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RESEARCH ARTICLE

Prediction and optimization of gene transfection and drug delivery by electroporation

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Although electroporation is widely used for laboratory gene transfection and gaining increased importance for nonviral gene therapy, it is generally employed using trial-and-error optimization schemes for lack of methods to predict electroporation's effects on cells. Therefore, we used a statistical approach to quantitatively predict molecular uptake and cell viability following electroporation and show that it predicts both in vitro and in vivo results for a wide range of molecules, including DNA, in 60 different cell types. Mechanistically, this broad predictive ability suggests that electroporation is mediated primarily by lipid bilayer structure and only secondarily by cell-specific characteristics. For gene therapy applications, this approach should facilitate rational design of electroporation protocols. Gene Therapy (2001) **8,** 1464–1469.

Keywords: *electropermeabilization; statistical correlation; cell viability; molecular uptake*

Introduction

Electroporation is routinely used for gene transfection in molecular biology laboratories. However, protocols are determined empirically and then collected in multivolume books of recipes that are often semi-anecdotal.[1–5] This situation, as well as recent and increasing application of electroporation to clinical chemotherapy and nonviral gene therapy,[6–8] suggest the need for methods to predict electroporation-enhanced molecular uptake, loss of cell viability and degree of transfection. To address this need, we (1) used a statistical approach to quantitatively predict electroporation-mediated gene transfection, molecular uptake and loss of cell viability; (2) validated it using a large independent data set from the literature; and (3) demonstrated how it can be used to rationally optimize electroporation protocols.

Electroporation is believed to make short-lived rearrangements to the structure of lipid bilayer membranes that have a lifetime of milliseconds to minutes.[1,5] Creating these pores in cell membranes provides transient access to the cytosol. This phenomenon is believed to occur universally in lipid bilayers when the transmembrane voltage is raised above a few hundred millivolts. DNA transfection of cells in culture has been known for many years; more recent studies have also demonstrated electroporation-mediated drug delivery across skin, gene delivery in animals and targeted chemotherapy in human patients.[6–9]

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Results

Development of predictive correlation

To develop mathematical expressions that predict electroporation-mediated uptake, loss of viability and gene transfection, we were guided by experimental observations[10–13] and theoretical calculations.[14,15] Combined, these suggest that molecular uptake has three important features: (1) uptake occurs only above a quasi-threshold field strength, which depends on pulse length and number; (2) uptake then increases with field strength; and (3) at large field strengths, uptake plateaus at a level which depends on pulse length and number. This suggests that an expression which describes uptake should have a sigmoidal shape. Using experimental measurements of electroporation-mediated molecular uptake (absolute number of calcein molecules per cell) for a large number of individual DU145 prostate cancer cells exposed to more than 200 different experimental conditions (Figure 1),[10] we used nonlinear regression to develop a mathematical expression to describe this large data set.

$$N = 7.0 \times 10^7\, y\tau^{0.31} n^{0.12} \nu\, [1 - e^{\left(-1.4\times10^{-3}\tau^{2.2} n^{2.1} \Psi_c^{4.8}\right)}] \qquad (1)$$

In this expression, y is the extracellular solute concentration (M), τ is the effective pulse length (ms), n is the number of pulses, ν is the cell volume (μm^3) and Ψ_c is the maximum transmembrane potential (V) (see Materials and methods). Note that the pre-exponential term represents the maximum, or plateau, amount of uptake and the exponent represents both the threshold and steepness with which uptake approaches the plateau.

