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Continuous Low-Voltage dc Electroporation on a Microfluidic Chip with Polyelectrolytic Salt Bridges

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A microfluidic electroporator operating under a continuous low dc voltage (7 to ∼15 V) is reported. The proposed electroporation microchip exploits the ionic conductivity of polyelectrolytic gel electrodes to precisely control the electric field that is applied to cells without bubble generation in the microchannel. In this study, pDADMAC (poly diallyldimethylammonium chloride) was used to efficiently apply the electric potential difference to the cells in the microchannels. Impedance analysis showed that the pDADMAC plugs could work as ionic conductors with a conductivity of approximately 16 S m^{-1}. In accordance with the calculation using CFD-ACE, an input voltage of only 10 V could generate an electric field of 0.9 kV cm^{-1} across the microchannel; this meets the requirements for electropermeation. The electropermeation of K562 human chronic leukemia cells was observed in the microchip from 7 V, and the efficiency increased up to 60% upon the application of an input voltage of 15 V with a viability of 80%. An amount of 10^5 cells could be transfected every minute under a constant potential difference. The transfection and expression of DNA plasmids were also successfully demonstrated in the suspension cell line.

Over the last few decades, many scientists have been trying to deliver various chemical and biological agents into cells without adverse effects.[1] However, the cell membrane is an elaborate barrier that sustains homeostasis by allowing highly restricted chemical access. Thus, bulky molecules pass through the barrier only when they are specially modified with chemical helpers[2−5] or when the cell is damaged. Electroporation is one of the methods used in genetics and molecular biology to transfer molecules such as DNA into cells. The cell suspension is electrically impacted to make a momentary and reversible damage on the cell membrane, and the molecules in the media diffuse into the cytosol through the transient pores of the cell membrane. The general consensus is that an electric field gradient of 0.3 to ∼1 kV cm^{-1} generates the transient pores.[6] Traditional electroporators suffer from a couple of problems. Some cells are destroyed and others are unaffected, resulting in the limited efficiency of gene transfection. A nonuniform and less stable electric field profile during the treatment is unavoidable due to the programmed short pulse period.[7] Moreover, Al electrodes are widely used in commercial instruments and can be a source of Al^{3+} ions that dissolve into the media, leading to unpredictable results in cells. Furthermore, special caution is necessary for experimenters to operate commercial electroporators at several hundred volts.

Recent experiments on microfluidic chips have demonstrated several outstanding performances by applying a uniform electric field to individual cells.[8] In most of the systems, cell permeation begins at an even lower input voltage due to the proximity between the electrodes and the cells. The competitiveness of microelectroporation chips is due to its applicability to single cell manipulation and electropermeation. Rubinsky et al. presented the first proof of concept with a relatively complex, trilayered silicon microfluidic chip with Al thin film electrodes.[9,10] Lee et al. designed simpler polydimethylsiloxane (PDMS) microchannels that focused the electric field on a spot of cellular membrane and investigated the electropermeation of a single cell by monitoring the current.[11] Those chips allow in situ monitoring for a single cell and claim a very high permeation rate. However, the viability of the treated cells is poor because the pores are relatively large and irreversible.

For general biological or medical applications, large amounts of cells need to be treated and retrieved in a resilient condition. Lin et al. aligned gold electrodes at the top and bottom of a microchannel through which cells passed. By synchronization of the electrical pulse and cell flow, 10 ms pulses of 10 V could

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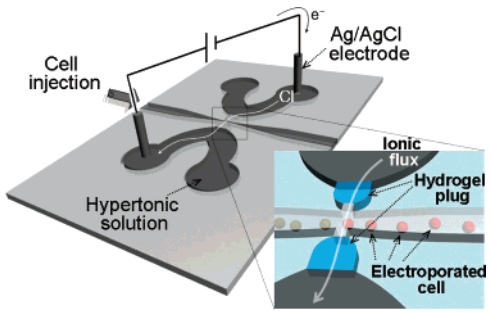

Figure 1. The schematic of the microelectroporation chip. Cells passing through the region between the salt bridges experience an electric field gradient.

