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DNA Introduction into Living Cells by Water Droplet Impact with an Electrospray Process

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Electrospraying is performed by applying a high voltage to a capillary tip. A highly charged droplet at the capillary tip splits into micro- or nanoscale droplets, and a charged fine-liquid aerosol is accelerated by a high-voltage electric field. This method has been used for soft ionization in the mass spectrometric analysis of macromolecules and biomaterials.[1] Moreover, in desorption electrospray ionization (DESI) for mass spectrometry, the electrically charged droplets hit the surface molecules and are ionized under ambient conditions.[2] Electrospraying is also used for the production of nanofibers[3] and protein chips.[4]

Delivery of a foreign nucleic acid into a living cell is an important technique in molecular biology and the medical field. Previously developed transfection techniques, including methods that use a cationic polymer,[5] lipofection,[6] a gene gun,[7] and electroporation,[8] have been reported. Moreover, Pui et al. used electrospraying to increase the momentum of particles in the gene-gun method.[9] However, these techniques sometimes cause severe damage to cells, especially mammalian cells, or require complex and expensive devices.

We hypothesized that liquid droplet impact by electrospraying would transport a nucleic acid into a cell by making a transient channel through mild damage on the cell surface. Herein, we describe a technique for the introduction of a gene into eukaryotic (mammalian) and prokaryotic (bacterial) cells and avian embryonic tissue by spraying water droplets produced by an electrospray device without any cytotoxic reagents.

Figure 1 shows a diagram of the electrospray equipment for gene delivery. Purified water is sprayed onto the cells as a charged aerosol generated by static electricity repulsion at the tip of the tube. Only water or phosphate-buffered saline (PBS) is used in this method, and the device is a simple structure basically consisting of a tube connected to a high-voltage power supply that is a constant potentiostat with the current limited to 100 μA. A stainless-steel capillary (caliber 0.1 mm, outside diameter 0.3 mm, length 40 mm) was impressed with a high voltage, and water was supplied at a flow rate of 100–200 μL min^{-1}. In this experiment, the stainless-steel tube was impressed from 7 to 18 kV. This voltage (over 7 kV) is higher than that used for mass spectrometry (1–3 kV), as an organic solvent, which is usually used in mass analysis for reduction of surface tension, cannot be applied to gene transfection because of its cytotoxicity. The sprayed dishes were placed on an electrically grounded square-plate electrode, and the inside of the culture dish was also grounded by attaching a small piece of metal foil to the plate.

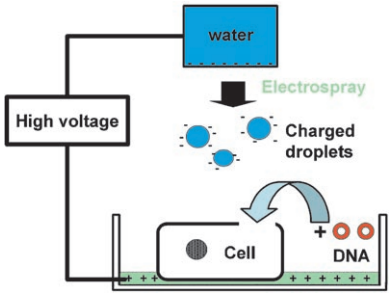

Figure 1. Diagram of the electrospray equipment for gene delivery. The plasmid DNAs located around the plasma membrane are introduced into cells by collision of water microdroplets.

Adhesive Chinese hamster ovary (CHO) cells and HeLa cells were tested as models, and almost the same results were obtained. CHO cells were cultured in minimum essential medium (α-MEM, Gibco, USA) supplemented with 10% fetal bovine serum. Cells were plated in a 35-mm culture dish (Falcon, USA) at 2.0×10^5 cells per dish and cultured at 37 °C under 5% CO_2 in air. Three days later, the cells were used for electrospraying. The culture medium was removed from the dish and an aqueous solution (100 μL) of plasmid vector pEGFP-N1 (100 μg mL^{-1} in water, Clonetech, USA), as green fluorescence protein (GFP) encoding DNA, was added to the dish. Water was electrosprayed onto the cells from a height of 2 cm at −10 kV, and culture medium was directly added to the dish. After 24 h of cultivation, GFP-positive cells were counted with a hemocytometer under a fluorescence microscope (Olympus, Japan).

