Microfluidic cell electroporation using a mechanical vavle

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Introduction

Electroporation is a technique that applies electric field to transiently permeabilize the cell membrane to deliver impermeant molecules into cells. Due to its wide applications to gene/drug delivery, electroporation has been adapted in microfluidic devices by fabricating microscale electrodes and structures that apply short electric pulses [1-3]. The fabrication of microscale electrodes presents a difficulty for lowering the cost of these microfluidic chips. Furthermore, an electric pulse generator is needed to generate pulses with durations ranging from microseconds to milliseconds. The pulse generator also adds to the cost and complexity of the overall system. In recent work, microfluidic channels with geometric variation were applied to electroporation and electrofusion of flowing cells using a common dc power supply [4, 5]. While the technique doesn't require a pulse generator and microfabricated electrodes, it cannot be applied to *in situ* electroporation of adherent cells. The adhesion to the substrate can be important for cellular functions of adherent cell types. Thus electroporating cells while they adhere to the substrate is often desired due to the minimum disturbance to the cell culture.

Here we demonstrate a microfluidic technique that electroporates both suspended cells and cells adhering to the bottom of a microfluidic channel using a common dc power supply, taking advantage of a fast-response microscale mechanical valve [6]. The mechanical valve turns on/off a dc electric field established in a microfluidic channel rapidly by physically connecting/separating the ionic buffer. By virtue of that, we are able to effectively produce electric pulses of milliseconds using this approach. We investigate the effects of the valve dimensions, the actuation pressure, and the voltage applied on the performance of the valve in terms of pulse generation. We also examine the viability of cells after electroporation using the device and demonstrate the electropermeabilization of cells by an impermeant dye SYTOX green.

Experiment

1. Microchip fabrication

The design of the device is shown in Figure. 1. The device was fabricated using multilayer soft lithography with substantial modifications[6]. The control layer master (photoresist/3 inch silicon wafer) was made using a negative photoresist SU-8 2025 (Microchem) with a thickness of 33 μm. The fluidic layer master was made using a positive photoresist AZ 9260 (Clariant) with a thickness of 25 μm. The fluidic layer master was baked at 120 ℃ for 2 min to generate a rounded cross sectio n for the channel. The thickness of the photoresist was translated into the depth of the microfluidic channel. Both control and fluidic layers of the device were molded using PDMS of the same composition (GE Silicones RTV 615, MG Chemicals, mass ratio of A:B=10:1). The fluidic layer had a thickness of 133 μm (formed by spinning of the liquid PDMS prepolymer at 1200 rpm for 30s) which left the thickness of the PDMS membrane between the fluidic channel and the control channel around 108 μm. The control layer had a thickness ~0.5 cm. The two layers were bonded together

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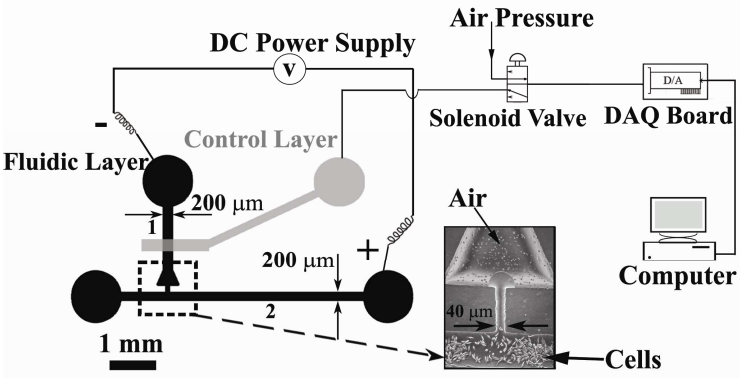

Figure 1. The design of the microfluidic electroporation device. The control channel (grey) is on top of the fluidic channel (black) with a PDMS membrane in between. The valve locates in the vertical fluidic channel 1 and the cell culture is in the horizontal fluidic channel 2. A common dc power supply is kept on during the operation of the device.

upon contact after oxidizing the two PDMS surfaces using a Tesla coil (Kimble/Kontes) in atmosphere and the combined layers were then bonded to a clean glass slide using the same oxidation method. Finally the entire device was baked in an oven at 80 ℃ for 2 hr. The PDMS device was exposed to UV light in a biological safety cabinet overnight for sterilization before use. The fluidic channels were also silanized by exposing to (Tridecafluoro-1,1,2,2-tetrahydrooctyl)trichlorosilane vapor for 2 hr in order to increase the insulation property of the valve when closed.