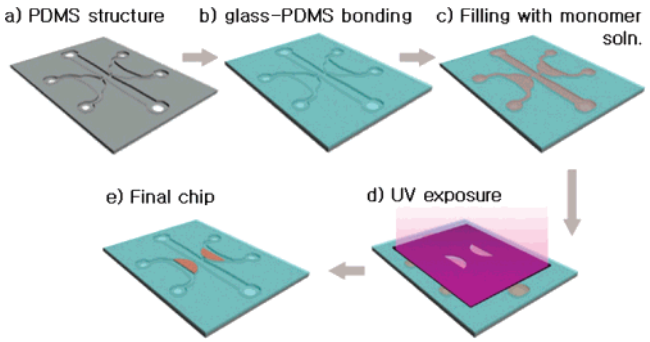

Figure 2. Fabrication process for the microchip, which is comprised of soft lithography and in situ photopolymerization.

transfect green fluorescent protein (GFP) genes into human hepatocellular carcinoma cells.[12] They proposed a microchamber for cell culture as well as gene transfection and demonstrated the electroporation of human umbilical vein endothelial cells.[13,14] Shin et al. and Wang et al. placed the electrodes at the ends of channels to avoid bubble generation, which is a fatal problem in microchannels. A high electric potential (>1 kV) was applied to Pt wire electrodes in simple PDMS chips, and the swelling of the cells was monitored during permeation.[15,16]

In the present study, polyelectrolytic salt bridges are constructed on a microfluidic chip for safe and efficient gene transfection with low-voltage (~10 V) dc electroporation. Diallyldimethylammonium chloride (DADMAC) is a water-soluble quaternary ammonium compound whose linear polymer (poly diallyldimethylammonium chloride, pDADMAC) absorbs water due to the high charge density along the polymer chain. Because of the mobile Cl^- ions, pDADMAC plugs have ionic conductivities as high as agarose salt bridges saturated with KCl.[17] The pDADMAC hydrogel structures can be constructed at specific spots of interest on the microfluidic chips by in situ photopolymerization.[18–20]

Figure 1 shows the schematic of cell electroporation on our microfluidic chip. A pair of pDADMAC plugs on both sides of the microchannel separate the cell suspension and the hypertonic solution, while the ionic flux completes the electric circuit from an external electrode to the other electrode across the microchannel. Cells flow through the intersection and are exposed to the electric field. In terms of the electric circuit, the chip is designed to have low impedance so that a large portion of the potential difference is exerted on the cell flux. Consequently, a small dc bias is expected to be sufficient to produce a high electric field gradient to accomplish efficient and safe electropermeation.

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For this purpose, the pDADMAC hydrogel was characterized by impedance analysis in the present study. With the obtained electric characteristics of the gel system, we investigated the electric field distribution near the intersection for various dimensions of the channels. Our microelectroporation chip was fabricated by in situ photopolymerization and evaluated with a suspension cell line, K562 human chronic leukemia cell.

EXPERIMENTAL SECTION

Electroporation Chip Fabrication. The electroporation chips in this study were fabricated by soft lithography and in-channel polymerization as shown in Figure 2. The chips commonly consisted of a 0.1 mm thick cover glass as the bottom substrate and cast PDMS along with a microchannel network. The molds for the microchannels were constructed using SU8 2050 (Micro-Chem Corp., USA) with a height of 75 μm on 4 in. silicon wafers. PDMS (Sylgard 184 Silicone Elastomer Kit, Dow Corning, USA) was degassed in a vacuum chamber and poured into the SU8 mold. The liquid PDMS was cured on a hot plate at 85 °C for 45 min. The solidified PDMS structure was cut and detached from the mold and punched for creating inlets and outlets of the microchannels. A 0.1 mm thick soda lime glass was bonded to the PDMS piece by plasma treatment. We applied corona discharge for 10 s to induce hydrophilicity on the PDMS surface for spontaneously introducing the DADMAC solution into the microchannel and stabilizing the hydrogel plugs as they were prepared.

