High-Throughput and Real-Time Study of Single Cell Electroporation Using Microfluidics: Effects of Medium Osmolarity

Hsiang-Yu Wang,1 Chang Lu1,2
1School of Chemical Engineering, Purdue University, Indiana 47907
2Department of Agricultural and Biological Engineering, Purdue University, 225 S. University Street, West Lafayette, Indiana 47907; telephone: 765-494-1188; fax: 765-496-1115; e-mail: changlu@purdue.edu

Received 20 April 2006; accepted 2 June 2006

Published online 30 June 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/bit.21066

Abstract: Electroporation has been widely accepted as an important tool for the delivery of exogenous molecules into cells. Previous mechanistic studies have been carried out by observing either the average behavior from a large population of cells or the response from a small number of single cells. In this study, we demonstrated a novel microfluidic method with high throughput (up to 30 Hz) for real-time studies of single cell electroporation events. Electroporation occurred when cells flowed through a section of a microfluidic channel defined by special geometry. A CCD camera was used to monitor the response of cells starting from the onset of the electroporation. We studied the swelling of Chinese hamster ovary cells and the rupture of cell membrane during electroporation using this technique. We applied buffers with different osmolarities to investigate the effects of medium osmolarity, based on results from a population of single cells. We were able to establish the distributions of the rates of swelling and membrane rupture in the cell population. We also explored establishing the correlation between the property (the cell diameter) and the behavior (the swelling rate) of single cells. Our results indicated that the processes of swelling and rupture occurred more rapidly in the hypotonic or hypertonic buffers than in the isotonic buffer. Statistical analysis did not reveal strong linear correlation between the cell size and the swelling rate. These proof-of-concept studies reveal the potential of applying microfluidics to study electroporation of a cell population at single cell level in real time with high throughput. The limitations associated with this approach were also addressed.

Keywords: soft lithography; membrane; electropermeabilization; lysis; osmolarity; swelling

INTRODUCTION

Cell electroporation has been an intensively researched topic due to its widespread applications in cell biology, biotechnology, and drug/gene delivery. Electroporation enables delivery of foreign molecules such as drugs and nucleic acids into cells, taking advantage of the transient membrane permeabilization upon applying an electrical field with intensity higher than the threshold (Aihara and Miyazaki, 1998; Jaroszeski et al., 1997; Neumann et al., 1982). While the apparatus and procedures have become refined and well established, the dynamics involved in the electropore formation and molecular transport are not fully understood. In general, most mechanistic studies on electroporation have adopted one of the two approaches: total population methods and single cell methods. Total population methods, such as measuring turbidity changes or following radioactivity of cell suspensions, yield information about average electroporative behavior of cells (Asgharian and Schelly, 1999; Bureau et al., 2004; Chi et al., 2004; Eynard et al., 1992; Frantescu et al., 2005). However, given the heterogeneity of the cell population, it is important to find out whether the average behavior is representative of the individual cells within the population or distinct subpopulations with different responses exist. In that light, single cell methods reveal responses from individual cells under electroporation conditions. Image analysis of individual cells has been frequently used (Gabriel and Teissie, 1997, 1999; Golzio et al., 2002; Hibino et al., 1991; 1993; Shirakashi et al., 2002). Real-time observation of electroporation events with high temporal resolution was possible using pulsed-laser fluorescence microscope (Hibino et al., 1991; Kinosita et al., 2002) or a fast imaging digital camera (Riske and Dimova, 2005). Single cell electroporation has also been investigated using electrical signal based techniques such as patch-clamp (Navarrete and Santos-Sacchi, 2006; Ryttser et al., 2000). However, most real-time single cell methods are restricted to interrogating only a few cells at a time due to the limitation in the size of the imaging device’s frame. The distribution of the interested property in the population has been difficult to obtain when the experiments are done in real time. Flow cytometry has been the tool of choice to bridge the total population approach and the single cell approach by providing a large number of quantitative measurements on individual cells with high throughput (Canatella et al., 2001;
Michie et al., 2000; Prausnitz et al., 1993, 1994). Unfortunately, such measurements have to be conducted after electroporation which means that the information about the kinetics of the process is missing.

