Electroporation Dependence on Cell Size: Optical Tweezers Study

ARTICLE *in* ANALYTICAL CHEMISTRY · JUNE 2011

Impact Factor: 5.64 · DOI: 10.1021/ac1019649 · Source: PubMed

5 AUTHORS, INCLUDING:

Xin Hu
Schlumberger Limited
33 PUBLICATIONS **265** CITATIONS

SEE PROFILE

analytical chemistry

ARTICLE

pubs.acs.org/ac

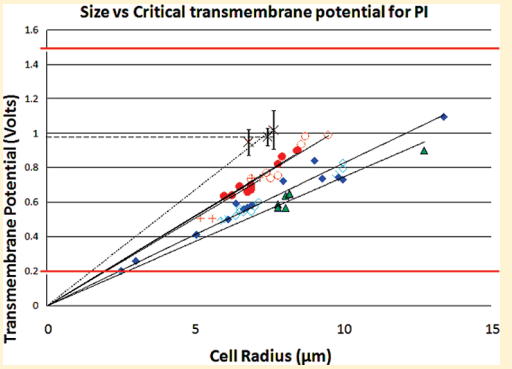
Electroporation Dependence on Cell Size: Optical Tweezers Study

Brian E. Henslee,[†] Andrew Morss,[‡] Xin Hu,[§] Gregory P. Lafyatis,[*,‡] and L. James Lee[*,†,§]

[†]Department of Chemical and Biomolecular Engineering, [‡]Department of Physics, and [§]NSF Nanoscale Science and Engineering Center for Affordable Nanoengineering of Polymer Biomedical Devices, The Ohio State University, Columbus, Ohio 43210, United States

Supporting Information

ABSTRACT: Electropermeabilization or electroporation is the electrical disruption of a cell's membrane to introduce drugs, DNA/RNA, proteins, or other therapies into the cell. Despite four decades of study, the fundamental science of the process remains poorly understood and controversial. We measured the minimum applied electric field required for permeabilization of suspended spherical cells as a function of the cell radius for three cell lines. Key to this work is our use of optical tweezers to precisely position individual cells and enable well-defined, repeatable measurements on cells in suspension. Our findings call into question fundamental assumptions common to *all* theoretical treatments that we know of. It is generally expected that, for individual cells from a particular cell line, large cells should be easier to electroporate than small ones: the minimum electric field to cause electropermeabilization should scale inversely with the cell diameter. We found instead that each cell line has its own *characteristic field* that will, on average, cause permeabilization in cells of that line. Electropermeabilization is a stochastic process: two cells which appear identical may have different permeabilization thresholds. However, for *all three cell lines*, we found that the minimum permeabilization field for any given cell does *not* depend on its size.



Despite the development of effective transfection protocols, fundamental questions about the basic science of electropermeabilization remain open.[1−5] In vitro electropermeabilization is usually carried out by placing cells between parallel electrodes in a buffered suspension with the genes or drugs to be delivered. A program of voltage pulses is applied to the electrodes which generate the electrical field experienced by the cells. Much research in the field has sought to optimize the amplitude, shape, number, frequency, and polarity of the pulses for efficient permeabilization and high cell survival rates.[6,2,5] For eukaryotic cells, applied fields are usually on the order of 1 kV/cm. Pulse lengths can vary considerably, with typical values ranging from tens of microseconds to several milliseconds.[7,2]

The widely accepted description of the process at the single-cell level begins by electrically modeling an intact cell membrane as a near-electrically insulating, near-spherical shell that separates the cell's interior from its exterior environment, both of which are electrical conductors.[5,8,9,1] Permeabilization occurs when the applied field causes the voltage across the cell membrane, the transmembrane potential, to exceed a threshold "breakdown" value. The actual value(s) of V_{perm} is a source of controversy. Some workers claim that V_{perm} has a near-universal value with only small variations even for cells from different kingdoms, as long as the cells' environments and electropermeabilization parameters are the same.[5,10] A survey of the literature shows that reported values for V_{perm} generally range between 200 and 1500 mV.

Quantitatively, a (square) voltage pulse of amplitude V applied to the apparatus at time $t = 0$ generates an applied field in the poration buffer medium, $E = V/x$, where x is the separation of the electrodes. Near a cell, the electric field is distorted and "concentrated" at the cell membrane. See Figure 1. For times short compared to the pulse width, a position-dependent voltage is induced across the cell membrane:

$$V_{\text{induced}} = 1.5fgrE(\cos\theta)\left[1 - \exp\left(\frac{t}{\tau}\right)\right] \qquad (1)$$

Here θ specifies the position on the cell membrane relative to the applied field. See Figure 2a. r is the cell's radius, f is a geometrical factor equal to 1 for a perfect sphere, and g allows correction for nonzero electrical conductivity of the cell membrane. The cell lines we work with are all spherical in suspension, and prior to permeabilization the cell membrane is a good insulator: f and g should both be 1. τ is the time constant for charging the cell membrane's capacitance.

