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Vortex-assisted DNA delivery†

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Electroporation is one of the most widely used methods to deliver exogenous DNA payloads into cells, but a major limitation is that only a small fraction of the total membrane surface is permeabilized. Here we show how this barrier can be easily overcome by harnessing hydrodynamic effects associated with Dean flows that occur along curved paths. Under these conditions, cells are subjected to a combination of transverse vortex motion and rotation that enables the entire membrane surface to become uniformly permeabilized. Greatly improved transfection efficiencies are achievable with only a simple modification to the design of existing continuous flow electroporation systems.

Introduction

Delivery of exogenous DNA payloads into cells (i.e. gene transfer or transfection) is a critical step in cell-based therapeutics, tissue engineering, and fundamental molecular and cell biology. This process is often accomplished by electroporation, a method that involves applying electrical pulses to open transient nanoscale pores in the cell membrane that permit entry of impermeant DNA. Electroporation methods are advantageous due to their simplicity and capacity for operation in a high-throughput continuous flow manner† and in microscale devices,‡§ but they are also extremely inefficient. These limitations arise as a consequence of the fundamental interactions between the cells and the applied electric field whereby favorable permeabilization conditions (i.e., transmembrane potential exceeding a critical threshold value of ~0.25–1 V) can only be achieved over a very small fraction of the total membrane surface. This can be seen by considering the transmembrane potential distribution over a spherically shaped cell (Δψ = 0.75 g(λ)E cosθ, where g(λ) is a complex function of the membrane and buffer conductivities, a is the diameter of the cell, E is the field strength and θ is the angle between the normal to the membrane surface and the field direction) which shows that DNA transfer is favored only near the poles (i.e., θ → 0) where the surface normal is closely aligned with the field direction, as has been observed experimentally.¶ The size of this zone cannot be expanded by increasing the electric field because the cells will become damaged and experience dramatically reduced viability. Applying periodic electric pulses of different polarities and directions was shown to improve gene transfer.¶ However the instrument complexity required to employ this approach is relatively high, with limited feasibility for scale-up to processing large numbers of cells.

In this report we introduce a new approach to overcome these limitations by harnessing hydrodynamic effects that arise in fluidic networks incorporating curved flow paths. Under appropriate conditions, the curvature-induced inertial force acting along the channel’s radius of curvature becomes strong enough to establish a transverse vortex flow superimposed over the fluid’s axially directed pressure driven motion. When a cell laden suspension encounters such a flow field, the cells are simultaneously subjected to a complex combination of transverse advection and rotation. In this way, a much larger fraction of the total cell surface is able to experience alignment with the electric field and become permeabilized, so that it can be accessed by exogenous DNA. We show that electroporation in this curved spiral-shaped channel design yields a two-fold increase in transfection efficiency compared to a straight microchannel.

Materials and methods

Materials

Fluorescent polystyrene beads with diameter 9.9 ± 0.05μm (s.d.) were purchased from Duke Scientific. The density of beads is 1.05 g/ml according to the protocol. Before experiment, the beads were diluted in phosphate buffered saline (PBS) at a concentration of 1.9 × 10⁶/ml. The 4.7 Kb plasmid vector pEGFP-C1 (Clontech) coding green fluorescent protein (GFP) was propagated in Escherichia coli and extracted in Tris-EDTA buffer with QIAfilter Plasmid Giga kit (Qiagen). The plasmid was stained with green fluorescent DNA dye YOYO-1(Invitrogen) with a molecular ratio of 1 dye per 5 bp when fluorescent labeling was necessary. Chinese hamster ovary (CHO-K1) cells were grown using protocols provided by ATCC. To observe flowing cells in microfluidic channels, CHO cells were stained by 5 μM nucleic acid dye SYTO 16 (Invitrogen) for 5 min followed by washing.

Microchip fabrication

Microfluidic devices were fabricated on polydimethylsiloxane (PDMS, General Electric Silicones RTV 615) using standard soft lithography as we demonstrated previously.¶

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Fluorescence microscopy

To show the flow pattern of cells and particles in microfluidic channels, CHO cells were labeled with SYTO 16 and 100~200 consecutive fluorescence images were taken with 40 ms exposure time for each of them and superimposed for image analysis. The delivery of YOYO-1 labeled pEGFP-C1 into cells was observed using a confocal fluorescence microscope (LSM 510, Carl Zeiss) with a 63X, 1.4 NA oil-immersion objective. In order to observe the delivery of the plasmid under confocal microscope, 500 μg/ml fluorescent plasmid was mixed with CHO cells in the electroporation experiment. These processed cells were moved to a centrifuge tube immediately after electroporation and washed twice to remove nonspecifically adsorbed DNA molecules from the cell surface before imaging.