The salt bridges were constructed from a solution containing 65% monomer (diallyldimethyl ammonium chloride), 2% photoinitiator (2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone), 5% crosslinker (*N*,*N*′-methylene-bisarylamide), and 500 mM KCl purchased from Sigma Aldrich. The solution in the middle of the channel was exposed to 17 mW s^{-1} of ultraviolet light by a mask aligner, MA-6 (Karl Suss GmbH, Germany), for 2 s. After the residual solution in the channel was removed, the chip was rinsed with deionized (DI) water and stored in an aqueous 3 M KCl solution. Pictures of the microelectroporation chip are shown in Figure 3.

Reagents and Cell Culture. K-562 (human chronic leukemia cell line) cells were incubated at 37 °C under 5% CO_2 in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, USA) and 1% penicillin and streptomycin (Gibco, USA). For electroporation tests, the density of cells was maintained at 1×10^7 cells mL^{-1}. The cell viability was determined before and after electroporation

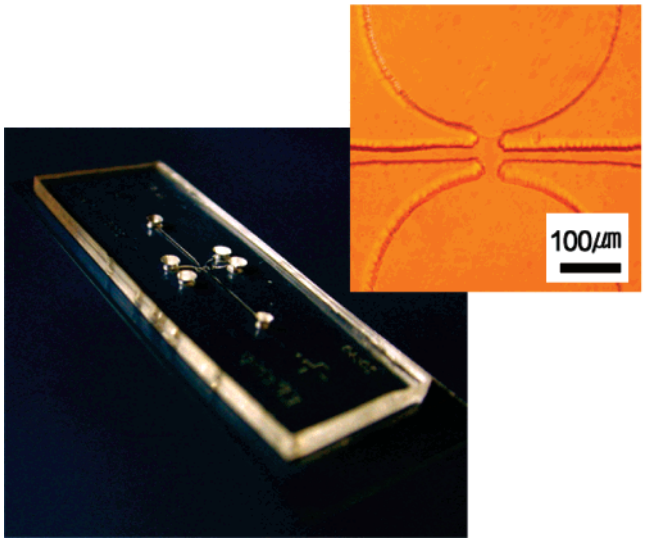

Figure 3. Pictures of the microelectroporation chip (2 cm × 6 cm) and a magnified photograph of the pDADMAC plugs. The microchannel is 75 μm high throughout the chip and 30 μm wide at the narrowest center. The pDADMAC plugs were 40 μm wide and 70 μm apart from each other at the cross section. The flow test with dye solutions confirmed negligible leakage or diffusion through the pDADMAC plugs.

using trypan blue exclusion, for which 0.25% trypan blue (Gibco, USA) was added to the cell media at a ratio of 1:1 (v/v). A solution of 1.0 mg mL^{-1} propidium iodide (Calbiochem, Germany) was used to monitor the permeation of cells mixed in the media at a ratio of 1:20 (v/v).

For DNA transfection, plasmids expressing EGFP (pEGFP-C2) were purchased from Clontech (Palo Alto, USA). Competent cells, DH5α, were transformed using the plasmid. The plasmid was extracted from an overnight culture of the transformed cells and was purified using a plasmid Miniprep kit (Qiagen, USA). For electroporation, the purified plasmid was diluted to the concentration of 0.1 g L^{-1} in the media. The cells were collected after electroporation and their gene expression was inspected in 16 to ~18 h by the fluorescence from the expressed GFP.

Instrumental Setup and Procedure. In order to characterize the properties of the salt bridge, a pDADMAC plug (1 cm × 0.5 mm × 0.5 mm) was constructed and the length was measured before and after swelling. The ionic conductivity of the plug was recorded using an electrochemical analyzer (CHI 750B, CH Instruments, USA) in the frequency range of 0.1 Hz to ~100 kHz. The experimental setup for impedance measurements during the electroporation is described in Supporting Information. For GFP transfection and protein expression, the chip and syringe were sterilized in a UV sterilizer (256 nm, 7W) for 2 h. Electroporation was performed before the cells from the outlet were immersed in the culture media and placed in an incubator. After 1 day, a fluorescence microscope, LSM 5 Pascal (Carl Zeiss, Germany) yielded images of the cells in the 12-well plate to evaluate the expression of the GFP. For detecting fluorescence from the expressed GFP, the excitation radiation was at 488 nm and the emission was observed at 527 nm.

