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Non-Viral Gene Transfection with Ultrasound: Is 100% Transfection Possible?

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Ultrasound has been used as a modality to enhance the transfection of cells, purportedly via entry of genetic material through defects in the cell membrane produced by bubble cavitation events. A mathematical model was created to simulate the cellular accumulation of damaging membrane insults due to stresses from shock waves produced by bubble collapse cavitation events. The simulation consists of randomly located cells subjected to shock waves from randomly distributed (in space and energy) collapse cavitations. The shock wave energy that is accumulated during more than 20,000 simulated events is compared to the threshold levels for killing and transfection, at which time the cell is assigned to be dead, transfected, or neither. The model results were compared to published experimental results, and the threshold levels were adjusted to match the model with the experiment. The results indicate that a population of cells has a distribution of killing thresholds. A lognormal distribution fits the experimental viability data adequately. Applying a similar distribution to the transfection threshold indicates that the transfection threshold has a higher value than the killing threshold. This model is based on the assumption that cell damage is cumulative; this and other assumptions are discussed. This model is also compared to the "blast radius" model published by others. The model indicates that cell fragility limits transfection of all cells.

Keywords: Ultrasound, Cavitation, Non-Viral Gene Transfection, Mathematical Model, *In-Vitro* Gene Delivery.

1. INTRODUCTION

Therapeutic gene delivery and subsequent transfection is a significant topic of interest because of the potential benefits to treating diseases, genetic or otherwise, in which chemotherapeutic intervention is not successful. Two general delivery methods are currently being investigated for gene therapy: viral and non-viral. While initially viral gene delivery seemed to promise easy gene delivery, its development has been plagued by several safety and production concerns, such as immunogenicity, low targeting ability, hepatic toxicity, and the cost of producing large amounts of pure virus.[1,2]

Non-viral gene delivery, however, suffers from a different set of problems that appear to be equally difficult to overcome. Many current methods for non-viral gene delivery involve packaging the genetic material into nanoparticles that can enter the cell by endocytosis.[3] Non-viral vectors include polysaccharides (e.g., Chitosan), liposome carriers, and cationic polymers. Their advantages include low cost of large-scale production, ease of use, and low immunogenicity. Although this approach is promising and has shown some *in vitro* and *in vivo* success,[3] the cationic polymers and lipids are often cytotoxic.[4,5] Furthermore, the transfection efficiency of these non-viral vectors is very low (compared to viral gene delivery). Directing the vector to the desired tissue is still a challenge. Thus novel and improved methods are needed. Several strategies have been used to enhance tissue specificity of non-viral vectors including attaching either cell-specific ligands to the non-viral vectors, electroporation, ultrasonication, or direct injection using a gene gun.[6]

One promising approach to non-viral gene (or drug delivery) is the use of ultrasound and contrast agents (gas bubbles) to permeabilize cell membranes, rendering them susceptible to entry of naked or complexed DNA plasmids (or chemical agents).[7–20] Ultrasound consists of pressure waves with frequencies greater than 20 kHz usually generated by piezoelectric transducers that change an applied voltage waveform into mechanical translation of the face of the transducer. Like optical or audio waves, ultrasonic waves can be focused, reflected, refracted, and propagated through a medium, thus allowing the waves to be directed to and/or focused on a particular volume of tissue. The technologies for ultrasonic wave control and delivery are well advanced and pervasive in the areas of biomedical imaging and flow measurement. The main advantage to using ultrasound is its non-invasive nature: the transducer is placed in contact with a water-based gel on the skin, and no surgery is required. Thus,

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ultrasonic gene delivery has been promoted particularly because it can be focused on a small tissue volume. But ultrasound itself does not produce gene delivery without the accompanying phenomenon of gas bubble cavitation.

Cavitation is defined as the oscillatory response of a gas bubble in an acoustic field.[21–23] As the pressure wave passes through the media, gas bubbles of any size expand and contract as the pressure is cycled. If the resulting oscillation in bubble size is fairly stable (repeatable over many cycles), the cavitation phenomenon is called "stable". As the ultrasonic intensity increases, the amplitude of oscillation increases to a point in which the inward moving wall of fluid has sufficient inertia that it cannot reverse direction when the acoustic pressure reverses, but continues to compress the gas in the bubble to a very small volume, creating extremely high pressures and temperatures.[21, 24–26] This type of cavitation (called transient, inertial or collapse cavitation) can be stressful to nearby cells because of the very high shear stresses in the region of the collapse, the shock wave produced by the collapse, and the free radicals produced by the high temperatures within the collapsed bubble. Furthermore, if the collapse is near a surface (such as a cell membrane) an asymmetrical collapse occurs which ejects a jet of liquid at sufficient speed to pierce nearby cells.[24, 27, 28] In general, the likelihood and intensity of collapse cavitation increases at higher intensities and lower frequencies,[24, 29, 30] and is somewhat correlated with the "mechanical index", $MI = P^{-}/\sqrt{f}$, where P^{-} is the peak negative pressure in MPa, and f is the frequency in MHz.

