Minimally invasive intracellular delivery based on electrokinetic forces combined with vibration-assisted cell membrane perforation

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1. Introduction

To achieve the goal of improving human health and quality of life, a comprehensive understanding of life processes at the cellular level is crucial. This information has a wide range of life, a comprehensive understanding of life processes at the cellular level is crucial. This information has a wide range of applications in various fields—from basic biology to biomedical sciences such as medical diagnostics, drug discovery, and tissue engineering—and is essential because cells, which are the structural and functional units of all known living organisms, play a significant role in fundamental life processes such as signal transduction, metabolism, intracellular trafficking, and protein degradation. For this reason, a new approach to single-cell analysis that enables the detailed investigation of individual cells is essential for the complete understanding of cellular heterogeneity in a dynamic cellular environment. Analysis of complex biological systems such as living biological cells on a microfluidic platform, also known as lab-on-a-chip (LOC), based on microelectromechanical system (MEMS) technology has opened up new possibilities for revolutionary approaches to the analysis of cellular functions at the single-cell level.

The regulation of cell functions is also essential for the development of effective cell-based therapies and is emerging as a promising approach for the treatment of various diseases. Recent research has revealed that induced pluripotent stem cells (iPSCs) have tremendous potential in personalized stem cell therapies (e.g., for ocular surface reconstruction and Parkinson’s disease) and in drug discovery and development (by using patient-derived iPSCs as a platform for human disease modeling). Targeted genome editing has also attracted considerable attention due to its potential for enabling the next great leap forward in genetic therapeutics and personalized medicine. A recently developed genome editing tool, the CRISPR-Cas9 system, has led to a new era in molecular biology due to its simplicity, high efficiency, and design flexibility. However, an efficient and high-throughput intracellular delivery technique is required to fully realize the potential of genome editing for gene and cell therapies and to establish iPS cell banks (similar to blood banks), which will accelerate research and development in a wide range of potential clinical applications.

Thus, one of the most challenging aspects of this goal is to develop a technique for the introduction of biomolecules such as DNA, RNA, proteins, or therapeutic drugs into a living cell and the extraction of intracellular biomolecules or components. As mentioned above, this technology is crucial not only for addressing fundamental questions regarding how living systems function as a whole and process dynamical information but also for enabling medical and pharmaceutical applications, e.g., highly advanced diagnoses and treatments in gene therapy, validation of cell culture in tissue engineering, cell-based screening of drug candidates in drug discovery, highly efficient and robust genome editing, and mass production methods to generate iPSC cells. Methods for the intracellular delivery of bioactive molecules into living cells can be broadly categorized into three fundamental strategies: viral-based methods, non-viral chemical methods, and physical delivery methods. Viral methods are generally highly efficient but may not be clinically applicable due to significant safety problems related to viral contamination. Chemical methods are characterized by low toxicity and low host immunogenicity, but have low transfection efficiency. In contrast, physical delivery methods (e.g., microinjection, electroporation, and sonoporation) have some advantages over viral and non-viral methods, such as absence of the side effects associated with viral vectors and the ability to deliver various types of molecules directly into almost all types of cells, particularly the so-called difficult-to-transfect cells such as primary, progenitor, and stem cells. However, their application is still limited by low transfection efficiency and poor cell viability.

Among the different physical delivery methods, microinjection is one of the most widely used techniques for directly delivering biomolecules. In this method, a glass micropipette with a diameter of approximately 1 µm is used to penetrate the cell membrane. However, manual operation under an optical microscope requires a very high degree of skill. Therefore, the method is associated with the potential drawbacks of low success rates and poor reproducibility.
In addition, this method has been limited in application to relatively large egg cells with a diameter of approximately 100 μm. It has not yet been performed in somatic cells with a diameter of around 10 μm, which are our target cells. Moreover, it would be difficult to use external hydrostatic pressure to introduce extremely small amounts of biomolecules into somatic cells in a controlled manner.

