

## Determination of cell electroporation from the release of intracellular potassium ions

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### Abstract

When cells are exposed to a strong enough external electric field, transient aqueous pores are formed in the membrane. The fraction of electroporated cells can be determined by measuring the release of intracellular potassium ions. The current work is the first study where such a method was employed successfully not only with cells suspended in the medium with a rather high concentration of potassium (4–5 mM) but also with cells that release some part of intracellular potassium responding, in this way, to the stress caused by manipulation procedures during the preparation of the cell suspension. Experiments were carried out on mouse hepatoma MH-A22 cells exposed to a square-wave electric pulse. The curves showing the dependence of the fraction of the cells that have become permeable to bleomycin, a membrane-impermeable cytotoxic drug, are close to the ones showing the release of intracellular potassium ions.

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When a strong enough external electric field is applied to the cell suspension, it causes the appearance of the transmembrane potential [1] that eventually leads to the increase of the membrane permeability (electroporation) [2]. This increase of the permeability of the cell membrane is consistent with the formation of transient aqueous pores, and the phenomenon is called electroporation [3].

For comparison of the theoretical models describing pore formation kinetics with the experimental data [4] or optimization of practical applications, such as loading of cells with various substances [5] or cell transformation [6], it is important to know whether and how many cells have become electroporated under the influence of certain electric field treatment (e.g., shape, frequency and number of pulses, amplitude, duration). If electrical treatment is not too strong, electroporation can be completely reversible [2,7] and electroporated cells can remain viable [8,9]. In such cases, cell viability tests (e.g., by cells clonogenic

or the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]<sup>1</sup> assays [10,11]) do not reveal all electroporated cells, and therefore other tests need to be used for the evaluation of the threshold of cell membrane electroporation [12].

The treatment of cells by electric pulses increases the membrane permeability to various substances to different extents [13–15]. It is known that the plasma membrane can become leaky for small ions (e.g., K<sup>+</sup>, Na<sup>+</sup>, Rb<sup>+</sup>) but still remain impermeable to larger molecules such as sucrose, the essential proteins, and/or enzymes within the cell [14–16]. Because of this, using the test molecules that are larger than the smallest ions (e.g., K<sup>+</sup>, Na<sup>+</sup>, Rb<sup>+</sup>), sucrose (MW = 342 Da) [2,15], calcein (MW = 623 Da) [17], propidium iodide (MW = 660 Da) [14,18], direct blue (MW = 992.8 Da) [19], bleomycin (MW = 1410–1430 Da) [10,12], and the like may fail to detect cell membrane electroporation [14,15,20].

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<sup>1</sup> Abbreviations used: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EDTA, ethylenediaminetetraacetic acid.

Because of this, electroporation must be considered with respect to the membrane permeability to the smallest substances, that is, ions such as  $K^+$ ,  $Na^+$ ,  $Rb^+$ ,  $Li^+$ , and  $Cl^-$  [21]. In other words, the cell can be regarded as electroporated when, after the electric treatment, the membrane permeability to these ions has increased noticeably.

However, not all of these ions are equally appropriate for this purpose. Although the flux of sodium ions can be used as an objective verification of the membrane permeability changes [22–24], it is generally easier to measure the release of the ions out of the cell than to measure their uptake due to more complicated determination of the intracellular concentrations. Potassium ions are particularly suitable for monitoring the changes of the membrane permeability because they are the abundant cations in the cells and their intracellular concentration usually is high enough (in the range of 100–170 mM in erythrocytes [25], 250–300 mM in yeasts [26], and up to 400 mM in *Streptococcus faecalis* [27]). In addition, several different techniques are available for determination of the concentration of potassium ions in cell suspensions, including emission flame photometry [23], fluorescent indicators [28], radioisotopic tracers [29], and potentiometric measurements with ion-selective electrodes [30].

