was 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 nonviable 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, Fisher, IN, d = 7.2 μm, lot #B00136) with known fluorescence intensity (Prausnitz et al. 1994).

Data analysis
As described previously (Guzmán et al. 2001; Cochrane 2000), WinMDI 2.8 (Windows Multiple Documents Interface, Joseph Trotter, Scripps Research Institute, La Jolla, CA) was used to analyze flow cytometry data to determine the fraction of cells that were viable and, among the viable cells, the number of calcein molecules per cell. Then, molecular uptake data were imported to a Microsoft Excel (version 9.0) spreadsheet using Ldata, 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. The mean absolute percent error associated with these fits was approximately 30%.

Sonoluminescence analysis
Light output data gathered by the digital oscilloscope (Hewlett Packard, 54603B) was downloaded to a computer (Ginstar Computer, Norcross, GA) for analysis. Light output generated during all US bursts applied to a given cell sample were combined to yield an average light vs. time trace of a single “representative” burst for each sample, such as shown in Fig. 2.

Statistical methods
To assess the relation of various aspects of sonoluminescence to cell viability and molecular uptake, we fitted several regression models incorporating restricted cubic splines in S-Plus (MathSoft, Seattle, WA) (Venables and Ripley 1999). Most models included a significant nonlinear component, and we used multiple R^2 values to compare the fits of the various models. This
Heterogeneous molecular uptake

Prostate cancer cells exposed to 24-kHz US over a range of different pressures, pulse lengths and total exposure times, internalized large numbers of calcein molecules from extracellular solution. However, the number of calcein molecules taken up by cells showed significant heterogeneity. Figure 3 displays 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 (Fig. 3A) shows a single peak with weak background fluorescence probably due to autofluorescence, membrane-bound calcein, and optical and electrical noise. Histograms of sonicated samples (Fig. 3B–E) show broad distributions of fluorescence, which indicates heterogeneous amounts of calcein uptake within each population 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 valley in between was seen in most samples. The exceptions were distributions that looked more similar to control samples (e.g., Fig. 3A).

As described previously (Guzmán et al. 2001), to facilitate analysis, we divided each uptake histogram into three subpopulations: nominal-uptake population (NUP), which corresponds to the low-fluorescence peak and is interpreted as indistinguishable from controls; low-uptake population (LUP), which corresponds to the valley between the peaks and is interpreted as cells with intermediate amounts of uptake; and high-uptake population (HUP), which corresponds to the high-fluorescence peak and is interpreted as cells with the maximum possible uptake (i.e., approaching equilibrium with the extracellular environment).

Although the relative sizes of these subpopulations depended on US conditions, the number of molecules taken up within each of the three subpopulations remained the same order of magnitude (see data and discussion below). Graphically, this means that the heights of the peaks changed, but their positions did not vary much. For all of the US conditions examined in this study, the average number of molecules, in millions per cell, taken up by NUP cells was $0.035 \pm 0.041$, LUP cells was $2.55 \pm 0.47$, and HUP cells was $8.70 \pm 0.88$ (average $\pm$ SEM). Based on a cell volume of $2 \times 10^{-9}$ mL per cell (measured using a Coulter counter), the average calcein concentration within HUP cells was 7 $\mu$M. Because calcein was supplied extracellularly at 10 $\mu$M, this supports the interpretation that HUP cell uptake approaches equilibrium.

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 Fig. 4. The overall height of each bar represents cell viability, and the three stripes within each bar represent the fraction of cells in NUP (gray), LUP (white) and HUP (black). Each graph shows the effect of pressure; each row corresponds to a different pulse length and each column to a different total exposure time. 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 atm. Except when cell viability is low (i.e., $< 25\%$), the sum of LUP and HUP cells in each graph is relatively constant at $21\% \pm 10\%$ (mean $\pm$ SD).

Figure 5 replots the data shown in Fig. 4 to better display the effects of total exposure time and pulse length. In each graph, pressure is held constant and the...
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. 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. (In Fig. 5, asterisk indicates viability that is lower than that measured at 1 ms, with statistical significance of $p < 0.05$ by Student’s $t$-test).

Correlation of sonoluminescence with molecular uptake and cell viability

Although the data presented in Figs. 4 and 5 characterize the effects of US 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 US conditions tested. The parameter we selected was sonoluminescence, or the light generated by cavitation. Our hypothesis was that, because cavitation is believed to cause the observed effects on cells, then a measure of cavitation (i.e., sonoluminescence) would correlate with these bioeffects over the broad range of US conditions used in this study.

To test this hypothesis, we have replotted the uptake and viability data, not as a function of US parameters as shown in Figs. 4 and 5 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 US exposure chamber. Figure 6 shows that the bioeffects measured in this study correlate with sonoluminescence. The many graphs shown in Figs. 4 and 5 can be reduced to the set of single curves shown in Fig. 6.