The transmembrane voltage, $U$, is typically described by the equation

$$ U = 1.5 \, r_{\text{cell}} E_e \cos \theta $$

(1)

Where $r_{\text{cell}}$ is the radius of the cell, $E_e$ is the external electric field, and $\theta$ is the angle between the site on the cell membrane where $U$ is measured and the direction of $E_e$. At the poles ($\theta = 0, \pi$), the transmembrane electrical field, $E_m$, is large compared to $E_e$.

$$ \frac{E_m}{E_e} = \frac{1.5 r_{\text{cell}}}{h} $$

(2)

Where $h$ is the membrane thickness. $E_m/E_e$ can be as high as $3 \times 10^3$ when $r_{\text{cell}}$ is around 10 μm and $h$ is about 5 nm (Weaver and Chizmadzhev, 1996). These simple equations suggest there could be considerable difference in the response to electric field in the cell population due to the difference in the properties such as the cell size and the membrane thickness. To make it even more complicated, the physiological states of cells and the properties of proteins in the cell membrane such as those forming ion channels may also play roles in the electroporation process (Chen et al., 1998; Chen and Lee, 1994; Weaver, 2000).

We have recently demonstrated a novel microfluidic device which performed electroporation of biological cells with high throughput (Wang et al., 2006; Wang and Lu, 2006). Briefly, a continuous DC voltage instead of electrical pulses was applied to produce the electroporation field in a microfluidic channel. There were alternating high- and low-field intensities in different sections of the channel due to its geometry. Electroporation occurred only in the predefined section with field intensity higher than the threshold. The duration for cells to expose to the electroporation field was varied by adjusting their velocity in the microscale flow. Both transient electroporation and irreversible electroporation were demonstrated in this device. In this study, we have conducted a proof-of-concept study of the response of Chinese hamster ovary (CHO) cells to electroporation at single cell level in real time using this microfluidic tool. We were able to monitor single cells in the microfluidic channel using optical and fluorescence imaging in real time, starting from the onset of the electroporation to the point they flowed out of the field of the camera. The kinetics associated with the swelling of cells and the disruption of the membrane in the electrical field was studied and the distributions in the population were obtained. Using this approach, we compared single cell electroporation behavior in buffers with different osmolarities and investigated the correlation between the cell size and the electroporation kinetics. The osmolarity of the electroporation buffer has been investigated in the literature as an important parameter which affects the transfection efficiency (Cegovnik and Novakovic, 2004; Golzio et al., 1998; van den Hoff et al., 1990). However, there have been conflicting reports about the effects and the mechanism is not totally clear.

This microfluidics-based technique allows us to obtain kinetics information about the electroporation process at single cell level while studying a population of cells with high throughput to obtain the distributions. In principle, both the temporal resolution and the throughput in this method can be significantly improved by applying a fast imaging digital camera (Riske and Dimova, 2005). Our technique will be a useful addition for mechanistic studies of electroporation and optimization of experimental conditions for electroporative delivery of foreign molecules. Since electrical stress may also cause dynamic responses from proteins in the cytoskeleton and introduce complex modulations to the biochemical and biophysical properties of cells, this tool will also allow studies of the time courses of these events (Akinlaja and Sachs, 1998; Navarrete and Santos-Sacchi, 2006).

**MATERIALS AND METHODS**

**Microchip Fabrication**

Microfluidic devices were fabricated based on poly(dimethylsiloxane) (PDMS) using standard soft lithography method (Duffy et al., 1998). The microscale patterns were first created using a computer-aided design software (FreeHand MX, Macromedia, San Francisco, CA) and then printed out on high-resolution (5,080 dpi) transparencies. The transparencies were used as photomasks in photolithography on a negative photoresist (SU-8 2025, MicroChem Corp., Newton, MA). There could be up to 5% error introduced to the width of the channel due to the quality of the photomask. The thickness of the photoresist and hence the depth of the channels was around 33 μm (measured by a Sloan Dektak3 ST profilometer). The pattern of channels in the photomask was replicated in SU-8 after exposure and development. The microfluidic channels were molded by casting a layer (~5 mm) of PDMS prepolymer mixture (General Electric Silicones RTV 615, MG chemicals, Toronto, Ontario, Canada) with a mass ratio of A:B = 10:1 on the SU-8/silicon wafer master treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Unichem Technologies, Bristol, PA). The prepolymer mixture was cured at 85°C for 2 h in an oven and then peeled off from the master. Glass slides were cleaned in a basic solution (H₂O₂: NH₄OH (27%): H₂O₂ (30%) = 5:1:1, volumetric ratio) at 75°C for an hour and then rinsed with DI water and blown dry. The PDMS chip and the pre-cleaned glass slide were oxidized using a Tesla coil (Kimble/Kontes, Vineland, NJ) in atmosphere. The PDMS chip was immediately brought into contact against the slide after oxidation to form closed channels.

