BIOEFFECTS CAUSED BY CHANGES IN ACOUSTIC CAVITATION BUBBLE DENSITY AND CELL CONCENTRATION: A UNIFIED EXPLANATION BASED ON CELL-TO-BUBBLE RATIO AND BLAST RADIUS

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Abstract—Acoustic cavitation has been shown to load drugs, proteins and DNA into viable cells as a complex function of acoustic and nonacoustic parameters. To better understand and quantify this functionality, DU145 prostate cancer cell suspensions at different cell concentrations (2.5 × 10⁵ to 4.0 × 10⁷ cells/mL) were exposed to 500 kHz ultrasound (US) over a range of acoustic energy exposures (2 to 817 J/cm²; peak negative pressures of 0.64 to 2.96 MPa; exposure times of 120 to 2000 ms) in the presence of different initial concentrations of Optison® contrast agent bubbles (3.6 × 10⁴ to 9.3 × 10⁷ bubbles/mL). As determined by flow cytometry, molecular uptake of calcein and cell viability both increased with increasing cell density; viability decreased and uptake was unaffected by increasing initial contrast agent concentration. When normalized relative to the initial contrast agent concentration (e.g., cells killed per bubble), bioeffects increased with increasing cell density and decreased with increasing bubble concentration. These varying effects of contrast agent concentration and cell density were unified through an overall correlation with cell-to-bubble ratio. Additional analysis led to estimation of “blast radii” over which bubbles killed or permeabilized cells; these radii were as much as 3 to 90 times the bubble radius. Combined, these results suggest that extensive molecular uptake into cells at high viability occurs for low-energy exposure US applied at a high cell-to-bubble ratio. (E-mail: mark.prausnitz@che.gatech.edu) © 2003 World Federation for Ultrasound in Medicine & Biology.

Key Words: Ultrasound bioeffects, Drug delivery, DU145 prostate cancer cell, Optison® contrast agent concentration, Cell density.

INTRODUCTION

Although prevention of ultrasound (US)-induced bioeffects during imaging has been studied for decades (Nybørg 2001), intentionally causing desirable acoustic bioeffects for drug delivery and other applications has only recently received attention. This interest is motivated by observations that US can deliver small compounds (Brayman et al. 1999; Guzmán et al. 2001a; Keyhani et al. 2002), macromolecules (Fechheimer et al. 1987; Miller et al. 1999; Guzmán et al. 2002), DNA (Bao et al. 1997; Greenleaf et al. 1998; Wyber et al. 1997) and other therapeutic compounds (Saad and Hahn, 1987; Harrison et al. 1996) into cell suspensions. US can also deliver protein (Mukherjee et al. 2000) and DNA (Amabile et al. 2001; Unger et al. 2001) into tissue.

These bioeffects are believed to be caused by oscillation and/or implosion of ultrasonically generated cavitation bubbles that reversibly break open cell membranes (Miller and Quddus 2000; Wu 2002). When not properly controlled, cavitating bubbles can also cause irreversible cell damage, resulting in significant cell death (Miller et al. 1996). For this reason, controlled amounts of contrast agent, CA, microbubbles have been added during sonication to serve as cavitation nuclei (Apfel and Holland 1991). These nuclei have been shown to enhance US-mediated bioeffects in cells and tissue (Miller et al. 1997; Greenleaf et al. 1998; Poliachik et al. 1999).

Because previous studies were carried out over a wide range of acoustic and nonacoustic conditions (e.g., varying cell densities, initial contrast agent concentration and cell type), observed bioeffects exhibit large variation and usu-
ally have not been unified by common descriptors. To partially address this, we recently showed that molecular uptake and cell death caused by acoustic cavitation correlated with a single acoustic parameter, acoustic energy exposure, for two cell types exposed to either 24 kHz or 500 kHz US over a broad range of different pressures (above the cavitation threshold), pulse lengths, exposure times and duty cycles (Guzmán et al. 2001a; Keyhani et al. 2002). In a related study, we also found that these bioeffects correlated with sonoluminescence generated during sonication (Cochran and Prausnitz 2001).

In this study, we built on the previous observation of energy as a unifying acoustic parameter to determine how nonacoustic parameters influence bioeffects, and if those parameters can similarly be unified. Because the mechanism for bioeffects is believed to involve cell-bubble interactions, we propose that both cell and initial CA concentration (i.e., [CA]i) should be important nonacoustic parameters and suggest that the ratio of these parameters (i.e., cell-to-bubble ratio) might serve as a unifying nonacoustic parameter.

