Anal. Bioanal. Electrochem., Vol. 11, No. 5, 2019, 598-609

Analytical & Bioanalytical Electrochemistry

2019 by CEE
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Full Paper

Conductometry in Pulsed Electric Field with Rising Strength: Bioelectrochemical Applications

Victor A. Shigimaga

Kharkiv Petro Vasylenko National Technical University of Agriculture ave. Moscowsky, 45, Kharkiv, 61050, Ukraine

*Corresponding Author, Tel.: +38-0505531257

E-Mail: vash105@gmail.com

Received: 9 April 2019 / Accepted with minor revision: 11 May 2019 / Published online: 31 May 2019

Abstract- It is known, that conductivity of liquid media as well as biological objects directly related to the mobility of ions, which in turn depends on electric field strength. This article describes general principles and applications of method and hand-made conductometry device for measurement conductivity of a single biological cells and liquid media in pulsed electric field with rising strength. The device allows to determine the conductivity in the range 0.1-10^5 μS/cm (with an error about 3%) in the field strengths 0-10 kV/cm, pulse duration 50 μs, repetition period 5-10 s. Conductometric measurements were carried out on mouse and cow oocytes in 0.3 M solution of sucrose and some 0.3M aqueous solutions: xylitol, sorbitol, mannitol, glucose, sucrose, conventional distillate and deionized apyrogenic water. It was found that with rising in the field strength, the conductivity of cells first increases gradually and almost linearly in the range 0.2-1.3 kV/cm, and then sharper and finally exponentially, with strength more 2.8 kV/cm and 3.3 kV/cm for mouse and cow oocytes respectively, i.e., electric breakdown of the cell membrane occurs. The conductivity of liquid media is almost independent of the field strength, but small variations in some media have shown the presence of conductive impurities in them, which are absent in the solvent. Thus, the cell conductivity changes in rising field strength can detect and investigate all stages of membrane electroporation (reversible and irreversible electric breakdown) and the method can serve for estimating the purity of the initial reagents as well as quality control of other liquid media.

Keywords– Conductometry, Conductivity, Pulsed electric field, Rising strength; Liquid medium.

1. INTRODUCTION

Conductometry is an electrochemical analysis method based on the electrical conductivity measuring of liquid media [1]. Recently, the field of conductometric analysis application has significantly expanded due to the transfer of researchers' attention to organic, in particular, biological and medical objects, namely, cell, suspensions, biological fluids and tissues [2-9]. The growth of interest in the study of biological objects using electrochemical methods is apparently determined by their informativity, ease of implementation and the possibility of non-destructive effects, which is very important, for example, for in vivo diagnosis of the cells and tissues condition [2,4]. In addition, these methods have fairly simple hardware and relatively easy to integrate into automated measuring systems, due to the fact that electrochemical information signal already has an electrical nature and does not need to be further converted [1]. Electrochemical signal is further easily processed on the basis of modern achievements of radio electronics and mathematical modeling. In this regard, conductometry is of particular value as one of the most common in many areas of scientific and practical activity, as well as easy to implement and interpret electrochemical methods.

It is known that the conductivity of liquid medium is directly related to the mobility of ions, which in turn depends on field strength [1]. Hence, there is an interest in the study of conductivity in a field with variable, or rather rising strength. First the conductivity (or resistance) of strong electrolytes solutions depending on the pulsed electric field (PEF) with rising strength (PEFRS) was investigated by German physicist M. Wien [10-13]. Since then, this productive idea for some reason nearly has not been used, at least in relation to biological objects. Regardless of M. Wien idea, it was possible to develop a simple method and device of conductometry in PEFRS to study the dynamics of the membrane electroporation process of a single *living* biological cell [14-16]. The cell can be in various liquid media (solutions) on the water basis, including well-conducting ones (cryoprotectors, polyatomic alcohols, sucrose, phosphate-buffered saline (PBS), nutrient media with hormonal protein additives, etc.) [16-21].

