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An *In Vitro* Study of the Correlation Between Bubble Distribution, Acoustic Emission, and Cell Damage by Contrast Ultrasound

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Abstract

The objective of this study was to investigate the influences of total exposure duration and pulse-to-pulse bubble distribution on contrast-mediated cell damage. Murine macrophage cells were grown as monolayers on thin polyester sheets. Contrast agent microbubbles were attached to these cells by incubation. Focused ultrasound exposures ($P_r = 2$ MPa) were implemented at a frequency of 2.25 MHz with 46 cycle pulses and pulse repetition frequencies (PRF) of 1 kHz, 500 Hz, 100 Hz, and 10 Hz in a degassed water bath at 10 or 100 pulses. A 1 MHz receive transducer measured the scattered signal. The frequency spectrum was normalized to a control spectrum from linear scatterers. Photomicrographs were captured before, during, and after exposure at a frame rate of 2000 fps and a pixel resolution of 960×720 . Results clearly show that cell death is increased, up to 60%, by increasing total exposure duration from 0 ms to 100 ms. There was an increasing difference in cell damage between a 10-pulse exposure and a 100-pulse exposure with increasing PRF. The greatest change in damage occurred at 1000 Hz PRF with a 53% increase between 10-pulse and 100-pulse exposures. For each pulse from 0 to 10, an overlay of the $2 \mu\text{m}$ bubble count with corresponding emission shows consistent behavior in its pulse-to-pulse changes, indicating a correlation between acoustic emission, bubble distribution, and cell damage.

I. Introduction

Contrast agents for diagnostic ultrasound consist of a suspension of stabilized gas bubbles (i.e., gas bodies). Owing to their microscopic size (typically 2 to 5 μm in diameter) these gas bodies have the ability to pass through the circulation and augment the echogenicity of blood-filled regions during diagnostic imaging. They have recently been the subject of extensive research and commercial development for diagnostic medical applications. Enhanced echogenicity is achieved because gas bubbles of this size strongly scatter ultrasound in the mega-Hertz frequency range. They can resonate at a fundamental frequency, producing increases in backscatter of up to 3 orders of magnitude [1]. Contrast agent gas bodies act as cavitation nuclei in the presence of an appropriate acoustic field, which can result in a strong enhancement of acoustic emissions available for imaging applications [2]–[4].

The bioeffects potential is presently not well understood, and it has been the subject of recent bioeffects research [5]–[7]. A useful *in vitro* system for research on bioeffects mechanisms consists of a cell monolayer grown on the upper inside window of an ultrasound (US) exposure chamber. Adenosine triphosphate release, which indicates cell membrane damage, was detected in monolayer cultures with added Alunex (Molecular Biosystems Inc., San Diego, CA) contrast agent during US exposure [8]. Membrane damage thresholds are less than the pressure amplitudes needed for nucleation of inertial cavitation suggesting a form of gas body

activation [9]. Both lethal and repairable sonoporation were observed in Jurkat lymphocytes in suspension with the addition of varying amounts of Optison (GE Healthcare, Chalfont St. Giles, UK) [10]. In addition, pulses from an actual diagnostic scanner have been shown to produce sonoporation of monolayer cells attached to Optison gas bodies [11]. Moreover, studies by Miller using Alunex showed that the strong nonlinear scattering of ultrasound by contrast agent gas bodies appears to provide useful indicators of gas body activity including cavitation bioeffects [8].

The objective of this study was to investigate the effects of total exposure duration on contrast-mediated cell damage. Several studies have examined contrast-related acoustic emissions [12], [13] and bioeffects [14], [15]. However, a comprehensive understanding of the relationship between these factors is lacking. Such an understanding can contribute significantly in the development of an indicator of potential exposure effects. An accurate and consistently reliable indicator might find immediate use in research on drug and gene delivery and clinical value for avoiding bioeffects in diagnosis.

The results of the previous study showed that cell damage following 10 pulses at 1 kHz pulse repetition frequency (PRF) is lower than that at 100 Hz, 10 Hz, 1 Hz, or 0.1 Hz [16]. That suggests that if subsequent pulses arrive rapidly, that is, a high PRF, there is then a reduced effect of cell damage for the same number of pulses. This, however, may only be applicable to situations where a limited number of pulses interacts with the bubbles. Should pulses continue to arrive, such as 100 pulses at a PRF of 1 kHz, then this reduced effect may not hold. Perhaps the reduced effect at high PRF is indicative of some evolution of the shelled microbubble and requires a longer total exposure duration (>10 ms but <100 ms). Therefore, tests were developed to examine:

- The effect of total exposure duration in the range of 10 ms to 1 s.
- Pulse-to-pulse bubble distribution, to understand the process of microbubble evolution and its contribution to acoustic emission and cell damage.
- The correlation between pulse-to-pulse bubble distribution, acoustic emissions, and cell damage.