For the buffer and cells used in this work τ is under 1 μs. To find the *total* transmembrane potential, the cell membrane's resting potential, V_{rest}, needs to be added to eq 1. The resting membrane potential for eukaryotic cells is generally <100 mV, with the inside of the cell lower than the outside, and it is usually taken to be independent of the position on the membrane. The exponential in eq 1 rapidly dies off, and barring permeabilization, within 1 μs after application of the voltage, the cell's total

Received: July 24, 2010
Accepted: April 7, 2011
Published: April 07, 2011

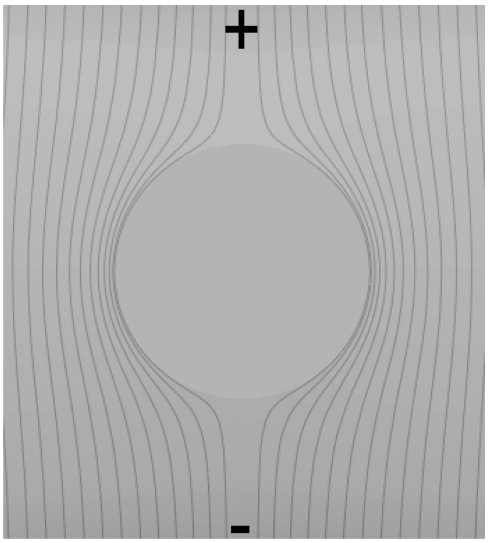


Figure 1. Electric field lines bending around a model spherical cell with an electrically insulating membrane.

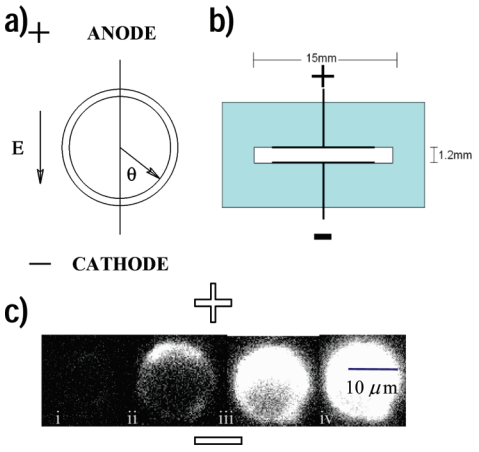


Figure 2. (a) Diagram of a cell in an applied poration field. The radial angle, θ, on the cell membrane is measured relative to the direction of the electric field. (b) Schematic top view of a permeabilization cuvette. Cells were held by the optical tweezers midway between the electrodes and about 20 μm above the coverslip defining the bottom sidewall. (c) Sequence showing propidium iodide fluorescence of a K562 cell permeabilized by a near-threshold electric field applied at $t = 0$. Shown are times (i) $t = 0$, (ii) $t = 5$ s, (iii) $t = 20$ s, and (iv) $t = 60$ s (note that the fluorescence signal shows saturation in (iii) and (iv)).

position-dependent transmembrane potential should settle to the steady-state limit:

$$V_{\text{total}}(\theta) = V_{\text{rest}} + 1.5Er\cos\theta \qquad (2)$$

Permeabilization occurs on the region of the cell membrane for which the magnitude of V_{total} exceeds the permeabilization threshold, V_{perm}. Equation 2 predicts (1) the induced potential is largest at the poles of the cells facing the electrodes, (2) cells with a negative resting potential become permeable first at the pole facing the anode, where the resting potential and the induced potential have the same sign, and (3) for cells with the same resting membrane potentials and permeabilization threshold

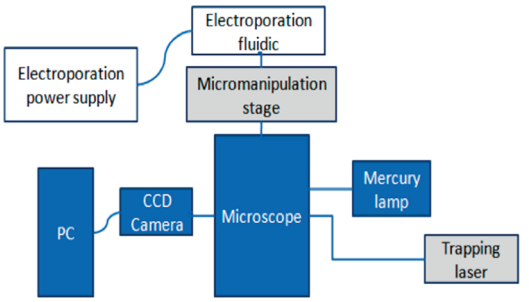


Figure 3. Schematic of the electropermeabilization and optical tweezers apparatus.

voltages but different radii, the minimum electric field for permeabilization scales inversely with the radius.