The stress and damage to cell membranes is thought to create direct channels through which genetic material can pass, including both free and complexed plasmids or oligonucleotides. Free plasmids are often successfully used in ultrasonic-mediated transfection.[16, 31–33] Excessive membrane damage, however, can lead to cell death. Thus there appears to be a fine line between a sufficient and an excessive amount of membrane damage during ultrasound-enhanced gene delivery (USEGD). Fortunately, the cells possess membrane repair mechanisms that patch the damaged membrane.[34] This allows a small time-window for transmembrane gene transfection.

Historically, the diagnostic ultrasound community has employed microbubbles of size 1–15 microns as ultrasonic contrast agents because they reflect US back to a receiver. In the past decade researchers have found that these same bubbles can be used to enhance gene delivery. Two main classes of microbubbles have been used:

(1) a gas bubble (usually air or a gas of low solubility) stabilized by a shell of crosslinked protein (e.g., Albunex® or Optison;[35]) and

(2) a gas bubble of low blood solubility stabilized by a layer of lipid-based surfactants (e.g., Definity,[36, 37] BR14,[38, 39] Sonovue,[40] etc.).

These ultrasonic contrast agents (UCAs) are traditionally used to visualize the coronary arteries or other vascular structures; but they have recently been used *in vitro* to successfully deliver genes or used *in vivo* to target sites by the localized (focused) application of ultrasound with bubbles present. In most published studies, a marker-gene such as green fluorescent protein (GFP), luciferase or β-galactosidase has been delivered to show the efficacy of the gene delivery technique.[16, 19, 32, 38, 41–48] The method has shown promise for *in vivo* applications.[49–52]

In theory, it may seem plausible to achieve high transfection efficiency. One would need to use bubbles of uniform size (so they produce uniform collapse phenomena) and distribute them fairly uniformly around the cells (such that all the cells were uniformly treated) in a suspension of a high concentration of DNA. Then the ultrasonic intensity could be adjusted to the level at which cavitation events were sufficient to sonoporate the membrane without causing lethal damage. Most reports of *in vivo* and *in vitro* ultrasonic-enhanced gene delivery claim substantial increases in transfection efficiency when US is applied, some even reporting several orders of magnitude increase.[46, 53] Despite the large increase compared to non-insonated controls, a careful reading shows that the actual percentage of transfected cells remains quite low.[54] Thus, one of the significant questions waiting to be answered is whether ultrasound-enhanced gene delivery (USEGD) can achieve high levels of transfection, even close to 100% transfection that is routinely accomplished by viral gene delivery, which is the ultimate goal for non-viral gene delivery.

This paper describes our investigation of why USEGD has such low transfection rates, and whether it is possible to achieve high transfection rates (approaching 100%) with this technique. In our investigation, we created a mathematical model of the ultrasonic excitation of contrast agents and their subsequent collapse to produce shock waves that stress cell membranes. We then correlated our model with *in vitro* experimental results and were able to make some important observations about the distribution of the sensitivity of the cells toward transfection, and the possibility of obtaining 100% transfection.

2. MODEL DEVELOPMENT

In most reports of *in vitro* transfection of cells, the cells are grown on a solid surface (in a cell culture dish or flask), trypsinized, and then resuspended in a container and placed at the focal point of the ultrasonic field. The cells are exposed to US for a given time, after which they are recovered and assayed for gene transfection. The careful experimental results of Zarnitsyn et al.[54] indicate that transfection is correlated with the mathematical product of ultrasound power density and time of insonation; or more simply said, longer exposure to low intensity US produces transfection similar to that from shorter exposure at a higher intensity. This observation suggests that a large number of less intense insults to the cell membrane may produce the same effect on gene uptake (or at least gene transfection) as a small number of more intense insults. In other words, the effects of the cavitation insults appear to be cumulative, and Zarnitsyn's data suggest that the scaling is linear with respect to both time and ultrasonic intensity.

We captured this linear cumulative effect in time and intensity by constructing a mathematical model consisting of cells randomly distributed in a volume in which collapse cavitation events occur in random locations. We constructed a 3-D cube with sides of length L and periodic boundary conditions.[55] The box edge length L was a function of the mean and standard distribution of the cavitation collapse energy as follows. We assume that there exists a minimum energy density, e_{min}, below which there is no cumulative effect on the cell. This assumption is based on observations that hours of sufficiently low intensity exposure have negligible effect on cell viability.[56] We designed the minimum lengths of the box to be such that a collapse energy of

maximum magnitude occurring in the center of the box (position $L/2$, $L/2$, $L/2$) would dissipate to a value of e_{min} at the center faces of the box. Assuming that the maximum event is four standard deviations above the mean energy, L is defined by the relation

$$e_{\min} = \frac{(E_{\text{ave}} + 4E_{\text{sd}})}{4\pi\,(L/2)^2} \quad (1)$$

where E_{ave} and E_{sd} are the mean and standard deviation of a normally-distributed cavitation energy distribution. In some simulations, the box was larger than this minimum size so that we could study the effect of changing the standard deviation of the collapse energy on boxes of equal size.