Previous studies have examined the effects of [CA]i and cell density, where increasing [CA]i was shown to correlate with increased cell lysis and intracellular uptake of macromolecules (Miller et al. 1997; Ward et al. 2000), and increasing cell density partially protected red blood cells from hemolysis (Ellwart et al. 1988; Miller and Thomas 1995; Brayman et al. 1996). However, previous studies have not varied both of these parameters over a broad range of different [CA]i and cell densities.

Motivated by this, the goal of the first part of this study was to determine the effects of [CA]i and cell density on molecular uptake and cell viability. We were guided by two hypotheses: bioeffects increase with increasing [CA]i (i.e., more bubbles can interact with more cells); and bioeffects increase with increasing cell density (i.e., more cells can interact with more bubbles). In the second part of this study, our goal was to develop a unified conceptual framework that could broadly explain the observed cell-to-bubble interactions. In this context, we hypothesized that the ratio of cells to [CA]i can serve as a single parameter that quantitatively correlates with the effects of changing both cell density and [CA]i. Moreover, we propose that calculating the efficiency with which individual CA bubbles affect neighboring cells and over what length scales those effects are felt (i.e., “blast radius”) provides a unified conceptual framework that facilitates analysis and can lead to mechanistic interpretations.

METHODS

Ultrasound equipment

US was applied, as described previously (Guzmán et al. 2001a), using a focused 500-kHz piezoelectric transducer with a 3.5 inch (9 cm) focal length and a beam width of 3 mm (~6 dB intensity area) at the focal beam point. A three-way micropositioner (1-mm resolution, Velmex, Bloomfield, NY) was used to place cell samples in the US focal region. Root-mean-squared pressure (P) and spatial-peak-temporal-peak (SPTP) negative pressure (P_SPTP^-2) were measured using a 0.2-mm aperture PVDF membrane hydrophone (NTR Systems, Seattle, WA, model No. MHA200A). Spatial-peak acoustic energy exposure (E) was approximated by the product of the spatial-peak-pulse-average (SPPA) acoustic intensity (I_SPPA) of a plane traveling wave and the total US exposure time, t.

\[ I_{SPPA} = \frac{P^2}{\rho c} \]  \hspace{1cm} (1)

\[ E = I_{SPPA} \cdot t \]  \hspace{1cm} (2)

where \( \rho \) is density of water, and \( c \) is speed of sound in water (Pierce 1994; Guzmán et al. 2001a).

Cell sample preparation

As described previously (Guzmán et al. 2001a), human prostate cancer cells (DU145; American Type Culture Collection, item No. HTB-81) were grown as monolayers in RPMI-1640 media supplemented with 10% fetal bovine serum (Cellgro, Mediatech, Herndon, VA). The cells were harvested by trypsin/EDTA digestion (Cellgro), centrifuged at 1000 \times g (Beckman Coulter, Fullerton, CA, model GS-15R) for 6 min, and resuspended in fresh cell media to their final cell concentration as determined by a Coulter counter (Coulter Multisizer II, Beckman Coulter, Fullerton, CA). Cell volumes were approximated using cell radius measurements obtained from a Coulter counter, assuming a spherical geometry. To promote cavitation, different concentrations of albumin-stabilized gas bubbles (Optison®, bubble diameter = 2.0 to 4.5 \( \mu \)m, Mallinckrodt Inc., St. Louis, MO) were added to the final cell suspensions (Guzmán et al. 2001a).

Calcein (623 Da, radius = 0.6 nm; Molecular Probes, Eugene, OR), a green-fluorescent molecule that cannot cross the membranes of intact cells, was used to quantitatively monitor the transport of molecules across the membranes of viable cells. The final concentration of calcein in the cell suspension was 10 \( \mu \)M. To test the effect of Optison® concentration, [CA]i was varied from 0.0056% v/v (approximately 4 \times 10^4 bubbles/mL) to 14.4% v/v (approximately 9 \times 10^4 bubbles/mL) at constant cell density (1 \times 10^6 cells/mL). To test the effect of cell concentration, cell density was varied from 2.5 \times 10^5 to 4.0 \times 10^7 cells/mL at a constant [CA]i = 1.7% v/v (approximately 1.1 \times 10^7 bubbles/mL).

Cell samples were slowly aliquoted via a 3-mL syringe (Becton Dickinson, Franklin Lakes, NJ) with a 22-
Ultrasound bioeffects unified by cell: bubble ratio ● H. R. Guzmán et al.