II. Methods

A. Cell Culture and Assay Methods

The cell monolayers were prepared using the same protocol as described previously [11]. Briefly, exposure chambers were constructed with 5- μ m thick Mylar sheets (DuPont Polymer Division, Circleville, OH) clamped in stainless steel rings with an o-ring seal and a 1.9-cm diameter cell culture area. A mouse macrophage-like cell line, RAW-264.7 (American Type Culture Collection, Rockville, MD) was cultured in monolayers on the chamber's Mylar holder under aseptic conditions. The contrast agent employed for this study was Optison (Perflutren Type A microspheres, Amersham Health, Princeton, NJ), which contains about 5 to $8 \cdot 10^8$ ml^{-1} gas bodies between 2 and 5 μ m in diameter. The monolayer was incubated for 15 min in culture medium (RPMI growth medium supplemented with 10% fetal calf serum and antibiotics) containing 5% Optison to allow for the adhesion of gas bodies to cells. Unattached gas bodies were removed by rinsing with phosphate-buffered saline.

B. Optical Equipment and Configuration

An illustration of the system employed for the detection of acoustic emission from microbubbles and cells with optical feedback is shown in Fig. 1. An inverted microscope (Leica DMIL, Bannockburn, IL) was used for mounting and viewing the specimen. A $20\times$ long-working-distance (LWD) objective (Model M Plan Apo $20\times$, Mitutoyo, Aurora, IL) was

adapted to be used in conjunction with the inverted microscope. Because the LWD objective was almost twice as long as a regular objective of the same magnification, the microscope stage had to be raised from its default position to allow the objective to fit properly under it. Two support adapters of appropriate length were fabricated and placed between the microscope base and the stage.

The experiments were conducted in a cylindrical Plexiglas tank (2.5 in. height $\times$ 6.75 in. diameter) with an optical glass window (1.5 in. diameter) centered at the bottom. The tank rested on the microscope stage. The proximity of the water-air interface to the target specimen at the bottom of the tank would have compromised the acoustic environment. Resulting standing waves from the water-air impedance mismatch would have reflected the US beam back and thereby increased the exposure. Fig. 2 displays the configuration employed to minimize this effect. The dark narrow strip on top of the stainless steel rings represents 2 Mylar membranes within which the cell monolayer, culture medium, contrast agent, and Trypan blue were confined. Note that a disc-shaped sound absorbing material (6 mm height $\times$ 10 mm inner diameter) was placed at the bottom of the tank with the disc's opening centered on the optical glass window. To absorb as much of the divergent US beam past the Mylar holder as possible, the opening of the disc was fabricated to be smaller than the exposure chamber window. The beam path in the figure shows that most of the divergent beam reflected by the surface of the sound-absorbing disc would have been diverted away from the transducer focus and the component reflecting to the focus would have been significantly attenuated. The exposure chamber was placed on the sound-absorbing disc such that the Mylar holder was farthest from the optical glass window. The LWD objective, with a working distance of 20 mm, allowed for the optical viewing of the target specimen even in the raised position.

A CMOS sensor-based camera (Phantom V9, Vision Research Inc., Wayne, NJ) was used during exposures to capture optical information. The Phantom V9 can capture 1000 frames per second (fps) at the highest resolution of 1600×1200 and frame rates up to 160 000 fps can be achieved at lower resolutions. Exposure times as small as 2 μ s can be realized. Images are in grayscale. The camera was attached to the microscope using an F-mount to C-mount adapter. Two extension rings (model Nikkor, Nikon, Melville, NY) and a $2\times$ teleconverter (model Nikkor, Nikon) were inserted between the adapter and the camera to obtain the desired magnification ($\sim 40\times$). The camera was controlled by a PC running software supplied by the camera manufacturer (Phantom Camera Control Version 7.0.605, Vision Research Inc.). The camera's capture button saves frames in cine loops to its volatile memory. The trigger button marks the cine loop's start point. The total number of frames and pre-trigger frames can be assigned for inclusion in the cine loop. The user transfers the cine loop to the computer hard drive at the end of the capture to save it. While the capture command is manually given by the user, the trigger signal can be sent either by the user (by way of the camera software) or by an external source.

For the purposes of this experiment, the capture mode was initiated by the operator, after which the camera awaited actuation by an external trigger (the leading edge of a square pulse). The external trigger was provided by the same function generator supplying the trigger for acoustic pulses (described below). The camera was set to capture frames at a resolution of 960×720 and a frame rate of 2000 fps. That frame rate afforded the allotment of at least 2 frames per interpulse interval at the highest PRFs used (1 kHz). Once the capture button was pushed on the camera's user interface (UI), the camera initiated storage of frames in a cine loop in its non-volatile memory. The external trigger from the function generator (described below), then, marked the time when the corresponding frame in the cine loop was frame number 0. Frames numbering -10 to -1 were preset in the UI as pre-trigger frames. The pre-trigger frames ensured that all ultrasound-mediated microbubble activity was optically captured and prevented loss

of data from delays between trigger and camera activation. The maximum number of captured frames was set at 2235.