Similarly, electroporation's effect on cell viability was also measured and fit using nonlinear regression.

$$V = 100\, [e^{\left(-0.14\tau^{0.92} n^{0.54} \Psi_c^{2.1}\right)}] \qquad (2)$$

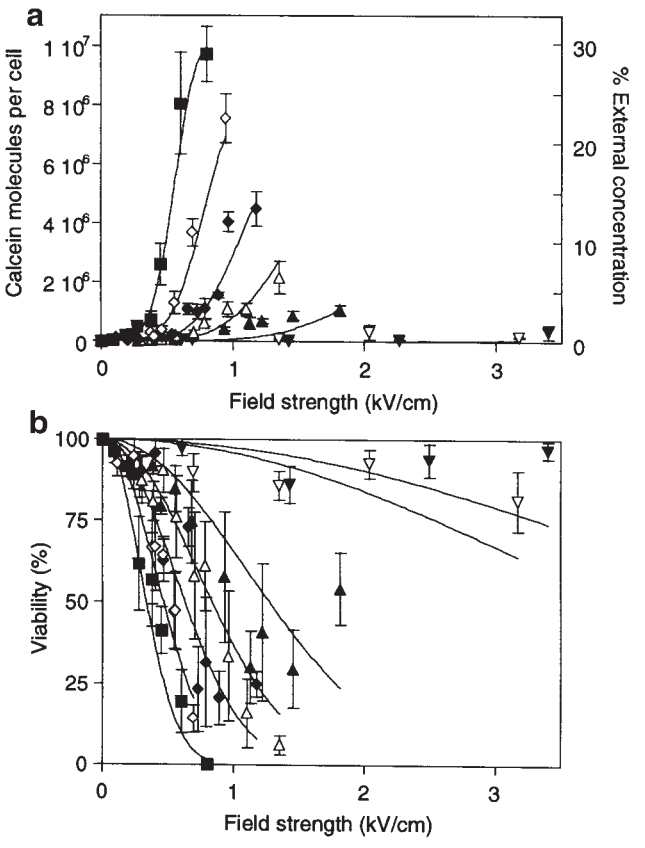

Figure 1 *Representative results from experimental data set used for statistical analysis. (a) Uptake and (b) viability are shown as functions of field strength and pulse length. Single pulses were applied at pulse lengths of 0.050 (▼), 0.090 (▽), 1.1 (▲), 2.8 (△), 5.3 (◆), 10 (◇) and 21 ms (■). Data points represent the average of between three and nine samples collected at 10^{-5} M calcein in the extracellular media and 10^6 cells/ml. Curves are generated from equations 1 and 2 for uptake and viability, respectively. Figure and data are reproduced[10] with permission.*

The correlation expressed in equations 1 and 2 describes the experimental data with an overall uncertainty approximately equal to the scatter in the data itself (Figure 1), as shown by *F*-values of 1.16 and 1.17 for uptake and viability, respectively. An *F*-value of unity indicates the best fit possible given the level of scatter in the data.[16] As a further characterization of this correlation's ability to describe the data, mean absolute percent error (MAPE) of the correlation was calculated as 71% and 27% for equations 1 and 2, respectively. Because MAPE values associated with experimental uncertainty were of similar magnitude – 40% and 32%, respectively – this further indicates that predictions were almost as good as permitted by experimental scatter. Although this statistical agreement shows the correlation's ability to describe our data, these expressions would be much more powerful if they could predict electroporation's effects broadly for cells and molecules not included in its development.

Validation of predictions

Based on the hypothesis that electroporation occurs universally in cell membranes under conditions largely independent of the detailed composition of those membranes and further bolstered by widespread experimental demonstration of electroporation in a diversity of cell types,[1–4] we tested the ability of equations 1 and 2 to predict uptake of a range of different molecules (mostly DNA) and viability for over 900 experimental conditions, including data from 60 different cell lines from 33 different literature studies (mostly *in vitro*, some *in vivo*)[11–13,17–48] (Figure 2). Good agreement between predictions and this large and diverse set of data would (1) provide a broadly applicable and quantitative means to analyze and optimize electroporation protocols and (2) support the above hypothesis regarding electroporation universality.

Mammalian transfection prediction: We first tested the ability to predict relative levels of DNA transfection for 34 different mammalian cell types from 12 different studies[17–28] (See Materials and methods for discussion of how these predictions were made). Of greatest importance for gene therapy applications is the ability to predict the set of electroporation conditions corresponding to the maximum level of transfection. Correlation predictions of optimal conditions over the range of conditions considered in each study were within an average of 15 ± 18% of experimentally determined peak values (eg Figure 2a–e). Because only a limited number of experimental conditions was collected in each study, predictions can be validated with an accuracy limited by the spacing between data points. In 23 out of 33 studies, the predicted optimum fell between the measured optimum and the next closest data point, which corresponds to the best possible agreement for the available data. In addition, 32 out of 33 predictions were less than two data points away from the measured optimum.

