An impulsive, electropulsation-driven backflow in microchannels during electroporation†‡

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Received 17th September 2007, Accepted 29th November 2007
First published as an Advance Article on the web 10th December 2007
DOI: 10.1039/b714371k

We describe for the first time an impulsive, electropulsation-driven backflow in microchannels for on-chip cell electroporation.

One of the advantages of on-chip electroporation is the *in-situ* visualization and monitoring of uptake phenomena of molecules into cells, which makes it possible to investigate in more depth the basic mechanisms of DNA transfer and intracellular response to the external electric pulses.[1,2] Moreover, a uniform electric field in these narrow regions helps enhance both cell viability and transfection efficiency with few side effects that are known to exist in the conventional devices.[3] For a few recent years, our group has made lots of effort to develop a versatile on-chip mammalian cell electroporation method using microchannels for a rapid, effective optimization of DNA transfection protocol with high efficiency.[3,4] More recently, we achieved a direct visualization of uptake mechanism of small and macro molecules into living mammalian cells in microchannels at the single-cell level.[5] Interestingly, these studies enabled us to observe the possibility of an impulsive pressure drag which happened oppositely to the direction of electroosmotic flow (EOF).

In this communication, we highlight the fact that the external energy can change microfluidic flow swiftly and in fact produce an impulsive response which is mostly ignored until now in the conventional EOF. In particular, we will try to explain the impulsive backflow within the microchannel during electropulsation. This phenomenon supports our interpretation on a new impulsive driving source of EOF within the microchannel. This could, in particular, be investigated by directly observing the motion of cells, molecules and the other aggregates flowing in the microchannel. As far as we know, however, such backflow could not be easily elucidated with conventional EOF only. Therefore, we derived the resultant EOF from two dominant factors to fully understand an easily-changeable EOF and then formulated it in a simple form. The concept was mainly focused on the direction of EOF induced in the microchannel during electropulsation. Thus, it could be derived from two dominant flow rates induced by (i) the electric double layer (EDL) of the walls and (ii) a sudden, violent gas evolution due to the electrolysis of water in the following

$$Q_{EOF} = Q_{EDL} - Q_G = C_1\Delta V - C_2\Delta p \quad \text{for } \lambda_D \ll \frac{1}{2}h \tag{1}$$

where C_1 and C_2 are coefficients for the EDL and gas evolution, ΔV is the electric potential drop across the microchannel with a height h, Δp is the pressure difference from gas evolution, and λ_D is the thickness of the EDL. Q_{EDL} stands for the flow rate induced by the EDL of the microchannel walls and Q_G stands for the flow rate made by both hydrogen (H_2) and oxygen (O_2) gases instantly evolved at two electrodes. Q_{EOF} indicates the resultant flow rate of EOF created from both the microchannel walls and the electrodes during electropulsation as illustrated in Scheme 1 (see the ESI I† for a more detailed formulation of eqn (1)). Without any surface treatment, the direction of EOF in the microchannel would be determined by the induced Q_{EOF} during electropulsation. There may be some opposing opinions about some of these potential factors, even if not dominant, on this backflow, but these issues will be discussed in more detail later.

Specifically, the main aim of this communication is to examine the "impulsive" backflow that can occur oppositely to the direction of the conventional EOF and to provide a standard for the direction of EOF in the microchannel during electroporation. No chemical and physical treatments which might change the EOF within the microchannel were made. Generally, the direction of EOF is the same as that of the electric field, if the surface is not treated. In principle, the liquid within the microchannel moves toward the cathode along the direction of the given electric field.[6] In the case of electrophoresis, however, the direction of the objects within the liquid strongly depends on their sizes and surface charge densities. Based on electrokinetic flows, we reinterpreted the "impulsive" backflow in the microchannel during electropulsation. Obviously, in our experiments, the liquid within the microchannel suddenly moved towards the anode, quite differently to that expected. Possibly, this phenomenon can be affected by the electrophoresis under a highly uniform electric field in a narrow region, but cannot be applied to the liquid motion itself. In our experiments, the liquid moved oppositely to the known direction of the EOF, but the uptake molecules normally worked within this liquid. Given the electric conditions, there cannot help being a current-dependent gas evolution at both platinum (Pt) wire-type electrodes in the microchannel.[7] In our experiments, the charge of cell-membrane impermeable molecules seems hardly to affect the direction of the EOF (see the ESI II & III†). For a high-voltage electroporation, in particular, the reaction occurs violently at the electrodes due to the electrolysis of water, which normally happens over 1.37 μA. In the electrolysis, in principle, the cathode produces hydrogen gas, while the anode produces oxygen. In general, the