Figure 2c shows the threshold electropermeabilization of a cell where permeabilization is identified via the penetration of a fluorescent dye. As predicted, the entry is through the cell's anode-facing pole (note that sometimes we observe dye entering a cell near-symmetrically, but we never have seen it enter preferentially from the cathode-facing side). *The principal result of this work is that our experimental observations are inconsistent with the predicted dependence of the threshold field strength on the cell radius.*

While the existing literature on electropermeabilization is truly vast, apparently, there have been no previous *definitive* experimental studies of the relationship between cell size and electropermeabilization parameters. We summarize relevant previous work below. Frequently, the cell radius dependence of eq 2 is *assumed* and used as a basis for subsequent analysis in experiments.

MATERIALS AND METHODS

We set about making as controlled and simple-to-interpret a study as we could design. Experimental considerations included (a) using cell lines with spherical cells and a large range of cell sizes, (b) working with cells in suspension in contrast to plated cells, and (c) using electropermeabilization conditions that are similar to those routinely use in applications. A major experimental difficulty is that the voltages required for electropermeabilization generate enormous numbers of bubbles due to electrolysis and these violently move cells, making it ordinarily impossible to track a single suspended cell. Our solution was to hold cells under study with optical tweezers. An optical tweezers uses a tightly focused laser beam to manipulate microscope objects on the basis of differences in the indices of refraction between the object and its environment.[11] Our apparatus was able to hold various types of cells against the electrolysis bubbles for permeabilization pulses shorter than 3 ms. Various diagnostic tests described below indicated that the optical tweezers itself has a negligible impact on the permeabilization process.

Figures 2b and 3 illustrate our experimental approach. A suspension of cells in a commercial phosphate-buffered solution was introduced into a custom-built poration cuvette mounted on the stage of an inverted microscope. The microscope was used both for observations and to provide the tight focus required for the laser tweezers beam. About 600 mW of laser power at 1064 nm was used to select and position a cell for study. The cell's radius was measured from pictures made by our imaging system. A sequence of voltage pulses would then be applied to

dx.doi.org/10.1021/ac1019649 |*Anal. Chem.* 2011, 83, 3998–4003

Table 1. Average Threshold Electric Fields Required for Permeabilization

cell line	av threshold field (V/cm)	std dev (V/cm)
K562	692	32.0
MES	555	30.1
3T3	509	22.2

the cuvette's electrodes such that successive pulses increased the field experienced by the cell until we observed permeabilization. Usually a pulse length of 1 ms was used, the voltage between successive pulses was increased in steps of either 8 or 16 V/cm, and the time between pulses was typically 1/2 min. After each pulse, the cell was watched to see if it had been permeabilized. To detect electropermeabilization, the cell impermanent dye propidium iodide (PI) was used. PI is commonly used to detect dead cells. Under normal circumstances, a cell has an intact cell membrane, this dye cannot pass through it, and as a result the cell exhibits a low fluorescence. If the cell membrane is compromised or damaged, the dye passes into the cell and combines with nucleic acids to exhibit a 30× enhancement of its fluorescence. In our case, most of the fluorescence is due to PI interacting with RNA in the cytoplasm.[12] In a typical sequence, between three and five cycles of pulsing and observation would occur before a cell was permeabilized. Once permeabilization was observed, the last pulsing voltage was recorded and the pulsing, observation cycle stopped. To prepare for the next measurement, the poration cuvette was flushed and reloaded with a fresh suspension of cells.

Electropermeabilization. Electropermeabilization was carried out in custom-made cuvettes. These consisted of a 15 mm × 1.2 mm channel milled in pieces of a 1.5 mm thick poly(methyl methacrylate) sheet. Parallel copper electrodes lined the lengths of the channel (i.e., perpendicular to the 1.2 mm dimension). The bottom sidewall of the channel faced the microscope objective and was sealed with a glass coverslip (no. 1 thickness) that allowed images to be captured and gave access to the optical tweezers. The electronic pulser from a Bio-Rad Gene Pulser Xcell electroporation system was used to provide the required voltage pulse sequences. Typical rise times for rectangular pulses were measured to be about 1 μs.

