Continuous-flow multi-pulse electroporation at low DC voltages by microfluidic flipping of the voltage space topology

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Concerns over biosafety, cost, and carrying capacity of viral vectors have accelerated research into physical techniques for gene delivery such as electroporation and mechanoporation. Advances in microfabrication have made it possible to create high electric fields over microscales, resulting in more efficient DNA delivery and higher cell viability. Continuous-flow microfluidic methods are typically more suitable for cellular therapies where a large number of cells need to be transfected under sterile conditions. However, the existing continuous-flow designs used to generate multiple pulses either require expensive peripherals such as high-voltage (>400 V) sources or function generators, or result in reduced cell viability due to the proximity of the cells to the electrodes. In this paper, we report a continuous-flow microfluidic device whose channel geometry reduces instrumentation demands and minimizes cellular toxicity. Our design can generate multiple pulses of high DC electric field strength using significantly lower voltages (15–60 V) than previous designs. The cells flow along a serpentine channel that repeatedly flips the cells between a cathode and an anode at high throughput. The cells must flow through a constriction each time they pass from an anode to a cathode, exposing them to high electric field strength for short durations of time (the “pulse-width”). A conductive biocompatible poly-aniline hydrogel network formed *in situ* is used to apply the DC voltage without bringing the metal electrodes close to the cells, further sheltering cells from the already low voltage electrodes. The device was used to electroporate multiple cell lines using electric field strengths between 700 and 800 V/cm with transfection efficiencies superior than previous flow-through designs. *Published by AIP Publishing.*

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Multiple strategies have been explored over the years to safely and efficiently deliver exogenous genetic materials into a living cell.¹ Although virus particles can efficiently deliver genes to a diverse range of cells (including primary cells), these vectors can be mutagenic, immunogenic, and expensive to produce, with strict limitations on the genetic load they can carry.² Physical methods, where genes pass across temporary pores in the membrane made with strong electric fields (electroporation),^{3–6} ultrasound⁷ (sonoporation), or shear stresses⁸ (mechanoporation), have emerged as viable alternatives to viral vectors. Electroporation is inexpensive, is not cell-type specific, and does not have limits on the size of the genes to be inserted.⁹ The trans-membrane potential induced by an external electric field ($\Delta\psi_E$) at a particular point on the cell membrane can be expressed using the following equation:¹⁰

$$\Delta\psi_E = -1.5g(\lambda)rE \cos(\theta), \quad (1)$$

where the cell is assumed to be a sphere of radius r , $g(\lambda)$ is a complex function of the conductivities of the membrane, the cytoplasm, and the surrounding buffer, E is the external electric field intensity, and θ is the angle between the direction of the electric field and the normal to the membrane at a particular point of the cell.

When the trans-membrane potential of a cell is increased to a critical threshold range (0.2–1 V), pores are formed on the membrane; these pores can reseal after the field is removed.¹⁰ The optimal electric field required depends on the buffer and membrane conductivity, cell size, and the strength and distribution of the external electric field.¹¹

Microscale electroporation, by producing uniform electric fields and minimizing the pH and temperature variations of the microenvironment around a single cell, has the potential to increase cell viability and improve transfection rates, compared to cuvette-based (“bulk”) electroporation.⁴ Microfluidic electroporation approaches, developed over the last decade, broadly fall into two categories: in the first category, individual cells are trapped against the micro- or nano-channels and an applied high electric field drives the DNA through the channels into the cells;^{12–16} in the second category, cells are exposed to high electric field pulses while flowing through microchannels that create pores and drive the exogenous DNA into the cells.^{17–24} Although the trap-based strategy can lead to high transfection efficiency with low voltages, the continuous-flow approach is more suited for the high throughputs needed in cell-based therapeutics. Continuous-flow electroporation methods have employed two main strategies: (a) flowing the cells between closely spaced electrodes in a microchannel placed lateral to the direction of flow,^{17,20,22,24} or (b) flowing the cells through the geometric constrictions that concentrates the electric field established by the electrodes placed at the two ends of the microchannel.^{18,21,25} The channel constriction design uses

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changes in microchannel geometry and flow rate to determine the pulse width and the field intensity.^{21,25} However, in this design, the cells are flowed down a voltage gradient, and therefore, in order for cells to pass through multiple high electric field regions (multiple pulses have been shown to increase the transfection efficiency¹²), the voltage required can be very high (several hundreds to a thousand V).²⁶