2. Cell Sample Preparation and Seeding in the Microfluidic Device.

Chinese hamster ovary (CHO-K1) were cultured in plastic tissue culture flasks at 37℃, under 5% CO_2 in the Dulbecco's modified Eagle's medium (DMEM, Mediatech Inc.) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), penicillin (100 units/ml, Sigma), and streptomycin (100μg/ml, Sigma). Cells were diluted at a ratio of 1:5 every 2 days to maintain them in the exponential growth phase (~1×10^6 cells/ml). They were harvested and resuspended in the culture medium at a concentration of 1×10^8 cells/ml for seeding in the microfluidic device. Before seeding, the horizontal channel 2 (Figure 1) was pre-coated with 1 mg/ml fibronectin (Sigma) (incubation for 0.5 hr at 80℃[7]), before flowing CHO cells into channel 2. The cells were cultured in the channel 2 for 12 hr in a 37℃, 5% CO_2 incubator while the culture medium slowly infused through the channel 2 under gravity, before the experiment unless otherwise noted. All the operations involving seeding and culture were done exclusively in channel 2 by keeping air in the channel 1. The cell viability was examined by flowing 1μM SYTOX green into the channels at 1 hr after electroporation.

3. Fluorescence Microscopy.

The microfluidic device was mounted on an inverted fluorescence microscope (IX-71, Olympus). The epifluorescence excitation was provided by a 100W mercury lamp, together with brightfield illumination. The excitation and emission from cells peameabilized with DNA dye SYTOX green (Molecular Probes) were filtered by a fluorescence filter cube (exciter HQ480/40, emitter HQ535/50, and beamsplitter Q505lp, Chroma technology Corp.).

4. Microchip Operation.

The layout and the setup of the microchip are shown in Figure 1. The valve was actuated by pressurizing the control channel (filled with water) using a fast-response solenoid valve (ASCO Scientific). The pressure pushed down the PDMS membrane to contact the glass bottom and closed the fluidic channel. The solenoid valve was controlled by a LabVIEW program which designated the time of the valve being opened.

After the cell culture, phosphate buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 250mM sucrose, pH=7.2) was used to replace the culture medium and fill in the entire fluidic channels (both channels 1 and 2) before electroporation. A dc power supply (1AA12P30, Ultravolt, with current monitoring) provided constant dc voltage when the chip was in operation.