**Reagents and Cell Culture**

Chinese hamster ovary (CHO-K1) cells have been employed in all our experiments. Cells were incubated at 37°C, under...
5% CO₂ in the Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc., Herndon, VA) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma, St. Louis, MO), penicillin (100 units/ml, Sigma, St. Louis, MO), and streptomycin (100 μg/ml, Sigma, St. Louis, MO). Cells were diluted everyday to maintain them in the exponential growth phase (~1 × 10⁶ cells/ml). They were harvested by adding Trypsin-EDTA (Sigma, St. Louis, MO) to the culture and centrifuged at 300g for 10 min to remove the supernatant. 

The electroporation buffer (8 mM Na₂HPO₄, 2 mM KH₂PO₄, and sucrose of various concentrations, pH 7.4) was used to suspend the cell pellet for the subsequent experiment. The cell density was typically around 10⁵ cells/ml before flowing into the microfluidic device. The osmolarity of the electroporation buffer was adjusted by adding different concentrations of sucrose: 125 mM (hypotonic), 250 mM (isotonic), and 375 mM (hypertonic) as previously reported (Golzio et al., 1998; Rols and Teissie, 1990). The cells were placed in the hypotonic/hypertonic buffer about 30 min before the electroporation experiments to allow enough time for regulatory volume decrease/increase (RVD/RVI) to take effect. To prevent clogging the channel, the electroporation buffer was filtered by a 0.2 μm filter.

In the experiments involving observation of the membrane disruption, cells were loaded with a fluorogenic dye, calcein AM (λem,max = 517 nm, Molecular Probes, Eugene, OR) at a concentration of 1 μg/ml in the isotonic buffer (8 mM Na₂HPO₄, 2 mM KH₂PO₄, and 250 mM sucrose, pH 7.4) for 15 min. The stained cell suspension was centrifuged to remove the isotonic buffer and cells were then resuspended in the electroporation buffers of interest. In live cells the nonfluorescent calcein AM is converted to green-fluorescent calcein, after acetoxymethyl ester hydrolysis by intracellular esterases. The leak of intracellular materials was observed using a fluorescent microscope during electroporation. The fluorescent images of cells also enabled us to obtain the velocity of cells under different field strengths and infusion rates. The velocity was typically obtained based on image series containing at least 10 cells.

We determined cell viability after electroporation using trypan blue exclusion. Cells were collected from the receiving reservoir (the outlet) immediately after electroporation and transferred to a microcentrifuge tube. After incubating the cells in the electroporation buffer for 1 h, 0.2% trypan blue was added to the solution and the staining of cells was examined after 10–15 min. Viable cells excluded trypan blue, while dead cells stained blue due to trypan blue uptake. The percentage of viable cells was typically determined by observing 500–1,000 cells on a hemocytometer under a microscope. It needs to be noted that we measured only short-term cell death (after 1 h) in this work. The long-term cell death (after 24 h or longer) may be different.

Phase Contrast and Fluorescence Microscopy

During the experiments, we observed the cells in the channel using a fluorescence microscope with phase contrast. The microfluidic device was mounted on an inverted fluorescence microscope (IX-71, Olympus, Melville, NY) with a 40× dry objective (NA = 0.40). The epifluorescence excitation was provided by a 100 W mercury lamp, together with bright-field illumination. The excitation and emission from cells loaded with calcein AM were filtered by a fluorescence filter cube (Exciter HQ480/40, emitter HQ535/50, and beamsplitter Q505lp, Chroma technology, Rockingham, VT). The images of the cells were taken with a CCD camera (ORCA-285, Hamamatsu, Bridgewater, NJ) at a frame rate of 16 or 33 Hz.