It is well known the traditional conductometry of biological cells (first of all in suspension) and conductometry based on electroporation of cell membrane in different liquid media [4-8, 22, 23]. But in all this cases the electric field was not continuously rising on strength. Only some works with using linear rising pulse voltage amplitude are known [24,25]. In these works the irreversible electroporation (electrical breakdown) of *artificial* bilipid membrane (BLM) was studied. It is known also researches of current-voltage characteristics of single living cells with rising in the pulse voltage amplitude [7,26]. However, in these studies only the fact of membrane electrical breakdown was stated and only the breakdown voltage was measured. But the conductivity of cell as an objective biophysical indicator of the development of membrane electroporation in PEFRS, was not determined.

In biophysics, reproductive biology, cell and genetic engineering, medicine and other fields

of knowledge are used different methods, based on the phenomenon of living cells membranes electroporation [9,27-29]. The membrane electroporation of a biological cell has been well known as a convenient, multipurpose and universal way of temporarily increasing its permeability in a PEF with certain parameters [29-31]. The influence of this field on the cell leads to reversible perforation or irreversible membrane rupture depend on applied field strength. By the way, PEF in those cases is usually no changeable on strength (monotonously!). To describe this process were built numerous electroporation models [5,6,21,32-35]. However, they do not take into account the monotonous rising in the field strength. Therefore, these models do not take into account the effect of a gradual increase in cell conductivity up to its sharp growth due to a gradual increase in the number of electropores in the membrane, as well as their avalanche-like growth during its breakdown. In own models of cell conductivity in rising field strength this was took into account [16,36,37].

The PEF is formed by special devices (including hand-made) in a liquid medium with cells [5,7,14-16,24-29,38-40]. The process and result of the membrane interaction with the PEF is greatly influenced by its heterogeneous biological structure, which has both native pores of various sizes and various protein inclusions. This leads to heterogeneity of the electrophysical properties. All this ultimately affects the cellular conductivity in PEFRS, which is both an indicator and an integral characteristic of the electroporation process of the membrane as a whole. As a result of own research it was found that many methods and processes, which are based on electroporation and traditional conductometry can be combined on the basis of the developed method of conductometry in PEFRS [14-16]. So, the main purpose of using method and pulsed conductometry equipment is to obtain experimental dependences of cells and liquid media conductivity in PEFRS. Then it is necessary to process the dependences by software to study the dynamics of the membrane electroporation process or quality characteristics of liquid media (purity, mineralization etc.).

2. EXPERIMENTAL

2.1. Reagents and materials

The main conductometric measurements in PEFRS were carried out on MII stage of meiosis oocytes of 6–8 weeks-old F1 mice (C57Bl×CBA). Superovulation was induced by hormonal treatment: injection of 5.0 IU of pregnant mare serum gonadotropin (Folligon, Netherlands) and 7.5 IU of human chorionic gonadotropin (HCG) (Chorulon, Netherlands) with 48 h interval. 13 h after HCG injection females were euthanized by dislocation of the cervical vertebrae. Oocytes were obtained by puncturing of the wall of oviduct ampulla in Dulbecco's PBS supplemented with 5% of fetal bovine serum (SIGMA, USA) using standard method [41]. Part of the conductivity measurements was carried out on cow oocytes to compare murine and bovine oocytes conductivity parameters. Immature oocytes were obtained from

antral follicles of cow ovaries (from slaughter-house). Oocytes were matured to metaphase II of meiosis by *in vitro* culture in the medium of TSM-199 (SIGMA, USA) supplemented with 10% fetal bovine serum and hormones (FSG/LH, 17β-estradiol) (SIGMA, USA) at the 38.5 ^{o}C and 5% CO_2 and high humidity for 24-26 h. Conductometric studies of cells were performed in 0.3 M solution of sucrose (SIGMA, USA). In order to provide the minimum influence of medium conductivity on results of measurements the oocytes were washed three times in 0.3M sucrose solution immediately before measuring. Also conductivity measurements were carried out on some 0.3M aqueous solutions: 1–xylitol, 2 – sorbitol, 3–mannitol, 4–glucose, 5–sucrose and conventional distillate. Qualification of chemicals: 1,2–Pharmacopoeia; 3,4,5–SIGMA, USA. As a solvent in all cases was used deionized apyrogenic water (DAW), unless otherwise specified. All experiments were performed at room temperature (22±2 ^{o}C).