In order to address this issue, we proposed a novel intracellular delivery method based on electrokinetically driven forces. In our previous work, we proposed a method for minimally invasive intracellular delivery of biomolecules into a living cell by using electrokinetic forces combined with vibration-assisted cell membrane perforation to improve cell viability. This technique is particularly appropriate for the establishment of a fully or semi-automatic delivery system. Moreover, to overcome the intrinsic problem of low microinjection throughput associated with the use of a single glass micropipette, we have been developing an array of out-of-plane, hollow silicon dioxide (SiO2) microneedles with a minimum diameter of a few micrometers, which will allow the large-scale parallel introduction of desired biomolecules into living cells."}

"2. Experimental methods"

Figure 1 shows a schematic diagram and photograph of the homemade apparatus for the intracellular delivery of biomolecules into a living cell, which was placed on an inverted fluorescent microscope (Nikon TE2000-U). A glass micropipette with an inner diameter of 300 ± 30 nm and an outer diameter of 500 ± 60 nm (mean ± SD, n = 20) was mounted on a three-axis piezoelectric stage (Physik Instrumente NanoCube P-611.3S), which was operated with a servo controller (Physik Instrumente Piezo Controller E-664.S3). The movement of the micropipette was controlled automatically using commercial software (LabVIEW) for the injection of biomolecules into a living cell. DC or AC voltage was applied to a pair of Ag/AgCl electrodes (0.2 mm in diameter); one electrode was placed inside the glass micropipette, and the other was immersed in a microchannel filled with a phosphate-buffered saline (PBS) solution. The resulting current was amplified and converted to voltage with a current amplifier (Keithley PROG-428). The amplified signal was then read by a data acquisition (DAQ) board (National Instruments PCI-6251) together with a homemade LabVIEW program to control the vertical movement of the glass micropipette during cell membrane perforation.

In the experiments, fluorescein-conjugated dextran (F-GL4-II, 19 bp) was dissolved in Tris–EDTA buffer (TE buffer: 10 mM Tris–HCl and 5 mM EDTA, pH 8.0) adjusted to a concentration of 40 μM and then drawn into the micropipette, which was previously rinsed with 0.1% bovine serum albumin (BSA) in a PBS solution for 15 min to prevent cell adhesion. HeLa cells were used as a representative somatic cells; they were cultured for at least 48 h in a 5% CO2 incubator at 37 °C in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Prior to the injection experiment, the culture medium was replaced with PBS. Note that in this study, the cells adhered to a coverslip without any treatment to enhance cell adhesion. Cell viability after cell membrane penetration was estimated with Calcein-AM (Dojindo Molecular Technologies); this dye stains only living cells, and causes the emission of a strong green fluorescence. After 10-min incubation with Calcein-AM, the cells were observed with the inverted fluorescent microscope.

3. Results and discussion

3.1 Vibration-assisted cell membrane perforation

In the present work, we propose a vibration-assisted insertion method for penetrating the cell membrane to reduce cell damage. First, the effects of vibration on cell mechanics were investigated using a commercially available finite element modeling (FEM) software (ABAQUS). In the FEM analysis, the power-law rheology (PLR) model was used to describe the viscoelastic deformation of a cell; the PLR model was developed to describe cell deformation during micropipette aspiration.21) Axisymmetric geometries were created for the micropipette (500 nm inner diameter and 700 nm outer diameter) and the hemispherical shape of a cell (20 μm diameter and 5 μm height) as shown in Fig. 2(a). The micropipette was considered to be analytical rigid, and the cell was attached to a rigid substrate at its bottom surface. In the simulations, the micropipette was moved downward up to an indentation depth of 150 nm, and it then started vibrating at a certain frequency with a peak-to-peak ampli-
Fig. 2. (Color online) FEM analysis of cell deformation behavior with mechanical oscillation; (a) axisymmetric FEM model for the micropipette and cell; (b) close-up view of the contact region between the tip of the micropipette and the surface of the cell showing equivalent stress distribution obtained by imposing sinusoidal deformation with an amplitude of 80 nm at 1 kHz.

Fig. 3. (Color online) Resulting stress–strain curves obtained by imposing a sinusoidal deformation with an amplitude of 80 nm at 1 Hz (a) and 1 kHz (b).

Fig. 4. (Color online) Induced maximum stress (a) and total strain (b) as a function of mechanical vibration frequency.