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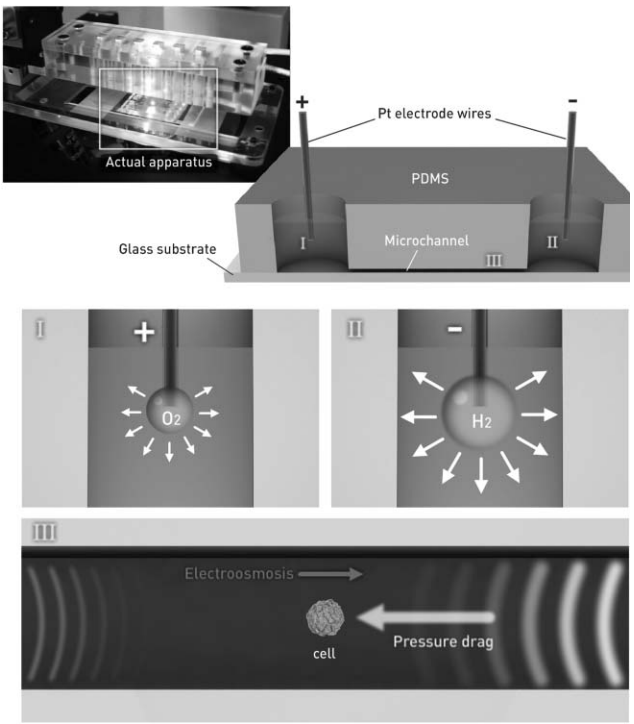
† Electronic supplementary information (ESI) available: A summary of formulation for eqn (1) and two movies showing an impulsive backflow in microchannels. See DOI: 10.1039/b714371k
‡ The HTML version of this article has been enhanced with colour images.



Scheme 1 Schematic illustration for an impulsive, electropulsation-driven backflow in the microchannel during electroporation. (Top) An actual apparatus for on-chip cell electroporation and its simple operation scheme: (I) the inlet reservoir with anode wire-electrode, (II) the outlet reservoir with cathode and (III) microchannel. (Middle) During electroporation, gas evolution happens violently at both electrodes like a bomb. While oxygen gas is formed at region (I), hydrogen is at region (II). Then, an instant pressure difference induced by such a gas evolution can make an impulsive pressure drag. (Bottom) The liquid experiences a swift, impulsive backflow against the conventional EOF. The thin arrow indicates the direction of the conventional EOF and the thick arrow indicates the direction of backflow on electropulsation.

number of hydrogen molecules produced here is nearly twice the amount of oxygen molecules. Under this same condition, the hydrogen gas has twice the quantity of moles as oxygen gas. Immediately after electropulsation, more gases can be evolved at the surface of the immersed cathode than that of the anode. Normally, the quantity of gas formed must be strongly dependent on the given electric condition such as the amplitude and sign ($\pm$) of the induced electric current. Here we also tried to focus on the profile of the electric current, induced from the square wave pulse of the voltage, to fully understand the "impulsive" backflow as shown in Fig. 1. This profile can be altered by a duty ratio of the given electric pulse. Since most cell electroporation methods using the electric pulse have hardly 50% duty ratio, it is natural that there should be a big discrepancy between experiment and theory for the current profile. Similarly, this disagreement can be observed for DC or AC electroosmosis. Particularly, this phenomenon clearly shows that the pressure difference due to the gas evolution instantly exceeds that of the EDL. These findings imply that a full understanding of the "impulsive" EOF is essential to enhance the efficiency of on-chip cell electroporation, separation and lysis at the single-cell level.[8,9] Furthermore, this is useful in investigating the effect of a local pH variation in the vicinity of the cathode on cell transfection and viability in electroporation using