RESULTS AND DISCUSSION

Characterization of the pDADMAC Plugs. Because of its intrinsic ionic abundance, the polyelectrolytic plugs play the role

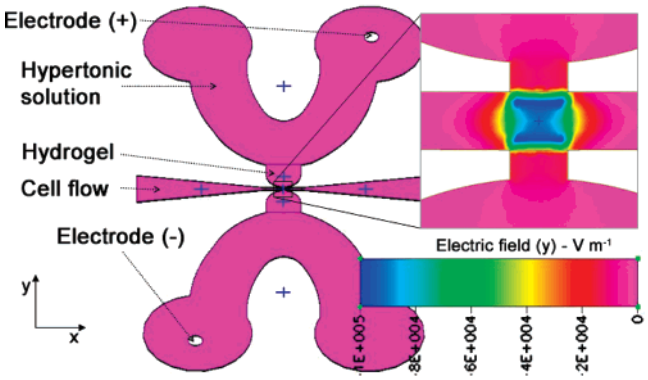

Figure 4. Electric field in the microfluidic channel, as calculated by using CFD-ACE. The magnified diagram shows the focused electric field around the intersection.

of salt bridges substantially reducing the potential gradient. Ionic conductivity of the pDADMAC plug with a cross-sectional area of 0.5 mm^2 was examined as a function of the crosslinker content, which was related to the mechanical stability of the salt bridges. The ionic conductivity was approximately 16 S m^{-1} when the crosslinker content was between 2 and 10%. The pDADMAC plugs and the hypertonic solutions showed similar conductivities, but the cell media solution was 10 times more resistive. The impedance of the polyelectrolytic plug was constant regardless of the frequency in the range of 1~100 kHz with a phase of −10° to ~10°, indicating a negligible capacitive component. The highly nonpolarizable electrode used for the measurement, such as Ag/AgCl in a Cl^- rich aqueous solution, minimized the potential drop at the electrode−solution interface. Ag/AgCl electrodes were also used as input electrodes for electroporation.

Another intrinsic property of the pDADMAC plug was its tendency to swell as it absorbed water. Over-swollen plugs may meet each other and block the microchannel. On the other hand, less-swollen polymers may allow leakage. Besides, the electroporation is sensitively affected by the distance between the hydrogel plugs facing each other. By the optimization of the crosslinker composition, 5% of the crosslinker was adopted for the fabrication of microelectroporation chips. The detailed experiments and results are provided in Supporting Information.

Calculation of Electric Field Acting on Cells. In order to predict the influence of the electric field gradient on the cells, the electric field profile in the microchannel was calculated as a function of the input voltage by using CFD-ACE (CFD Research Corporation, Alabama). The structure of the simulation was implemented with an asymmetric, steady-state model. The electric field was calculated with the boundary conditions of the fixed voltage at the edges of the electrodes. Conductivities of solutions and hydrogels were designated according to the measurements (data shown in the Supporting Information). Figure 4 illustrates the resulting distribution of the electric field in the electroporation chip. The widths of both the microchannel and hydrogel plugs were set to be 40 μm at the intersection. Apart from the intersection, the hydrogel plugs were rapidly widened to reduce their resistance and to make their contribution to the resistance of the entire circuit negligible. As shown in the magnified inset, the electric field was focused across the microchannel between the salt bridges. For a 10 V external dc bias, the electric field