The experiments with animals were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (*Strasbourg*, 1986).

2.2. Instrumentation

Conductometric measurements were carried out using 2-microelectrodes bodiless cage (own design). Technical requirements for the geometric parameters, design and material of microelectrodes are established based on the possibility of determining the conductivity of single living reproductive cells (oocytes) and embryos with 80-150 μm diameter in the micro volume of liquids (100-150 μl).The material of microelectrodes must therefore be biologically inert, withstand the effects of various liquid media, have high conductivity and resistance to electrochemical dissolution under the action of PEFRS and high density currents. In order to be able to measure the conductivity of various, including aggressive, liquids in the drop, as well as to reduce the level of polarization, microelectrodes must be made of the appropriate metal and completely isolated, except for a small contact end surface for the cell or liquid test. Taking into account these technical requirements, microelectrodes were made of suitable biologically inert metals: gold Ø55 μm (Au 99.99) and tungsten microwire Ø100 μm (W 99). The wire was soldered into a glass Pyrex capillary by hot glass covering [42]. The working ends of microelectrodes are presented in Fig. 1.

A B

Fig. 1. Working ends of microelectrodes made from wire: Au (99.99) Ø55 μm (A) and W (99.0) Ø100 μm (B), sealed in a glass Pyrex capillary and grinded from the end (×150)

To measure the conductivity of cell, microelectrodes were immersed in a droplet of liquid medium until they touch the cell surface. Then, a series of rectangular voltage pulses increasing in amplitude with a given step was fed to the microelectrodes.

To measure conductivity of single cells and liquid media the simple method and inexpensive hand-made hardware of conductometry in PEFRS were developed [14-16]. Due to universality of the developed equipment allows us to determine the conductivity of cells and liquid media in the range 0.1-10^5 μS/cm (with an error about 3%) in the field strengths 0-10 kV/cm and some biophysical parameters of cell e.g., the membrane electric breakdown field strength in various liquid media [16-20] and cell capacity [43].

The main equipment consists of measuring and accessory devices, Fig. 2. The measuring device contains the hand-made generator of rectangular pulses sequence (GRPS) with rising amplitude, calibrated resistor *R*, microelectrodes and dual-channel storage oscilloscope RIGOL DS5022M (China).

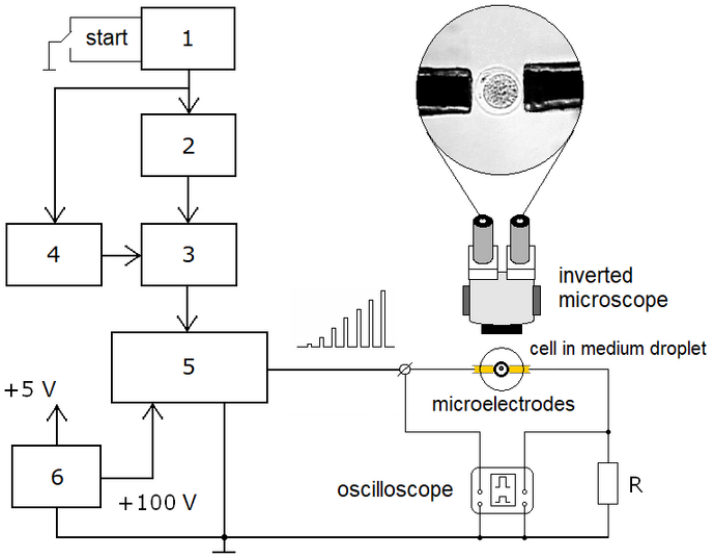

Fig. 2. Block diagram of the GRPS and the functional diagram of the conductometer