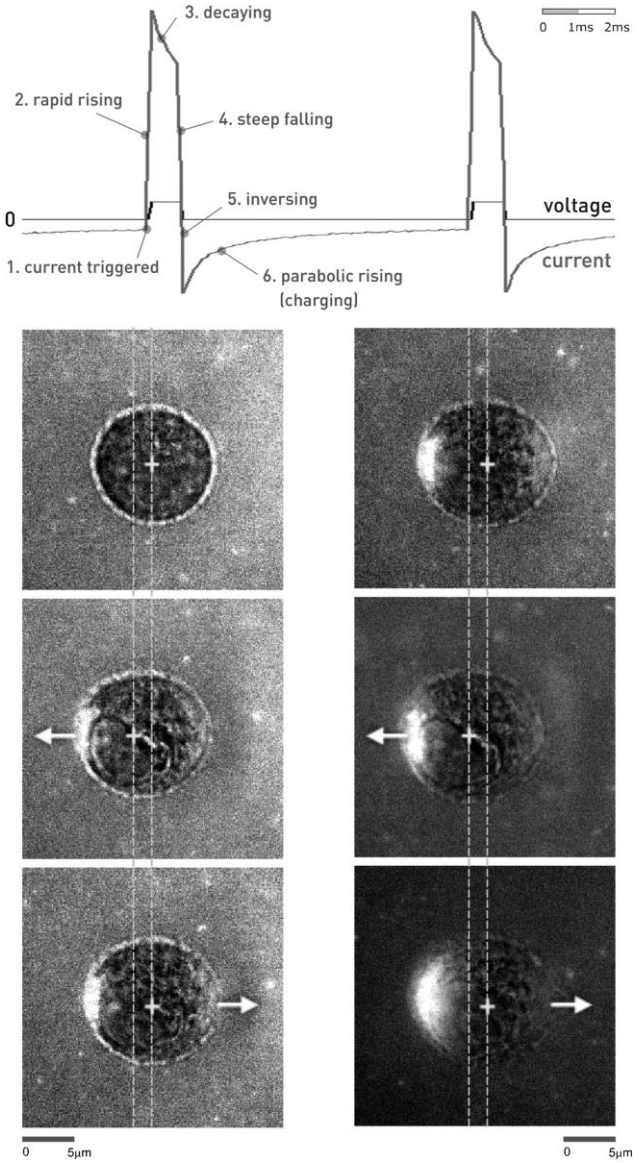


Fig. 1 Experimental profiling of the induced electric current within the microchannel during electropulsation of two square-wave pulses. (Top) This profile shows a hidden turnover of polarity of the current for some time. The electric current has two polarities for the external voltage input. The positive current occurs between Regions 1 and 4, while the negative one occurs at Regions 5 and 6 before the next pulse is triggered. The gas evolution normally happens over 1.37 μA, so our experimental conditions strongly agree with this rule. In particular, the creation and evolution of gas can be violently made at a higher voltage with a larger duty ratio. In that case, a sudden formation of gas will play a significant role in driving EOF instantly like a bomb in water as illustrated in Scheme 1. (Bottom) The left column shows that HeLa cell experiences the impulsive backflow, reflecting the EOF on the first electropulsation and the right column shows that of the second electropulsation. Here molecular uptake of propidium iodide (PI) into living HeLa cells was achieved at the single-cell level. The arrow indicates the direction of motion of HeLa during electropulsation. In our experiment, the motion of objects in the liquid was in good agreement with the current profile (see the ESI II & III†). Note that the above current profile and cell images were independently obtained, so the movement mapping was made with sequential images corresponding to each portion of the current profile. In general, cell images can be synchronously recorded using an external trigger signal.

microstructures. For example, if the cathodic flow is consistently observed, the pH level will be observed to decrease and become locally acidic near the cathode. Particularly, the EOF created in the region which has nearly neutral EDL can be explained by this impulsive backflow. Normally, the degree of electrode passivation is an important factor that can affect the quantity of gas evolution. Thus, replacement and cleaning processes of the electrodes prior to each experiment are required. The electrodes can be easily reset by changing the polarity of the voltage source, *i.e.*, both reduction and oxidation react at the opposite electrodes. In a very narrow microchannel, the effect of gas evolution on the EOF can be limited because its high equivalent resistance tends to hinder the backflow. The position and the immersed surface of electrodes should also be well-aligned prior to the electropulsation. Note that the distribution of these forces can be expressed as a function of position within the microchannel (see the ESI I†).