C. Acoustical Equipment and Configuration

The timing diagram of the control sequence for the equipment used in this study is shown in Fig. 3. A programmable function generator, function generator 2 (Model 3314A, Hewlett Packard, Palo Alto, CA), produced sinusoid pulses containing 46 cycles (pulse duration of 20.4 μ s) at a center frequency of 2.25 MHz. Sufficient signal-to-noise ratio and narrow bandwidth for the subharmonic and ultraharmonics was established by using long pulses. The HP 3314A (function generator 2) was triggered by function generator 1 (Model 33120A, Hewlett Packard) responsible for generating bursts of 10-cycle or 100-cycle square waves. The pulsing parameters for function generator 1 included 10 cycles at 1 kHz, 500 Hz, 100 Hz, and 10 Hz and 100 cycles at 1 kHz, 500 Hz, and 100 Hz. The equivalent of low PRFs occur in clinical settings as in the case of interval imaging where frame rates can be lower than 10 Hz. Interval imaging is employed to allow refill of tissue with contrast agents. The use of 10 Hz and 100 Hz is well within the scope of B-mode imaging, while 1 kHz is within the range of Doppler PRF settings. Each cycle of this function generator corresponds to a burst of 46 cycles generated by the HP 3314A (function generator 2) at the carrier frequency of 2.25 MHz. The pulsing parameters correspond to total exposure times of 10 ms, 20 ms, 100 ms, 200 ms, and 1 s. A 55 dB RF power amplifier (Model A-300, MKS, ENI Products, Rochester, NY) amplified the output from Function Generator 2.

The amplified signal was supplied to a 2.25 MHz transmit transducer (A305S, Panametrics, Waltham, MA). The scattered signals from the bubbles/cells within the chamber were detected using a 1 MHz transducer (A314S, Panametrics) which was most sensitive to the subharmonic response of the bubbles. The receiver has a peak frequency of 1.03 MHz and a -6 dB bandwidth of 66%. The lateral beam width is 2.3 mm (-6 dB FWHM) at the focus of each transducer as was determined using a calibrated planar hydrophone (Reference Shock Wave Hydrophone; Sonic Industries, Hatboro, PA). The received signals were amplified by 50 dB (Model MR 106 receiver; MetroTek, Beaverton, OR) before being digitized (described below). Both the exposure and receiver transducers were 1.9 cm in diameter and spherically focused at 3.8 cm. To limit the received signal to the monolayer scattering, the transducers were placed in planes perpendicular to each other and at an approximate angle of 45° to the plane of the Mylar membrane where the cells were attached. The transducers were aligned to overlap their foci at the center of the exposure window of the chamber (Fig. 1). Five exposures at a P_r of 2 MPa were made at each pulsing parameter in degassed water bath at 37°C . Five sham exposures were performed with exposure chambers containing monolayers, Optison, and Trypan blue placed in the water bath without ultrasound exposure. Photomicrographs were captured first, immediately after placement in the tank and second, 5 min later.

D. Data Acquisition Equipment and Processing Algorithms

Briefly, a high-speed digitizing board (PDA12A-125 MHz, Signatec, Corona, CA) in conjunction with Lab-VIEW (National Instruments, Austin, TX) was employed in the acquisition of the scattered signal $s(t)$ from the microbubbles and cells as sensed by the receive transducer. The data set for each exposure was transferred to a computer for analysis using Matlab (MathWorks, Inc., Natick, MA). A Hamming window of 16 μ s duration (35 cycles for a 2.25 MHz pulse) for the 46-cycle exposures was used to range gate for the central portion of the received pulse from the chamber. The frequency spectrum $s(f)$ was obtained using fast Fourier transform (FFT) on the windowed signal. The range gating ensured that the transient response of the receive transducer (ring up and ring down) was excluded in the analysis and 35 cycles (as used with most exposures) ensured high signal-to-noise ratio. The power spectrum $s^2(f)$ was calculated. Ten reference measurements (100 pulses at each of 10 independent

locations) from Zerdine (CIRS, Norfolk, VA) were obtained for P_r of 2 MPa, where each pulse represented a reference signal $r(t)$. Zerdine is a material used in tissue-mimicking phantoms and is used here as a model for linear scattering. The power spectrum $r^2(f)$ was calculated for each measurement pulse. The reference power spectrum was averaged over 100 pulses and 10 measurements locations. The relative integrated power spectrum (RIPS) was then computed as

$$\text{RIPS} = \frac{\int_{f_1}^{f_2} s^2(f)}{\int_{f_1}^{f_2} r^2(f)}.$$

The RIPS represents the acoustic power scattered by the microbubbles and cells when normalized to that from the static linear scatterers. The RIPS was calculated for a frequency bandwidth ($f_2 - f_1$) of 1 MHz, centered on the subharmonic ($1/2f_0 = 1.125$ MHz) and the ultraharmonic ($3/2 f_0 = 3.375$ MHz).

E. Cell Count Methodology and Procedures

A photomicrograph was captured before each exposure and 5 min following each exposure. A generally accepted protocol for Trypan blue exclusion test of cell viability states that 5 min allows sufficient time for the damaged cells to be stained while viable cells continue to exclude the dye [17]. The damaged cells, which are normally displayed as blue regions in color images, were not easily identifiable because all images acquired were in grayscale. A red gelatin filter in front of the camera addressed the problem by using optical filtering. Normally filters in the red/orange/yellow range absorb blue light and cause blue regions to appear lighter on a grayscale image than surrounding regions represented by other colors, thus increasing contrast. Cells that were damaged and stained blue were almost unidentifiable from the background (Trypan blue) while the viable ones appeared darker and showed clear membrane boundaries. Viable cells were quantified for damage assessment. A set of photomicrographs (pre-exposure, and 5 min post-exposure) corresponding to each exposure was used for cell counts and for a rough quantification of bubble-size distribution associated with each pulse. Representative photomicrographs of microbubble-attached cells is shown in Fig. 4. Pre-exposure in Fig. 4(a), pulse 5 in Fig. 4(b), pulse 10 in Fig. 4(c), and 5-min post-exposure in Fig. 4(d) photomicrographs are shown. The dark spherical objects in Fig. 4(a)–(c) are contrast microbubbles which, following the ultrasound exposure, are absent from Fig. 4(c). The viable cells (with membrane boundaries) can be clearly identified in these images.