We used cell line K562 (a leukemia cell line, CCL-243) in the experiments. Cells were procured from the American Type Culture Collection (ATCC; Manassas, VA). Invitrogen (Carlsbad, CA) supplied the culture media and additives. Cells were grown in nutrient medium consisting of RPMI 1640 (D-MEM/F-12, catalog no. 21870-076), with the addition of 10% (v/v) fetal bovine serum (FBS; heat inactivated, catalog no. 10082-139), 2 mM L-glutamine (catalog no. 25030), and 1 mM sodium pyruvate (catalog no. 11360). Cells were cultured in 25 cm^2 T-flasks incubated at 37 °C under an atmosphere of 5% CO_2. We passaged cells every 2−3 days by placing a portion in a fresh medium filled flask.

Leading up to an experiment, we harvested cells in the growth phase (<80% confluence) from suspension. We centrifuged cells into a pellet and washed them with Dulbecco's phosphate-buffered saline (D-PBS; catalog no. 14190). Ten minutes prior to electropermeabilization experiments, the pellet was resuspended in D-PBS containing 100 mM PI (Invitrogen, catalog no. P3566) and incubated. Electropermeabilization measurements were carried out on the cells in this solution.

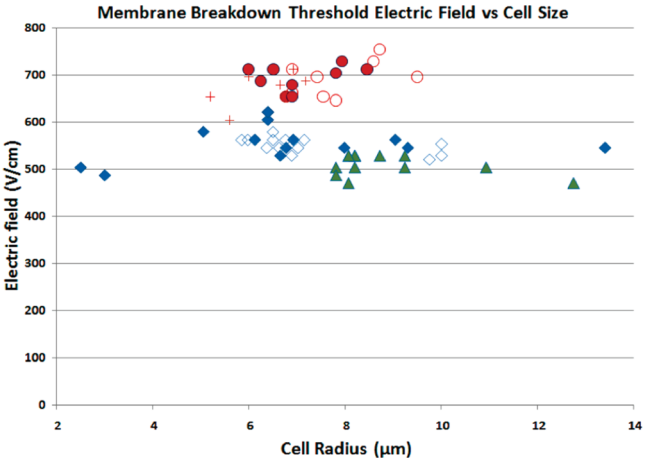


Figure 4. Measured minimum applied electric field causing permeabilization vs cell radius: red, K562 cells; blue, MES cells; green, NIH-3T3 cells; ○, K562 single 1 ms pulse; ●, K562 sequence of 10 1 ms pulses; +, K562 single 100 μs pulse; ◇, MES single 1 ms pulse; ◆, MES sequence of 10 1 ms pulses; ▲, 3T3 single 1 ms pulse.

Optical Tweezers and Microscopy. Cell manipulation was accomplished using an optical tweezers built from a 3 W, 1064 nm laser (Crystal Laser) and an inverted microscope (Nikon). Typically 600 mW of power was used to hold a cell, although diagnostic tests were carried out at lower powers. An acousto-optic deflector (InterAction-DTD-272D6) was used to rapidly turn the laser trap off and on. The microscope was also used for observation. An electron-multiplying charge-coupled device (CCD) camera (Photometrics Cascade II: 512 EMCCD) detected PI fluorescence.

RESULTS

Electropermeabilization Threshold Fields. Figure 4 and Table 1 summarize our principal results. We initially studied K562 cells, a line of immortalized leukemia cells. In addition to the 1 ms, single-pulse measurements described above, we also measured permeabilization thresholds for single 100 μs pulses and for a program consisting of 10 1 ms pulses spaced by intervals of 100 ms. Rols and Teissie[7] claim that while the basic fact of permeabilization is determined solely by the *size* (voltage or field strength) of an applied pulse, the degree of permeabilization is determined by the length and number of pulses. The 100 μs measurements probe the sensitivity of our results to the pulse length. Our 10-pulse program checks whether the single-pulse measurements miss successful permeabilizations simply because the degree of permeabilization is below our detection sensitivity. In fact (see Figure 4), both the 100 μs measurements and the 10-pulse program results are indistinguishable from the single-pulse measurements. In all successful permeabilizations the ultimate fluorescence signal was at least an order of magnitude larger above our minimum detection sensitivity. Hence, we believe that our conclusions are robust with respect to the pulse length and number and, as far as permeability to PI is concerned, that we observe all successful attempts. Two features are immediately clear from the K562 cell data. First, electropermeabilization is a stochastic process in the sense that two seemingly identical cells can have different measured permeabilization thresholds, and second, there is no apparent dependence of the permeabilization