RESULTS

In this study, DU145 prostate cancer cell suspensions at different cell concentrations were exposed to 500-kHz US over a range of acoustic energy exposures in the presence of different initial concentrations of CA bubbles. At all exposure conditions tested, levels of molecular uptake by cells within each sample showed significant cell-to-cell heterogeneity (data not shown), consistent with previous observations (Guzmán et al. 2001b; Cochran and Prausnitz 2001; Keyhani et al. 2002). Typically, cell samples exposed to US showed a distribution in levels of molecular uptake containing a clustered subpopulation of cells with nominal uptake (i.e., just above the detection limit), another clustered subpopulation of cells with high uptake, and a broad subpopulation with intermediate levels of uptake. Because a single mean value of intracellular uptake would not accurately reflect this distribution, the cells from each sample were divided into three groups termed nominal (NUP), low (LUP) and high (HUP) uptake subpopulations (Guzmán et al. 2001b).

The mean intracellular concentrations of calcein within each uptake subpopulation were 0.013 ± 0.007, 1.49 ± 0.26, and 11.2 ± 1.7 μM for NUP, LUP and HUP, respectively (mean ± SEM, n = 110 samples). The extracellular concentration of calcein was 10 μM,
signifying that cells in the HUP subpopulation contained intracellular calcein that was apparently in equilibrium with the extracellular solution; LUP cells contained subequilibrium uptake and NUP cell uptake was indistinguishable from zero. The distribution of cells within each uptake subpopulation correlated with energy exposure by nonlinear regression \((p < 0.05; \text{data not shown})\), consistent with previous observations (Guzmán et al. 2001b).

**Dependence of bioeffects on initial CA bubble concentration**

To determine how \([\text{CA}]_i\) affected molecular uptake and cell viability, US was applied at four energy exposures (2 to 817 J/cm\(^2\)) over a large range of \([\text{CA}]_i\) (3.6 \(\times\) 10\(^4\) to 9.3 \(\times\) 10\(^7\) bubbles/mL). The resulting bioeffects are shown in Fig. 1a, where the height of each bar represents overall cell viability; white stripes = cells in NUP, gray stripes = cells in LUP and black stripes = cells in HUP. Cell viability decreased with increasing \([\text{CA}]_i\) and energy exposure (except in the absence of CA). NUP decreased, and LUP and HUP generally did not change with increasing \([\text{CA}]_i\) at constant energy exposure. \([\text{CA}]_i\) values were: a = 0, b = 0.04, c = 0.29, d = 1.6, e = 11, f = 93 \(\times\) 10\(^6\) bubbles/mL. These data were reanalyzed to express bioeffects on a per-bubble basis. The resulting “efficiency” of a CA bubble at (b) transiently permeabilizing cell membranes (i.e., the sum of LUP and HUP cells) and (c) making cells nonviable, decreased with increasing \([\text{CA}]_i\). Further analysis yielded estimates of (d) the maximum effective blast radius around each bubble and (e) the maximum effective killing radius (normalized relative to bubble radius), both of which decreased with \([\text{CA}]_i\). Cell density was 1 \(\times\) 10\(^6\) cells/mL and the applied energy exposure was \(\bigcirc = 2, \Delta = 49, \Box = 221\), and \(\diamond = 817\) J/cm\(^2\). Data expressed as mean \(\pm\) SEM.
subpopulations, respectively. In support of our hypothesis, cell viability decreased with increasing \([\text{CA}]\), and increasing energy exposure \((\rho < 0.05)\). However, the molecular uptake of calcein, which is of primary interest to drug delivery, showed no dependence on \([\text{CA}]\); the calcein-loaded fraction of cells in LUP and HUP remained constant with increasing \([\text{CA}]\) \((\rho > 0.05)\), and the fraction of cells in NUP decreased \((\rho < 0.05)\). In the absence of any CA, cell viability was unaffected by US \((\rho = 0.65)\) and molecular uptake did not occur, consistent with a cavitation-based mechanism (Guzman et al. 2001a).

