Electroporation Introduction of Diclofenac Sodium into Human Erythrocytes and Its Determination

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Abstract
A method of electroporation for introducing diclofenac sodium (a drug) into human erythrocytes was developed. The conditions (the pulse strength, pulse duration and pulse number) for the introduction of diclofenac sodium into erythrocytes by electroporation were investigated. The diclofenac sodium in single human erythrocytes introduced by the electroporation was analyzed qualitatively and quantitatively by capillary zone electrophoresis (CZE) with end-column ampermetric detection at a carbon microdisk array electrode.

Keywords: Electroporation, Diclofenac sodium, Human erythrocytes, Capillary zone electrophoresis

1. Introduction

Experiments done on cells have the potential to replace most animal testing, provided that the data acquired from cells can be made to reflect the response of the whole organism. This requires developing new methods to introduce chemicals. In recent years, electroporation of cell membrane has become one of the methods. Its general principles and mechanisms of the delivery of drugs, genes, and DNA to cell interior have been reviewed [1–4]. Many theories have also been proposed to explain the complex process [5–7]. Since the first report on gene transfer by electroporation [8], it has been widely used as a universal method of genetic transformation [9, 10]. However, this technique also has a great potential in biochemical and pharmacological studies [11, 12]. The most interesting biotechnological and biomedical application include improving transdermal drug delivery [13, 14] or electrochemotherapy – in situ delivery of an electric pulse after intravenous injection of a chemotherapeutic drug, which was found to enhance significantly the efficiency of tumor treatment in human subjects [15, 16]. Electric pulses with moderate electric field intensity can lead to rapid genetic transformation and manipulation in a wide variety of cell types including bacteria [17, 18], yeasts [19, 20], animal and human cells [21, 22], and so forth.

In this work, we developed a method of introducing a drug (diclofenac sodium, [*o*-(2,6-dichloroanilino)phenyl]acetate) with pronounced antirheumatic, antiinflammatory, analgesic and antipyretic properties [23] into human erythrocytes. The laboratory-made electric pulse producing equipment and the electroporation cell are illustrated. Diclofenac sodium in individual erythrocytes introduced by electroporation is detected by capillary zone electrophoresis (CZE) with end-column electrochemical detection at a carbon fiber microdisk array electrode.

2. Experimental

2.1. Apparatus

2.1.1. Electroporation Equipment

Figure 1 shows the laboratory-made RC electric-pulse generator. This generator included a charge part and a discharge part. The charge part comprised a direct current (DC) power supply of 108 V, a potentiometer of 470 kΩ, a resistance of 1 MΩ and an electrolyte capacitor of 10 μF (which also was the power supply of the discharge part). The discharge part contained the electrolyte capacitor and electroporation cell. The electroporation cell shown in Figure 2 included two parallel platinum wire electrodes (300 μm diameter, 20 mm length) with a distance of ca. 100 μm. The two platinum wires were welded with two copper wires, respectively. The Pt electrodes were immobilized by a Plexiglass circle (10 mm i.d., 3 mm thickness) adhered to the bottom of a slide.

In Figure 1, K_1 was the DC power switch and K_2 was the charge/discharge switch. When K_1 was turned on and K_2 was switched to the charge mode (place 1 in Fig. 1), the DC power charged the electrolyte capacitor to a constant voltage through a potentiometer and a resistance. Therefore, the voltage of the electrolyte capacitor charged could be adjusted by using the potentiometer. When K_2 was switched to the discharge mode (place 2 in Fig. 1), the capacitor released its charge through the electroporation cell.