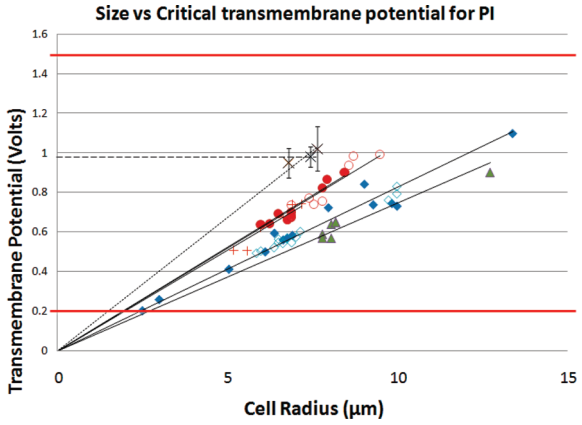


Figure 5. Transmembrane potential thresholds for permeabilization inferred from our data using eq 3. Also shown are the "double Coulter counter" data of Zimmermann et al. Almost *all* previously reported experimentally determined transmembrane potential threshold values lie within the range defined by the red lines. "Conventional theory" predicts that cells of a given variety will all have nearly the same *transmembrane potential*, independent of the cell radius, and should be fit by a horizontal line. Data fit by a line passing through the origin indicate the constant threshold *electric field* dependence on the cell radius that we claim. Key: ○, K562 single 1 ms pulse; ●, K562 sequence of 10 1 ms pulses; +, K562 single 100 μs pulse; ◇, MES single 1 ms pulse; ◆, MES sequence of 10 1 ms pulses; ▲, NIH-3T3 single 1 ms pulse; ×, Zimmerman data. Zimmermann et al. report their results in terms of cell volumes. For this figure, we calculated cell radii and error bars for those data by assuming spherical cells.

threshold on the cell size. Certainly the predicted inverse dependence on the cell radius is not observed. The stochastic nature of the process is to be expected[8] on the basis of similar observations in studies on the breakdown of planar lipid membranes. The observed independence of the cell size was entirely unexpected. Consequently, we carried out further measurements on two other lines of spherical cells that were available to us: mouse embryonic stem cells (MESc's) and NIH-3T3 (3T3) cells. These additional data confirmed the essential K562 observations. The MESc's allowed measurements for over a factor of 5 variation in radius but even then showed no relationship between the cell size and permeabilization threshold field. In Table 1 we summarize our data by calculating the mean threshold field and its standard deviation for each cell line studied, where we have include *all* measurements made for a cell line in the average. The mean permeabilization threshold fields for the three lines are similar but measurably different. The variation in the mean permeabilization field for these three cell lines is about 30%.

Transmembrane Potentials. An alternative way of looking at our data is to use each individual data point to estimate the threshold *transmembrane potential* needed for permeabilization of a cell. The basic idea is that if in eq 2 it is assumed that the membrane resting potential is small enough to be ignored, threshold permeabilization occurs at the poles of a cell when

$$V_{\mathrm{perm}} = 1.5E_{\mathrm{c}}r \qquad (3)$$

where E_{c} is the minimum applied field causing permeabilization. This way of looking things is often used because it is generally felt that the threshold transmembrane permeabilization potential is fairly constant for different types of cells and *very* constant for a given cell line. To be clear, in fact, we have just shown that this appears *not* to be the case, but we can nevertheless use our measured values of r and E_{c} in eq 3 to calculate from each measurement an *inferred* transmembrane permeabilization threshold. We have done this in Figure 5. Now it is *not* surprising that data for the individual cell lines show excellent linear fits. This just reflects our previous observation that E_{c} in eq 3 is nearly constant and independent of the cell size. What *is* interesting is that each of the individual measurements yields an entirely reasonably appearing value for the transmembrane potential threshold in the sense that all lie within the 200−1500 mV range in the literature. Are we actually measuring electropermeabilization transmembrane threshold values? We discuss this question below.

Diagnostic Tests of the Possible Optical Tweezers' Impact. Crucial to this work is our checking to see if the optical tweezers themselves significantly affect the permeabilization process either by heating the cells or by some other, unforeseen laser−cell interaction. We carried out three experimental tests. First, using K562 cells, we reduced the laser power used to hold cells by a factor of 10 and remeasured permeabilization thresholds as above. Normally, we keep the trapping laser power at about 600 mW to hold the cell as tightly as we can. This is especially necessary for the 10-pulse sequences, and for the lowered laser power trap, we were only able to get single-pulse data. Four trials were carried out for variously sized cells at reduced power, and the mean threshold field was measured to be 685 V/cm. This is in harmony with the 692 V/cm average reported for the full power measurements in Table 1. As a second test, we made additional threshold measurements for K562 cells in which we turned off the trapping laser for the 1 ms period that the permeabilization electric field pulse was applied. The average measured threshold using this procedure was 683 V/cm. Again these measurements are statistically indistinguishable from the full power measurements. Our final test was to use 3T3 cells that plated out on a surface of our permeabilization cuvette. We compared electropermeabilization threshold measurements made with the tweezers laser blocked from the apparatus with measurements in which the tweezers laser was focused on the plated cell under study. To summarize our results, in no case did we detect statistically significant correlations between measured threshold permeabilization thresholds and the presence or strength of the tweezers laser.