Microfluidic Electroporation

Prior to the experiments, the microfluidic channels were flushed with the electroporation buffer for 15 min to condition the channels and remove impurities. When a slow velocity (0.1–1 mm/s) was desired for obtaining clear cell images, the flow of cells was induced by a liquid level difference between the two reservoirs. The microfluidic device was mounted on an inverted fluorescence microscope (IX-71, Olympus, Melville, NY) with a 40× dry objective (NA = 0.40). The epifluorescence excitation was provided by a 100 W mercury lamp, together with bright-field illumination. The excitation and emission from cells loaded with calcein AM were filtered by a fluorescence filter cube (Exciter HQ480/40, emitter HQ535/50, and beamsplitter Q505lp, Chroma technology, Rockingham, VT). The images of the cells were taken with a CCD camera (ORCA-285, Hamamatsu, Bridgewater, NJ) at a frame rate of 16 or 33 Hz.

RESULTS

Device Design

In this study, we applied a novel microfluidic device for electroporation based on continuous DC voltage. We applied geometric modification to a microfluidic channel to create local high field in a geometrically defined section. We controlled the overall voltage across the channel so that only the field intensity in the defined section would produce electroporation. We were able to adjust the duration for the cells to be in the high field by controlling the velocity of the cells and the length of the electroporation section.

The schematic of the microfluidic electroporation device is shown in Figure 1. The devices used in this study have the following dimensions: L₁ = 2.5 mm, L₂ = 2.0 mm, W₁ = 213 μm, and W₂ = 33 μm. Based on Ohm’s law, when a DC voltage is applied at a conductor (in this case, a buffer-filled channel) the potential drop at individual sections of the conductor is proportional to its resistance within the section. Like any conductor, the resistance within a certain section of a microfluidic channel is determined by the conductivity, the length, and the cross sectional area. For a channel with a

DOI 10.1002/bit
uniform depth and a varying width as shown in Figure 1, the field strength $E$ is different in different sections. The field strength in the wide section ($E_1$) and in the narrow section ($E_2$) can be described using the below equations.

$$E_1 = \frac{V}{2L_1 + L_2 \left(\frac{W_1}{W_2}\right)}$$

$$E_2 = \frac{V}{2L_1 \left(\frac{W_1}{W_2}\right) + L_2}$$

$$\frac{E_2}{E_1} = \frac{W_1}{W_2}$$

The width $W_2$ was always much smaller than $W_1$ in our design. This resulted much higher field strength in the narrow section compared to those in the other two sections when a DC field was established across the whole length of the device. $W_1$ can always be further widened if the weak electrical field $E_1$ appears to contribute to electroporation. Electroporation started only when cells entered the narrow section. In this design the cross section of the narrow section (33 $\times$ 33 $\mu$m) determined that cells entered the narrow section one by one. It was possible to realize single cell analysis to investigate the heterogeneity in a cell population. Given the cell density used in this study and the fact that both the channel’s width and depth were almost two times larger than the diameter of a single cell, the presence of cells should not create significant change in the electric field in the narrow channel.

In this report, proof-of-concept studies were carried out to investigate a population of CHO cells under electroporation in buffers of different osmolarities. We studied the swelling of cells and irreversible disruption of cell membrane in the electric field by taking time-sequence images of each cells flowing through the electroporation field in the narrow section with a temporal resolution of tens of milliseconds. The duration of exposure in the electroporation field, which is equivalent of the pulse width in electropulsation, was in the range of 40 to several hundred milliseconds. We were able to study up to 30 cells per second with this approach.

**Kinetics of Cell Swelling in the Electrical Field**

The diameter of cells has been known to increase immediately following electroporation (Deng et al., 2003; Ferret et al., 2000; Golzio et al., 1998; Kinosita and Tsong, 1977b; Pavlin et al., 2005; Shirakashi et al., 2002; Sukhorukov et al., 2005; Tsong, 1991). Swelling of permeabilized cells is believed to be caused by the difference in the permeabilities of ions and larger molecules (macromolecules) inside the cell, which results in an osmotic pressure that drives water into the cells and leads to cell swelling (Kinosita and Tsong, 1977b; Tsong, 1991). Swelling of cells has been mostly studied after electrical pulses in previous reports. The cell diameter was observed to continuously increase during a course of tens of seconds after pulse(s) of submillisecond was applied (Ferret et al., 2000; Pavlin et al., 2005).