As a more comprehensive test of predictive ability, we also determined average percent error for each data set, which yielded an average correlation MAPE of 61 ± 47%. Because most literature studies did not provide errors bars for their data, we can use the experimental MAPE value of 61% determined from our data (ie uncertainty of N·V; see above) as a basis for comparison. The similarity of correlation and experimental MAPE values further suggests that predictions are generally as good as possible, given the limitations on validation imposed by data scatter.

Mammalian viability predictions: We tested the ability of equation 2 to predict cell viability for 35 different mammalian cell types from 18 different studies.[11,17–19,24,25,27–38] To validate the correlation, we compared experimental data to predictions of electroporation conditions that yield 50% viability, since this criterion is often used as a 'rule of thumb' to crudely identify optimal laboratory transfection conditions.[1–4] Overall, predictions for 50% viability were within an average of 29 ± 28% of experimentally determined values (eg Figure 2a,c). In 20 out of 39 studies, predicted conditions fell between the experimental data points that bounded 50% viability, which corresponds to the best possible agreement for the available data. In addition, viability predictions give an MAPE value of 31%, which compares favorably with an MAPE of 32% for the uncertainty in experimental data.

Other predictions: Although much less data are available in the literature, we validated predictions of transfection *in vivo* (eg Figure 2e). Based on comparison with five