As an alternative approach to interpret the results from Fig. 1a, cells affected by US \((i.e., \text{nonviable cells and cells having molecular uptake})\) were replotted on a per-bubble basis as a function of \([\text{CA}]\), \((\text{Fig. 1b–c})\). Note that this normalization is relative to the number of CA bubbles initially present in the sample and does not account directly for the dynamic bubble population generated during sonication (this point is discussed further below). To simplify analysis, cells previously classified as LUP and HUP where combined into a single parameter: permeabilized cells. As shown in Fig. 1b, the number of cells permeabilized per bubble decreased with increasing \([\text{CA}]\) \((\rho < 0.01)\). Likewise, Fig. 1c shows the number of cells rendered nonviable per bubble \((i.e., \text{killed})\), also decreased with increasing \([\text{CA}]\) \((\rho < 0.05)\). Thus, neighboring bubbles influenced each other so that bubble “efficiency” \((i.e., \text{the number of cells affected by each bubble})\) decreased with increasing \([\text{CA}]\).

Fitting power functions to the data in Fig. 1b and c yields an average dependence of cells permeabilized per bubble on CA concentration to the power \(-1.16 \pm 0.20\) \((\text{average } R^2 = 0.98)\), and an average dependence of cells killed per bubble to the power \(-0.87 \pm 0.04\) \((\text{average } R^2 = 0.98)\). Although fitting to power functions has no mechanistic basis, the negative values of both power dependences mean that the presence of a neighboring bubble (which is increasingly likely at high \([\text{CA}]\)) reduces the ability of a bubble to affect a nearby cell. Moreover, the greater absolute power-dependence of permeabilization vs. killing suggests that the presence of neighboring bubbles reduces a bubble’s ability to permeabilize a nearby cell to a greater extent than its ability to kill.

The observed dependence on \([\text{CA}]\) in generating bioeffects has implications for drug delivery. Molecular uptake of calcein \((i.e., \text{percentage of permeabilized cells})\) was shown to be independent of \([\text{CA}]\) and cell viability was shown to have a strong inverse dependence on \([\text{CA}]\) \((\text{Fig. 1a})\). This result suggests that low \([\text{CA}]\) is desirable for drug and gene delivery into cells while maintaining high viability. In contrast, if cell death is beneficial, high \([\text{CA}]\) may be used, while at the same time maintaining high levels of drug delivery. Additionally, Figs. 1b–1c show that the efficiency of CA bubbles at causing bioeffects decreased with \([\text{CA}]\), which means that adding more bubbles increases bioeffects, but with diminishing returns.

To aid in the physical interpretation of the results of Fig. 1b–c, we calculated estimates of the “effective” length scales over which a bubble acts. According to our simplified model shown in Fig. 2, cells within the “blast radius” \((R_b)\) of a bubble are affected by that bubble \((i.e., \text{killed or permeabilized})\) and those within the “killing radius” \((R_K)\) are rendered nonviable. Cells that are outside the blast radius are not affected \((i.e., \text{cells classified as NUP})\).

To calculate \(R_K\), ordinate values obtained from Fig. 1c were divided by initial cell density \((i.e., 1 \times 10^6 \text{ cells/mL})\) to provide the volume around each bubble containing the cells killed by each bubble. From this volume, \(R_K\) was determined, normalized relative to initial CA bubble radius and presented in Fig. 1d. Similarly, \(R_B\) was obtained from the sum of the ordinate values in Fig. 1b and c, and the resulting data are presented in Fig. 1e, normalized relative to initial CA bubble radius.

The advantage of this approach is that through normalization it does not require knowledge of the radii, or distribution of radii, within the dynamic population of bubbles created during sonication. However, its weakness is that it assumes that the total amount of gas present within bubbles is constant. Although it is unlikely that large amounts of perfluorocarbon gas from the original CA bubbles would be lost, given its low aqueous solubility (Skyba et al. 1996), it is quite likely that gas dissolved in the liquid sample \((e.g., \text{nitrogen, oxygen})\) would partition into bubbles by rectified diffusion (Leighton 1994). For this reason, calculated values of \(R_K\)
and $R_B$ should be taken as upper estimates that would be reduced by increased gas partitioning into bubbles.

$R_K$ and $R_B$ depended strongly on $[CA]$, as evidenced by their decreasing radii ($p < 0.01$), suggesting that the length scale over which a bubble acts is reduced by neighboring bubbles. This result is consistent with results from Fig. 1a–c, which shows that the effectiveness of a bubble is reduced by the addition of more bubbles, and further supports the idea that CA bubbles present at low concentration are more efficient at causing bioeffects than CA bubbles at high concentration, due to their increased effective blast radii.