Cells of radius R were assigned random positions within the box until a specified cell density was achieved. Also at random locations in the box, cavitation events were generated that had a normal distribution of energies around a mean energy level. The transfer of energy from a collapse event to the surrounding cells was calculated by modeling a shock wave with total energy E that expanded spherically such that its energy density was $E/4\pi r^2$, where r is the distance from the center of the collapsed bubble. At the location of each cell in the box, the energy density for each shock wave was multiplied by the cell surface area and then summed to keep a running total of accumulated energy density injury, reported in units of J. The cavitational energy released within the box is characterized as the sum of the collapse energies of all the events divided by the box volume, reported as nJ/cm.[3]

At the end of a specified number of collapse cavitation events (usually around 2×10^4), values were selected for "thresholds" of accumulated energy; i.e., the minimum (threshold) value required to produce a biological event. We selected a threshold for cell transfection and a threshold for cell death. As will be evident from the results section below, comparison with experimental data suggested that not all cells are created equal, in that there may not be a single threshold value that applies equally to all cells; rather there appears to be a distribution in the susceptibilities of the individual cells toward permeation and death. Therefore in some simulations, the cells were assigned individual threshold levels that represent a distribution of susceptibilities according to a statistical distribution function.

The literature is rather sparse regarding the energy released in a shock wave associated with a collapse cavitation event. Experimental measurements by Vogel and Lauterborn et al.[57] indicate that during bubble collapse, 84% of the total energy is lost (26% is recovered elastically for the next cycle), and of that 84%, about 73% is emitted as sound. This value of 61.3% experimental sound emission ($0.84 \times 0.73 = 0.613$) is similar to a value of 61.5% calculated theoretically for sound emission in a shock wave of a collapsing bubble.[58] For this study, we used a value of 61.3% emission of total bubble energy in the shock wave.

The question still remains as to what is the total energy encompassed within a bubble collapse event. The dynamics of bubble expansion and collapse in an oscillating pressure field are very complex and dependent upon bubble size, pressure, frequency and amplitude, as well as the thermodynamic properties of both the gas phase and the surrounding liquid phase. As the bubble collapses, there is a conversion of potential energy and pressure-volume work to the kinetic energy of the bubble. As a first approximation of the total energy, we considered the maximum kinetic energy during the bubble collapse, since this value was more readily accessible. We admit that the use of kinetic energy probably underestimates total energy, because even at maximum kinetic energy, there is still some potential and thermal energy.

The kinetic energy during bubble collapse was calculated by solving the Rayleigh-Plesset equation[24, 26] for an air bubble of 1.625-μm radius in an acoustic field at 500 kHz and 24 kHz. The Rayleigh-Plesset equation was solved in a MatLab environment (MathWorks, Natick, MA) using a Runga-Kutta 45 algorithm. The kinetic energy was calculated as $1/2mv^2$, where v is the bubble wall velocity and m is the effective mass of the liquid, which is 3 times the mass of the liquid displaced by the bubble.[24–26] Note that the effective mass decreases as the bubble collapses, so the maximum in kinetic energy does not occur at the maximum in wall velocity.

For the computer simulation of the interaction of shock waves and cells, the equations were non-dimensionalized by scaling lengths to the cell radius and scaling energies to the mean energy of a single cavitation event. Thus the model was executed in dimensionless space so that the results could be scaled to any cell size and cavitation energy. The code was written in C++ and executed on a personal computer. For the present study, at each "experimental condition" at least 3 simulations were done with different random initial configurations of cell and bubble positions. The mean average and standard deviations were calculated and plotted as shown below. These results were compared to the experimental work of Zarnitsyn et al.[54] which in our opinion are the most thorough and complete set of data showing both viability and transfection under various ultrasonic conditions.