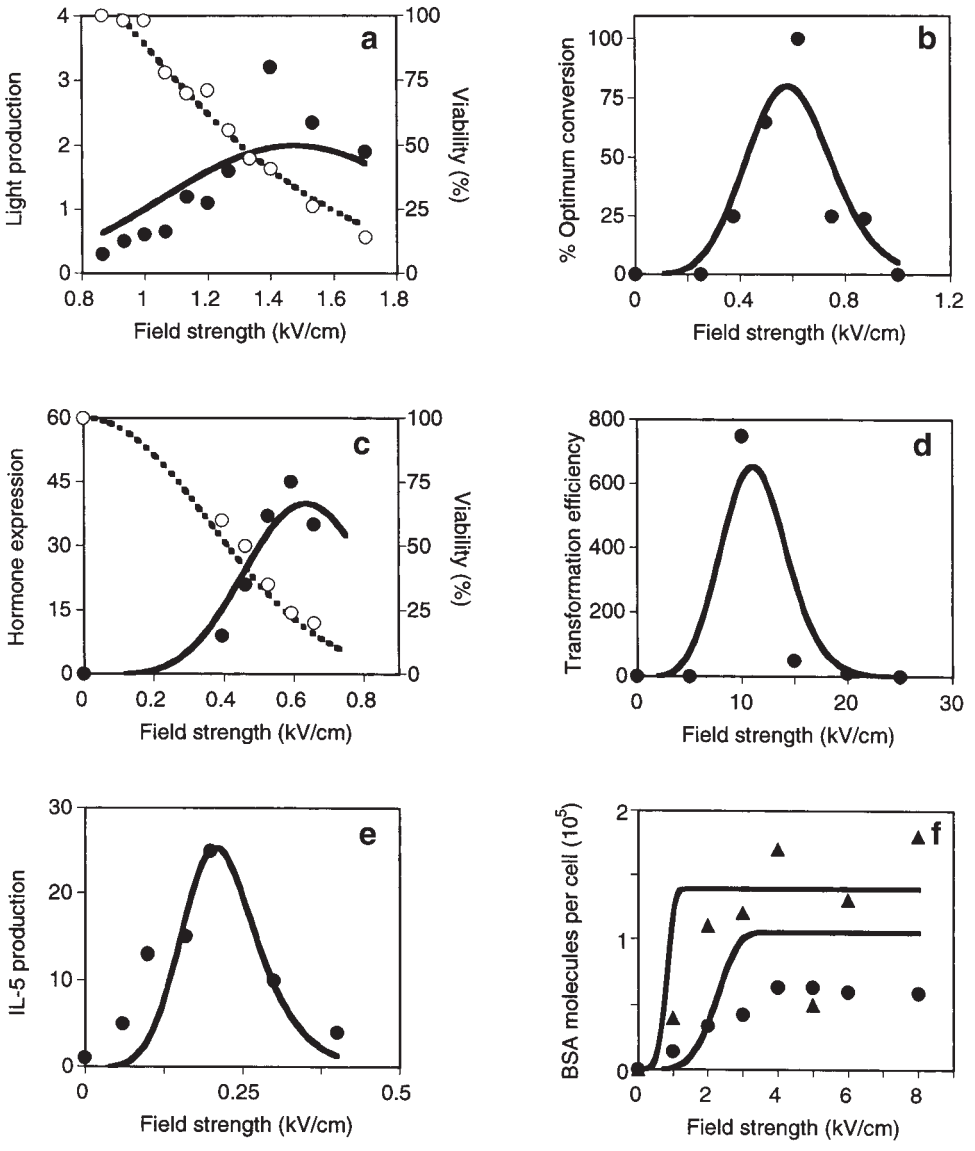

Figure 2 *Representative studies showing correlation predictions of electroporation-mediated gene transfection, viability and molecular uptake for the delivery of (a) luciferase gene to PC-12 cells;[23] (b) CAT gene to U-937 cells;[31] (c) human growth hormone gene to IMR-90 cells;[17] (d) pNC9503 plasmid to Rhodococcus opacus bacteria cells;[48] (e) IL-5 expressing plasmid to in vivo mouse muscle;[40] and (f) bovine serum albumin to human red blood cell ghosts for (●) one pulse and (▲) 10 pulses.[12] Points represent experimental data; solid curves represent predictions of molecular uptake (equation 1) and transfection (equation 1 × equation 2) (see Materials and methods); and dashed lines represent predictions of cell viability (equation 2).*

studies, correlation predictions of peak transfection conditions *in vivo*[39–43] were of similar accuracy as for cell suspensions (MAPE = 51 ± 53%), which suggests that this correlation may be of importance in developing clinical applications. Predictions of peak transfection conditions for bacteria and yeast[22,44–48] (MAPE = 48 ± 62%) were not as good as for mammalian cells, but predictions of 50% viability conditions were similarly accurate (MAPE = 21 ± 8%) (eg Figure 2d). Finally, we validated predictions by equation 1 for uptake of small compounds and macromolecules[11–13,28] (eg Figure 2f). For these studies that predicted the absolute level of molecular transport using five different molecules and three cell types, the overall correlation MAPE value was 101 ± 118%, which shows some discrepancy when compared with the experimental MAPE of 40%.

Optimization

To use this correlation as a tool for laboratory transfection and possibly clinical gene therapy, equations 1 and 2 can be employed to optimize electroporation conditions for a given application. For example, most gene transfection studies require high levels of both DNA uptake and cell viability. In this scenario, it may be useful to maximize the product of the number of molecules per cell, N, and cell viability, V, to yield the most molecules in the most viable cells. Correlation between values of N·V and relative transfection level was supported by our previous analysis (eg Figure 2). Note that N·V correlates on a relative scale with transfection, since the absolute relationship between DNA uptake and expression should depend on cell type and expression system (see Materials

and methods).

As an example of the utility of this correlation, predictions of N·V as a function of electroporation parameters are shown in Figure 3. Long, high-voltage pulses give poor results because too many cells are killed, while short, low-voltage pulses also give poor results because they transport too few molecules. Good results are seen along a narrow corridor, starting with short, high-voltage pulses and becoming progressively better for pulses of longer duration and lower voltage. The best response is achieved with multiple long pulses at relatively low field strength. These conditions, however, are constrained by a practical limitation: the peak becomes very sharp and especially sensitive to small changes in field strength for long, low-voltage pulses. Because field strength is often difficult to control due to voltage drops at electrode interfaces,[49] heterogeneity in cell size and the frequent mismatch between voltages selected on most commercial electroporation units and the actual voltages they deliver, a more conservative approach would be to use multiple short, high-voltage pulses. In this region, optimal conditions are less sensitive to field strength and more sensitive to pulse length and number, both of which are more easily controlled.