We used nonmoving equipment in this experiment, which resulted in a limited spraying area on the dish. We evaluated the transfection rate of isolated GFP-positive cells to total cells, which included cells located in the external region of the sprayed zone, and obtained a transfection rate of 0.05 to 1.6%. The number of cells showing fluorescence increased with an increase in applied voltage, which indicates that the

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kinetic energy of the microdroplets is an important factor in this method (Figure 2a,b). The impressed voltage between the capillary and culture dish without water spraying caused almost the same current as that in electrospraying, which suggests that corona discharge occurs with only the application of voltage, but corona discharge alone without water spraying failed to introduce the genes into the cells.

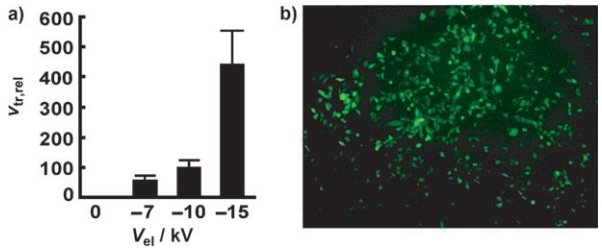

Figure 2. a) Relative value of GFP transfection rate ($\nu_{tr,rel}$) to electrospray voltage (V_{el}). The transfection rate increased with electrospraying in a voltage-dependent manner. Many GFP-expressing cells were observed at a high voltage (−15 kV). b) GFP expression in CHO cells induced by electrospraying. Many GFP-transfected cells were observed after 24 h of incubation.

We studied the parameters that affect transfection efficiency and found at first that an increased volume of added solution significantly decreased transfection efficiency (Figure 3a). Moreover, the transfected cells were found in the region in which the sprayed aerosol particles had collided, and the proportion of GFP-positive cells increased with increase in cell density (Figure 3b). These results clearly indicate that direct contact between the microdroplets and cell is important for gene introduction by electrospraying.

Another important factor affecting transfection efficiency is swelling of the cell caused by intracellular pressure generated by the hypotonic environment. The use of water instead of PBS for preincubation before electrospraying led to a significant increase in the number of GFP-positive cells. Moreover, although 15 min incubation with water resulted in death of all of the cells, 10 min incubation resulted in the highest transfection efficiency, which suggests that the high osmotic pressure induced by the swollen state of the cell is important for transfection (Figure 3c,d).

The charged microdroplets generated by electrospraying collide with the highly tensioned plasma membrane and may penetrate the membrane by momentum transfer from the high-speed droplet, by microfractures caused by blowing surface molecules on the plasma membrane mediated by a process known as the DESI mechanism, or by electroporation. After making a small hole in the cell membrane, DNA may enter the cell through this hole by a diffusion mechanism. The efficiency of transfection was also affected by the timing of the addition of DNA and electrospraying: addition of DNA prior to electrospraying resulted in high transfection efficiency, whereas addition of DNA soon after electrospraying resulted in a significant decrease in efficiency (see Figure 3e), which suggests that the hole persists for only one minute.

Furthermore, using a chicken embryo, we also clearly demonstrated that this method can be applied to a developmental specimen. White Leghorn fertile eggs were incubated at 38 °C. After 1.5 days, an embryo was cultured by the modified New's method.[10] The embryo was placed in an agarose dish, and GFP plasmid (4.3 μg mL^{-1}, 1 μL) with 2% Fast Green (Wako, Japan) was put on the target region of the embryo. The PBS was electrosprayed onto this target region from a height of 10 cm for 20 s at 10 kV with a flow rate of 120 μL min^{-1}. After electrospraying, the PBS was removed from the agarose dish and the embryo was incubated overnight. The results of gene transduction are shown in Figure 4a.