We investigated whether our data were sensitive to the interval between permeabilization attempts and measured no dependence at all for intervals ranging from 15 s to 2 min. Additionally, using a microthermistor, we measured the Joule heating of the poration medium by the permeabilization pulses to make sure that cells were not being damaged by heating. We found that, for even the longest sequence of 10-pulse trials, the increase in temperature was less than 2 °C.

Impact of the Pulsing History on the Measured Threshold. In the above, we determine a cell's electroporation threshold by exposing it to a series of incrementally increasing voltage pulse attempts until we observe electroporation. A possible systematic effect in this way of doing things is that a cell's measured threshold might depend strongly on its history. For example, the early "below threshold" pulses might weaken or toughen a cell's membrane so that it porates at a field significantly different from that at which it would if it were instead exposed to a single pulse. To carry out a systematic study of the dependence of the electroporation threshold on the pulsing history as a function of

Table 2. Summary of the Study of the Effect of the Pulsing History on Electroporation

measurement	applied field (V/cm)	number of cells	mean size (μm)	smallest cell (μm)	largest cell (μm)	results
below threshold	~415	6	17	13	22	one 17 μm cell porated on first pulse; all others intact after >8 pulses
above threshold	~575	8	16	12	20	all cells porated on first pulse

the cell size would require generating an enormous amount of data for this type of experiment. Instead, we performed an additional set of measurements to check if such an effect could have a large impact on our results and found that it does not.

The measurements were as follows. We used our incremental technique as above to establish the electroporation threshold and its standard deviation for a population of K562 cells. This work was performed about a year after the work described earlier. We used a different population of K562 cells and measured a different threshold field. We then carried out two types of measurements. First, we exposed cells to a pulse sequence, waiting 1 min between pulses, but this time, instead of incrementally increasing the voltage, *all* of the pulses were at the same voltage, ~2 standard deviations (~20% in voltage or field) *below* the nominal threshold. Next we made measurements in which we exposed cells to pulses ~2 standard deviations *above* the nominal threshold. The results are given in Table 2, and details of the measurements are included in the Supporting Information. Summarizing, the data indicate that the measured threshold poration field of a cell does *not* depend strongly on a previous history of smaller pulses. Of the six cells measured for the below threshold case, one average-sized cell porated on the first pulse and the rest stayed intact until they were lost by the optical tweezers—typically eight or nine pulses. For the eight cells in the above threshold case, all cells were porated by the first pulse. We conclude that our incrementally determined threshold differs (if at all) by much less that 20% from a putative "history-free" threshold in which each cell is exposed to just a single pulse. Most importantly, in *no* case did we observe any correlation between the cell size and the electric field required for poration.

DISCUSSION

In looking at earlier work, three observations are useful: (1) The permeabilization literature is vast, and experimental parameters other than cell size—especially the pulse length and number—vary considerably. Indeed there are claims[5,13] that entirely different physical mechanisms are at work depending on whether short ($\ll$1 ms) or long pulses are used. However, *everyone* uses the formulation of the problem outlined above as a starting point both for theoretical treatments and in analyzing experiments. (2) In vitro electropermeabilization, as it is usually carried out, frequently leaves important experimental parameters only loosely controlled. A typical electropermeabilization apparatus will have fringe fields, cells sticking to surfaces, and electrolysis bubbles that move cells and distort fields. Correlations between these factors and the cell size can possibly show up as secondary correlations in electropermeabilization with cell size. (3) Different studies use different criteria to identify "permeabilization". These include changes in the cells' electrical properties,[14] observed penetration of variously sized molecules into the cytosol,[15] the successful electrofusion of two adjacent cells,[10] or the observed expression of reporter genes.[16]

There are very few previous experiments studying the role of cell size in electropermeabilization, and these mostly measure *statistical* properties of cell *populations*. The results are mixed. For example, Puc et al.[17] measured the permeabilization yield as a function of the applied electric field for populations of DC-3F cells (Chinese hamster fibroblasts). Assuming that cell size was the *only* source of threshold field variation, they found good agreement with a model based on the accepted theory outlined above. In contrast, Hojo et al.[18] measured the permeabilization efficiency of *Saccharomyces cerecisiae* (yeast) cells as a function of the cell size for various fields and found that permeabilization rates for small cells were significantly higher than for large cells—*precisely the opposite* of the expectations based on accepted theory. Most recently, the University of Pittsburgh group carried out single-cell studies[19,20] on the role of cell size on electroporation; however, a direct comparison with our work is not presently possible.