**Dependence of bioeffects on cell density**

Having observed that the presence of additional bubbles influences acoustic bioeffects, we next wanted to determine the effect of adding more cells. Figure 3a shows the effects of US on cells at densities ranging from $2.5 \times 10^5$ to $4.0 \times 10^7$ cells/mL. Levels of cell viability were significantly higher with increasing cell density and decreasing energy exposure ($p < 0.05$). The fraction of cells having uptake of calcein (i.e., LUP and HUP) also increased with increasing cell density ($p < 0.05$), but the fraction of cells in NUP was independent of cell density ($p > 0.05$), except at the highest energy exposure (i.e., $332 \text{ J/cm}^2$, $p < 0.05$).
Because increasing cell density increases both cell viability and molecular uptake, applications that seek to deliver large numbers of molecules into viable cells would benefit from using cells at high density.

Considering these effects on a per-bubble basis, the efficiency of CA bubbles in causing bioeffects was determined as a function of cell density, as shown in Fig. 3b and c. In Fig. 3b, the number of permeabilized cells per bubble increased with increasing cell density ($p < 0.01$) for all energy exposures. Similarly, the number of cells killed per bubble, shown in Fig. 3c, increased with cell density at high energy exposures (i.e., $=98$ J/cm$^2$, $p < 0.01$), but was statistically independent of cell density at low energy exposures (i.e., $=42$ J/cm$^2$, $p > 0.05$). This indicates that the presence of additional cells allows a bubble to interact more efficiently with a greater number of cells, especially at high levels of energy exposure.

We originally hypothesized that increasing cell density would increase bioeffects (i.e., decrease cell viability and increase molecular uptake). The hypothesized increases in molecular uptake are supported by Fig. 3a-b. However, the hypothesized decreases in cell viability appear to be contradicted by data in Fig. 3a, which shows increasing cell viability with cell density. This can be better understood by examining Fig. 3c, which presents the absolute number of cells killed. Because the number of cells killed did not increase in proportion to cell density, cell death expressed as a fraction of cells decreased in Fig. 1a, but the absolute number of cells killed, nevertheless, increased. With this understanding, the data are consistent with the hypothesis.

To show how the upper estimate of blast radius was affected by cell density, the data from Fig. 3b-c were replotted in Fig. 3d-e. In contrast to the previous observation that $R_k$ and $R_b$ were strongly dependent on [CA]$_i$ (Fig. 1d-e), Fig. 3d-e shows that $R_k$ and $R_b$ were statistically independent of cell density ($p > 0.05$). This suggests that the blast radius of CA bubbles is only a function of [CA]$_i$ at constant energy exposure and is not influenced by the presence of cells over the range studied. This observation also illustrates why bubble efficiency increases with increasing cell density: if blast radii remain constant while cell density is increased, then these radii encompass a greater number of cells and, thus, affect more cells.

The observed dependence of cell density on US-mediated bioeffects has implications for drug delivery into cells and tissue. The data show that increased molecular uptake and cell viability (on a percent basis) can be achieved by increasing cell density. Thus, for laboratory applications using US, cells at high density might be best. This analysis could be extended to tissues, where cells are at very high cell density and, therefore, uptake and viability is predicted to be high. However, at such high cell densities found in tissues, which are typically beyond the range studied here, bubble penetration, growth and oscillation may be impeded by the lack of space between closely packed cells. Moreover, the complexities of intact tissue and the in vivo environment may also make extrapolation of results from this study to tissues more difficult.

**Single unifying parameter for bubble and cell density dependence**

Figures 1 and 3 show the dependence of cell viability and molecular uptake on [CA]$_i$ and cell density, respectively. Because these bioeffects are believed to be caused by a cell-to-bubble interaction, we hypothesized that there is a single unifying parameter that correlates with the effect of changing both cell density and [CA]$_i$. Previous studies have proposed parameters that characterize this cell-to-bubble interaction: 1. the nearest distance between a cell and bubble (Ward et al. 2000) and 2. the ratio of the number of cells to bubbles (Miller et al. 1997). Because the present study has quantified the effects of both cell density and [CA]$_i$ over a broad range of conditions, we believe that these data should provide a good means to test this hypothesis.