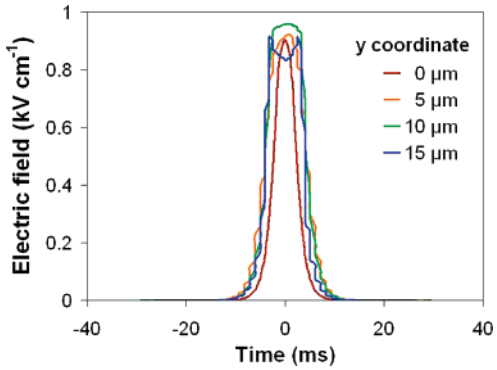

Figure 5. Pulsatile electric field acting on cells passing through the intersection; the electric field is calculated with an input voltage of 10 V and a flow rate of 1 cm s^{-1}.

gradient at the intersection was 0.9 kV cm^{-1}; this was sufficiently strong to meet the general requirements for cell permeation (0.3 to ~1 kV cm^{-1}). The transient electric field across a single cell was calculated for an input voltage of 10 V and a flow velocity of 1 cm s^{-1}, and it is illustrated in Figure 5. In this case, each cell was predicted to experience an 8 ms long pulse of electric field. The pulse height was dependent on the *y* position of the cells within a 10% variation. The critical parameters of the transient electric field seemed to be easily controlled by the input voltage and flow rate. Since the electric field was uniform in the *z* direction, the configuration resembled a parallel plate model and provided an ideal environment for high efficiency and well-distributed pore generation.[21]

Electroporation Efficiency and Cell Viability. Electropermeation on the microelectroporation chip was demonstrated using the K562 human chronic leukemia cell line. Researchers have been unable to transfect plasmids into many hard-to-transfect suspension cells by conventional methods, e.g., lipid based reagents, and have relied on alternative methods such as electroporation. The K562 cell line is one of the most typical model systems for leukemia researchers, and its transfection efficiencies are known to be poor. A mixture of propidium iodide (PI) solution and cell suspension was loaded and injected into the microchip, while a constant potential was applied between the chlorinated Ag wires. After being allowed to run through the microchannel, the cells were collected in the reservoir for 1 min and inspected. The comparison between the images obtained from white light and fluorescence allowed us to distinguish the permeated live cells from the nonpermeated cells or dead cells. As the cells were electropermeated, PI diffused into the cell cytosol and emitted fluorescence by intercalating into the nucleic acids. The dead cells were excluded during counting by staining the cells with trypan blue after electropermeation (Figure 6). The electroporation efficiency was defined as the number of live permeated cells divided by the number of live cells after electroporation. The viability was evaluated by using the ratio of live cells to dead cells and normalizing with the cell viability before the experiment.

The electric field strength was one of the most critical parameters in cell permeation, and it was controlled by the input

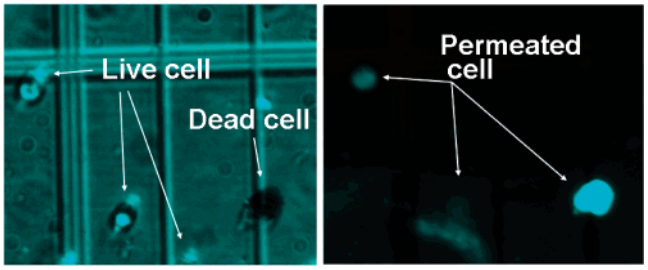

Figure 6. Classification of the treated cells by comparison between the images obtained from white light and fluorescence. Viability was judged from a general optical image (left), and electropermeation was assessed from a fluorescence image (right).

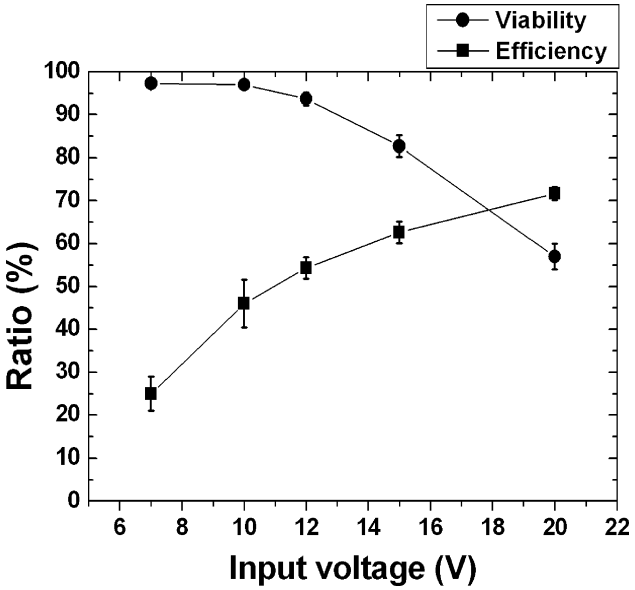

Figure 7. Electroporation efficiency and viability as functions of input voltage.