Microchip operation

The microfluidic electroporation device was mounted on an inverted fluorescence microscope (IX-71, Olympus) (as shown in ESI Fig. S1†). The inlet of the channel was connected to a syringe pump (PHD infusion pump, Harvard Apparatus) by plastic tubing through which cells or beads were pumped into the microfluidic devices at a constant flow rate. A high voltage power supply (PS350, Stanford Research Systems) was used to generate a constant direct current (DC) voltage between the sample and the outlet reservoirs. Electroporation occurred when cells flowed through the narrow section of the channel where the field intensity was higher than the electroporation threshold.1,12

Transfection

Cells were centrifuged at 260 g for 5 min and resuspended at 2 × 10^6 cells/mL in the electroporation buffer (8 mM Na_2HPO_4, 2 mM KH_2PO_4, and 250 mM sucrose, pH = 7.2) containing 10 μg/ml plasmid. After electroporation under certain electric parameters in the microfluidic devices, the cells were cultured in DMEM medium for 2 days before quantification of transfection frequency and viability. Transfection efficiency is the percentage of EGFP expressing cells among living cells. The cell viability after electroporation was assessed by staining cells with propidium iodide (PI, Invitrogen) and calculated by dividing the number of viable cells by the total number of cells. All data points were generated by experiments in triplicate and ~5,000 cells were examined in each trial. Two-tailed student’s t test was applied to evaluate the data.

Results

We first examined the effects of hydrodynamics on the migration of cells and particles (including Chinese hamster ovary (CHO) cells with a~14.6 ± 2.2 μm (s.d.) and polystyrene beads with a~9.9 ± 0.05 μm (s.d.)) in microfluidic channels incorporating a central spiral section of reduced cross-section (i.e., the electroporation zone). The migration of cells or beads in the spiral channel is affected by a balance between (1) transverse lift forces that act to stabilize them at distinct equilibrium positions within the microchannel cross section, and (2) a centrifugal Dean force that acts to entrain them in a counter-rotating transverse vortex flow pattern.13 The interplay between these effects can be seen in Fig. 1a, where a suspension of CHO cells is loosely focused at the channel centerline throughout the entire spiral section (height ~ 75 μm, width ~ 35 μm) when the flow rate is low (3.75 μl/min). As the flow rate increases to 75~150 μl/min the Dean drag begins to dominate and the focused stream of CHO cells becomes displaced, crossing the centerline at multiple locations along the spiral section (Fig. 1a). This transverse motion indicates that the CHO cells are entrained in the Dean vortex flow pattern at high flow rates. Similar transverse advection can be observed more readily when a suspension of the fluorescent polystyrene beads flows through the same spiral-shaped channel at 150 μl/min (Fig. 1b). As a comparison and control, we also studied migration of the cells and polystyrene beads in a device where the spiral channel is replaced with a straight section of identical length, width and depth. In this geometry, CHO cells gradually focus to the centerline as they travel through the narrow section at the low flow rate of 3.75 μl/min (Fig. 2). At higher flow rates (37.5~150 μl/min) the CHO cells become focused into two streams
section, rendering the electric field intensity higher by the same factor in the narrow section than that in the wide sections when a constant voltage is applied across the channel (with two platinum electrodes in the inlet and outlet reservoirs). When a mixture of cells and plasmid DNA is pumped through the microchannel network, the cells are exclusively electroporated in the narrow section because the field intensity in the wide sections (28–56 V/cm) is substantially lower than the electroporation threshold for most mammalian cells (>300–400 V/cm). We observed the delivery of exogenous DNA molecules (fluorescently labeled with YOYO-1) into the CHO cells using confocal fluorescence microscopy. Because the DNA molecules can only penetrate the cell membrane within the electroporated zone, and since they are uniformly distributed throughout the micro-channel, the fluorescence on or inside the cell membrane essentially “maps” the area that has been electroporated to the extent that allows DNA entry. In a straight microchannel with an electroporation duration of 0.5 ms (i.e., the time for cells to travel through the narrow electroporation section), DNA delivery is confined to a narrow zone in the vicinity of one pole of the cell (Fig. 3a). This result is in agreement with comparable results obtained in static electroporation systems.6,19 and is consistent with a unidirectional flow profile through the electroporation section without cell rotation. Longer electroporation durations (5 ms) in the straight microchannel produce DNA delivery within a thin circular strip around the cell equator due to the onset of rotation about a fixed axis (Fig. 3b). When identical flow conditions and electric field parameters are applied in a spiral microchannel of the same dimensions, however, the confocal images appear dramatically different with DNA delivery uniformly distributed over the entire cell surface as a consequence of the combined effects of rotation and transverse advection during electroporation (Fig. 3c). ESI Fig. S2† shows the epi-fluorescence images of the larger cell populations which indicate that the images in Fig. 3 are representative of the cell populations.

The enhanced DNA uptake based on flow-through electroporation in curvilinear channels has important implications. In Fig. 4a, we show the transfection of CHO cells by pEGFP-C1 vector using both straight and spiral shaped microchannels of the same dimensions. Under the same flow rate and electric field conditions, the transfection efficiency in the spiral geometry is about two-fold higher than that achieved in the straight channel devices. Cell viability after electroporation is similar in both microchannel geometries (Fig. 4b). The cell viability was not compromised by electroporation significantly due to the fairly short field duration and medium field intensity. The shear stress that cells experience here is not excessive compared to in other established cell-handling techniques such as flow cytometry. The similar viability in the straight and spiral channels suggests that the cell viability is independent of the membrane area that is permeabilized. Instead, the cell viability is possibly much more affected by Joule heating which is determined only by the field intensity/duration and identical in the two devices.