The only relevant previous single-cell measurements to our knowledge that directly address this issue are the "double Coulter counter" work of Zimmermann et al.[14] carried out shortly after the discovery of electropermeabilization or "reversible dielectric breakdown" of cell membranes. We discuss this work in detail because, similar to our work, they measure threshold field values for individual cells in suspension that had also been sized. A Coulter counter detects and sizes single cells by measuring the reduction in the electrical resistance of a volume of buffer medium as a cell passes through the medium. It was observed that, if high fields were used for the resistance measurement, sometimes a lower-than-expected reduction in the resistance was observed. This was identified with the high fields causing electrical breakdown of the cell membrane: electropermeabilization. A double Coulter counter apparatus was developed in which a cell was first detected and sized in a low-field Coulter counter and then passed to a second, high-field counter that measured its breakdown voltage. This device was used to measure the size dependence of the threshold breakdown voltage of guard cell protoplasts in *Vicia faba*. Their results are included in our Figure 5. First, these measurements are certainly *consistent* with the accepted theory: that the permeabilization threshold *transmembrane potential* is independent of the cell size and the data *may* be fit with a horizontal line. However, the data may also be fit by a line passing through the origin and, we argue, are equally consistent with our conclusion that permeabilization occurs at a constant threshold *electric field* value that is independent of the cell size. Thus, while we think that the experimental approach taken in that work is reliable, the results do not have the precision to distinguish between the prediction of the conventional theory and the alternative that we suggest.

We next suggest some possible explanations of our observations. To the extent that the cell may be modeled as an insulating membrane surrounding a conducting interior, eq 2 certainly correctly describes a cell's steady-state transmembrane potential. It is generally assumed that the permeabilization threshold potential is pretty much the same for all cells of a given cell line (assuming constant experimental conditions: buffer, temperature, ...), but maybe this is not the case. Our observations could be explained if smaller cells *actually do* have smaller threshold potentials as inferred from our data in making up Figure 5. For example, smaller

cells often have a higher surface tension in their cell membranes, and a higher surface tension has been shown to reduce the required permeabilization potential. *If* the dependence of the permeabilization threshold due to cell membrane tension exactly compensated the radius dependence of eq 2, our data would be explained with a only a moderate addition to the accepted theory.

On the other hand, it is generally expected that the transmembrane potential electropermeabilization threshold is reasonably constant for a given cell line, and to the extent that eq 2 and the analysis used to generate Figure 5 also produced a wide range of thresholds—almost a factor of 5 for one cell line—one questions the validity and relevance of that analysis. Have we reliably measured (for permeability to PI dye) the value of the threshold transmembrane potential at the *relevant time and location* on the cell membrane? It seems perhaps not. However, similar analysis is also used in most previous experiments that report transmembrane potential permeabilization thresholds[5,10] for cells, so we may ask the question more generally: how valid is the picture that effecting permeabilization of a cell is just a matter of applying a strong enough field to get a transmembrane potential above some threshold value? Note that eq 2 does *not* accurately describe the cell during its initial capacitive charging, *nor* is it accurate after regions of the cell membrane have "broken down" in the sense that they are electrically conductive. For the former, the initial electric field within a cell is *independent* of its radius. Large current densities flow and screening ion gradients form within the cell as the membrane charges. Perhaps a process crucial to permeabilization that requires a large electrical field within the cell needs to occur during this initial charging period, making the overall permeabilization process independent of the cell size. Alternatively, while it is generally thought that the field-induced breakdown of the *electrical* insulation of the cell membrane is closely *related* to its permeability to medium-sized molecules such as PI—both are identified as "electropermeabilization"—it has not been shown experimentally that these occur coincidentally. Certainly, after electrical breakdown, departures from eq 2 are expected and have been observed by Hibino et al.[21] Perhaps electrical breakdown is indeed described accurately by the accepted theory outlined above but that additional mechanism(s) are important to effect permeability to PI and these lead to the dependence on the field that we find. Finally, cell membranes are *not* homogeneous insulators.[22] They are richly populated with channels, transporters, other protein structures, and variant lipid rafts. Within the generally accepted theory, these presumably have a role in determining threshold permeabilization potentials for a particular cell line. We suggest that perhaps these have a broader role that is missed in the first-order modeling of the cell membrane as a homogeneous insulator and that is what is being seen in our measurements.