Fig. 5(a) shows the profile of an ion current, defined as the “time-current curve”, passing through a glass micropipette tip under various tip–sample distances during penetration of a living HeLa cell adhered to a coverslip. Here, each ion current data point represents the average value of 4096 data points measured at a sampling frequency of 10 kHz. First, the micropipette approached the surface of the cell at a feed rate of 68 nm/s (28 nm/step) with an applied voltage of 350 mV; it was also driven by a 20-Hz sine wave with a peak-to-peak amplitude of 100 nm during its approach in order to measure the ion current with greater stability. The time-current curve indicated that the ion current dropped steeply when the micropipette approached the cell surface owing to the decrease in the tip–sample distance. It has been...
shown that ion current starts to drop significantly when tip–sample distance is in the range of the inner radius of the micropipette tip. After the indentation depth of the tip into the cell reached approximately 1 µm, the micropipette movement was stopped and subsequently, it was vibrated by a 20-Hz sine wave with a peak-to-peak amplitude of 200 nm; with this process, the indentation depth into the cell can be adjusted automatically to a desired position by monitoring the change in ion current. After mechanical oscillation for 30 s, the micropipette was promptly retracted ∼5 µm from the cell surface. As shown in Fig. 5(a), the ion current was kept almost constant during the mechanical oscillation at 20 Hz.

However, when the micropipette was vibrated at 1 kHz, the ion current increased at almost the same time (less than at least 0.4 s), as shown in Fig. 5(b). This result indicates that the micropipette tip was inserted through the cell membrane, and consequently, the cell membrane was successfully penetrated with a vibration amplitude as small as 200 nm (peak to peak) at an indentation depth of approximately 1 µm in a cell with a height of approximately 3.5 µm. The probability of the perforation of the cell membrane was dramatically improved by up to 77% (36/47) with a 1-kHz mechanical oscillation, as compared with a 20-Hz oscillation (0%). Cell viability after the cell membrane perforation was also significantly improved by up to 77% (36/47) with a 1-kHz mechanical oscillation, as compared with a 20-Hz oscillation (0%).

In our previous work, we successfully demonstrated the electrokinetic injection of a fluorescent DNA (19 bp) and dye solution (rhodamine B) into a living HeLa cell through a glass micropipette (approximately 1 µm in diameter) at applied DC voltages of 5 and −5 V, respectively. In this technique, two types of “electrokinetic phenomena” should be considered. One is the electroosmotic (EO) flow, where the fluid moves toward the negative electrode. The other is the electrophoretic (EP) flow, where electrically charged molecules move toward the oppositely charged electrode. The net velocity of a charged molecule, $U_P$, is calculated using the sum of the EP velocity ($U_{EP}$) and the EO velocity ($U_{EO}$) with the following equation:

$$U_P = U_{EP} + U_{EO} = (\mu_{EP} + \mu_{EO})E,$$

where $E$ is the applied electric field, $\mu_{EP}$ is the EP mobility of the molecule in the solution, and $\mu_{EO}$ is the EO mobility of the channel wall. Neutral molecules, including rhodamine B, move toward the negative electrode by generating an EO flow, whereas the EP flow becomes dominant over the EO flow for negatively charged DNA molecules; thus they are ejected toward the positive electrode. This phenomenon based on the electrokinetic ejection of biomolecules through a nanopipette tip has also been utilized for the direct writing of DNA molecules and proteins onto a glass substrate and the delivery of probe molecules to a desired position on the plasma membrane of a living cell.

As described in the previous section, our vibration-assisted insertion method for penetrating the cell membrane has the potential to reduce cell damage. However, DNA cannot be delivered into the cell via the EP-driven flow at an applied DC voltage of 350 mV. To enhance the rate and amount of DNA molecules delivered, the applied DC voltage was increased to 5 V after penetrating the cell membrane, as we described in our previous work. Nevertheless, the delivery of DNA into the cell was not successful. Figure 6(a) shows that there was a change in the ion current during the insertion and injection processes. The ion current increased as the micropipette was vibrated at 1 kHz with an amplitude of 200 nm (peak to peak), resulting in the penetration of the cell membrane, as described in Fig. 5(b). The ion current increased immediately after increasing the DC voltage further to 5 V. However, the ion current then decreased rapidly again, contrary to our expectations. This transient current is probably due to cell capacitance.