Software provided by the camera manufacturer (Cine-View 630; Vision Research Inc.) permits the frame-by-frame playback of cine loops associated with each exposure. An effective magnification of $40\times$ ($20\times$ objective + $2\times$ teleconverter) was used while capturing the images. The chamber was allowed to sit for 5 min outside the bath to permit complete staining of the permeabilized cells after which the last photomicrograph of each exposure was captured. The percentage of cell death was calculated as 100 times the difference between the total number of viable cells in the pre-exposure and post-exposure photomicrograph divided by the total number of viable cells from the pre-exposure photomicrograph. The effect of detachment of cells during exposure on the quantification of cell death was not material. Exposures performed in investigating this behavior showed minimal detachment (1%–3% of cells exposed). The counting procedure, therefore, did not take into account cells that may have been detached from the Mylar during exposure.

F. Bubble Distribution Analysis Methodology

Previous studies have shown that most of the bubble response, or resonant bubble behavior in an acoustic field, occurs within the first 10 pulses [16]. Further elucidation of bubble response is provided in Section IV. Bubble distribution was, therefore, quantified for the first 10 pulses of each exposure at 100 Hz, 500 Hz, and 1000 Hz PRFs. Images from the cine loop were superimposed with micrometer images (both horizontal and vertical alignment of the micrometer) at the same magnification using the merge layer feature of Photoshop (Version CS2, Adobe Systems Inc., San Jose, CA). A grid was then drawn on the image using the graduations of the micrometer. Each square on the grid represented $4 \mu\text{m}^2$. The number of squares (including fractions) overlapping each bubble was tabulated for quantification of pulse-to-pulse bubble distribution.

III. Results

Fig. 5(a) shows the median RIPS for all pulses at PRFs of 1000, 500, 100, and 10 Hz. The pulse number is presented on a log scale. This allows representation of all 100 pulses while clearly showing the acoustic response during the first 20 pulses. On a pulse-to-pulse basis, the response of the bubbles and cells to insonation is sustained longer with increasing PRF. Twenty pulses at 1000 Hz PRF are required for the emission to reach noise levels whereas at 10 Hz PRF that level is reached in 3 pulses. Fig. 5(b) shows the RIPS of the acoustic emission from the microbubbles and cells as a function of time. Again, the acoustic emission at various PRFs (1000, 500, 100, and 10 Hz) is compared. Each data point corresponds to a pulse at the particular PRF. Therefore, each 1000 Hz data point corresponds to 1 ms pulse repetition period (PRP), while that at a 100 Hz corresponds to a 10 ms PRP. The range for the 100 Hz response is the only one shown to prevent clutter from numerous data points. The range presented, which is $\pm 25\%$ of the data value, is representative of all data points.

With the exception of the 10 Hz response, each plot shows an increase in RIPS value for the second or third pulse. At the 500 and 1000 Hz PRF, the acoustic emission reduces to noise around 35 ms. Greater and more sustained levels of emission at the 100 Hz PRF require about 100 ms to reach the noise floor. Due to the relatively large time period for the 10 Hz PRF, only 2 data points are presented in this plot. The emission corresponding to the second pulse of the 10 Hz PRF is lower than the first (unlike the corresponding pulses at 1000, 500, and 100 Hz), albeit at a higher level than emissions at the same time point for the other PRFs.

Fig. 6 represents the percentage of cells stained by the exclusion dye, Trypan blue, indicating a loss of membrane integrity and cell damage. Results of sham exposures are presented in comparison with the 46-cycle exposures. The percentage of stained cells increases with total time and reaches a plateau at approximately 100 ms. The damage is 27% for 10 ms of exposure, 47% for 20 ms, and 60% for 100 ms and longer. Data points corresponding to various PRFs are also identified in the figure. Note that the results for 10-pulse exposures at 1000 Hz, 100 Hz, and 10 Hz PRFs is consistent with results of the previous study (Samuel *et al.*, 2006). Damage at 1000 Hz was significantly lower ($P < 0.01$) than that at 100 or 10 Hz PRF in both studies. In addition, damage between the 100 Hz and 10 Hz PRFs was statistically similar in both studies ($P = 0.81$). The difference in cell damage between a 10-pulse exposure and a 100-pulse exposure (at the same PRF) increases directly with increasing PRF. Thus, at 100 Hz PRF, the change from 10 pulses to 100 pulses results in a 7% increase in damage. At 500 Hz, the increase is 21%, while at 1000 Hz, the increase is 53%.