3. RESULTS

A computer program was written to simulate the accumulation of energy insults on a random distribution of suspended cells in a random field of collapse cavitation events. The first task was to obtain the energy of a shock wave as a function of the acoustic amplitude. Figure 1 shows the maximum kinetic energy for a collapsing air bubble (resting radius = 1.625 um) at 24 kHz and 500 kHz for acoustic amplitudes of 0.35 to 2.0 MPa. Maximum kinetic energy values at 24 kHz and 0.6 MPa, and at 500 kHz and 1.25 MPa are 4.1×10^{-15} J and 1.5×10^{-12} J respectively. These amplitudes were selected as the middle of the ranges used in the experimental work of Zarnitsyn et al.[54] Both conditions produce collapse cavitation, as evidenced by their mechanical indices of 3.9 and 1.8, respectively.[59] As described above, these kinetic energies were scaled to 61.3% to estimate the average

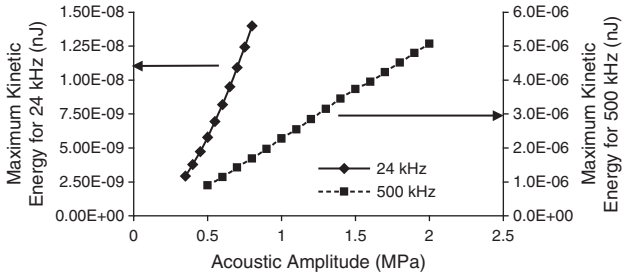

Fig. 1. Maximum kinetic energy of a collapsing bubble of 1.625-μm radius during the first cycle of acoustic insonation, calculated using the Raleigh-Plessett equation. Diamond: 24 kHz insonation from 0.35 to 0.80 MPa. Square: 500 kHz insonation from 0.5 to 2.0 MPa.

energy in a shock wave of 2.5×10^{-15} J and 9.2×10^{-13} J, at 24 and 500 kHz respectively. The estimated shock wave energy at 500 kHz is about 2.5 orders of magnitude greater than that at 24 kHz because the resonant frequency of the bubble (about 2 MHz) is much nearer to 500 kHz. These values are very small in terms of absolute energy, but the proximity to cells and the cumulative effects can add up to substantial energy exposure to the cells.

Data from simulations at 500 kHz and 1.25 MPa are plotted in Figures 2(a and b), showing the fraction of cells permeabilized and the fraction of living cells for 4 different thresholds, in which a single threshold was applied uniformly to every cell within its simulation. It is noteworthy that the data for cell viability (Fig. 2(b)) shows that the cells survive for a time at nearly 100% viability followed by a steep drop to nearly zero viability. Furthermore, the amount of cell transfection can approach 100%, as long as the value of the permeabilization threshold is less than that of the killing threshold. This stands in stark contrast to experimental data, such as that provided by Zarnitsyn et al.[54] in Figure 3, and of other researchers whose data are not shown here,[19, 60, 61] in which the cell viability decreases immediately, even at very low values of energy exposure. The shapes of these plots are very different. In our simulations (Fig. 2), even if the killing threshold is placed at very low values, a sigmoidal shape is always produced for the simulation of viability. However, the experimental shapes of the viability plots most often appear to be more like a decaying exponential curve, in that viability decreases immediately, and the experimental killing "rate" decreases as time or energy increases.

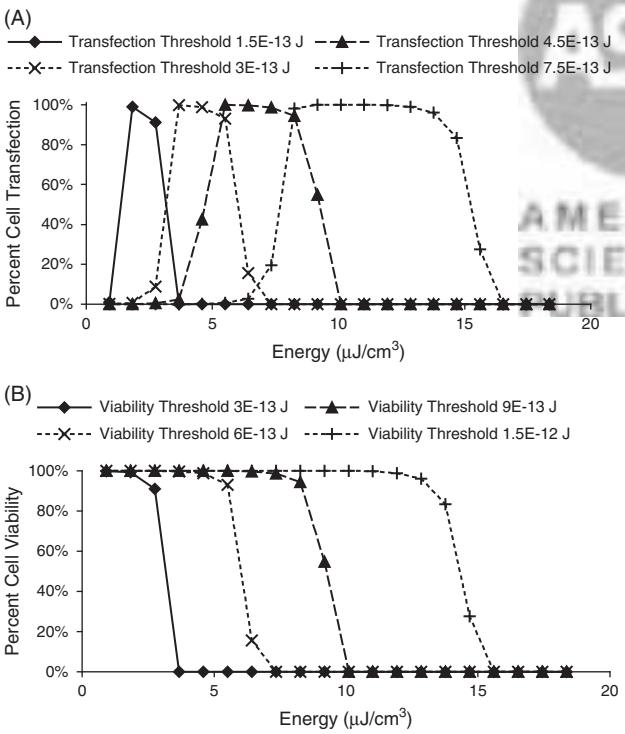

Fig. 2. Cell transfection (top) and viability (bottom) as a function of acoustic exposure for simulations in which all the cells have a uniform value of transfection threshold (top) or killing threshold (bottom). The various threshold levels are indicated on the figures. These simulations were generated at 500 kHz with a cavitation event energy centered at 9.2×10^{-13} J with a standard deviation of 5%.