1 - the generator of a single pulse; 2-emitter follower; 3-preamplifier; 4-automation unit; 5-high-voltage power amplifier; 6 - power supply; *R* – precision resistor, selectable for scale from a range of 0.01-100 kΩ (0.05%) by decade switch. The oscilloscope was connected to the PC via USB port

The inverted microscope OLYMPUS IX71 (Japan) was used to observe the cell and the microelectrodes. In one of the microscope oculars was integrated the scale for measuring the geometric parameters of the cell and the bodiless conductometric cage formed by two microelectrodes in medium droplet. Microelectrodes were fixed in micromanipulators Eppendorf (Germany). During the measuring the microelectrodes were coaxially adjusted in the field of the microscope view. Figure 2 also shows the arrangement of mouse oocyte during measurement of electrical characteristics (conductivity and/or capacity) [16,43].

Mathematically, the series of output rectangular voltage pulses of rising amplitude are wrote as a sequence of paraphase single jumps with the growth increment of amplitude ΔU and repetition period T in the measurement time t [15]:

$$U(t) = n\Delta U \cdot \left[1\left(t + (n-1)T\right) - 1\left(t + (n-1)T - \tau\right)\right] \quad (1)$$

where: n - number of pulses in the series, τ – pulse duration, $1(t)$ – unit step function.

$1(t) = \begin{cases} 0, t < 0 \\ 1, \ t \geq 0 \end{cases}$ is the Heaviside unit step function.

The pulse duration of 50 μs and the repetition period of 5-10 s were selected according to the recommendations in the works [27,29]. The growth increment of the amplitude ΔU is set to 2 or 5 V depending on ultimate output pulse voltage of the GRPS (35 or 100 V).

Further, from the output of the GRPS, the series of rising amplitude pulses (1) follows and a pulse current passing through the cell and/or liquid medium. It produces a voltage drop on the resistor *R*. Voltage drop carries information about the conductivity of the object between the microelectrodes.

2.3. Measurement technique and processing of primary data

The cell for the study was placed on the slide on the table of the inverted microscope in a droplet of liquid medium between two coaxial microelectrodes. Next, the cell was exposed to a gradually rising amplitude of the pulsed voltage with a given pulse duration and a certain follow-up period (see formula 1), depending on the purpose of measurement. The conductivity of cell (or liquid medium) was determined by the method of series-connected resistor *R* with microelectrodes by measuring the voltage drop on it.

The conductance of the object in the inter-electrode space was calculated using Ohm's law by the formula [14,16]:

$$G = U_R / R\left(U_{out} - U_R\right), \quad (2)$$

Where: G - conductance, S; U_{out} - output pulse voltage, V; U_R - voltage drop measured on a precision resistor, V; R - nominal value of the precision resistor, Ohm.

The amplitude U_{out} of the GRPS output pulse voltage was measured on one channel, and the voltage on the precision resistor on the other channel of the oscilloscope.

To calculate the specific conductivity G_s, the obtained value (2) must be multiplied by the geometric parameter of microelectrodes k, which was calculated by the following formula [12,13,16]:

$$k = 12\left(1+\sqrt{1+\frac{4L^2}{d^2}}\right) \Big/ \pi\left[3\frac{d^2}{L}\left(1+\sqrt{1+\frac{4L^2}{d^2}}\right)+4L\right], \quad (3)$$

Where: L - the distance between the microelectrodes, μm; d – the diameter of one of them, μm. So, $G_s = G \cdot k$, μS/cm.

First, the overall conductivity of the cell and the medium was determined in a pulsed field with a linearly rising strength from zero (rising the output voltage of the GRPS). Then, with the same method, the conductivity of the medium was determined only, taking the microelectrodes away from the cell. For the same algorithm, the conductivity of both the cell with medium and the cell-free medium was calculated using equations (2) and (3).