2.2.2. Capillary Zone Electrophoresis

Details of the CZE separation system used in this work was similar to our description previously [24]. Briefly, the apparatus consisted of a fused silica capillary with dimensions of 30 μm i.d., and 375 μm o.d. (J&W Scientific Incorporated Company, CA, USA), which was cut to a length of about 70 cm and placed between two buffer reservoirs. A high voltage was applied at the injection end, while the reservoir containing the electrochemical detection cell was held at ground potential. Separation was carried out at an applied voltage of 20 kV generated by a high-voltage power supply (Model 9323-HVPS, Beijing Institute of New Technique, Beijing, China). The electrochemical detection at a constant potential was performed using the end-column amperometric approach with an electrochemical analyzer (Model CHI800, CH Instruments, Austin, TX, USA). The detection cell and the detector were housed in a Faraday cage in order to minimize the interference from external sources of noise. Electrochemical detection was carried out with a three-electrode system. It consisted of a carbon fiber microdisk array electrode as the working electrode, a coiled Pt wire as the auxiliary electrode

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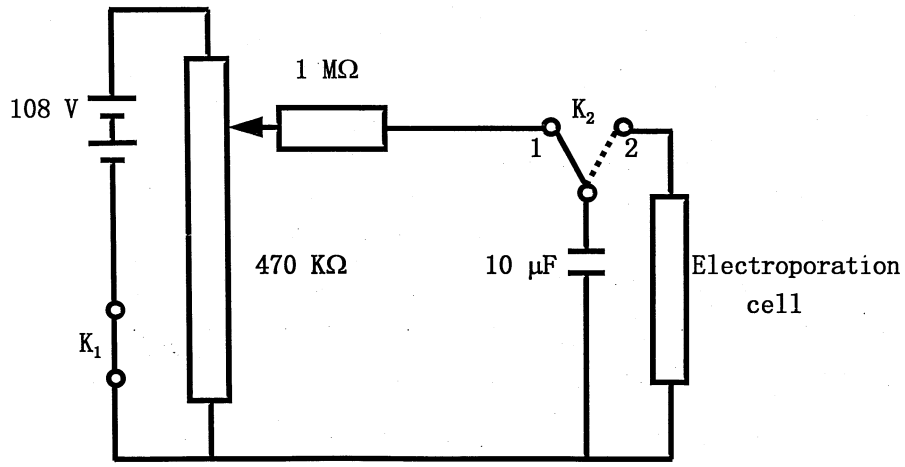

Fig. 1. Circuit diagram of the RC electric pulse generator.

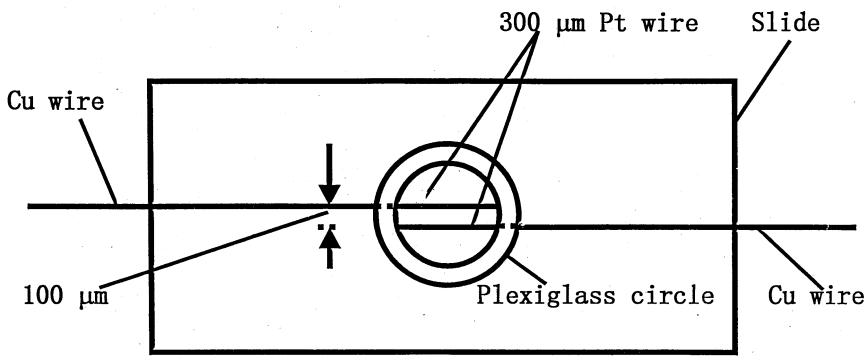

Fig. 2. Sketch map of the electroporation cell.

(which also served as the ground for the potential drop across the capillary) and a saturated calomel electrode (SCE) as the reference electrode. The carbon fiber microdisk electrode cemented onto a microscope slide has to be aligned with the detection end of the capillary under a microscope. The arrangement of the electrochemical detection cell was previously illustrated in detail [24]. The carbon fiber microdisk array electrode was described previously [25].

2.2. Reagents and Solutions

A 1.0×10^{-2} mol/L stock solution of diclofenac sodium or a 0.5 % (w/v) trypan blue stock solution was prepared by dissolving an appropriate amount of diclofenac sodium (pharmaceutical grade, Yanzhou pharmaceutical factory, Yanzhou, China) or trypan blue (Sigma, St. Louis, MO, USA) in water. Dilute solutions were obtained by serial dilution of the stock solution with PBS. The PBS consisted of 0.135 mol/L NaCl and 0.02 mol/L NaH_2PO_4–NaOH (pH 7.4). All reagents were of analytical grade except for diclofenac sodium. All solutions were prepared with double distilled water.