These predictions may be useful to guide *in vivo* electroporation. A recent study of *in vivo* gene therapy determined experimentally that long, low-voltage pulses give the greatest transfection,[43] in agreement with our prediction. In contrast, clinical electrochemotherapy protocols have used multiple, short pulses at moderate field strengths.[6–8] This clinical protocol may be successful because the greater reliability of using protocols less sensitive to small variations in field strength outweighs the need to maximize uptake, since the drug used in these studies, bleomycin, is extremely potent at low intracellular concentrations.[8]

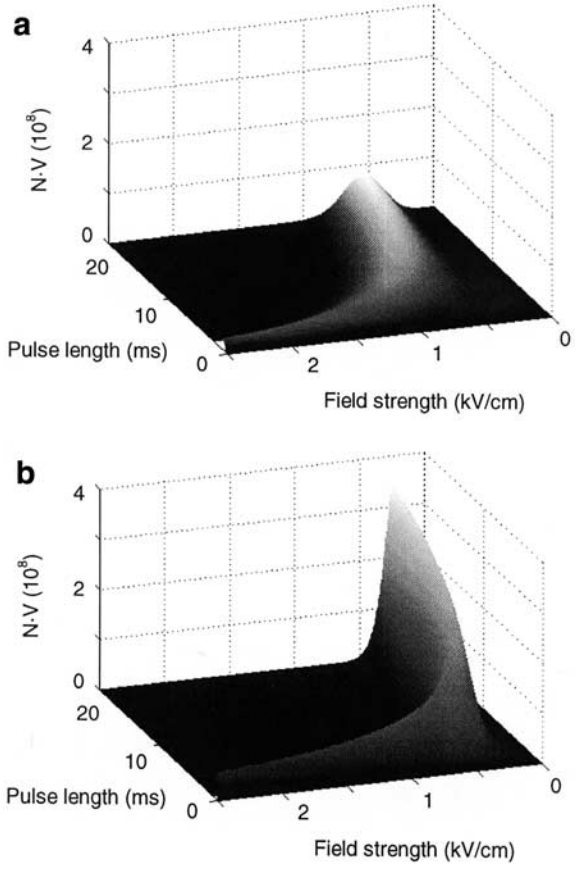

Figure 3 *Optimization using correlation predictions (equations 1 and 2) to maximize the product of uptake and viability (N·V) for (a) one pulse and (b) 10 pulses as functions of field strength and pulse length. The greatest response is predicted for multiple, long pulses at relatively low field strengths, although multiple, short pulses at high field strength may be better in some cases (see text for discussion).*

Discussion

The preceding analysis shows that although the correlation was developed through statistical characterization of results generated using a single cell type, it is nevertheless a good predictor of electroporation's effects on dozens of different cell types. Although viability, uptake and transfection levels vary over many orders of magnitude depending on electroporation conditions, correlation predictions generally have an accuracy better than a factor of 2 (ie MAPE $<100\%$). We believe this good agreement exists because electroporation is mediated predominantly by the existence of lipid bilayer structures. Cell-specific variation in cell membrane lipids and protein composition, as well as different local electrical, mechanical and chemical environments, likely influence electroporation, but appear to be of secondary importance. Other effects, such as addition of free radical scavengers, use of hyperosmotic conditions and presence of nonspherical cells, have not been considered here and may require additional analysis.