**Discussion**

In order to better understand how these hydrodynamic effects act to produce enhanced transfection efficiency, we consider the
The physics of microscale particle migration in curvilinear channels. Suspended particles are subjected to the laminar flow field inside a curved microfluidic channel and experience a combination of inertial lift and drag forces due to the transverse Dean flow. When the magnitude of these forces is similar, the convected particles migrate to occupy lift-induced equilibrium positions that minimize the Dean flow effects. On the other hand, when the drag is substantially larger than the lift force, the particles become displaced from these equilibrium positions and begin to follow the Dean vortex pattern. In comparison, particles that flow in a straight channel are focused into multiple equilibrium positions due to inertial self-ordering. The strong dependence of these effects on particle size has led to interest in harnessing Dean flow effects for particle sorting and separation applications. However, these effects have not been explored in connection with manipulation of particle orientation or DNA delivery into cells. There are two important Reynolds numbers that characterize the flow of particles in a channel. The Dean Reynolds number \( R_D \) and the particle Reynolds number \( R_e \) are defined by:

\[
R_D = \frac{2 \pi U_m a^2}{\nu D_h}, \quad R_e = \frac{a^2}{D_h^2} \left( \frac{a}{D_h} \right)^2,
\]

where \( U_m \) is the maximum velocity in the cross section, and \( a \) is the diameter of the particle. \( \nu \) is the kinematic viscosity and \( D_h \) is the hydraulic diameter which is defined as \( 2wh/(w+h) \) (with \( w \) and \( h \) being the width and depth of the channel). The Dean number \( D_e \) is used to characterize the curvature effects in a curvilinear channel and is defined as:

\[
D_e = \frac{U D_h}{\nu} \left( \frac{D_h}{2R} \right)^{1/2} = \left( \frac{D_h}{2R} \right)^{1/2} \left( \frac{U D_h}{\nu} \right),
\]

where \( R \) is the radius of curvature of the channel, \( U \) is the mean axial velocity, and \( \nu \) is the kinematic viscosity. Assuming the particle will cover a circular path \( L_c \) in the transverse direction with a length of \( \frac{3}{2} D_h \), the channel length \( L_c \) covered during the period that the particle travels through a complete circular path along a Dean vortex will therefore be

\[
L_c = \frac{U D_h}{\nu} \left( \frac{3}{2} D_h \right)^{1/2}. \quad \text{The equation shows that } L_c \text{ becomes short (i.e., more frequent centerline crossings) close to the spiral center and at high } D_e, \text{ consistent with our observations.}
\]

Based on the pattern of DNA delivery shown in Fig. 3, we can now infer the nature of the cell migration phenomena occurring in different microchannel geometries during flow-through electroporation. When flow-through electroporation occurs in a curved microchannel, the DNA delivery over the entire cell surface is a result of the induced cell motion in the spiral channel segment, which involves a combination of rotation and...
transverse advection following the Dean vortex pattern. Fig. 6a illustrates how this process would allow cells to experience electroporation over their entire surface. When a cell simultaneously experiences rotation due to the near-wall shear field coupled with transverse motion due to the Dean vortex flow, different parts of the cell surface are continuously exposed to a sufficiently high transmembrane potential to permit electroporation. In comparison, in a straight channel cells experience rotation about an axis that is parallel to the vertical walls as they travel through the electroporation section (Fig. 6b). These effects begin to influence electroporation when the electric pulse duration is long enough (e.g. ~5 ms) by expanding the zone of DNA delivery from the pole to a circular strip. Previous literature indicates that such free rotation of particles occurs in response to imposed shear in a Poiseuille flow, and that the cell-wall friction due to hydrodynamic interactions can also affect the rotation rate. The rotation rate is estimated to be ~830 revolutions/s at the flow rate of 150 μL/min in our device, based on experimental results in the literature. This is consistent with the fact that an electroporation duration of 5 ms in the straight channel generates a circular DNA delivery zone while 0.5 ms duration constrains the delivery to a much smaller area, as shown in Fig. 3. Finally, it needs to be noted that entrainment in the secondary Dean flow may not be entirely necessary. It is possible that similar effect is achievable by imposing conditions whereby the cells become inertially focused at prescribed equilibrium positions but are still exposed to torque generated by the secondary flow, thereby inducing different rotation characteristics than those due to shear field.

While curvature-induced inertial flows have been widely studied in single-phase fluids, these phenomena are only beginning to be fully understood and appreciated in particle laden suspensions. With proper design, these effects can be harnessed to greatly enhance gene delivery efficiency with only a simple modification to the design of existing continuous flow electroporation systems. This method is inherently robust and readily amenable to both scaling up and down for processing cell samples spanning a wide range of volumes, including small volume samples from scarce sources.

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