CONCLUSION

While we have experimentally turned up a surprising scaling relationship for electropermeabilization, at this point we can only speculate as to what it is telling us about the process. The simplicity of the scaling of the applied threshold permeabilization *field* with the cell radius—it is constant—leads us to suspect that there is a fundamental feature important to the permeabilization process that has yet to be uncovered. Experimentally, an important future direction is to investigate other parts of the "parameter space" for the electropermeabilization process. Results using other permeabilization markers, other buffers, and shorter time scales would be especially interesting. The use of optical tweezers for establishing tightly controlled and reproducible experimental conditions should enable additional fundamental experimental investigations.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lafyatis@mps.ohio-state.edu (G.P.L.); leelj@chbmeng.ohio-sate.edu (L.J.L.).

ACKNOWLEDGMENT

This work was partially supported by the National Science Foundation sponsored Nanoscale Science and Engineering Center for Affordable Nanoengineering of Polymeric Biomedical Devices (grant number: EEC-0425626).

REFERENCES

(1) Escoffre, J.-M.; Portet, T.; Wasungu, L.; Teissié, J.; Dean, D.; Rols, M.-P. *Mol. Biotechnol.* **2009**, *41*, 286–295.
(2) Sukhorukov, V.; Reuss, R.; Zimmermann, D.; Held, C.; Muller, K.; Kiesel, M.; Gessner, P.; Steinbach, A.; Schenk, W.; Bamberg, E.; Zimmermann, U. *J. Membr. Biol.* **2005**, *206*, 187–201.
(3) Tarek, M. *Biophys. J.* **2005**, *88*, 4045–4053.
(4) Teissie, J.; Golzio, M.; Rols, M. *Biochim. Biophys. Acta, Gen, Subj,* **2005**, *1724*, 270–280.
(5) Zimmermann, U.; Neil, G. A. *Electromanipulation of Cells*; CRC Press, Inc.: Boca Raton, FL, 1995.
(6) Teissie, J.; Escoffre, J. M.; Rols, M. P.; Golzio, M. *Radiol. Oncol.* **2008**, *42*, 196–206.
(7) Rols, M.; Teissie, J. *Biophys. J.* **1998**, *75*, 1415–1423.
(8) Weaver, J.; Chizmadzhev, Y. *Bioelectrochem. Bioenerg.* **1996**, *41*, 135–160.
(9) Krassowska, W.; Filev, P. D. *Biophys. J.* **2007**, *92*, 404–417.
(10) Teissie, J.; Rols, M. *Biophys. J.* **1993**, *65*, 409–413.
(11) Ashkin, A.; Dziedzic, J.; Yamane, T. *Nature* **1987**, *330*, 769–771.
(12) Kotnik, T.; Pucihar, G.; Miklavcic, D. *J. Membr. Biol.* **2010**, *236*, 3–13.
(13) Benz, R.; Conti, F. *Biochim. Biophys. Acta* **1981**, *645*, 115–123.
(14) Zimmermann, U.; Groves, M.; Schnabl, H.; Pilwat, G. *J. Membr. Biol.* **1980**, *52*, 37–50.
(15) Tekle, E.; Astumian, R.; Chock, P. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11512–11516.
(16) Golzio, M.; Teissie, J.; Rols, M. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1292–1297.
(17) Puc, M.; Kotnik, T.; Mir, L.; Miklavcic, D. *Bioelectrochemistry* **2003**, *60*, 1–10.
(18) Hojo, S.; Shimizu, K.; Yositake, H.; Muraji, M.; Tsujimoto, H.; Tatebe, W. *IEEE Trans. Nanobiosci.* **2003**, *2*, 35–39.
(19) Agarwal, A.; Zudans, I.; Weber, E. A.; Olofsson, J.; Orwar, O.; Weber, S. G. *Anal. Chem.* **2007**, *79*, 3589–3596.
(20) Olofsson, J.; Bridle, H.; Jesorka, A.; Isaksson, I.; Weber, S.; Orwar, O. *Anal. Chem.* **2009**, *81*, 1810–1818.
(21) Hibino, M.; Itoh, H.; Kinosita, K. *Biophys. J.* **1993**, *64*, 1789–1800.
(22) Gowrishankar, T.; Weaver, J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3203–3208.