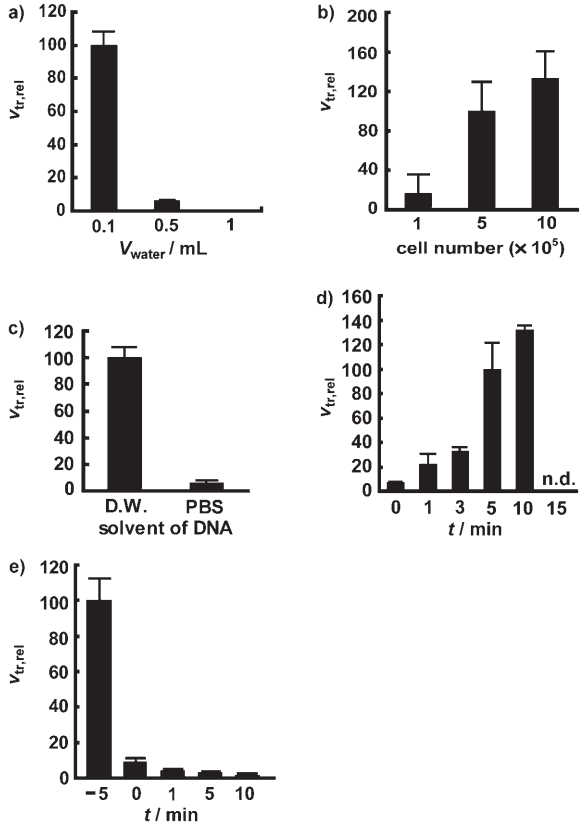

Figure 3. a) Effect of solution volume covering CHO cells on transfection rate $\nu_{tr,rel}$. b) Effect of number of culture cells on transfection efficiency. c,d) Swollen state of CHO cells and transfection efficiency. e) Relationship between transfection rate and timing of DNA addition. D.W. = deionized water; n.d. = not detected.

This method was also applicable to bacteria. Noncompetent *Escherichia coli* (K12 strain) solution was plated on a 1.5% agar/Luria–Bertani (LB) dish and incubated at 25 °C for 2 days. Then the plasmid pUC19 (10 μg) in Tris/ethylenediaminetetraacetic acid (TE) buffer (100 μL) was spread on the *E. coli* lawn, and water (100 μL) was electrosprayed at −7 kV onto the colonies at a flow rate of 100 μL min^{-1}. The colonies were collected in a 2-mL tube and washed with LB medium. The *E. coli* was seeded on a 1.5% agarose dish with ampicillin (50 μg mL^{-1}), and many ampicillin-resistant *E. coli* colonies were found on the agarose dish (Figure 4b). The gene introduction efficiency was 10^4–10^5 cells per μg.

In conclusion, we have clearly shown that the water or PBS droplet impact technique by electrospraying can be

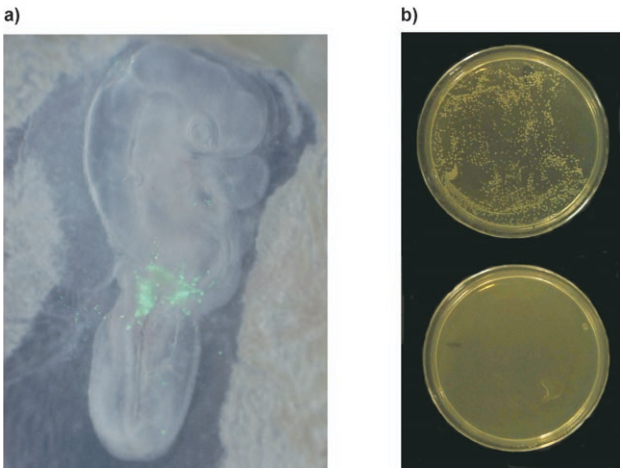

Figure 4. a) Region-specific GFP expression in a chicken embryo, which was localized in the E2.5 embryo. b) Electrospray transformation in *E. coli*. Upper plate: −10 kV impress; many ampicillin-resistant colonies were found. Lower plate: 0 V impress.

applied to gene transfection to eukaryotic cells, embryonic tissue, and prokaryotic cells. This method has the following advantages:

1. The physical-force-dominant method can be applied to many kinds of cells and tissues.
2. The equipment is simple and portable, and can be downsized.
3. Cytotoxic reagents are not necessary, and this may lead to a high survival rate of cells. With refinement of the configuration of the spray tubes or scanning of the whole surface of the dish, expansion of the exposed area or homogeneous electrospraying of the aerosol is expected. Moreover, it might be possible to focus the aerosol flow by a field lens to introduce a gene into a very small area.

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