In this study, using the microfluidic electroporation device we observed cell swelling during electroporation by flowing a small population of cells through the electroporation field of 200–500 V/cm and observed the change in the cell size in the initial 200–400 ms after the cells entered the electroporation field (the narrow section of the microfluidic channel). It needs to be noted that the duration for cells to expose to the field was one or two orders of magnitude longer than the pulse width typically applied in drug delivery. The cells would be dead after this long exposure when the field strength was higher than the electroporation threshold (as will be revealed in Section 3.3). We used durations in this range mainly due to the fact that the temporal resolution of our CCD camera (33 Hz or lower) was not high enough to capture events at millisecond level. A population of around 30 cells was interrogated one by one using our microfluidic device in each experiment. Buffers with different osmolarities were applied to explore the effects of medium osmolarity. Since the electropores were present in the membrane during the whole period of the observation due to the continuous field, our data of the swelling kinetics may provide some information about the molecular transport during electroporation.

Figure 2 shows a series of snap shots of the same cell entering and flowing in the narrow section of the microfluidic channel when the field strength in the narrow section was 400 V/cm and the buffer was isotonic (10 mM phosphate and 250 mM sucrose). In these experiments, cells were flowing through the channel due to the difference in the liquid level between the inlet and outlet reservoirs with a velocity of 0.1–1 mm/s. We were able to capture 5–8 images before the cells went out of the sight of the CCD camera with a frame rate of 16 Hz. When the field strength in the narrow section was 400 V/cm or higher in all three buffers, the size of cells...
visibly increased during the period. This value (400 V/cm) matches very well with the electroporation threshold field intensity previously reported with CHO cells under electropulsation and in the same buffer system (Golzio et al., 1998). We did observe swelling (to about 111%) at 300 V/cm in the hypotonic buffer. Figure 3 shows averaged swelling rate traces over the first several hundred milliseconds after the cells entered the narrow section with varying field intensities in hypotonic, isotonic, and hypertonic buffers. Each trace in the figure was the average of 30 cells. In general, we did not see significant swelling when the field strength was 200 and 300 V/cm except in the hypotonic buffer. We did not observe any change in the cell size when cells were exposed to the low field $E_1$ in the wide section ($E_1 < 1/6 E_2$) even when the electroporation field $E_2$ was up to 500 V/cm. Several traces appear to start to reach plateau after 300–400 ms. This was not caused by the constraints of the channel walls since the cell size was still significantly smaller than the channel width. When buffers of different osmolarities were used, the rates of the swelling were noticeably different. The swelling was the most dramatic in the hypotonic buffer. The rate was the lowest in the isotonic buffer. For example, when the field intensity was 400 V/cm, the average percentile cell diameter increased to 128% in the isotonic buffer at 300 ms, compared to 149% in the hypotonic buffer and 145% in the hypertonic buffer. At 500 V/cm, the rates in the isotonic and the hypertonic buffers were similar and the rate in the hypotonic buffer was substantially higher than them. In principle, the data here will allow one to estimate the properties characteristic of the cell membrane such as hydraulic conductivity, electrolyte, and sugar permeabilities based on modeling and data fitting, which is out of the scope of this paper (Kleinhans, 1998; Reuss et al., 2004).

Using the high throughput microfluidic device, we were able to investigate the properties of single cells in a heterogeneous population and determine whether there are correlations between the properties and the behaviors. As a proof-of-concept, we studied the relationship between the rate of swelling and the cell size (the original size before entering the electroporation field). It is well established that the transmembrane potential is linearly related to the cell diameter (Kinosita et al., 1988; Weaver and Chizmadzhev, 1996). In our study, we obtained the percentile change in the cell size for individual cells after a short period in the electroporation field. We chose the data points at 180 and 240 ms since in general these time points were well into the growth stage and not reaching the plateau in terms of the cell size. We also chose data taken at $E_2$ of 400 and 500 V/cm since we saw obvious increase trend at these field intensities in all three buffers. For a given buffer, a given time point, and a given field intensity, we collected data of the percentile increase from 30 cells and calculated the Pearson’s correlation coefficient ($r$) between the original cell size and the percentile increase, based on Equation (6).