The percentage of stained cells to the cumulative function of the RIPS (CRIPS) was plotted to examine the correlation of acoustic emissions to cell damage (Fig. 7). CRIPS is calculated by summing the RIPS over all pulses for a given exposure. The observed damage is the result of all 10 or 100 pulses transmitted during an exposure because the percentage of stained cells is

quantified by comparing the pre-exposure photomicrograph with one captured 5 min post-exposure. The figure shows a correlation ($R^2 = 0.48$) between emissions and cell damage. There is an increase in cell damage with increasing CRIPS. The data points on the scatter plot are differentiated in terms of their corresponding pulsing parameter. Besides the 10-pulse exposures at 1000 Hz and 10 Hz, the data points are clustered along the trendline.

Most bubbles were attached to the cells before exposure. Displacement of these bubbles in an acoustic field due to the primary and secondary Bjerknes forces could potentially exert enough stress to rupture the cell membrane. Average displacement was, therefore, calculated for bubbles attached to a macrophage (Fig. 8). At each PRF (10, 100, 500, and 1000 Hz), the position of 15 different bubbles, each at a different location, was monitored from pulse to pulse over the course of 10 pulses. The average displacement was calculated as the mean of the distance traveled by the 15 bubbles for each of the 10 pulses. There is a decrease in displacement of the bubbles with increasing PRF. The difference in displacement between the 10 Hz PRF and the 1000 Hz PRF is statistically significant ($P < 0.05$).

Most of the microbubble activity occurred within the first 10 pulses at each PRF. The relationship between pulse-to-pulse bubble distribution and acoustic emission was investigated to better understand this activity. Fig. 9(a)–(c) shows the pulse-to-pulse bubble distribution as a function of the total number of bubbles available for the subsequent pulse at PRFs of 100 Hz, 500 Hz, and 1000 Hz, respectively. Fig. 9(d)–(f) shows each bubble size as a percentage of the total number of bubbles in each pulse. This allows visualization of the evolution of bubble distribution from pulse 1 to pulse 10. Fig. 9(a)–(c) demonstrates a general trend of reduction in the total number of bubbles from pulse 1 to pulse 10. Fig. 9(d)–(f) provides the relative contribution of each bubble size to the total number present in the field of view (exposure zone) before each pulse. Prior to exposure, most bubbles are generally in the $2 \pm 0.5 \mu\text{m}$ range. The bubble population tends to shift to the sizes below $1.5 \mu\text{m}$ and above $2.5 \mu\text{m}$ in each subsequent exposure. The most dramatic effect is at 100 Hz. The investigation of the pulse-to-pulse bubble distribution suggested that there may be a unique bubble size that produces the maximum response, both in terms of acoustic emission and cell damage. The arrows in each figure identify the 1 and $2 \mu\text{m}$ bubble sizes as the subpopulation of interest based on the following reasoning:

1. They tend to have the largest number before exposure.
2. Their number initially increases, similar to previously observed emissions results.
3. After reaching the maximum, their number decreases by the tenth pulse.

Overlaying the pulse-to-pulse bubble count with the corresponding RIPS data at PRFs of 100, 500, and 1000 Hz further distills the bubble size of interest to the $2 \mu\text{m}$ diameter bubbles shown in Fig. 10(a)–(c). The pulse-to-pulse evolution of the $2 \mu\text{m}$ bubble appears to behave similarly to the corresponding acoustic emission at each PRF. Note that the highest bubble count and corresponding maximum emission occurs at a later pulse for the 1000 Hz PRF when compared with the 100 Hz and 500 Hz PRFs.

There is good correlation ($R^2 = 0.76$) found by plotting the RIPS as a function of the $2 \mu\text{m}$ bubble count (Fig. 11). Table I compares the correlation of other bubble sizes to emission. Remaining bubble sizes are correlated poorly with emission, with $1 \mu\text{m}$ being the closest ($R^2 = 0.17$). The correlation of the $2 \mu\text{m}$ bubble size to RIPS is significantly different than the correlation of the other bubble sizes to RIPS ($P < 0.05$).

Briefly, results indicate an increase in RIPS from the first pulse to the second or third. Cell death increases with total time and reaches a plateau at ~ 100 ms. There is a correlation between emissions and cell death with the $2 \mu\text{m}$ bubbles most strongly correlated with emissions. The increase in cell death corresponds to an increase in CRIPS.

IV. Discussion

The effect of total exposure duration on cell damage was examined. Cell damage increases with total duration of exposure between 10 ms and 100 ms beyond which it plateaus at 60%. There is a correlation between emission and cell damage with greater damage associated with higher CRIPS values. The evolution of the microbubble was investigated using high-speed photography and indicated by the pulse-to-pulse bubble distribution. Microbubbles of 2 μm diameter size are the most responsive to the acoustic field under the conditions set up for this study (2.25 MHz transmit frequency, 2 MPa peak rarefactional pressure, 46 cycles, 10 or 100 pulses). A good correlation was found between bubble size and emission ($R^2 = 0.76$).