2.3. Preparation and Electroporation of Cells

Human red blood from a normal adult was collected in a 1.5 mL microcentrifuge tube and centrifuged at 1000 rpm for 5 min to separate erythrocytes. The supernatant liquid was removed. In order to wash the erythrocytes, a six-fold physiological buffer saline (PBS) was added into the microcentrifuge tube. After vibrating lightly, the mixture was centrifuged again, and then the supernatant liquid was removed again. This step was repeated over five times until the supernatant was clear and transparent. After the supernatant liquid was removed, the erythrocytes were obtained.

About 5 μL erythrocytes and an appropriate amount of diclofenac sodium were mixed. After the solution was diluted to 0.5 mL with PBS, the erythrocyte suspension was obtained. Then the erythrocyte suspension of 0.5 μL and the 0.5 % (w/v) trypan blue solution of 0.5 μL were dropped into the place between the two platinum electrodes of the electroporation cell, which had been put on the platform of an inverted biological microscope. After both solutions were mixed adequately, the colorless erythrocytes suspending in the blue solution can be seen clearly under the microscope. After that, the power supply switch K_1 of the electroporation generator was turned on and its charge/discharge switch K_2 was switched to the charge mode (place 1). After five seconds, K_2 was switched to the discharge mode (place 2). Such, one electric pulse was added to the erythrocytes. This step was repeated until the cells were electroporated. The erythrocytes electroporated were blue and they could be monitored under the inverted microscope. The erythrocyte suspension was taken out from the electroporation cell and put into a 0.5 mL microtube. The volume of the erythrocyte suspension obtained was less than 1 μL. In order to obtain enough volume (about

20 μL at least) for about ten single cell injection, the process mentioned above was repeated more than 20 times. The drug in the erythrocyte suspension should be washed down before an erythrocyte was injected into the separation capillary. For this, the erythrocyte suspension collected in 0.5 mL microtube was diluted about 25 times by PBS. After being mixed fully, let the suspension stands for more than 2 hours. When the cells subsided to the bottom of the tube, the supernatant liquid was removed. This process was repeated more than 4 times.

Before each run, the capillary was flushed with 0.1 mol/L NaOH for 5 min, and then with water and the corresponding separation buffer for 5 min, respectively, by means of a syringe. After the electroosmotic current reached a constant value (after about 10–20 min), the electromigration injection of the whole cell was carried out. The washed erythrocyte suspension of 5 μL was then placed on a clean microscope slide of the inverted biological microscope, the injection end of the capillary filled with electrophoresis buffer was gently immersed in the droplet under the guidance of a three dimensional micromanipulator. In order to see the opening of the injection end, a ca. 5 mm section of the polyimide coating at the injection end of the capillary was removed by burning before use. A platinum wire was placed in the cell suspension to serve as the electrophoresis anode. As soon as the erythrocyte was drifting towards the injection end, an injection voltage of 3.0 kV was applied to transport the whole cell into the capillary tip.

2.4. Single Cell Analysis

After the whole cell was injected into the capillary, the capillary was manipulated up, out of the erythrocyte suspension and then immersed in the CZE running buffer as the cell lysate solution. Once the individual erythrocyte was lysed, the separation voltage of 20 kV was applied across the separation capillary, the detection potential was applied at the working electrode and the electropherogram was recorded.

3. Results and Discussion

3.1. Optimum Electroporation Conditions for Erythrocytes in PBS and Trypan Blue Medium

The cell membrane can be considered as a capacitor with two extremely thin plates. Excessive voltages can lead to the breakdown of its dielectric by overcoming its permittivity. If a proper pulse-shaped electric field is applied across the cell membrane, the permittivity of the membrane will be exceeded and its conductivity dramatically increased. At the same time, the structure of the phospholipid bilayer is disrupted locally and the pores on the cell membrane are formed, which enhance permeability of the membrane for macromolecules [1, 4]. When the electric field is removed, the cell membrane is resealed automatically and the pores disappear. In this case, the cells are still alive. This process is called reversible electroporation. When the applied external electric field exceeds 4–6 times of a critical value or when the duration of the electric pulse is longer than 20–100 μs, the cell membrane can not be resealed, in other word, the cells are smashed to piece. This situation is called irreversible electroporation. It is clear that the reversible electroporation should be expected, i.e., the cells should be still alive after being electroprated. Therefore, the optimum reversible electroporation conditions of erythrocytes should be researched. The optimum electroporation conditions include electric pulse strength, pulse duration and pulse number.