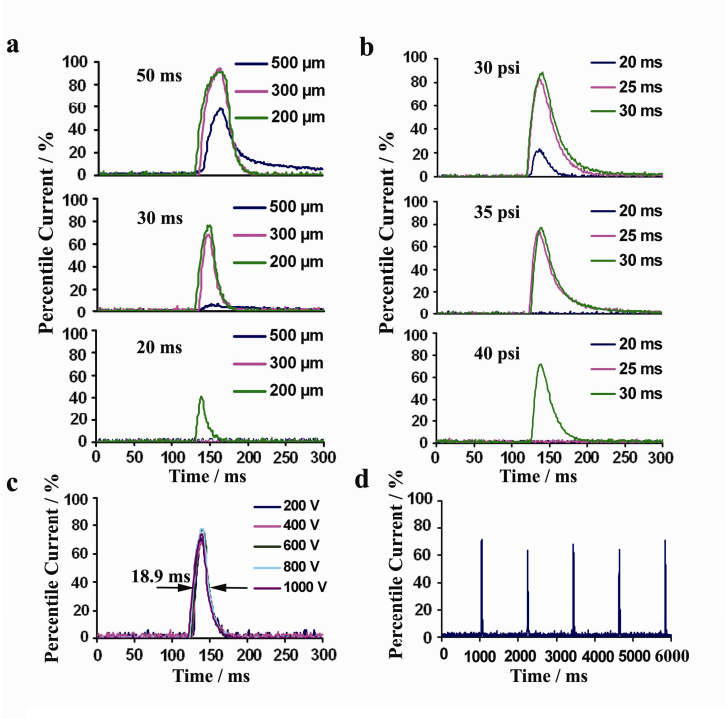

Figure 2. The effects of the control channel width, the actuation pressure, and the applied voltage. **(a)** The percentile current-time curves with different control channel widths and valve opening times. The actuation pressure is 40 psi and the voltage is 400 V. **(b)** The percentile current-time curves with different actuation pressure and valve opening times. The control channel width is 300 µm and the voltage is 400 V. **(c)** The percentile current-time curves with different voltages provided by the power supply. The control channel width is 300 µm, the valve opens for 30 ms, and the actuation pressure is 40 psi. **(d)** A series of pulses generated by opening the valve for 30 ms within an interval of 1.2 s for 5 consecutive times. The control channel width is 300 µm, the voltage is 400 V, and the actuation pressure is 40 psi.

Results and Discussion

We used a simple multilayer PDMS device and a common dc power supply to carry out the electroporation of cells (as shown in Figure 1). The multilayer valve effectively creates an open-circuit while it is closed due to its insulating nature. While the power supply provided constant and continuous voltage, the valve was operated to have a close-open-close sequence to generate a pulse which electroporated the cells in the channel 2. It is found that long time soaking of the PDMS valve with the culture medium could weaken its electric insulating ability and increase the background current significantly. As shown in the inset image in Figure 1, the triangle shape structure was used to keep the culture medium out of channel 1 during the whole cell seeding process, taking advantage of the hydrophobicity of PDMS. The cells were exclusively cultured in channel 2.

We examined the effects of the width of the control channel, the actuation pressure, and the dc voltage across the channel on the generation of the pulse. The current was monitored between the two electrodes while the valve was operated to close, then open for a designated period (milliseconds), and close again. To present the current in percentile, we designated the current with the valve fully open as 100% and that with two electrodes connected to an insulator as 0 under a certain voltage. In Figure 2a, the overall voltage applied was 400 V and the valve was actuated by 40 psi pressure. The width of the control channel was varied from 200 to 500 µm. As the width of the control channel increases, the response of the valve gets more retarded. A 500 µm valve failed to generate a pulse when the valve was actuated to open for less than 30 ms by the computer. On the other hand, a 200 µm valve seems to response most promptly to the changing pressure. It generated a pulse as short as 20 ms and always gave the highest percentile current at its peak. The high peak percentile current indicates that the 200 µm valve was closest to be fully open during the operation compared with the other valves with wider control channels. We conducted tests with varied pressure and voltages using devices with the control channel of the width of 300 µm. In Figure 2b, the effect of the actuation pressure is shown. Higher pressure produced less “tail” for the pulse. However, it also became harder to