To overcome the voltage drop, a 5 Vpp sine wave AC voltage at 20 MHz with a DC offset voltage of 2.5 V was superimposed on the DC voltage (350 mV) that had been previously applied for ion current measurements. Note that the application of the DC offset voltage is required to maintain positive polarity on the electrode immersed in simulation results. The experimental results also indicated that it will be possible to construct a fully or semi-automatic system for minimally invasive intracellular delivery by further combining a method for the precise planar positioning of a glass micropipette tip within a resolution of 0.5 µm by using image-based visual servoing of the horizontal movement of the micropipette with a homemade LabView program (see Fig. S1 in the online supplementary data at http://stacks.iop.org/JJAP/56/017001/mmedia).
the microchamber to prevent the inward migration of DNA molecules into the micropipette.\(^{18}\) After the application of AC voltage, the resulting ion current increased over at least 100 nA (the ion current exceeded the possible measurement range) as expected [Fig. 6(b)]. As a result, fluorescently labeled DNA was successfully delivered into a living HeLa cell (see Fig. 7). The probability of DNA delivery was increased up to 50% (16\(^{\pm}\)32) for the AC voltage, compared with 0% for the application of DC voltage alone. This result indicates that the DC-biased AC electrophoretic flow of DNA molecules proposed here has the potential to enable the quantitative introduction of biomolecules with high precision. In addition to intracellular delivery, the application of reversed-polarity voltage should allow us, in principle, to extract an extremely small number of biomolecules or cellular components. Furthermore, the electrophoretic delivery method presented here offers the advantage of preventing the electrolysis of water even at potentials higher than 2.5 V because the voltage drop occurs at the last few microns of the micropipette tip.\(^{18,26}\) This means that the potentials of the pair of electrodes can be kept very low.

Below is an explanation of why the application of AC voltage is effective for DNA delivery. As shown in Fig. 8, we performed a numerical analysis of the electric field distribution around the micropipette tip inserted into the cell using a commercially available FEM software (COMSOL Multiphysics). In the FEM analysis, the cell was modeled as a hemispherical shape with a diameter of 20 µm and a height of 10 µm surrounded by a thin cell membrane (7 nm thickness). The glass micropipette tip, which had an outer diameter of 1.5 µm and an inner diameter of 0.5 µm, was inserted into the cell to a depth of 1 µm. At an applied voltage of DC 5 V, the voltage drop occurred very close to the tip [Fig. 8(a)]. However, there was dramatic improvement when a 5 V\(_{pp}\) sine wave AC voltage at 20 MHz with a DC offset voltage of 2.5 V was applied [Fig. 8(b)]. The magnitude of the electric field induced by the application of AC voltage was eight times that induced by DC voltage, and the range to which the electric field extended into the depths of the cell was roughly estimated to be more than twice that induced by the DC voltage [Fig. 8(c)]. The simulation results suggest that the very high resistance of the seal between the cell membrane and the outer surface of
the micropipette can be maintained even after penetration, resulting in a voltage drop inside the cell during the application of DC voltage. In other words, minimally invasive cell membrane perforation can be performed.

In addition, the ion current ratio before and after insertion was almost constant, as shown in Fig. 9(a). This means that the insertion process can be performed with high reproducibility. Figure 9(b) shows the total fluorescence intensities inside the cells as estimated using publicly available software (ImageJ); these intensities represent the total amounts of fluorescently labeled DNA molecules introduced into the cells. However, despite our expectations, the fluorescence intensity varied greatly. The reproducibility of electrokinetic DNA delivery into cells requires further improvement. We are currently working to improve the efficiency and reproducibility of our proposed intracellular delivery method, e.g., by studying the effects of various waveforms of the applied voltage (sinusoidal AC vs square-wave voltage). Our numerical analysis results indicate that the application of a square-wave voltage will lead to an improvement in the reproducibility of our proposed intracellular delivery method.

The glass micropipette provides another useful function for enabling scanning ion conductance microscopy (SICM), which is a scanning probe microscopy (SPM) technique. In the SICM, the imaging of biological cells can be achieved without damaging them by monitoring ion current changes without any mechanical interaction between the micropipette and the cell (see Fig. S2 in the online supplementary data at http://stacks.iop.org/JJAP/56/017001/mmedia). By combining delivery with the SICM topographic imaging technique, it is possible to not only perform intracellular delivery with high spatial resolution but also to image changes in cell morphology after the delivery of biomolecules.