Because our simulations could never match the shape of the experimental viability data, we concluded that this simple model—that each cell has the same killing threshold—does not match experimental reality. It appears that in real systems, some cells are fragile and die with little exposure, while other cells are more robust and remain viable even at high levels of exposure. Therefore we modified our experimental model to allow the cell population to have a distribution of "fragilities" or "susceptibilities" to cumulative insonation insults.

Figure 4 shows the results of our numerical simulations in which each cell was assigned a random threshold value within a particular distribution for transfection and viability, in this case a lognormal distribution with a mean threshold of 9.18×10^{-14} nJ/cm^3 for viability and 1.84×10^{-12} nJ/cm^3 for permeabilization. Comparison of Figures 3 and 4 shows that by proposing that the cell population has a distribution of susceptibilities to acoustic insults, the model can more accurately match the experimental data.

A very interesting aspect of this analysis is that the transfections rates are very low—less than 1% for the data of Zarnitsyn et al. as shown in Figure 3. To achieve such low levels of transfection, the model surprisingly predicts that the mean killing threshold is less than the mean transfection threshold.

Another important issue that our model addressed is the effect (upon transfection and viability) of distributions in the energies of the cavitation events. We do not believe that all cavitation events have equivalent energies. We ran the model at differing values for the standard deviation of the energies of the cavitation events (0, 5 and 25% of mean), always employing normally distributed cavitation energies. The results showed that for the same mean energy, the range of energy distribution had very little effect upon the distribution of viable and transfected cells, as shown in Figure 5.

4. DISCUSSION

In the quest for better technology to achieve high levels of gene transfection, one often wonders if novel technologies such as ultrasound-induced poration of cell membranes can achieve nearly 100% transfection without killing the cells in the process. We developed a simple computer model to address this question. However, the foundation of the model is the assumption that the stress on cells leading to transfection and death is cumulative; i.e., a large number of small stresses produces the same effect as a small number of larger stresses. We believe that this assumption is generally correct, at least for those cell lines and experimental conditions which show that cellular response correlates with the mathematical product of the ultrasonic acoustic power and the time of exposure, which we call acoustic energy. Many reports show that there is a correlation between acoustic energy and various cellular responses.[19, 54, 60, 62–67] Such a correlation implies that as the power of sound wave increases, then either the number of events increases proportionally at constant energy of the collapse event, or the energy of the event increases proportionally at constant number of events per time, or a combination of both. We have no data to reject either postulate, and thus allow either or both possibilities of increases in collapse energy and/or increases in the number of events as the acoustic power increases.

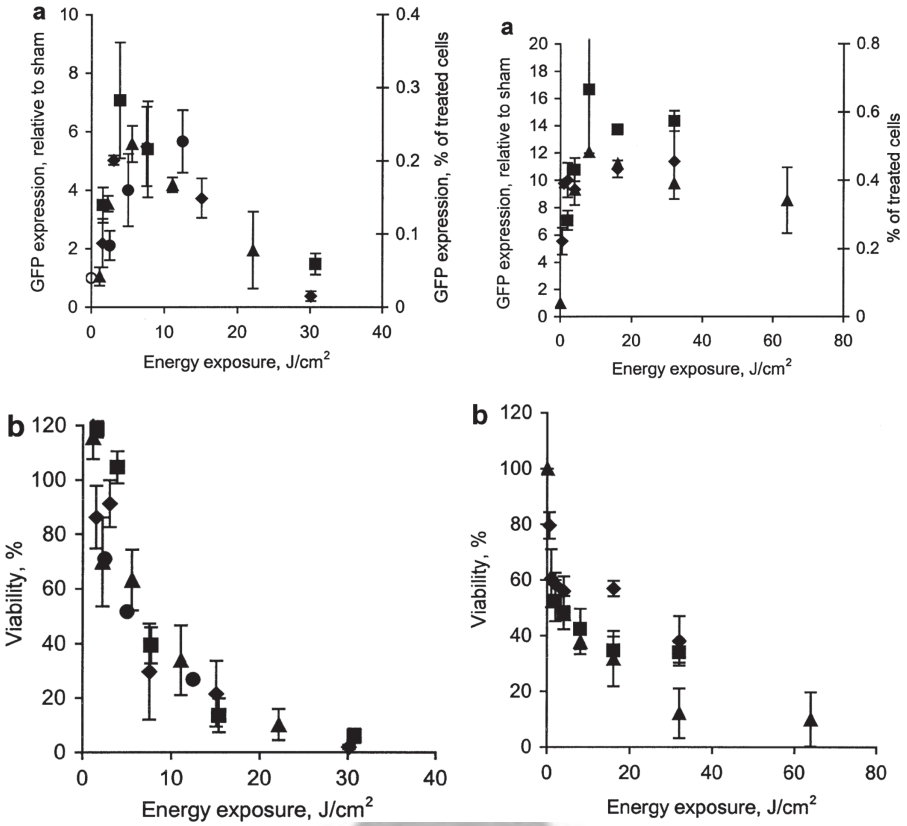

Fig. 3. Data from Zarnitsyn et al.[54] Top left: Transfection of DU145 cells exposed to 24 kHz insonation. Top right: Transfection of DU145 cells exposed to 500 kHz insonation. Bottom left: Viability of DU145 prostate cancer cells exposed to 24 kHz insonation. Bottom right: Viability of DU145 cells exposed to 500 kHz insonation. The various symbols refer to various levels of acoustic power density, which values can be found in the referenced paper.