Figure 2. The time-sequence images of the same CHO cell at different time points in the electroporation section of the device (in the isotonic buffer: 10 mM phosphate and 250 mM sucrose; $E_2 = 400$ V/cm). These images were captured at a rate of 16 Hz. The velocity of the cell was in the range of 0.1–1 mm/s.

Figure 3. The average percentile changes in the size of CHO cells during electroporation. Each trace is the average of the data from 30 cells. The field strength refers to $E_2$ in the narrow section. (a) In the hypotonic buffer (10 mM phosphate, 125 mM sucrose), (b) in the isotonic buffer (10 mM phosphate, 250 mM sucrose), and (c) in the hypertonic buffer (10 mM phosphate, 375 mM sucrose).
\[ r = \frac{N \sum_{i} x_i y_i - (\sum_{i} x_i)(\sum_{i} y_i)}{\sqrt{N \sum_{i} x_i^2 - (\sum_{i} x_i)^2 \sqrt{N \sum_{i} y_i^2 - (\sum_{i} y_i)^2}}} \]  

Where \( x \) and \( y \) are the two properties being linearly correlated and \( N \) is the number of data points. The probability of correlation \( p \) was calculated for each \( r \) based on Equations (7) and (8).

\[ p_r(r; v) = \frac{1}{\sqrt{\pi}} \left[ \frac{(v + 1)/2}{\Gamma(v/2)} \right] (1 - r^2)^{(v-2)/2} \]  

\[ p = 2 \int_{|r|}^{1} p_r(r; v) \, dv \]  

Where \( v = N - 2 \) is the number of degrees of freedom for an experimental sample of \( N \) data points (Bevington and Robinson, 2003). \( P \) gives the probability that the observed data could have come from an uncorrelated parent population. A small value of \( p \) implies that the observed variables are probably correlated.

The values of \( r \) and \( p \) are listed in Table I. The absolute values of \( r \) range from 0 to 0.66. We realize that the relatively low values of \( r \) could be due to the fact that the relationship between the two variables is more complicated than simple linear relationship. However, the presence of both negative and positive correlations suggests that there could be increase in the swelling rate with larger cell size or smaller cell size. We conclude that there is no strong correlation between the cell size and the swelling rate. However, there is the probability that the osmolarity might make a different in the significance of the correlation. The hypotonic buffer appears to yield the lowest significance of the correlation. Most \( r \) and \( p \) values from the experiments in the isotonic and hypertonic buffers suggest significant correlations with \( P < 0.05 \).

**Cell Viability After Electroporation**

In our method, the duration for cells to be exposed to the electroporation field was varied by adjusting the length of the narrow section and the velocity of cells. In principle, a duration as short as 1 ms can be realized by having a narrow section length of 100 \( \mu \)m and a velocity of cells around 10 cm/s. Limited by the temporal resolution of the CCD camera, we used durations in the range of 30 to several hundred milliseconds. These durations were significantly longer than the typical pulse durations used in electropulsation (microseconds to milliseconds). We studied how the duration in the electroporation section affected the cell viability after the electroporation process. We first determined the velocity of cells under known field strengths and varying infusion rates of the syringe pump using image analysis (data not shown). Then we varied the velocity of cells at a given field strength to study how the duration affected cell viability. In a typical experiment, 500–1,000 cells were processed by the microfluidic device and examined for viability afterwards with given duration and field strength.