It was found that only a part of erythrocytes can be reversibly electroporated in experiments. In order to evaluate degree of the reversible electroporation, the electroporation efficiency, η_{effi} is defined as follows:

$$\eta_{\text{effi}} = N_{\text{re}}/N_{\text{all}}$$

where N_{re} is the number of reversible electroporation and N_{all} is the total number of erythrocyte in the experiments of electroporation. In other word, the electroporation efficiency is the percentage of cell occurring reversible electroporation among all the cells.

Trypan blue is a dyestuff to distinguish cells electroporated reversibly. If trypan blue goes into the cells, these cells with blue color can be observed under the microscope. It was founded that erythrocytes could not be dyed by trypan blue, when the cells were incubated in trypan blue solution for 24 hours. This means that trypan blue can not be introduced into erythrocytes by pinocytosis or diffusion. However, erythrocytes can be stained by trypan blue, if cells are electroporated reversibly. In this case, "pores" are generated on the cell membrane. Trypan blue can penetrate into the cells through these reversible "pores" and dye the cells. The pre- and post-electroporated erythrocytes are shown in Figure 3. Therefore, one can count the number of blue cells and the number of colorless cells to calculate η_{effi}.

For the pulse duration of 40 μs and the pulse number of 4, when the pulse strength is lower than 8 kV/cm, η_{effi} increases with increasing pulse strength. However, when the pulse strength is higher than 8 kV/cm, η_{effi} decreases with increasing pulse strength. The maximum η_{effi} is at 8 kV/cm for the pulse strength. Under the pulse strength of 8 kV/cm and the pulse number of 4, when the pulse duration was less than 40 μs, η_{effi} increases with increasing the pulse duration. However, η_{effi} decreases, when the pulse duration was more than 40 μs. 40 μs was the optimal pulse duration. Pulse numbers can also affect the electroporation efficiency of the erythrocyte. For the pulse strength of 8 kV/cm and the pulse duration of 40 μs, when the cells are applied one electric pulse, about 70 % cells, not whole, are electroporated. η_{effi} increases with the pulse number. About 90 % cells can generate reversible pores, when 3–4 electric pulses are applied.

3.2. The Optimum Electroporation Conditions for Erythrocytes in PBS, Trypan Blue and Diclofenac Sodium Medium

If the optimum electroporation conditions for the solution containing PBS and trypan blue without diclofenac sodium mentioned above (8 kV/cm for pulse strength, 40 μs for pulse duration, 4 for pulse number) is applied to the solution with 1.6×10^{-3} mol/L diclofenac sodium, nearly 100 % cells are electroporated irreversibly. This may be due to the colloidal osmotic pressure of the cytoplasm micromolecules [26]. It was found that erythrocytes can be lysed when they were incubated in 2.5×10^{-3} mol/L diclofenac sodium solution for 30 min. Diclofenac sodium may affect the erythrocyte membrane structure and lead the cells to lyse easily. Table 1 lists the electroporation efficiency of erythrocytes obtained at different pulse strengths and pulse numbers. It can be noted that all the cells cause irreversible electroporation at the pulse strength of 7.0 kV/cm or 8.0 kV/cm for the pulse duration of 40 μs. In other words, the electric pulse conditions have exceeded the critical values for