Thus the experimental observation that a longer exposure (producing a higher number of events) at a lower acoustic power (lower energy events as evidenced by Fig. 1) produces the same biological effect as a shorter exposure (fewer events) at a higher acoustic power (higher energy events), supports our view that cellular stress is cumulative, at least within the time period of these experiments. To state it simply, two holes in the membrane are more effective than one (for transmembrane gene transfection), and are assumed to produce twice the probability of successful gene transfection. Cells appear to be able to repair the membrane damage inflicted by this type of insonation over time periods on the order of several seconds to minutes.[34, 68, 69] We assume that the time scale for membrane repair is much longer than the time scales for generation ($\sim \mu$sec) and accumulation ($\sim$sec) of membrane damage, thus providing a window for gene transport into the cell.

Having established the foundation of cumulative damage, we produced a simple mathematical model of random cavitation events occurring within a field of suspended cells, and then proposed that cells having accumulated more than a given threshold

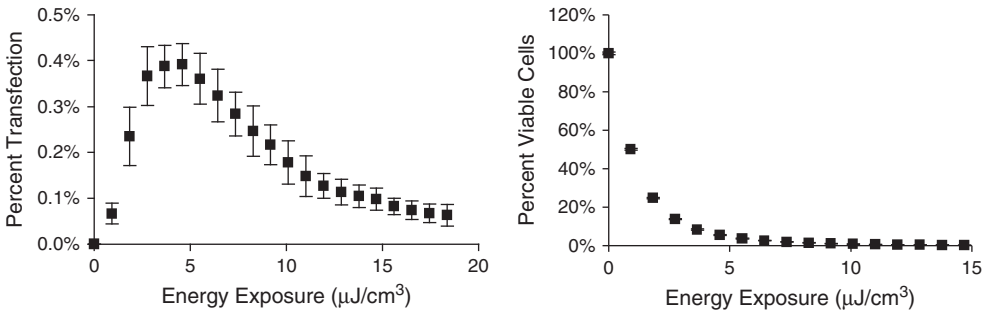

Fig. 4. Data from 500 kHz simulations showing the cell transfection (left) and the cell viability (right). The simulations was done with 10,000 cells of 10-μm diameter in a box of 1.0 mm.^{3} 20,000 cavitation events were simulated, having an average event energy of 9.2×10^{-13} J, and a Gaussian distribution standard deviation of 4.6×10^{-14}. The cells were modeled as having a transfection threshold following a lognormal distribution with an expected value of 1.84×10^{-12} J and the log standard deviation of 1. The viability threshold was also modeled as a lognormal distribution with a mean of 9.2×10^{-14} J the log standard deviation of 1. These distributions produce data comparable to the experimental data in Figure 3. The data points represent the mean average of at least 3 simulations, and the error bars indicate the standard deviations. In the viability plot the error bars are smaller than the size of the data marker.