Recently, it has been reported that the streaming potential could make the direction of the liquid opposite to the conventional EOF in microchannels for a DC power.[10] However, it did not imply a swift, impulsive backflow in microchannels during electropulsation. For the experimental setup and preparation, HeLa cells were tested to observe real-time molecule uptake and its motion during on-chip electroporation (see the ESI III†). All of the cells were cultured and seeded on the culture flask two days before the experiment. The cells were detached with Trypsin-EDTA (Sigma-Aldrich, USA) and washed using Dulbecco's Phosphate Buffered Saline (DPBS, 1×) with Ca^{2+} and Mg^{2+}. Then the cells were centrifuged and resuspended in the DPBS buffer at the final concentration of 1.5×10^6 cells ml^{-1}. For fluorescent imaging, PI and YOYO1-labeled plasmid enhanced green fluorescent protein (pEGFP) with 1 μg/100 μl mixture ratio for pEGFP/cells were used to directly observe the directionality of electrophoresis. Specifically, the PI was used for cathodic electrophoresis and the pEGFP for anodic one, but it was not used at the same time. Cell viability was measured to be around 97% prior to the experiments (statistical data not shown). Forty microliter of the sample was injected into the inlet of a single microchannel (50 μm × 400 μm × 2 cm, in height, width and length). The rectangular cross-sectional channel was fabricated with a poly(dimethylsiloxane) (PDMS) and glass slide by simply using the MEMS-based softlithography. Here, oxygen plasma treatment was used once to close the microchannel. The effect of oxidized or native PDMS surface on electroosmosis has been well investigated in the previous study.[11] Note PDMS is generally gas-permeable, so there may happen to be a little gas evolution at the PDMS surface. In our experiment, however, it showed little possibility of changing the direction of EOF. After balancing the quantity of the sample drop into the reservoirs, we put two Pt wire-electrodes into these reservoirs. Then, little flow occurs in the microchannel, if any, there is microflow occurring by a small Siphon's effect, a submicron-liter difference of the liquid drop into the inlet and the outlet. The microchip was aligned in the electroporation apparatus and then placed in the microscope before the electropulsation. The electric pulse is given to the cells and molecules in the microchannel through two Pt wire electrodes from a square-wave pulse generator. During the experiment, all images were taken using a 12-bit cooled charged coupled device (CCD) (Cooke SensiCam QE) on an inverted microscope (IX71, Olympus) with 100× oil-immersion objective lens (NA 1.4) for single-cell level imaging and 40× (NA 0.6) for the motion capture of all objects on the whole window. The camera exposure time was adjusted at 10 ms and a time-delay was not applied. The acquired images were quantitatively analyzed using an interactive processing of the JAVA program (data not shown).§ All electrical parameters (electric field strength: 335 V cm^{-1}, pulse width: 35 ms, pulse count: two times, pulse period: 1 s) were pre-optimized to the general electrotransfection protocol of HeLa cells. Note that the degree of electrotransfection strictly relies on dimensional properties of instrumental environments: the electrode type and the size and material of the chamber.

In brief, this communication reports an important gas-acoustic force, which could be a dominant force for EOF research, depending on the microchannel scale and the location of gas evolution but could be easily ignored during theoretical derivation. Here we tried to focus on a new reinterpretation on the direction of the conventional EOF in the microchannel by introducing a new driving source of EOF, an "impulsive" backflow of the liquid during electropulsation in microchannels. In conclusion, we have summarized two dominant flow rates consisting of the EOF in the microchannel: One can be induced by the EDL and the other can be formed by the gas created at both electrodes near the exit and the entrance of the microchannel. Thus, the direction of the EOF in the microchannel should be reconsidered and it can help explain the EOF often created within the structure which has a neutral or small EDL. Furthermore, we believe these findings will contribute to the *in-situ* visualization and observation of electroporative uptake for initial protocol optimization of multi-electrostatic functional gene delivery using microchannels or microstructures at the single-cell level, especially under microscale volatile circumstances with a local pH variation.

We especially thank J. A. Kim for sample preparation and Dr K. Cho for system evaluation, Digital Bio Technology, Inc., S. H. Lee, Korea Institute of Science Technology (KIST), Korea for electrical work, and Y. S. Shin, California Institute of Technology, USA for helpful discussions. This work was supported by a grant from the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (02-PJ10-PG4-PT02-0042).

Notes and references

§ The relative magnitude of each of these factors was measured using JAVA interactive processing. The measurement algorithm and data of the program was limitedly described for further commercial development.

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