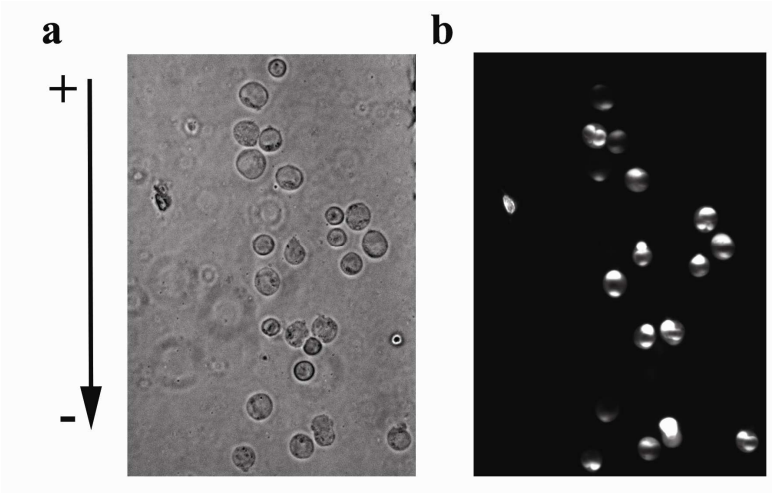

Figure 3. Electropermeabilization of suspended CHO cells (before culturing) with a pulse of 30 ms and a pulse intensity of 209 V/cm. The channel contains 5 µM SYTOX green during the electroporation. The images were taken 5 min after the electroporation. **(a)** Phase contrast. **(b)** Fluorescence.

generate short pulses (e.g. with 40 psi no pulses were produced when the duration was set at 20 and 25 ms.). The percentile current profiles generated by the same device were fairly reproducible at different voltages from 200 V to 1000 V (Figure 2c). The average full width at half maximum (FWHM) of the pulses was 18.9 ms with a standard deviation of 1.1 ms when the valve opened for 30 ms. In Figure 2d, a series of pulses was generated by opening the valve for 30 ms within an interval of 1.2 s for 5 consecutive times. The relative standard deviation (RSD) in the peak height was 5.7% and the RSD in the FWHM was 1.2%. The uniformity of a pulse series is better when the valve opens for longer periods. We were able to model the field intensity in the device using software package COMSOL 3.2 when the valve was entirely open and the effects of cells were ignored. The local field intensity is proportional to the electrical current based on Ohm's law. Therefore we could calculate the field intensity variation experienced by the cells in channel 2 based on the percentile current data in Figure 2. For example, using the data in Figure 2b, it is found that the peak intensity of the pulse generated by 30 psi was 327 V/cm with the valve opening for 30 ms, while it was 309 V/cm and 87 V/cm with the valve opening for 25 and 20 ms, respectively. The current data indicate that the actuation of the valve is effective in generating a pulse with reproducible duration and magnitude.

The devices with the control channel width of 300 µm were chosen for CHO cell electroporation tests. All these cell electroporation experiments were done by having the valve open once for 30 ms with an actuation pressure of 40 psi. SYTOX green was the model molecule we used for testing the electropermeabilization. It is a DNA counterstain that is impermeant to live cells and becomes green-fluorescent when bound to DNA. Figure 3 shows that the suspended cells were stained by SYTOX green at 5 min after a single pulse. The cells mostly had their two opposite poles stained that were aligned with the field direction. Similar results were obtained using electroporation based on a pulse generator and are characteristic of electroporative delivery [8, 9]. Figure 4a shows the time sequenced images of electropermeabilization of adherent CHO cells after applying one pulse using the valve at time 0. The images reveal that the delivery occurred first at the cathode-facing hemispheres of the cells. Such asymmetric transport during electroporation has been described before [8]. For the electroporation of adherent CHO cells, we further examined the viability of cells (the percentage of live cells) and the SYTOX green permeabilization rate among live cells after the electroporation under various pulse intensities (peak field intensities experienced by the cells). When the viability was measured, we carried out the electroporation and then flowed SYTOX green into the channel 1 hr later. The stained cells were the dead ones (with leaky membrane) in this case. When the permeabilization rate was measured, we first obtained the percentage of cells being stained by having 1µM SYTOX green in the channel during the electroporation.

The percentage of stained cells included both dead cells and live/electropermeabilized ones in this case. By deducting the percentage of dead cells based on the viability data, we obtained the permeabilization rate that represented the live and permeabilized cells among the starting cell population. The optimal permeabilization rate was reached at the pulse intensity of 279 V/cm with 51% of the cell population permeabilized and alive. Electroporation in this experiment occurred at field intensities lower than those commonly reported in the literature for CHO cells (~400 V/cm)[5, 10]. This is possibly related to the long pulse duration (the electroporation threshold is a slowly decreasing function of the pulse duration) and the shape of the pulse.