Our goal was to design a device that would (a) transfect cells in a continuous-flow mode and therefore be scalable for cell-therapeutic applications, (b) require minimal instrumentation and low voltages, (c) enable higher transfection efficiency by providing the multiple high electric field pulses, and (d) ensure high cell viability by shielding cells from the deleterious effects of pH change, heat, and electrolysis-generated bubbles.^{4,5} In our electroporation device, variations in the cross-sectional area give rise to a field strength change as the cells flow through constrictions at each turn of a serpentine channel (Fig. 1). The electroporation pulse duration is determined by the time taken by a cell to pass through the constriction channel.¹⁸ The potential drop (V) in a single turn of the channel (from the anode to the cathode) is given by Ohm's law and described by the equation

$$V = E_c L_c + 2E_w L_w, \quad (2)$$

FIG. 1. The continuous-flow electroporation device. (a) Schematic illustrating the concept of high electric field generation in a constriction along the fluidic path of the cells in one unit of the serpentine channel. (b) A 16-turn continuous-flow electroporation device made of PDMS and filled with the poly-aniline gel (dark green) in the binary-distributed network and Allura Red dye in the central serpentine channel. (c) Magnified picture of the Allura Red dye flowing through the channel, while an electric field is applied.

where E_c and L_c are the electric field strength and length of the constriction, respectively, and E_w and L_w are the electric field and the length of the wider regions, respectively, on either side of the constriction.

The electric field strengths are inversely proportional to the cross-sectional areas of the conductor (buffer-filled channel in this case). Therefore, the electric field E_c in the constriction can be expressed in terms of the externally applied voltage V and the ratio ρ_A of the cross-sectional area of the constriction channel (A_c) to that of the wider channel (A_w) ($\rho_A = A_c/A_w$) as follows:

$$E_c = \frac{V}{2\rho_A L_w + L_c}. \quad (3)$$

The key improvement in our design has been to create a channel topology and a method to fabricate an *in situ* polymerized binary network of conductive hydrogel, which distributes the voltage along the cell flow path in such a way that the cells move between the voltage polarities at each turn. While the field strength remains the same at every passage of the cell through a constriction, the direction of the field with respect to the cell's frame of reference reverses at every turn of the serpentine path, if we assume that the cell follows the laminar flow streamlines and does not significantly rotate. In reality though, due to the contraction-expansion, turns, and differential inertial forces, the cell will undergo some rotation when flowing from one constriction to the next.²⁷ By reversing the hyperpolarized and depolarized halves of the cell at every turn, a larger surface area of the cell is getting electroporated in our device. Although the induced trans-membrane potential depends on the angle of the membrane normal and the field direction and therefore varies with the location on the cell surface (Eq. (1)), the process of pore formation is independent of the polarity. Therefore, our design is able to expose the cells to an almost indefinite number of pulses without requiring an increase in the applied external voltage.

The continuous flow electroporation device, fabricated in poly-dimethylsiloxane (PDMS), had 8 or 16 turns of a central serpentine channel, 240 μm wide and 90 μm high, with constrictions (40 μm wide, 40 μm high) in between that are 50–400 μm long. The central flow-channel was connected to a binary-distributed channel network (200 μm high and 240 μm wide) on either side with a set of 100 μm long constrictions (40 $\times$ 40 μm) that function as passive capillary valves^{28–31,35} (see Note 1 in the [supplementary material](#)). The binary distributed network was filled with a very conductive hydrogel, poly-aniline. The PDMS device with the binary network filled with the dark-green pAni hydrogel, and the central cell-flow channel filled with Allura Red dye (Sigma-Aldrich) is shown in Fig. 1(b). The device is fabricated using standard soft lithography (schematically illustrated in Fig. S1 and described in Note 2 ([supplementary material](#))). The *in-situ* formation of the electroconductive poly-aniline hydrogel in the binary network is adapted from a previously described method.³² Allura Red, a dye with a charge/mass ratio (2 negative charges/490 Da) similar to DNA's (2 negative charges/650 Da), was useful in observing how electrophoretic mobility, flow, and diffusion affect the concentrations along the channel when an external voltage is applied (Fig. 1(c)). The dye distributes uniformly at

the entry port, gets increasingly asymmetrical as the flow moves downstream, and then distinctly concentrates on the side of the channel that was proximal to the anode by the output (Fig. 1(c)).