The determination of cell membrane electroporation by measuring the loss of intracellular potassium has been used successfully on human erythrocytes [13,16,20,22,23,31] and mouse spleen lymphocytes [32]. However, to the best of our knowledge, no studies attempting to use this approach in electroporation experiments on other types of cells have been reported. This can be attributed to the following main problems that might arise when trying to employ this method.

Due to technical (e.g., small chambers), financial (e.g., expensive chemicals), or other reasons, the experiments are performed with small-volume samples (40–100  $\mu$ l) [23,33–35] that might be much smaller than the ones required for accurate analysis (in the range of a few milliliters) by some of the methods such as emission flame photometry [23].

The majority of cell culture media already contain 4–5 mM potassium [11], and some cells might not tolerate the potassium-free medium. Because of this, electroporation often is carried out in high-potassium media [8,10,14,36–38].

When subjected to osmotic, oxidative, metabolic, or other stresses, cells sometimes respond by opening plasma membrane channels and/or activating transporters, causing the efflux of some cytosolic potassium out of the cells [39–42]. Meanwhile, during the preparation of the cell suspension, the cells are exposed to a variety of stresses (e.g., trypsinization, centrifugation, changes of the medium composition).

The first problem can be overcome by choosing an appropriate method, for example, by using a mini potassium-selective electrode that allows determination of the potassium concentration in samples as small as 40–50  $\mu$ l

[20]. The current work is the first study where the determination of cell electroporation by measuring the release of intracellular potassium ions was employed successfully not only with cells that were exposed to electric pulses in the medium with a rather high concentration of potassium (4–5 mM) but also with cells that did not tolerate potassium-free medium and release some part of intracellular potassium responding, in this way, to the stress caused by manipulation procedures during the preparation of the cell suspension.

## Materials and methods

### *Electroporation, growth, and other media*

The culture medium consisted of Dulbecco's modified Eagle's medium (cat. no. D5546, Sigma–Aldrich Chemie, Steinheim, Germany) supplemented with 10% fetal bovine serum (cat. no. F7524, Sigma–Aldrich Chemie), 1% L-glutamine (cat. no. G7513, Sigma–Aldrich Chemie), 100 U/ml penicillin, and 125 mg/ml streptomycin (cat. no. P0781, Sigma–Aldrich Chemie). As an electroporation medium, either the culture medium or minimum essential medium Eagle (cat. no. M8167, Sigma–Aldrich Chemie) was used.

Bleomycin hydrochloride (Bleocin, Nippon Kayaku, Tokyo, Japan) was obtained as a crystalline powder and dissolved in sterile 0.9% NaCl solution (Balkanpharma–Troyan, Troyan, Bulgaria) at a concentration of 1 mM. Further dilutions were also made in sterile 0.9% NaCl solution.

Calibration solutions containing 0.2–100 mM KCl were prepared by diluting a stock solution of 100 mM KCl and adding 150 mM sodium chloride and 8 mM sodium benzoate [43]. The NaCl was added to keep sodium ion concentration close to that in electroporation medium, and the sodium benzoate is a mold inhibitor.

### *Cell culture*

Experiments were performed with the cells of mouse hepatoma cell line MH-A22. Cell culture was obtained as a kind gift from V. Kirvelienė (Vilnius University, Lithuania). The cells were grown in monolayer culture in 75-cm<sup>2</sup> (200-ml) flasks (Greiner Bio-One, Frickenhausen, Germany) at 37 °C and 5% CO<sub>2</sub> in water-jacketed incubator IR AutoFlow NU-2500E (NuAire, Plymouth, MN, USA).

When cells reached confluence, they were trypsinized for 2 to 10 min. with 2 ml of 0.25% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) solution (cat. no. T4049, Sigma–Aldrich Chemie). When cells detached from the flask bottom, cell suspension was supplemented with 2 ml culture medium to protect cells from further action of trypsin. After centrifugation of the suspension for 5 min at 1000 rpm at room temperature, cells were resuspended in the culture medium at approximately  $2\text{--}5 \times 10^7$  cells/ml and kept for 60–70 min at room temperature (20–21 °C).

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