The increase in RIPS for the second pulse may be attributable to some evolution of the shelled microbubble after the first pulse. That pulse renders it active including the ability to pulsate more actively in an acoustic field. Dayton *et al.* observed similar association. They established that the echo amplitude increases from the first pulse to the second one for echoes returned from an Optison bubble at a transmitted frequency of 2.25 MHz. It was also indicated that the albumin shell of an Optison bubble breaks down during insonation, and gas subsequently escapes from the shell [18]. Postema *et al.* termed this phenomenon sonic cracking and captured clear images of escaping gas from a cracked shell upon insonification [19]. In another approach, Guan and Matula employed a modified Mie theory of light scattering to investigate the evolution of insonified microbubbles. Their results show that an Optison bubble is compromised after 2 or more pulses at peak rarefaction pressures as low as 340 kPa. Notably those pressure amplitudes are significantly lower than those applied during this study (2 MPa). A pulse-to-pulse examination of power spectral densities (PSDs) from the light scattering data revealed an increase at the resonance frequency (2.25 MHz) from pulse 1 to pulse 2 before reducing for pulse 3 [20]. Chatterjee and Sarkar have developed a model for the dynamics of an encapsulated microbubble contrast agent using a modified Rayleigh-Plesset equation and Newtonian interfacial rheological parameters [21]. Comparing a free bubble and an encapsulated one, the model of the latter showed that a shell considerably dampens a bubble's oscillations and, consequently, its nonlinear response to acoustic stimulus. Stride and Saffari demonstrate that this damping influence of the shell on contrast microbubble behavior, however, may be relatively short lived because of the very high stresses experienced during insonation [22]. This difference in behavior between an encapsulated bubble and a free one in an acoustic field may be the reason for the increase in the RIPS from the first pulse to the second one as noted in this study's results.

Because the released gas bubbles proceed to dissolve, fragment, or coalesce, their ability to acoustically scatter diminishes over time as shown in the RIPS data. However, greater and more sustained emission levels can be seen at the relatively lower PRFs (100 and 10 Hz). The rate of bubble dissolution resulting from acoustically driven diffusion may possibly play a part in this [18]. The diameter of a contrast agent has been observed to decrease due to a net diffusion of gas out of the bubble with a sinusoidal pressure field. At higher PRFs, acoustically driven diffusion for any given time period is more rapid because acoustic pulses arrive more frequently. This is consistent with the observations of this study wherein the emission reduces to noise by ~20 ms at 500 and 1000 Hz PRF whereas ≥ 100 ms is required at a PRF of 100 Hz or lower.

Total exposure duration is more of a factor in cell damage than PRF. There is an increase in cell damage from 0 to 60% with increased total exposure duration from 0 to 100 ms. This suggests that, up to a certain point, damage may be increased by increasing total duration of exposure. However, a plateau in damage exists at ~100 ms and there is no further damage (beyond 60%) as exposure time is increased. Perhaps this is because no resonant bubbles are available in the exposure region after this time. The bubbles had either disappeared (diffusion)

or were too small (fragmentation) or too large (coalescence) to resonate at 2.25 MHz insonification. The difference in cell damage between a 10-pulse exposure and a 100-pulse exposure (at the same PRF) increases directly with increasing PRF. There is >50% increase in damage when the number of pulses at 1000 Hz PRF is increased from 10 to 100 pulses. This range in induced damage may be advantageous for use in therapeutic applications that require distinction between minimal or significant damage.

Video clips were captured during the exposure of cells and bubbles to acoustic pulses and show the behavior of the bubbles during each pulse. The phagocytic cells are only given sufficient time to attach to, not engulf, the bubbles before exposure. Both primary and secondary Bjerknes forces act on the bubbles during acoustic exposure. The primary Bjerknes force, the force exerted on an air bubble due to the primary radiating sound field, causes the bubbles to move in the direction of the sound wave propagation. Concomitant with this movement, secondary Bjerknes forces, the force between 2 air bubbles due to the secondary sound fields radiated by the bubbles, either cause the bubbles to attract each other (pulsating in phase) or to repel each other (pulsating out of phase). The acoustic intervention on the bubbles causes them to shrink (acoustically driven diffusion or dissolution) [18], generate smaller bubbles from a large bubble (fragmentation) [23], or form a large bubble from a group of small ones (coalescence) [24]. The bubble movement causes them to attempt detachment from the cells, possibly stretching the cell membrane and causing membrane rupture and subsequent cell death. Therefore, displacement of the bubbles may also contribute to cell damage, and resonant bubbles may experience more displacement than nonresonant ones. Results show that there is greater displacement at 10 Hz PRF than at 1000 Hz PRF (Fig. 8). This may explain the observations in Fig. 7 where results from 10-pulse exposures at 10 Hz and 1000 Hz do not follow the trendline. Although the emissions corresponding to these pulsing parameters are similar, the damage is different with greater damage seen at 10 Hz PRF than at 1000 Hz PRF. The difference in average displacement between the 10 Hz PRF and 1000 Hz PRF may be more pronounced if the bubble size of interest is reduced to 1.5 to 2.5 μm (resonant bubbles).

It is well known that radius is an important factor determining the resonance of bubbles to an acoustic field at a particular center frequency. An investigation of the video clips and corresponding pulse-to-pulse bubble distribution reveals: 1) total bubble count decreases with each subsequent pulse, 2) bubbles either decrease in size through acoustically driven diffusion/fragmentation or increase in size through coalescence, 3) bubbles become less responsive to each subsequent incident acoustic pulse due to change from resonant size, and 4) the changes in bubble count associated with the 2 μm bubbles are consistent with changes in emission (RIPS). Similar behavior is shown in their pulse-to-pulse changes in an overlay of the 2 μm bubble count with corresponding RIPS for each pulse from 0 to 10. A clear correlation has also been observed between acoustic emission and cell damage. This corroborates the correlation between bubble size (in this case 2 μm), emission (RIPS), and associated cell damage for this experimental setup and agrees with previous theoretical and experimental work.