We used finite-element modeling (COMSOL 4.3a) to simulate the voltage distribution, electric field strengths, and DNA distribution in the channels under syringe-pump driven flow (see Note 3 in the [supplementary material](#)). The binary gel network distributes the voltage equally to each turn of the serpentine channel (Fig. S2A). Although the electric field is higher near the entry and exit points and the edges of the constrictions, it is largely uniform in the central region of the constriction (inset of Fig. S2A). The high conductivity of the hydrogel and the topology of the gel network with respect to the cell flow path significantly reduced the voltage required (~ 15 to 60 V) for generating the multiple pulses of reversible electroporation capable field strengths (400 – 1000 V/cm) in the constrictions (Fig. 2(b)). The average velocity of the fluid in the constrictions is at least 2.5 times larger than in the wider sections (Fig. S2A in the [supplementary material](#)). Due to the combined effects of electrophoretic migration, diffusion, and convection, there is an increased accumulation of the negatively charged species on the anode side of the channel in more downstream regions (Fig. S2B in the [supplementary material](#)). This pattern of migration results in a concentration

gradient of DNA orthogonal to the electric field direction in the constrictions. According to the Krassowska-Filev pore formation model,³³ while the number of pores is highest at the poles, the number of large ($\gg 1$ nm-diam.) pores is higher close to the border of the depolarized and hyperpolarized halves of the cell, where the induced trans-membrane potential is just above the electroporation threshold. The large pores in the equatorial region that coincide with higher concentration of DNA are particularly relevant to the success of transfection, as DNA plasmids (~ 100 nm in diameter) require larger pores to get inside the cells.

We used two output metrics to determine the performance of our device: (1) transfection efficiency, or the percentage of live cells that are expressing the transfected plasmid and (2) viability, or the percentage of cells that survive the electroporation process. The parameters that we experimentally varied for this study were (a) the input voltage and consequently, the electric field strength in the constrictions, and (b) the time of exposure of the cells to high electric fields, as determined by the length of the constriction. We kept other parameters like plasmid DNA concentration, electroporation buffer conductivity, flow rate, and number of pulses constant. Depending on the tissue and species of origin, cell types vary in their size, volume, membrane, and cytoplasmic electrical characteristics, as well as their sensitivity and susceptibility to thermal, pH, and osmotic stresses.⁹ The optimal parameters for efficient transfection using electroporation strongly depend on the above characteristics of a cell and therefore differ significantly across cell types. In particular, it has been empirically observed that there is a narrow window of electric field strength and pulse width that optimizes the transfection efficiency and viability in a specific cell type.^{9,34}

We tested the immortalized cell lines from Chinese hamster ovary (CHO-K1), mouse myoblast (C2C12), and human T-cells (Jurkat). Before electroporation, the cells were suspended to a density of 1×10^6 cells/ml in a low-conductivity electroporation buffer (8 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 250 mM sucrose, pH 7.4), mixed with $40 \mu\text{g/ml}$ of plasmid DNA (pMaxGFP), and flowed through the cell-channels at $48 \mu\text{l/min}$. This flow rate resulted in an average cell residence time of $2 \mu\text{s}/\mu\text{m}$ of a $40 \times 40 \mu\text{m}$ microchannel. At this flow rate, the cross sectional area of the constriction was large enough to ensure that there was no clogging of the channels over time (see Note 4 in the [supplementary material](#)). Voltage was applied using a DC power source through platinum wires embedded into the polyaniline gel at the electrode ports. The cells after electroporation were diluted 1:5 with pre-warmed media and cultured. Cell viability was ascertained through ethidium homodimer exclusion assay. Green fluorescent protein (GFP) expression was recorded after 24 h using epifluorescence microscopy.

Electric field strengths in the 600 – 900 V/cm range have been shown to be optimal for electroporation of various mammalian cells.⁵ A longer time of exposure also leads to an increased delivery of DNA into the cells.¹² However, too long an exposure or too high a electric field can lead to irreversible electroporation and subsequent cell death.⁵ Therefore, in order to find the optimal conditions for cell

FIG. 2. Finite-element modeling of the device. (a) Color plot of the simulated voltage distribution in a 16-turn device with $50 \mu\text{m}$ constrictions, when a voltage of 20 V is applied to the electrodes. (Inset) Color plot of the simulated electric field strengths in the constriction. The slices are taken every $10 \mu\text{m}$. (b) Plot of the average electric field strength in the constrictions with respect to the voltage applied across the device.