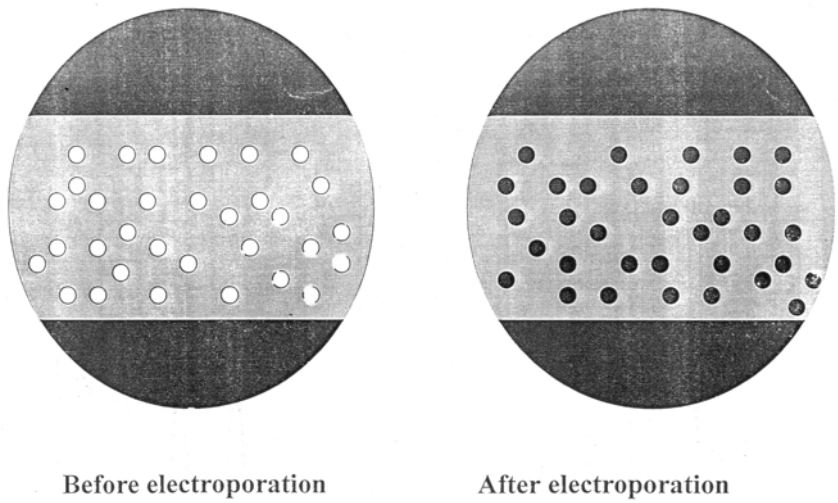

Fig. 3. Sketch map of the single cell electroporation under the microscope visual field.

Table 1. Electroporation efficiency at different pulse number and pulse strength (pulse duration: 40 μs).

Pulse number	*Pulse strength [kV/cm]*	*Electroporation efficiency [%]*
1	4.0	10
	4.5	20
	5.0	80
	5.5	60
	6.0	50
	6.5	30
	7.0	0
	8.0	0
2	4.0	20
	4.5	30
	5.0	90
	5.5	70
	6.0	0
	6.5	0
	7.0	0
	8.0	0
3	4.0	30
	4.5	30
	5.0	90
	5.5	50
	6.0	0
	6.5	0
	7.0	0
	8.0	0

reversible electroporation. It can be included that the critical values of the reversible electroporation conditions of erythrocytes in this medium with diclofenac sodium are different from that in the medium without diclofenac sodium. The optimum electroporation conditions of erythrocytes should be investigated again for the medium of introducing diclofenac sodium into cells.

When the pulse strength decreases from 8.0 kV/cm to 4 kV/cm, η_{effi} increases. If one electric pulse with the pulse strength of 6.0 or 6.5 kV/cm is applied to the cells, η_{effi} can retain 50 % or 30 %. If two and more electric pulses are applied, all cells were irreversibly electroporated. When one or two electric pulse with 5.5 kV/cm is applied to the erythrocytes, η_{effi} is 60 %–70 %. However, if more than two electric pulses are applied, more than 50 % of the cells rupture after several minutes. The pulse strength of 4.0 or 4.5 kV/cm can lead to only 20–30 % of the cells to be reversibly electroporated, even if several pulses are applied. Only at the pulse strength of 5 kV/cm, nearly 90 % of the erythrocytes were obtained and no rupture generated after 2–4 pulse were applied. Therefore, the optimum electroporation conditions in PBS, trypan blue and diclofenac sodium medium was 5 kV/cm for the pulse strength, 3 for the pulse number, and 40 μs for the pulse duration.

3.3. Determination of Diclofenac Sodium in Single Erythrocytes Introduced by Electroporation

The voltammetric characteristics of diclofenac sodium on glassy carbon electrode have been investigated by several researchers [27, 28]. We found that diclofenac sodium can also be oxidized at the carbon fiber array electrode in Na_2HPO_4–NaH_2PO_4 buffer of pH 7. An oxidation peak of diclofenac sodium at ca. 0.70 V (vs. SCE) was observed and no reduction peak appears on its cyclic voltammogram. This behavior has been used in determination of diclofenac sodium by CZE/end-column amperometric detection in our laboratory [29]. The separation was carried out with a separation voltage of 10 kV in 4.90×10^{-3} mol/L $Na_2HPO_4 - 3.10 \times 10^{-3}$ mol/L NaH_2PO_4 and the single carbon fiber microelectrodes of 8 μm diameter were used in the detection. The detection limit was 3.8×10^{-6} mol/L (or 7.8 fmol) for $S/N = 3$ and the linear range was 9.90×10^{-6} mol/L to 5.00×10^{-4} mo1/L. If the buffer is used in our experiments, the electrophoretic peaks of diclofenac sodium with tailing will widen (Fig. 4, curve 1). When 1.25×10^{-2} mol/L borax $- 3.13 \times 10^{-3}$ mol/L NaOH was used, the peaks of diclofenac sodium were improved much more (Fig. 4, curve 2). In addition, the carbon fiber microdisk array electrodes were used instead of the single carbon fiber microelectrodes in our experiments because of lower detection limit. In this case, the detection limit was as low as 1×10^{-6} mol/L (or 2.1 fmol) for $S/N = 3$. The linear relationship holds between the peak current detected and the concentration in the range of 1.00×10^{-6} to 1.00×10^{-3} mol/L.