Figure 4 shows the data about percentile cell viability after electroporation under different medium osmolarities. The duration tested was in the range of 30–100 ms. When the field strength \( E_2 \) was lower than 400 V/cm, the percentages of viable cells after the operation were generally very high (87–98%) and essentially identical to those of the control experiments (without the electrical field) in the isotonic and hypertonic buffers. In the hypotonic buffer the percentage dropped from 98% at 62 ms to 87% at 88 ms at 300 V/cm. When the field strength increased beyond 400 V/cm, the percentage of viable cells dropped with increasing field intensity and duration. For example, in the isotonic buffer, the percentage at the duration of 47 ms decreased from 93 to 31% when the field strength increased from 400 to 500 V/cm. At 500 V/cm, the percentage changed from 51 to 7% when the duration increased from 40 to 92 ms. The osmolarity of the buffer played a role in the preservation of cell viability after electroporation. Cells had the highest survival rates when the isotonic buffer was used. The hypotonic and hypertonic buffers yielded similar survival rates. Low survival rate has been a serious problem for electroporation in general. The data indicate that it is possible to achieve relatively high survival rates with our microfluidic device.

**Kinetics of Membrane Rupture (Cell Lysis) in the Electrical Field**

Complete rupture of cell membrane was observed within 150 ms for 100% of cells when the electroporation field \( E_2 \) was 600 V/cm or higher. Figure 5 shows a time series of images taken of the same cell entering and flowing in the narrow section with \( E_2 \) of 1,200 V/cm in the isotonic buffer.

### Table I. Linear correlation between the cell size (before electroporation) and the percentile increase in the cell diameter.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Hypotonic</th>
<th>Isotonic</th>
<th>Hypotonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field strength (V/cm)</td>
<td>400</td>
<td>500</td>
<td>400</td>
</tr>
<tr>
<td>Time points (ms)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td>( r(p) )</td>
<td>0.01 (0.95)</td>
<td>–0.21 (0.27)</td>
</tr>
<tr>
<td>240</td>
<td>( r(p) )</td>
<td>0.00 (0.97)</td>
<td>–0.07 (0.74)</td>
</tr>
</tbody>
</table>

The numbers not in bold are the values of Pearson’s correlation coefficients \( r \) and the probability of correlation \( p \) calculated using Equations (6)–(8).
Cells were loaded with a fluorogenic dye, calcein AM, for the convenience of observation. The cell experienced a rapid increase in the size within the first 30 ms and the majority of the intracellular materials were depleted due to membrane rupture within the first 60 ms. The membrane rupture could be observed at single cell level. We were interested in obtaining the distribution of rupture kinetics in the cell population. In Figure 6 we plotted the histograms of percentiles of cells ruptured at different time intervals at varying field strengths. Each histogram was generated based on the image analysis of 30 cells flowing through the device. In general, higher field strength shifted the whole distribution toward the low end of the time axis which suggested shorter average time for cell lysis to occur. The osmolarity affected the lysis time in a similar fashion as it affected swelling. Cell lysis occurred more rapidly in the hypo/hypertonic buffers than in the isotonic buffer. The hypotonic buffer appears to yield the most rapid lysis among the three buffers.

**DISCUSSION**

**Evaluation of the High Throughput Microfluidic Device for Real-Time Studies of Electroporation Processes at Single Cell Level**

In this study, we have demonstrated a high throughput microfluidic device to study electroporation of single cells in real time. This technique allows kinetics studies of electroporation-based processes at single cell level and yields information about the distributions in the cell population. The throughput we demonstrated in this study was up to 30 Hz. High throughput is important to yield results with statistical significance and relevance to cell populations. There is space for further improvement in several aspects of the technique. First, the temporal resolution is determined by the speed of the imaging device. A state-of-the-art fast digital camera can achieve a frame rate up to 30,000 fps (Riske and Dimova, 2005). In principle, we will be able to push the temporal resolution to tens of microseconds, three orders of magnitude better than our current one using such a camera. Second, the duration of exposure in the high field (the pulse width) can be shortened to submillisecond by decreasing the length of the narrow section to 100–200 μm with the same velocity for cells. The pulse width in this range will be directly relevant to current practice of electroperorative drug delivery. Third, a potential benefit of applying microfluidics to single cell studies is the possibility of integrating with other microfluidic devices to enable delivery of chemical and biological molecules during cell electroporation and monitoring such events in real time. Finally, in the current device
the leaked intracellular materials may adsorb on the surfaces of the channel to induce cell adsorption and clogging, which affects the performance of the device in an extended period. This issue will be addressed in the future research by surface modification of PDMS (Hu et al., 2004a,b).