To determine how the efficiency of the cell-to-bubble interaction is affected by cell-to-bubble distance and cell-to-bubble ratio, the data from Fig. 1b-c and Fig. 3b-c were combined and replotted in Fig. 4a-d. For these comparisons, cell-to-bubble distance and cell-to-bubble ratio were calculated based on [CA]$_i$, and do not account for the dynamic bubble population present during sonication. When plotted vs. cell-to-bubble distance, Fig. 4a shows that cells permeabilized per bubble increased with cell-to-bubble distance for the data collected at varying [CA]$_i$, but decreased in the data collected at varying cell density. Similar contrasting trends were also observed in Fig. 4b, which shows cells killed per bubble as a function of cell-to-bubble distance. We, therefore, conclude that cell-to-bubble distance is not a unifying parameter. This might have been expected, based on the data in Figs. 1 and 3. Increasing either cell density or [CA]$_i$ decreases cell-to-bubble distance. However, Figs. 1 and 3 show that bubble efficiency is decreased by increasing [CA]$_i$, but increased by increasing cell density.

When the two datasets are, instead, plotted vs. cell-to-bubble ratio, they collapse into single relationships, suggesting that this may provide a single unifying parameter. Figure 4c-d shows that the number of cells permeabilized and killed per bubble increased with cell-to-bubble ratio, when either cell density or [CA]$_i$ was varied. Moreover, the dependence of bioeffects on cell-to-bubble ratio showed similar functionality for all of the data. For cells permeabilized per bubble, power functions with dependence on cell-to-bubble ratio to the power $1.22 + 0.15$ provided good descriptions of the data at each energy exposure (average $R^2 = 0.98$). Similarly, cells killed per bubble were de-
scribed with a dependence on cell-to-bubble ratio to the power $0.84 \pm 0.05$ (average $R^2 = 0.97$). These correlations are consistent with the trends presented in Figs. 1 and 3, in which increasing cell density increased bubble efficiency, and increasing $[\text{CA}]_i$ decreased bubble efficiency. Because cell-to-bubble ratio includes the quotient of cell density and $[\text{CA}]_i$, this unified parameter captures the inverse dependence of bioeffects on these two parameters.

The above analysis supports our hypothesis that there is a single unifying parameter that correlates with the effects of changing both cell density and $[\text{CA}]_i$, and suggests that this parameter is the cell-to-bubble ratio. To test this hypothesis further, four different cell density and $[\text{CA}]_i$ combinations yielding the same cell-to-bubble ratio (tested at cell-to-bubble ratios of 10 and 0.1) were exposed to US at 98 J/cm$^2$ energy exposure and analyzed for bioeffects. The results of this test, shown in Fig. 4e-f, further support the hypothesis that cells permeabilized...
and cells killed per bubble were unchanged as long as the ratio of cells to bubbles was held constant, even when cell density and [CA]$_i$ were varied over orders of magnitude ($p > 0.05$).

**Nonlinear regression of data to show overall dependence of bioeffects on energy, CA bubble concentration and cell density**

In a previous study (Guzmán et al. 2001a), we showed that applied energy exposure was a unifying acoustic parameter and, in this study, we have shown that cell-to-bubble ratio is a unifying physical parameter that governs US-induced bioeffects. Recognizing this, we performed a multiple nonlinear regression on all of the data collected in this study to provide an overall picture of the combined effects of varying these two parameters. The result is presented graphically in Fig. 5 and shows that bubble efficiency depends on both energy exposure and cell-to-bubble ratio ($R^2 = 0.84$ and $0.94$ for cells permeabilized and killed, respectively). The number of cells permeabilized and cells killed per bubble both increased strongly with cell-to-bubble ratio and both increased weakly with energy exposure. This indicates that bubble efficiency is more sensitive to the nonacoustic parameter of cell-to-bubble ratio than it is to the acoustic parameter of energy exposure. This observation suggests that, for practical applications, it may be less important to accurately control energy exposure than to control cell-to-bubble ratio to achieve a desired amount of drug delivery and cell viability.

To determine the dependence of our upper estimates of bubble blast radii (i.e., $R_K$ and $R_B$) on acoustic and nonacoustic parameters, a similar multiple nonlinear regression was performed using the data from Fig. 1d-e and Fig. 3d-e, and is shown in Fig. 6 ($R^2 = 0.97$ and 0.92 for $R_K$ and $R_B$, respectively). In this case, $R_K$ and $R_B$ are shown as functions of energy exposure and [CA]$_i$, and cell density was held constant at $1 \times 10^6$ cells/mL. The unified parameter of cell-to-bubble ratio could not be used here because, to convert bubble efficiency data (i.e., cells per bubble) into blast radii (i.e., radius per bubble), it was necessary to divide by the cell density (i.e., cell per volume) and, thereby, introduce a third parameter. Figure 6 shows that $R_K$ and $R_B$ depended strongly on [CA]$_i$, and less strongly on energy exposure. Although not shown, $R_K$ and $R_B$ depended only weakly on cell density, which is consistent with Fig. 3d-e.