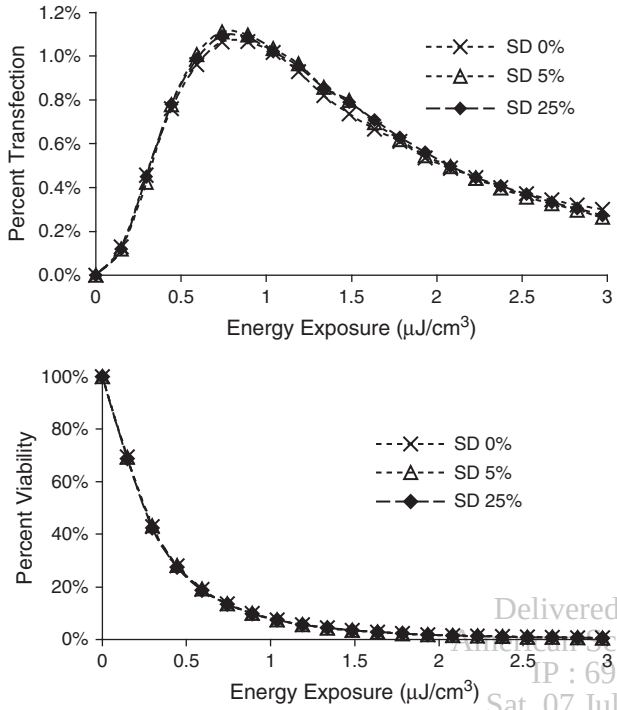

Fig. 5. Cell transfection (top) and viability (bottom) simulated at 3 different values for the standard deviation of a Gaussian distribution of cavitation energies with a mean value of 9.2×10^{-13} J. The simulations were done with 10,000 cells of 10-μm diameter in a box of 10.1 mm.[3] The cells were modeled as having a transfection threshold following a lognormal distribution with an expected value of 8.2×10^{-12} J and the log standard deviation of 1. The viability threshold was also modeled as a lognormal distribution with a mean of 5.5×10^{-13} J and the log standard deviation of 1. For clarity, error bars are not shown because they all overlap.

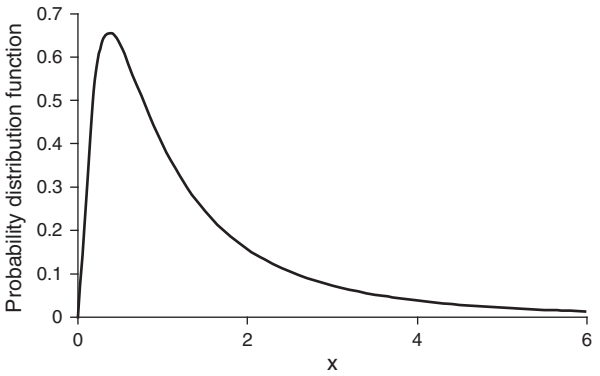

Fig. 6. Lognormal distribution function with a mean expected value of 1.65 and standard deviation of 1.

of energy insults would be transfected or would die. However, this simple model did not match experimental observations, particularly with regard to viability. Real cell populations appear to commence significant die-off from the very first application of ultrasound, even if that ultrasound is at low levels of exposure (See Fig. 3(b)). Other cells appear to be much more robust. The only way for this to be possible is if the population of cells contains some "weak" members that die upon low energy exposure, and some "strong" members that can endure to high exposure levels. We tried fitting the data to several statistical distributions (Gaussian, F, Chi-squared) and found that the data were best fit by the lognormal distribution with a standard deviation of 1.[70] This particular distribution, shown in Figure 6, is skewed toward many cells with low threshold values.

Other experimental data show an immediate commencement in cell death with insonation. We cannot re-publish all that data herein, but we can point out some examples, such as Figure 4 in Keyhani et al.[60] and Figure 2 in Duvshani-Eshet et al.[7]

Because it fits the cell viability so well, the lognormal distribution was applied to create a distribution of transfections thresholds, and we were able to match fairly well the experimental data of Figure 3. We note that these transfection values are very low, both in the experiment and the simulation. The most noteworthy result of this fitting exercise is that the best fit occurred when the average transfection threshold was larger than the average killing threshold. How can this be? Recall that these thresholds are the mean average of the distribution, and each cell has individual values somewhere above, below or at the average threshold. For any given accumulated energy insult, there is a small fraction of cells within the lognormal distribution whose natural resistance to cell killing is above the accumulated energy, and they survive. Within this small survival fraction, there is another subset whose transfection threshold, randomly distributed according to the lognormal distribution, is below the level of accumulated energy insults, so they are transfected, but not yet dead. Although this model is based on many assumptions that cannot be verified, (such as cells possessing the lognormal distribution and independent transfection and killing thresholds), it does match the experimental data.

There are other experimental data in the literature[7, 19, 60, 61] that show data on transfection and viability with values similar to those of Zarnitsyn et al. These data can also be fit with our model when using the lognormal distribution, but cannot be fit without employing some type of distribution in the susceptibilities of the cells to killing by ultrasound.

To return to the original question regarding whether 100% transfection is possible using USEGD to accomplish transfection, we do not think this is possible in practice. A hypothetical model that proposes that all cells in a population have a uniform killing threshold and another uniform transfection threshold also implies that nearly 100% of the cells can be transfected as Figure 2(a) shows. (The maximum is only "nearly100%" transfection because there is a random spatial distribution of locations for cells and cavitation events, and so there will always be a very small percentage of cells that are killed by very close cavitations.)

However, in real experiments the viability data indicate unequivocally that at least for viability, the cells do not all possess the same killing threshold. With any kind of distribution of cell viability fragilities, let alone the lognormal distribution that fits the data best, there will be some cell killing before all cells are transfected. It is no wonder then that experimentalists have not succeeded in achieving 100% transfection using ultrasonic methods for gene transfection, despite more than a decade of effort.