Conclusion

We demonstrated that a simple microfluidic device could effectively produce cell fusion under continuous DC voltage, when coupled with chemical linking method. This technique offers efficiency comparable to that of conventional electrofusion technique which requires expensive and complicated apparatus. Cell fusion was conducted at single pair level in the microfluidic channel and the physical dimensions of the channel effectively prevented more than 3 cells from fusing together. These features are important when cell fusion needs to be conducted based on scarce cell sources such as primary cells. This demonstration will help establish microfluidics as a viable platform for studying a number of important biological problems involving cell fusion. Further studies are needed to characterize the properties of fused cells produced by this technique.

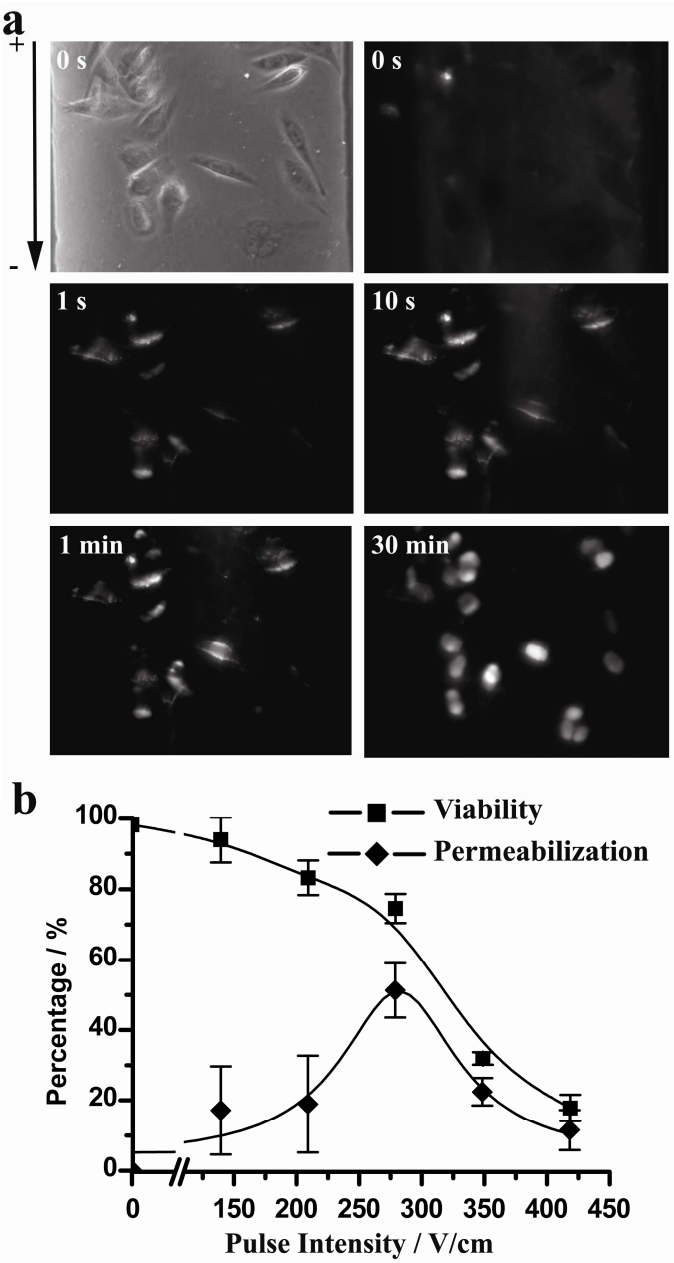

Figure 4. (a) Electropermeabilization of adherent CHO cells with a pulse of 30 ms and a pulse intensity of 349 V/cm. The pulse is applied at time 0. **(b)** The viability and permeabilization rate of adherent CHO cells at different pulse peak intensities. The viability represents the percentage of live cells among the starting cell population. The permeabilization rate represents the percentage of both live and permeabilized cells among the starting cell population.

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