This study does not provide rigorous modeling that captures the detailed physics of electroporation processes or describes them mechanistically. This is both a strength and weakness. The weakness is that mechanistic interpretation using this correlation is limited. The strength is that despite its simplicity and statistical nature, the correlation provides useful and fairly accurate predictions to guide gene therapy and other applications. Previous attempts to model electroporation have been limited to highly idealized systems, such as artificial planar bilayers.[14,15] These model predictions have primarily addressed changes in membrane electrical properties, with some attention to predicting mass transport but no ability to predict viability or transfection. Moreover, these models require the use of numerous unknown parameters that must either be fitted or estimated with significant uncertainty. Mechanistic physical models are important, but using them to make predictions of practical utility is difficult.

Overall, the broad predictive ability of the correlation presented supports the hypothesis that electroporation is a universal physical phenomenon that acts primarily on the lipid bilayer structure of cell membranes. Using equations 1 and 2 as a guide, researchers can mathematically predict optimal electroporation conditions to maximize gene expression or to achieve desirable levels of uptake and/or viability according to other criteria.

Materials and methods

Creation of correlation

Using previously collected data,[10] the nonlinear regression function on the MATLAB Statistical Toolbox

(MathWorks, Natick, MA, USA) was used to yield the empirical constants of nonlinear statistical expressions for electroporation-mediated molecular uptake (equation 1) and cell viability (equation 2). For uptake, the empirical constants were all statistically significant (P-value <0.05): $7.0 \times 10^7 \pm 1.8 \times 10^7$, 0.31 ± 0.08, 0.12 ± 0.07, $1.4 \times 10^{-3} \pm 0.7 \times 10^{-3}$, 2.2 ± 0.3, 2.1 ± 0.3 and 4.8 ± 0.6 in order of appearance in equation 1. For viability, all parameters were also statistically significant (P-value <0.05): 0.14 ± 0.01, 0.92 ± 0.07, 0.54 ± 0.07 and 2.1 ± 0.1 in order of appearance in equation 2.

In equations 1 and 2, τ is the effective pulse length (ms) defined for an exponential decay pulse as the time required to reduce the voltage by 1/e. The equivalent pulse length for a rectangular-wave pulse (ie equivalent to an exponential decay pulse) was determined based upon pulses that deliver the same energy, in the absence of a more rigorous way to equate pulses of different waveforms. For this reason the equivalent pulse length for a rectangular-wave pulse is twice the actual pulse length. ν is cell volume, which accounts for the ability of cells of different volumes being able to take up different numbers of molecules. Ψ_c is the maximum transmembrane potential (V), where $\Psi_c = 1.5\ U_c r$ and U_c is peak field strength achieved in the bulk solution around a dilute suspension of cells and r is cell radius.[10]

Validation of correlation

The correlation's ability to describe the data used to generate it and to predict other independent data is expressed using F-values and/or mean absolute percent errors (MAPE). An F-value is the ratio of prediction uncertainty due to lack of fit by the correlation *versus* that due to scatter in the data. If its value is unity, then the prediction uncertainty associated with the lack of fit is equal to that associated with data scatter; this is the best level of certainty possible due to the scatter in the data.[16]

MAPE is the percentage of the absolute value of the residual divided by the actual value. Experimental MAPE values represent the scatter of individual data points about their averages. Correlation MAPE values represent the differences between correlation predictions and average experimental data. The 'detection limit' was set at 10% viability and 50000 molecules per cell (for 10 μM solute concentration and an 11 μm cell radius for the DU145 cells used in the experimental study[10]); these values were considered indistinguishable from zero based on the sensitivity observed in experiments.

Prediction of transfection

Although the correlation explicitly predicts uptake (N) using equation 1 and viability (V) using equation 2, it can also be used to predict relative rates of transfection efficiency. We hypothesized that levels of transfection should correlate with the total amount of DNA delivered intracellularly. As an additional simplifying assumption, the relationship between the number of DNA molecules delivered (the prediction) and expression of an encoded marker signal (the experimental measurement) was assumed to be linear, but highly dependent on the cell and plasmid used, as well as other conditions. Using this approach, the product N·V (ie the number of molecules $\times$ the percent viable cells) was used as a relative measure of transfection (T) and a fitted-scalar (m) was used to account for differences between different expression systems, yielding $T = m \cdot N \cdot V$.

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