3.3 Fabrication of stepped hollow SiO₂ nanoneedles in an array format

In order to adapt the proposed intracellular delivery technique for large-scale industrial application, the intrinsic problem of low throughput in microinjection with the use of a single glass micropipette must be overcome. Thus, we developed a fabrication process for stepped hollow SiO₂ nanoneedles with a well-defined tip as shown in Fig. 10. The stepped hollow needle tips have an outer diameter of 1.5μm, an inner diameter of 0.8μm, a length of 22μm, and a pitch of 80μm; they were fabricated by a combination of electron beam lithography and anisotropic deep reactive ion etching (DRIE) processes followed by thermal oxidation. Each nanoneedle tip was successfully connected to another hollow SiO₂ supporting microstructure with an outer diameter of 15μm and a total length of 78μm, which was fabricated on the backside of the Si wafer by conventional photolithography and DRIE processes. Moreover, a Si pyramidal microstructure (44μm wide and 23μm high) was also selectively fabricated at the center of the needle base to provide sufficient mechanical strength. The fabrication processes for the hollow SiO₂ microstructures and selective Si pyramidal microstructures are described in detail in our previous papers.

Figure 11(a) shows a plain-view fluorescent microscopy image showing the ejection of a very small volume of a solution containing fluorescently labeled DNA molecules (19 bp) through the fabricated stepped nanoneedle tips into a PBS solution filling a microchamber at an applied DC voltage of 1.5 V. Although the fabrication process parameters need to be further optimized to reduce the tip diameter to less than 1μm and to increase the success rate for making a connection between each needle tip and the supporting structure, a small
amount of DNA molecules can be ejected electrokinetically through some, but not all, of the fabricated stepped nanoneedles. Figure 11(b) shows the estimated relative flow rate as a function of the applied voltage. The ejection volume was calculated from the diameter of the ejected DNA solution under the assumption that it formed a sphere. The resulting flow rate increased linearly with the applied voltage. This result indicates that the electrophoretic delivery method has the potential to enable the quantitative delivery of biomolecules into living cells with much better controllability than the conventional method, in which the ejection process is performed by the application of external hydrostatic pressure.

To transform the developed nanoneedle array into an application for high-throughput intracellular delivery, however, several technological problems must be solved. For example, individual cells should be aligned with the pitch of the nanoneedle array. Therefore, we are now developing an array of microchambers with a concave pyramidal shape for cell arrangement; this structure would enable automatic placement of single cells exactly at the center of each chamber so that they will align with the center of the nanoneedle tip.

4. Conclusions

With the aim of developing an effective platform for the in vitro manipulation and analysis of biological cells at the single-cell level as well as the regulation of cellular functions, we propose a novel electrophoretic intracellular delivery method for biological macromolecules (such as DNA, RNA, and proteins) into living cells and combined it with a vibration-assisted insertion method for penetrating the cell membrane to reduce cell damage. Preliminary insertion tests and FEM simulations revealed that the application of mechanical oscillation allows minimally invasive cell membrane perforation, resulting in dramatic improvement in the probability of cell membrane perforation by up to 77% without causing significant cell damage. Moreover, fluorescently labeled DNA was successfully delivered into a living HeLa cell using DC-biased AC electrophoretic driven flow; this method can overcome the voltage drop due to cell capacitance after insertion, resulting in an improved probability of DNA delivery by up to 50%, as compared with 0% when only applied DC voltage is used. In addition, the change in ion current during the insertion process allows the detection of the instant when the micropipette tip penetrates the cell membrane as well as precise control of the gap between them. In other words, by using the change in ion current as the feedback signal, it will be possible to construct a fully or semi-automatic intracellular delivery system. In addition, we propose a fabrication process for stepped hollow SiO$_2$ nanoneedles in an array format, demonstrating that an extremely small amount of DNA molecules can be ejected electrokinetically through the fabricated stepped nanoneedle tips. This approach will enable the large-scale parallel introduction of desired biomolecules into living cells to overcome the limitation of intrinsic low throughput of conventional microinjection methods using a single glass micropipette.

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Fig. 11. (Color online) Experimental results for electrophoretically driven DNA ejection: (a) plain-view image showing fluorescently labeled DNA flowing through nanoneedle tips; (b) the relative flow rate as a function of the applied voltage.

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