There are a couple of limitations associated with this approach. First, the pulse width is limited by the velocity of cells in the channel and the length of the narrow section. A pulse width around 500 μs or longer is readily achievable using this approach (e.g., the pulse width is 500 μs when the velocity is 10 cm/s and the length of the narrow section is 50 μm). However, the demonstration of short durations (e.g., low microseconds to nanoseconds) will be challenging since the velocity of cells needs to be reasonably low to avoid damage to cells due to mechanical forces. Second, when the cell density is very high in the electroporation buffer, the electric field in the narrow section may be disturbed by the presence of cells and become more complicated than what was suggested by Equations (3)–(5). Cells before permeabilization are essentially non-conducting and their conductance increases dramatically upon electroporation (Zimmermann et al., 1974). Such change will affect the local field strength in the narrow section.

Effects of Medium Osmolarity on the Electroporation of CHO Cells

In this study, we first determined the electroporation threshold by observing the swelling of cells in the field. It is generally believed that the threshold is a slowly decreasing function of the pulse width, both for the cell membranes and lipid membranes (Benz and Zimmermann, 1980; Kinosita and Tsong, 1977a). We observed substantial swelling at 300 V/cm in the hypotonic buffer, but not in the isotonic or hypertonic buffer.

We observed the swelling and the rupture of cell membrane during electroporation in real time at single cell level. Since these observations were made with cells exposed to the high field during the entire period, no resealing process was involved. The swelling rates measured here reflected the water influx into the cells when the electropores were open. In comparison, if the swelling kinetics are measured after electrical pulse(s) as in most previous reports, the subsequent process may have involved RVI/RVD or alternation of the cell cytoskeleton (Rols and Teissie, 1992). These effects may interfere with the measurement of the kinetics and affect the results. In both the cases of cell swelling and membrane rupture, we consistently observed that the osmolarity of the buffer played an important role in the kinetics of electroporation. The processes of swelling and rupture occurred more rapidly in the hypotonic or hypertonic buffers than in the isotonic buffer. These facts indicate that the hypo-/hyperosmolarity of the medium contributes to the formation of electropores with higher density and/or larger size. There have been reports in the literature of increased transfection rate in hypotonic electroporation buffers (Cegovnik and Novakovic, 2004; Golzio et al., 1998) or hypertonic electroporation buffers (van den Hoff et al., 1990), compared to the rate yielded in isotonic buffers. Our results indicate that the increased electropore density or size in hypotonic/hypertonic buffers could contribute to the increased delivery of biomolecules. However, we do realize...
that the increase in the transfection rate may not follow the same trend as the increase in the size and density of electropores due to the fact that larger pore size and higher pore density contributes not only to improved delivery of biomolecules but also to cell death. Our results here are different from the swelling rates measured after electroporation (Golzio et al., 1998) since the swelling is known to continue after the electrical pulse with a time scale of minutes (Ferret et al., 2000).

We were able to obtain kinetics information from single cells and establish the distribution of the population. We also studied the possible correlation between the swelling rate and the cell size. There was no indication that the swelling rate was strongly correlated to the cell size. However, it appears probable that the swelling rate in the hypotonic buffer has a less significant correlation to the cell size compared to the other two buffer systems. We believe that the low correlation coefficients or negative correlations between the swelling rate and the cell size were possibly a result of a balance between two counteracting mechanisms: the increased transmembrane potential with larger cell size and the higher volume of liquid needed to swell a larger cell. The transmembrane potential is linearly proportional to the cell diameter which makes larger cells experience higher transmembrane potential. As previously proposed, the cell membrane is electroporated to keep the transmembrane potential to be at or below a critical value (between 200 mV and 1 V) (Kinoshita et al., 1992). This mechanism suggests that larger cells may have higher pore density and/or larger pore size in the membrane, compared to smaller cells. On the other hand, a large cell needs to take in more water to expand its diameter to the same percentage compared to smaller cells. More comprehensive data combining with statistical analysis and modeling will hopefully bring more conclusive determination on this topic in the future.

This research was supported by Purdue Research Foundation (PRF) Fellowship and Purdue University. We thank Dr. Dabao Zhang in the Department of Statistics at Purdue University for helpful discussions about the data analysis.

References