voltage in this system. The input voltage varied from 5 to 20 V, which was an accessible range with regard to the general power supply. The cell velocity was fixed at 3.2 cm s^{-1}, corresponding to 8 μL min^{-1} of cell suspension. Fluorescence from cell permeation was observed from 7 V, and the efficiency increased with the input voltage up to 72%, as shown in Figure 7. On the other hand, the viability of the treated cells gradually decreased with the input voltage because the strong electric field supposedly damaged the membranes of unsound cells irreversibly. If the pDADMAC plugs were fabricated and positioned as described in the simulation, input voltages of 7 and 20 V should result in electric fields of ~0.6 kV cm^{-1} and ~1.8 kV cm^{-1} at the cross section, respectively. However, the pDADMAC plugs were located slightly indented from the microchannel in order to safely avoid interference with the stream while they expanded toward the hypertonic solution side to safeguard the structure from the pressurized flow. When compared with the calculated values in Figures 4 and 5, the structural changes supposedly lowered the focused electric field that was effectively applied to the cells.

Another important parameter is the pulse duration for the electroporation of cells. In the present study, the pulse duration was in the range of 0.8 to ~8 ms, which was calculated from the cell velocity and the width of the intersection (40 μm). Figure 8 shows the cell velocity effect when the input voltage is 15 V. As

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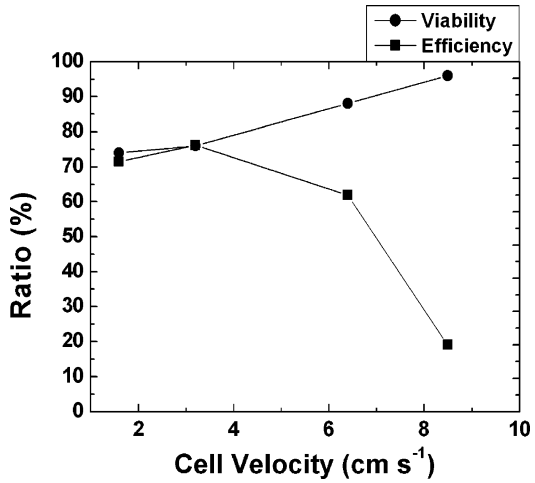

Figure 8. Electroporation efficiency (■) and viability (●) versus cell velocity. The cell velocity of 8.5 cm s^{-1} corresponds to a pulse duration of 0.5 ms.

the velocity of cells was increased (i.e., short pulse), the cell viability proportionally increased by up to ~20%, while the electroporation efficiency significantly decreased. Figure 8 indicates that a pulse duration of 2 ms or larger, which corresponds to a cell velocity lesser than 4 cm s^{-1}, is appropriate for the K562 cell line.

In this system, the uniform electric field and controllable cell velocity are favorable factors for finding the optimum conditions for electropermeation. Since the width of the microchannel was comparable to the diameter of the cell, the cells were expected to pass through the region in a row and the electric field acting on a cell was rarely disturbed by other cells. The microchannels rarely suffered from a clogging problem at a density of 10^7 cells per mL due to the gradual narrowing of the microchannel. As shown in Figure 3, the microchannel was 500 μm wide at the inlet and outlet, but it tapered to approximately 40 μm around the intersection, which was only 1.2 mm long.