- The theoretical model developed by Chatterjee and Sarkar predicted that at a driving frequency of ~2 MHz the resonant size of an encapsulated bubble, modeled after the interfacial properties of Optison, was 1.75 μm [21].
- Likewise, Morgan *et al.* observed experimentally that the mean frequency of the radial oscillation of a 2 μm encapsulated bubble was 2.3 MHz [25].

Thus, results show that cell damage may be induced through cavitation activity (inertial and/or noninertial) as well as displacement of the bubbles in response to the acoustic intervention.

V. Conclusion

This experimental setup demonstrates a correlation between bubble size (2 μm), emission (RIPS), and associated cell damage:

- Results clearly show that damage is increased, up to 60%, by increasing total exposure duration from 0 ms to 100 ms.
- There is an increasing difference in cell damage between a 10-pulse exposure and a 100-pulse exposure with increasing PRF. The greatest change in damage occurred at 1000 Hz PRF with a 53% increase between the 10-pulse and 100-pulse exposures.
- For each pulse from 0 to 10, an overlay of the 2 μm bubble count with corresponding RIPS shows strong correlation in their pulse-to-pulse changes.

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Biographies

Stanley Samuel was born in Bhopal, India, in 1974 and came to the United States in 1992. He received his B.S degree in biomedical engineering in 1996 from Oral Roberts University, Tulsa, OK, and received his M.S. and Ph.D. degrees in biomedical engineering in 1999 and 2007 from the University of Michigan, Ann Arbor, MI.

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Jeffery Brian Fowlkes (M'94–A'94) received his B.S. degree in physics from the University of Central Arkansas in 1983, and his M.S. and Ph.D. degrees in physics from the University of Mississippi in 1986 and 1988, respectively.

He is an associate professor in the Department of Radiology and associate professor in the Department of Biomedical Engineering. He is currently directing and conducting research in medical ultrasound, including the use of gas bubbles for diagnostic and therapeutic applications. His work includes studies of ultrasound contrast agents for monitoring tissue perfusion, acoustic droplet vaporization for bubble production in cancer therapy and phase aberration correction, effects of gas bubbles in high-intensity ultrasound, and volume flow estimation for ultrasonic imaging.

Dr. Fowlkes is a fellow of the American Institute of Ultrasound in Medicine (AIUM) and has served as secretary and as a member of its Board of Governors. He also received the AIUM Presidential Recognition Award for outstanding contributions and service to the expanding future of ultrasound in medicine. As a member of the Acoustical Society of America, Dr. Fowlkes has served on the Physical Acoustics Technical Committee and the Medical Acoustics and Bioresponse to Vibration Technical Committee. As a member of the IEEE, he has worked with the IEEE I&M Society Technical Committee on Imaging Systems. Dr. Fowlkes is a fellow of the American Institute of Medical and Biomedical Engineering.

Douglas L. Miller (M'99) was born in Steubenville, OH in 1947. He received the B.Sc. and M.S. degrees in physics in 1969 and 1971 from Ohio State University, Columbus, OH, and received the Ph.D. degree in physics in 1976 from the University of Vermont, Burlington, VT.

He worked in the area of biological effects of ultrasound at the University of Vermont, Department of Physics, from 1976 to 1987. From 1987 to 1998, he worked at Battelle Pacific Northwest National Laboratory as a staff scientist on ultrasonic bioeffects and bioelectromagnetics research. Presently, he is a research professor in the Radiology Department, University of Michigan Health System, Ann Arbor MI, pursuing research on biological effects and therapeutic applications of medical ultrasound.

Dr. Miller is a member of the IEEE, the Radiological Society of North America, and a fellow of the Acoustical Society of America and of the American Institute of Ultrasound in Medicine, which presented him the Pioneer Award in 2006.

Fig. 1.

Illustration of the system employed for the detection of acoustic emission from microbubbles and cells with optical feedback. The exposure chamber was placed in a degassed water bath at 37°C. To limit the received signal to just the scattering from the cells and microbubbles, the transducers were placed in perpendicular planes to each other and at an angle of 45° to the plane of the Mylar membrane where the cells were attached. An inverted microscope with a 20× long working distance objective was used. A high-speed camera captured images at a rate of 2000 fps.

Fig. 2.

Configuration used in minimizing the effect of standing waves at the bottom of the tank. A disk-shaped sound absorbing material was placed at the bottom of the tank over which the exposure chamber rested. The Mylar holder of the chamber sat farthest from the optical glass window at the tank bottom. As the beam path shows, most of the divergent beam reflected by the surface of the sound absorbing disc would have been diverted away from the transducer focus, while the component reflecting back to the focus would have been significantly attenuated.

Fig. 3.