**DISCUSSION**

The data collected in this study over a broad range of [CA]$_i$, cell density, and energy exposure supported the governing hypotheses of this study: 1. bioeffects increased with increasing [CA]$_i$; 2. bioeffects increased with increasing cell density; 3. the efficiency of CA bubbles at causing bioeffects increased with cell-to-bubble ratio; and 4. the effective blast radius of CA bubbles decreased with increasing [CA]$_i$, and decreasing energy exposure.

**Effects of CA bubble concentration**

Increasing [CA]$_i$, which generates “more” cavitation, caused cell viability to decrease and did not affect molecular uptake. This finding is similar to our previous observation that “more” cavitation generated by increasing acoustic energy exposure also generally decreased cell viability without affecting molecular uptake (Guzmán et al. 2001b).

The observed insensitivity of molecular uptake on
Effects of cell density

In contrast to the dependence of bioeffects on [CA]i, increasing cell density yielded an increase in both cell viability and the fraction of cells permeabilized. This is consistent with previous results in which the fraction of red blood cells that were hemolyzed decreased with increasing cell density (Williams 1983; Ellwart et al. 1988). The present study also showed that the absolute number of cells killed increased with increasing cell density, in agreement with the arguments of Brayman et al. (1996). This effect is readily seen in Fig. 3c, which shows that the number of cells killed per bubble increased with cell density.

These observations may be used to guide biological and biomedical applications of US. Although increasing cell density increased both molecular uptake and absolute levels of cell death, it increased molecular uptake at a greater rate than cell death (i.e., the slope in Fig. 3b is steeper than that in Fig. 3c). Thus, detrimental effects of increased cell death generated by increasing cell density may be outweighed by the much larger increase in cells with molecular uptake. These different “slopes” result in conditions that are close to 100% viability and yield 30% to 40% of cells with uptake (LUP + HUP) at low energy exposure (e.g., 12 J/cm²) and high cell density (e.g., ≥10⁷ cells/mL) (Fig. 3a). These may be useful conditions for drug and gene delivery applications.

Cell-to-bubble ratio

Consistent with the third hypothesis tested in this study, the effects of varying [CA], over more than three orders of magnitude and cell density over more than two orders of magnitude were all described by a single unifying parameter. Cell-to-bubble ratio correlated well with the number of cells killed and permeabilized per bubble, which can be thought of as a measure of bubble efficiency (Fig. 4). This correlation suggests that the ability of a bubble to affect cells is governed by the probability that a cell will “encounter” that bubble. The more cells there are near a bubble (i.e., larger cell-to-bubble ratio), the greater the odds for a bubble to affect more cells.

In a related test, we found that bubble efficiency did not correlate universally with cell-to-bubble distance. The probabilistic model proposed above is consistent with the prediction that cells having a cell-to-bubble distance less than the blast radius will be affected by a bubble. Increasing cell density makes cell-to-bubble distances closer and, therefore, increases bioeffects per bubble (Fig. 3). In contrast, increasing bubble concentration also makes cell-to-bubble distance closer, but decreases blast radii to an even greater extent (Fig. 1d-e), which gives the net effect of decreasing bioeffects per bubble.

Relative contributions of acoustic and nonacoustic parameters

In this study, both acoustic (energy exposure) and nonacoustic ([CA]i and cell density) parameters were
varied. Although both types of parameters influenced bioeffects, Figs. 5 and 6 show that the effect of acoustic energy exposure was less important than nonacoustic parameters over the ranges studied. In Fig. 5, variation of cell-to-bubble ratio over 4 decades changed the number of cells killed or permeabilized by up to 100-fold. In contrast, variation of energy exposure over 3 decades changed bioeffects by just a few fold. Similarly, Fig. 6 shows [CA], altering the upper estimates of blast and killing radii by more than an order of magnitude, but energy exposure changed these radii by less than a factor of two. This suggests that applications of US-mediated drug delivery need to carefully control both acoustic and nonacoustic parameters.

**Strengths and limitations of cell-to-bubble ratio, bubble efficiency and blast radii calculations**

One of the strengths of this study is that it examines bioeffects on a per-bubble basis. From a practical standpoint, the use of bubble efficiency to describe the observed results provided a normalization that facilitated analysis over the wide ranges in cell and bubble concentrations. Specifically, it led to the broad correlation of bubble efficiency with cell-to-bubble ratio; no such correlation existed for the raw, nonnormalized data (e.g., percent cell viability).