Another barrier to 100% transfection is the presence of another membrane barrier surrounding the nucleus. Once it enters the cytosol, the strand of DNA or RNA must evade a gauntlet of nucleases and binders on its journey to the nucleus. It is estimated that entry to the nucleus is achieved by only a small fraction of polynucleotides entering the cytosol. While in theory it might be possible load sufficient DNA or RNA into the cytosol through

a membrane rupture to obtain 100% transfection, in practice the low transport efficiency within the cell cytosol may reduce the observed rate of transfection. Unfortunately our model can not account for this transport inefficiency within the cell cytosol since the required physical parameters are not available.

Another deficiency in our model is that it does not account for cell damage due to microjets from asymmetric bubble collapse adjacent to the cell membrane. These events have been shown to pierce cell membranes.[27] They are difficult to include in this model because we have no comparison of the magnitude of their effect on membrane permeability with the effect of the shock waves from symmetric bubble collapse. We can only qualitatively state that inclusion of asymmetric collapse would increase the transmembrane uptake of genetic materials, and would probably also increase cell death.

Our simulations also explored the effect upon transfection of the existence of a distribution in the energies of the cavitation events themselves. Preformed gas bubbles (ultrasound contrast agents) and naturally occurring gas cavities do not all have the same size. Thus there will always be a distribution in the energy of the shockwave produced by a collapse event. We do not know how large this distribution is, but Figure 5 shows that the results of the simulation is not sensitive at all to the size of the standard deviation of a Gaussian distribution of collapse energies (at a constant mean value). This lack of sensitivity to this parameter occurs because the cells already receive a distribution of energies based on their distance from the collapse event. A higher energy event at a further distance produces a similar effect on a given cell as does a low energy event at short distance. As the standard deviation of the distribution is increased, there are a small number of larger energy events, but only a very small fraction of cells will experience the higher energy event at a very close distance; at the same time there is a similar small number of lower energy events that will cause less cumulative damage. Thus the spread of energy on both the high and low end balance each other out, and the result is not a substantially different overall distribution of the received energies, at least not within the scatter produced by the random locations of cells and cavitation events themselves.

Finally, we wish to compare our model to another model in the literature, that of the "blast radius".[62] This model also proposes cavitation events in a field of cells, and that the shock wave energy of the collapse event decreases with distance squared. This model proposes a single threshold (not a distribution) for cell transfection and a higher single threshold for killing. There is no accumulation of stress to the cell. This model can simulate the observation of low fractions of transfected cells and the high fractions of dead cells, and should also be considered when evaluating the interaction of cells with cavitation events. As with our model, it also shows that there will always be cell death, and so 100% transfection is not possible, at least in the geometry of cells and cavitation bubbles suspended randomly in 3 dimensions.

Thus far our model has validated what was painfully obvious from experiments, that it is difficult to obtain high transfection efficiency. So is this model at all useful in pointing out a way to increase transfection efficiently? Perhaps not in the scenario of random cells and random cavitation events disturbed in 3-D space. However, there are some scenarios in which high transfection could be simulated with our model. For example, if the cells were on a surface and the bubbles were attached to the cells at the cell/media interface, and if cavitation bubbles were all exactly the same size, then it might be possible to stimulate the bubbles to collapse with just the right energy to permeabilize and transfect the cells without exceeding the killing threshold. Theoretically, such a transfection might be done. It is probably worth doing the experiments to test this hypothesis. Such a transfection procedure might be very useful in treating vascular endothelial cells *in vivo*, or in transfecting large numbers of cells grown *in vitro* as a monolayer on a surface.

Miller et al. have published experimental observations of transfections via bubbles attached to cells grown on a flat surface.[71–73] This study showed that increasing insonation intensity increased cell death, but there was no investigation of gene transfection. However, such an experimental model might be very useful. We are currently modifying our computer code to model such an experiment. Hopefully higher transfections can be accomplished.

5. CONCLUSIONS

Our proposed model of ultrasonic-activated transfection of cells, after fitting to experimental data, indicates that it is very unlikely, if not impossible, to achieve transfection efficiencies approaching 100% when the transfection is done in a 3-dimensional suspension of cells. Comparison of our model to experimental data of others indicates that there is not a single value for the killing threshold of cells in the experimental population. Instead, the data are best fit by a population of cells having a lognormal distribution of killing thresholds. If a similar distribution is also applied to the transfection thresholds, the model can replicate the experimental data fairly well, at least within the scatter of the experimental data and the scatter of the simulation produced by the random position of cells and cavitation events. Interestingly, the model also suggests that the mean transfection threshold is higher than the killing threshold, at least for the experimental data that were fit to the model, that of DU145 cells and ultrasound at 24 and 500 kHz, and other data in the literature. While the results of the model are very sensitive to the distribution used for cell thresholds, they are not sensitive to the distribution of energies of the collapse cavitation events.

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