Timing diagram describing the control sequence of the equipment. Function generator 1 was responsible for generating bursts of 10-cycle or 100-cycle square waves. The pulsing parameters for function generator 1 included 10 cycles at 1 kHz, 500 Hz, 100 Hz, and 10 Hz and 100 cycles at 1 kHz, 500 Hz, and 100 Hz. Function generator 2, triggered by function generator 1, produced sinusoid pulses containing 46 cycles (pulse duration of 20.4 μ s) at a center frequency of 2.25 MHz. Long pulses were used to ensure sufficient signal-to-noise ratio for the subharmonic and ultraharmonics. Each cycle of function generator 1 corresponds to a burst of 46 cycles generated by function generator 2 at the carrier frequency of 2.25 MHz. Function generator 1 was also responsible for triggering the camera and the digital acquisition board.

Fig. 4.

Representative photomicrographs of microbubble-attached cells. Phagocytic cells were cultured as monolayers on Mylar sheets and incubated for 15 min with 5% Optison: (a) pre-exposure, (b) pulse 5, (c) pulse 10, and (d) 5-min post-exposure photomicrographs are shown. The viable cells, ones with intact membranes, can be clearly identified in these images. The scale bar is 50 μm .

Fig. 5.

Acoustic emission, expressed as median relative integrated power spectrum (RIPS) at pulse repetition frequencies (PRF) of 1000, 500, 100, and 10 Hz. (a) Median RIPS shown for all pulses. Pulse number is presented on a log scale to clearly show the acoustic response for the first 20 pulses. (b) Median RIPS as a function of total exposure duration. Each data point corresponds to a pulse repetition period (PRP). At 500, and 1000 Hz PRF, the acoustic emission reduces to noise around 30 ms. There are greater and more sustained levels of emission at the 100 Hz PRF.

Fig. 6.

Percentage of cells that are stained by the exclusion dye, Trypan blue, indicating loss of membrane integrity and cell damage. Results of sham exposures are presented in comparison with the 46-cycle exposures. The percentage of stained cells increases with total time and reaches a plateau around 100 ms. The difference in cell damage between a 10-pulse exposure and a 100-pulse exposure (at the same PRF) increases with increasing PRF. At 100 Hz PRF, the change from 10 pulses to 100 pulses results in a 7% increase in damage. At 500 Hz, the increase is 21%, while at 1000 Hz, the increase is 53%.

Fig. 7.

Correlation of acoustic emissions (cumulative function of the RIPS) to cell damage (percent stained cells). The CRIPS is calculated by summing the RIPS over all pulses for a given exposure. Because the percentage of stained cells is quantified by comparing the pre-exposure photomicrograph with one captured 5 min post-exposure, the observed damage is the result of all 10 or 100 pulses transmitted during an exposure. The figure shows a correlation between emissions and cell damage ($R^2 = 0.48$). The trend shows an increase in cell damage with increasing CRIPS. The data points are differentiated in terms of their corresponding pulsing parameter.

Fig. 8.

Average displacement of bubbles per pulse as a function of PRF. Average displacement was calculated over 10 pulses at PRFs of 10 Hz, 100 Hz, 500 Hz, and 1000 Hz. There is a decrease in displacement of the bubbles with increasing PRF. The difference in displacement between the 10 Hz PRF and the 1000 Hz PRF is statistically significant ($p < 0.05$). Therefore, displacement of the bubbles may also contribute to cell damage, along with cavitation activity, and resonant bubbles may experience more displacement than nonresonant ones.

Fig. 9.

Pulse-to-pulse bubble distribution from pre-exposure through pulse 10. The left column shows the bubble distribution as a function of the total number of bubbles available for the subsequent pulse at (a) 100 Hz PRF, (b) 500 Hz PRF, and (c) 1000 Hz PRF. The right column shows each bubble size as a percentage of the total number of bubbles in each pulse at (d) 100 Hz PRF, (e) 500 Hz PRF, and (f) 1000 Hz PRF and, therefore, provides the relative contribution of each bubble size to the total number present in the field of view. The arrows in each figure identify the 1 and 2 μm bubble sizes as the subpopulation of interest. They tend to have the largest number before exposure and their number initially increases, similar to the emissions results. Also after reaching the maximum, their number decreases by the tenth pulse.

Fig. 10.

Overlay of the 2 μm bubble count with the corresponding RIPS at (a) 100 Hz PRF, (b) 500 Hz PRF, and (c) 1000 Hz PRF. The pulse-to-pulse evolution of the 2 μm bubble seems to behave similarly to the corresponding acoustic emission at each PRF. Note that the highest bubble count and corresponding maximum emission occurs at a later pulse for the 1000 Hz PRF when compared with the 100 Hz and 500 Hz PRFs.

Fig. 11.

Plot of the RIPS as a function of the 2 μm bubble count. There is good correlation between the two with an $R^2 = 0.76$ suggesting that the acoustic emission depends on the availability of bubbles that are resonant at the insonating frequency.

TABLE I

Correlation Bubble Sizes to Emission.

Bubble Size (μm)	Correlation (R^2)
1	0.1662
2	0.7552
3.5	0.0263
5	0.0542
7.5	0.0542
10	0.0511
12.5	0.0539

All, except the 2 μm bubble size, are poorly correlated with emission. The correlation of the 2 μm bubble size to RIPS is significantly different than the correlation of the other bubble sizes to RIPS ($P < 0.05$).