From a mechanistic standpoint, analysis on a per-bubble basis is also advantageous. This approach allows one to think physically about the interaction between bubbles and cells. Although the concept of a blast radius is a highly simplified representation of complex bubble physics, the resulting measures of characteristic length scales for the effects of bubble activity lay the groundwork for physical models.

This approach does, however, have limitations. Calculation of cell-to-bubble ratio, bubble efficiency or blast radii requires knowing the number of bubbles present and/or their radii. It is straightforward to know the initial number of CA bubbles introduced into each sample, and this is the number of bubbles used in all calculations. However, after sonication begins and the dynamic cavitation process of oscillation, implosion and re nucleation of bubbles occurs, the number of bubbles is probably different from that at the outset. For example, bubble concentration might decrease if the initial CA bubbles of 1.6 μm average radius were broken apart during sonication and reassembled into fewer bubbles of 5.7 μm radius, which corresponds to the resonant bubble size at 500 kHz (determined for octafluoropropane bubbles suspended in water as calculated using the Minnaert resonant frequency equation, Leighton 1994, with a constant pressure heat capacity of 114.71 J/mol K; Praxair, Danbury, CT). Similarly, gas dissolved in the liquid sample could partition into bubbles by rectified diffusion; thereby, increasing the gas-phase content of the sample. For these reasons, cell-to-bubble ratios and bubble efficiencies are quantitatively correct only before sonication and should serve as qualitative measures during sonication. In addition, blast and killing radii should be regarded as upper estimates that would be reduced by dissolved gas partitioning into bubbles, as discussed in the Results section.

**Physical interpretation of the data**

Together, the data and analysis presented in this study suggest possible physical interpretations of how bubbles permeabilize and kill cells. The proposed model (Fig. 2) views cavitation bubbles as concentrators of energy that is rapidly released, possibly upon bubble implosion. This energy propagates away from the bubble, or site of bubble implosion, affecting cells in the near vicinity and having progressively weaker effects as energy dissipates farther away. We have modeled this energy dissipation as occurring in a spherically symmetrical fashion, although asymmetrical propagation is also possible.

The form of energy release is not shown by the present data, but could involve a local shock wave or jet of fluid, both of which are known to be associated with cavitation bubble collapse (Leighton 1994). High-pressure shock waves have been shown to disrupt cell membranes (Doukas and Flotte 1996) and direct impact and/or shear forces of fluid jets have been shown to induce a number of different bioeffects (Sass et al. 1991; Kodama and Takayama, 1998).

The maximum length scales over which energy release affects cells was estimated in the form of blast and killing radii. At greater energy exposure, these length scales were longer, suggesting that more energy was released from each bubble and, therefore, reached more distant cells before dissipating. Increasing [CA], decreased blast radii, which could indicate that each bubble had less energy due to distribution of the total energy exposure over more bubbles. It also could indicate that energy released by one bubble was absorbed by other bubbles, thereby limiting energy propagation past neighboring bubbles. This idea is supported by the observation that the range of normalized blast radii calculated for this study (3 to 90 bubble radii) was similar to normalized bubble-to-bubble spacings (14 to 189 bubble radii). Finally, addition of more cells did not significantly influence blast radii, suggesting that energy is not dissipated due to absorption by cells but, rather, by other loss mechanisms. Additional experiments and theoretical treatments are needed to validate these proposed mechanisms.

**CONCLUSIONS**

Exposure of DU145 prostate cancer cells to acoustic energy in the presence of CA bubbles transported large
numbers of molecules into as much as 43% of exposed cells and, under different conditions, rendered as much as 98% of cells nonviable. By varying cell density, [CA]i and acoustic energy, each over two to three orders of magnitude, we determined that increasing [CA]i decreased cell viability and had no significant effect on molecular update, but increasing cell density increased both cell viability and uptake. These varying effects of [CA]i and bubble density were unified through an overall correlation with cell-to-bubble ratio. Results were presented in the form of bubble efficiency (e.g., cells permeabilized per bubble), which facilitated physical interpretation of the data and led to estimates of maximum "blast radius" length scales over which the effects of a bubble were felt. Over the range of conditions tested, these radii spanned from 3 to 90 times the bubble radius, which was a similar length scale to the bubble-to-bubble spacings. For applications, these findings suggest that low-energy exposure US applied at a high cell-to-bubble ratio is recommended to produce extensive molecular uptake into cells at high viability.

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