Before diclofenac sodium was introduced into erythrocytes by electroporation, the single erythrocytes suspended in PBS were

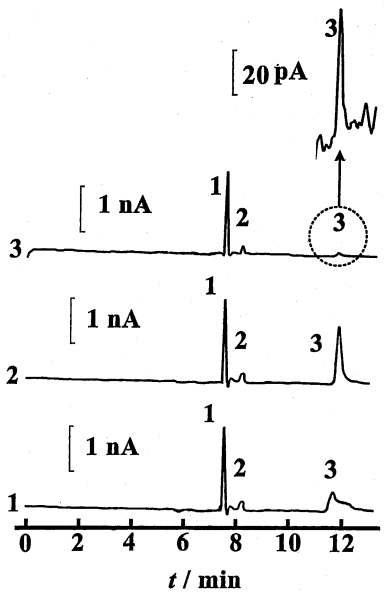

Fig. 4. Electropherograms of PBS, diclofenac sodium in PBS, and individual erythrocyte in PBS. 1) and 2) 1.0×10^{-4} mol/L diclofenac sodium; 3) individual erythrocyte with diclofenac sodium introduced by electroporation. Buffer: 1) 4.90×10^{-3} mol/L Na_2HPO_4—3.10×10^{-3} mol/L NaH_2PO_4; 2) and 3) 1.25×10^{-2} mol/L $Na_2B_4O_7$—3.13×10^{-3} mol/L NaOH. Capillary, 70 cm length, 30 μm i.d.; injection, 5 kV for 10 s; separation voltage, 20 kV; detection potential, 1.0 V.

detected at the carbon-fiber electrode with a constant potential of 1.0 V (vs. SCE). Only two peaks (peak 1 and peak 2 in Fig. 4, curve 2) appear in the electropherogram, which is the same as the electropherogram of PBS. This result indicates that the substances in the cells can not be detected under these conditions. Figure 4, curve 2 shows the electropherogram of 1.0×10^{-4} mol/L diclofenac sodium in the PBS. Peak 1 and 2 are the electrophoretic peaks of PBS, and peak 3 is the electrophoretic peak of diclofenac sodium. Figure 4, curve 3 shows the electropherogram of one individual erythrocyte after diclofenac sodium introduced in by electroporation. A very small peak (peak 3) appears. In order to distinguish the electrophoretic peaks clearly, Peak 3 in Figure 4, curve 3 was magnified 50 times. It can be found that the electromigration time of the peak 3 shown in Figure 4 is the same as that of the peak of the standard solution of diclofenac sodium in PBS. Therefore, it can be concluded that this peak is just the peak of diclofenac sodium introduced into the erythrocytes by electroporation. The concentration of diclofenac sodium introduced into the erythrocyte by electroporation was analyzed quantitatively by the calibration plot of the standard diclofenac sodium. The mean concentration of diclofenac sodium introduced into erythrocytes was 4.21 μmol/L for ten erythrocytes. The relative standard deviation of these data was 10 %.

4. Conclusions

We have found that the electric pulse can cause the reversible breakdown of the erythrocyte membranes and much enhanced permeability for molecules such as trypan blue and diclofenac sodium. The reversible electroporation conditions in PBS and trypan blue solution containing diclofenac sodium are slightly different from the solution without diclofenac sodium. In PBS, trypan blue and 1.6×10^{-3} mol/L diclofenac sodium medium, about 90 % erythrocytes will be electroporated reversibly at 5 kV/cm for the pulse strength, 40 μs for the pulse duration and 3 for the pulse number and then diclofenac sodium is introduced into erythrocytes. The diclofenac sodium introduced into cells could be determinated qualitatively and quantitatively by CZE with end-column ampermetric detection at a carbon microdisk array electrode.

5. Acknowledgements

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6. References

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