As mentioned above, molecular uptake associated with each subpopulation is significantly different (i.e., nominal, low or high), but uptake within each of the three subpopulations is generally of the same order of magnitude, although there is some scatter (Fig. 6A). Fitting these data with nonlinear regression models yielded multiple $R^2$ values of 0.09 (NUP), 0.13 (LUP) and 0.26 (HUP). These poor correlation coefficients indicate that the number of molecules per cell did not depend strongly on sonoluminescence light output. However, with increasing integrated light output, there were 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 value, we separated each population into nominal uptake (NUP), low uptake (LUP) and high uptake (HUP) subpopulations by fitting three Gaussian distributions, as illustrated in (C) (Guzmán et al. 2001). Ultrasound exposures were performed at 24 kHz for 2-s total exposure time and 10-ms pulse length at 10% duty cycle.

Calcein fluorescence is shown in arbitrary units.
statistically significant decreases in LUP and HUP molecules per cell and an increase in NUP molecules per cell (p < 0.001 for each association).

Figure 6B–E indicates an apparent threshold at an integrated light output near $10^{-2} \text{ V} \times \text{s}$, 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% to 30%) are in the LUP or HUP subpopulations. The observed associations between integrated light output and cell viability (Fig. 6B; $R^2 = 0.75$) and percent cells in NUP (Fig. 6C; $R^2 = 0.74$) were much stronger than those with percent cells in LUP (Fig. 6D; $R^2 = 0.21$) or HUP (Fig. 6E; $R^2 = 0.29$).

In Fig. 6, effects on cells are seen to correlate strongly with total integrated light output, which is defined as the average integrated light output during a pulse.
Fig. 5. Data from Fig. 4 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. Asterisks indicate viability lower than at 1 ms, with statistical significance, $p < 0.05$, by Student’s $t$-test. See Fig. 4 caption for more information.
(e.g., the integral of the curve shown in Fig. 2) multiplied by the total number of pulses applied during the given exposure ($R^2 = 0.75$ for a fit to cell viability). We also assessed the relation of other features of the measured sonoluminescence to cell viability, but none correlated as well as integrated light output. For example, we examined the peak level of light output (e.g., $10^{-2} \text{ V} \times \text{s}$). (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., $10^{-2} \text{ V} \times \text{s}$). All of the data from Figs. 4 and 5 collected over a broad range of US conditions are re-plotted here and shown to correlate with sonoluminescence light output.

Another approach was to correlate bioeffects with sonoluminescence only above a threshold, because weak cavitation that created low levels of sonoluminescence might not produce bioeffects. However, the association of viability with light output integrated above a number of different thresholds (see Fig. 2) was progressively stronger as the threshold was lowered (e.g., for a threshold of $-10 \text{ mV}$ light intensity, $R^2 = 0.37$, data not shown), but the correlation was highest at a threshold of zero (i.e., total integrated light output shown in Fig. 6,

Fig. 6. Uptake and cell viability shown as functions of sonoluminescence light output. (A) Calcein molecules per cell in each of the three subpopulations are relatively independent of light output. (B) Cell viability decreases sharply above a threshold level of light output (e.g., $10^{-2} \text{ V} \times \text{s}$). (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., $10^{-2} \text{ V} \times \text{s}$). All of the data from Figs. 4 and 5 collected over a broad range of US conditions are re-plotted here and shown to correlate with sonoluminescence light output.
DISCUSSION

The most significant observation from this study is the overarching correlation between cavitation-induced sonoluminescence and US effects on cell viability and molecular uptake. Although previous studies have measured sonoluminescence in the context of studying acoustic bioeffects (Miller et al. 1989; Carstensen et al. 1993), we believe that the data in Fig. 6 represent the first demonstration of a broad correlation. 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 US 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 US exposure. For example, a tissue in the body or suspension of cells in the lab could be exposed to US while simultaneously monitoring sonoluminescent output. After the total integrated light output reached a desired level (e.g., $10^{-3} \text{V} \times \text{s}$ 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 US, can be applied and, thereby, potentially achieve more reproducible bioeffects. A possible limitation is that sonoluminescence-based feedback may be limited to catheter- or endoscope-based scenarios, where US can be applied and assessed in darkness within the body. In addition, the low gas content and physically constrained environment within tissues may make generation of acoustic cavitation difficult in vivo, although appropriate acoustic conditions, possibly coupled with the use of cavitation nuclei (e.g., contrast agents), may generate sufficient cavitation for cell membrane disruption.

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 valley in between. This type of distribution has been observed before using a similar protocol, but involving US applied at 500 kHz (Guzmán et al. 2001), which leads us to believe that this heterogeneity in uptake may be a general feature of US-disrupted cells. It is not currently clear whether heterogeneity is due to cell-based differences in their response to US or physically-based differences due to nonuniformities in US-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 (NUP). Additional work is needed to provide a more complete 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 US parameters. Generally, larger 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; Guzmán et al. 2001). This result is consistent with a cavitation-based mechanism, because 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 often had significantly less effect on cell viability than 10- or 100-ms pulses (Fig. 5). 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 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. (2001) did not observe a dependence on pulse length for prostate cancer cells exposed to 500 kHz US for pulse lengths between 20 $\mu$s (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. (2001), which served as nucleation sites and, thereby, facilitated more rapid onset of cavitation (Flynn and Church 1988).

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