FIG. 3. Electroporation of cells. The phase and fluorescence micrographs of transfected cells 24 h after electroporation, together with graphs plotting the viability and transfection efficiency at different electric field strengths, and different pulse-widths (corresponding to different constriction lengths). (a) CHO-K1, (b) C2C12, and (c) Jurkat cells.

transfection, we experimentally varied the electroporation parameters for each cell type. To study the effect of electric field strength, we used our simulation results to vary the applied voltage, so as to generate average field strengths ranging from 600 to 900 V/cm in the constrictions. To recreate a pulse-train of high field exposures, we used an 8-turn device and flowed cells through the constrictions at 48 μ l/min. The operating flow rates through the device did not compromise the viability of cells (Fig. S3 in the [supplementary material](#)). To change the time of high-field exposure, we used devices with different constriction lengths, since the average time a cell spent in the high electric field region linearly depended on the length of the constriction and ranged from 100–800 μ s (in the 50–400 μ m long constrictions).

We were able to electroporate three different cell types with a GFP plasmid, without significantly compromising cell viability or proliferation over 24 h (Fig. 3). At 600 V/cm, while the viability was high for all cell types (60%–95%), the transfection efficiency was very low (less than 20%), irrespective of the length of constriction. For CHO-K1 cells, the viability only marginally decreased with increase in voltage from an average of $93.5 \pm 2.4\%$ at 600 V/cm to $71 \pm 5.5\%$ at 900 V/cm (Fig. 3(a)). At longer pulse durations (800 μ s), the transfection efficiencies at 800 V/cm and 900 V/cm were $72 \pm 12.5\%$ and $78 \pm 10.4\%$, respectively, and the viabilities were $85 \pm 5.3\%$ and $77 \pm 6.1\%$, respectively (Fig. 3(a)). Therefore, these two conditions were the most optimal for CHO-K1 cells. For C2C12 cells (Fig. 3(b)), the viability decreased with increasing voltages (for all pulse durations), with very low survival (less than 20%) at 900 V/cm. However, the average transfection efficiencies at 700–900 V/cm were higher (65%–74%) than what we observed with CHO-K1 cells (43%–47%). The optimal conditions for C2C12 cells were 400 μ s pulses of 700 V/cm (viability of $58 \pm 5.1\%$ and transfection efficiency

of $78 \pm 11.5\%$) and 100 μ s pulses of 800 V/cm (viability of $67 \pm 12.1\%$ and transfection efficiency of $76 \pm 8.2\%$). The viability of Jurkat cells (Fig. 3(c)) too decreased with higher voltages, with less than 20% survival at 900 V/cm. The overall transfection efficiency and GFP expression in Jurkat cells were found to be lower than the C2C12 and CHO cells. At 700 V/cm, the devices with 50 μ m and 100 μ m constrictions (100 μ s and 200 μ s, respectively) gave similar results ($72 \pm 7.9\%$ viability and $34 \pm 4.3\%$ transfection efficiency). While higher transfection efficiencies were observed at 800 V/cm and 900 V/cm with 100 μ m and 200 μ m constrictions ($59 \pm 17.3\%$ and $67 \pm 8.6\%$, respectively), the viability of the cells at these conditions was low ($34 \pm 9.1\%$ and $12 \pm 7.1\%$, respectively). Therefore, 100 μ s and 200 μ s pulses at 700 V/cm were the optimal conditions for Jurkat cells.

In conclusion, we have shown efficient plasmid DNA transfection using a conductive-gel integrated, continuous-flow electroporation device. By exploiting an alternating voltage space topology, we have generated multiple pulses with relatively low voltages (~ 15 to 60 V). We can further reduce the applied voltages by selecting a more electroconductive polymer and optimizing the gel-fluid interface area (see Note 5 in the [supplementary material](#)). We obtained an almost two-fold improvement in transfection efficiency with CHO-K1 cells compared to earlier continuous-flow microfluidic designs.²⁶ We envision future applications of this continuous-flow electroporation module for cell therapies such as autologous stem-cell transplantation and adoptive T-cell immunotherapy.

See [supplementary material](#) for device fabrication details and schematic, finite-element modeling details and results, and additional characterization of cell viability.

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