When the cell velocity was 3.2 cm s^{-1}, the applied pressure at the inlet was 20 kPa, which was slightly greater than the arterial pressure. Thus, no adverse effect was expected from the fluidic condition, at least for the blood cells, and it was probably fine for most of the suspended cells. Since the cell density was fixed at 10^7 cells per mL, each chip could theoretically handle 8×10^4 cells per minute, which was calculated from the corresponding volume rate. Most of the chips were intact on continuous usage for 30 min, and the proposed configuration was sufficiently stable for electroporating approximately 10^6 cells for 10 min. The path for the charge flow was designed to possess as low impedance as possible. The resistance between the two Ag/AgCl electrodes was 35 kΩ, and the resulting current was 400 μA for a 15 V input. This condition possibly leads to electrolysis or bubble generation, which might disturb the electric field and cell flow. However, we did not encounter such a problem. It can be explained that electrolysis could have occurred on the wire electrodes, but the generated bubbles were easily vented. Heat shock was another concern under high current conditions, and the temperature was a critical factor for the cell viability. No sensible change was observed while palpating the chip. The calculated power from an input voltage of 15 V was 6.4 mW, and its major portion must have been concentrated around the cross section region. We believe that the rapid flow of the media effectively dissipated the heat.

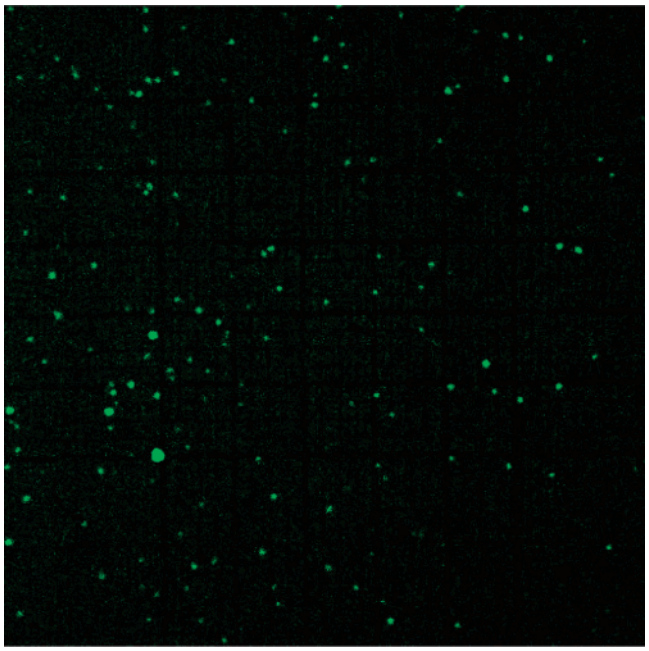

Figure 9. Fluorescence images of K562 cells transfected with GFP plasmids. The image was taken 24 h after electroporation.

Gene transfection and expression were also demonstrated by using a fluorescent protein. Green fluorescence protein (GFP) plasmids were transferred to the K562 cells by applying a voltage of 12 V and a cell velocity of 3.2 cm s^{-1} (1.25 ms pulse duration). Figure 9 shows the fluorescence images of the K562 cells transfected with GFP plasmids. The majority of the cells radiated fluorescence in a day owing to the expression of the GFP, and they were successfully cultured for more than a week after electroporation.

CONCLUSIONS

Electropermeation of human cells was accomplished with a low dc input voltage in a novel microfluidic system. The high current density and strong electric field did not suffer from the typical and critical problems in cell permeation such as bubble generation, heating shock, and chemical contamination. Polyelectrolytic salt bridges separated the metal electrodes from the cells, and their resistive characteristics allowed the precise control of both the electric field applied to each cell and pulse duration. The permeation efficiency reached 60% for an input voltage of 15 V, at which the cell damage was less than 20%. The throughput of the microelectroporation chip was 8×10^4 cells per minute under the given conditions. Moreover, human chronic leukemia cells were transfected with DNA plasmids and the expression of the protein was verified using fluorescence images for several days of cell culture. By taking advantage of simple and cost-effective fabrication processes, our chip is expected to evolve toward disposable devices and integrated system with other complex functional units in a monolithic configuration.

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SUPPORTING INFORMATION AVAILABLE

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

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