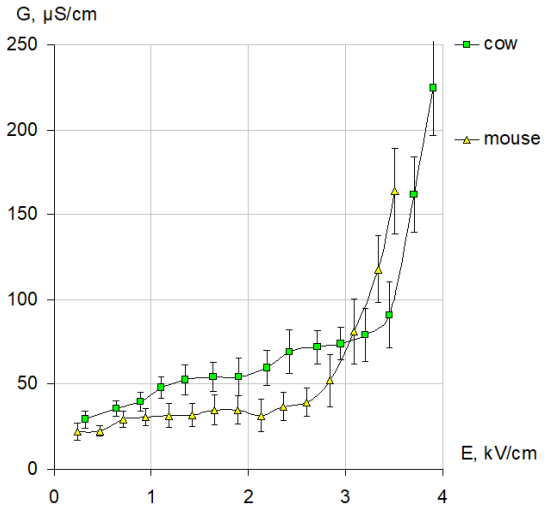

Fig. 3. Dependence of the conductivity on the PEFRS of different animals' oocytes: mouse and cow in 0.3 M sucrose (on DAW, pH 7.0, 23 ºC); pulse duration 50 μs, repetition period 10 s, ultimate output pulse voltage 60 V and 30 V for cow and mouse oocytes respectively; diameter of oocytes: cow – 167.2±3.5 μm, mouse – 82.5±1.7 μm

Then points G_s was plotted on the graph $G_s=f(E)$ depending on the field strength E, which is calculated by the formula:

$$E = (U_{out} - U_R)/L \quad (4)$$

Where: L - the distance between the microelectrodes, μm; U_{out} - output pulse voltage, V; U_R - voltage drop measured on a precision resistor, V;

To obtain the conductivity of only the cell, the corresponding ordinates of overall and medium conductivities on the plot were subtracted or the conductivity was calculated directly analytically according to the formula entered in the conductivity calculation algorithm [14,16]. Statistical processing of the results was performed using the parametric data analysis in

software Microsoft Excel 2010, and Student t-test to evaluate the significance of differences. Data were presented as M±se.

3. RESULTS AND DISCUSSION

With the help of the dependence of conductivity on the field strength, it is possible to determine all electroporation parameters of the cell membrane of different species of animals. Figure 3 shows as an example, the conductivity curves for oocytes of some mammalian–mouse and cow. The plot shows that the reversible electroporation of the membrane is noticeable already at field strength of 0.3-0.8 kV/cm. This can be used, for example, for the transfer of molecules into the cell that does not penetrate the membrane normally (drugs, some cryoprotectors, etc.).

With an increase in the field strength in the range 1.0-1.9 kV/cm, a phase of membrane stabilization occurs - a quasi-linear region [14,16]. The further increase in the field strength to more than 1.9 kV/cm up to 2.5 kV/cm for mouse oocytes and up to 2.95 kV/cm for cow oocytes leads to the fact that the conductivity of cells grows again – the second phase of reversible electroporation of membranes occurs. In this case (in our opinion) the cytoskeleton is most likely destroyed and the cell enters a state of electrical hyper stimulation [44,45]. When the field strength is more than 2.8 kV/cm for mouse oocytes and more than 3.3 kV/cm for cow oocytes, the last, but already irreversible electroporation with electric breakdown of membranes and cells lyses takes place.

Mathematical analysis of these curves can be carried out by previously developed methods using linear and nonlinear approximation models [16,36,37]. The analysis of these curves allows us to calculate the field strength of the electrical breakdown of oocyte membranes: 2.83 kV/cm (mouse) and 3.37 kV/cm (cow), which are more resistant to PEFRS action. To obtain such information about reproductive and embryonic cells of any other animal species, it is sufficient to build similar dependences of conductivity in PEFRS and to analyze them by the proposed methods [16,36,37]. Then it is necessary to decide on the application of a particular mode of PEF exposure to obtain a specific effect - reversible electroporation, fusion, stimulation or irreversible breakdown of the membrane.

Figure 4 shows the dependence of the conductivity on the field strength of 0.3 M aqueous solutions: xylitol, mannitol (in distillate (d)), sorbitol, glucose, conventional distillate, sucrose, mannitol (in DAW) and DAW. Conductivity of liquid media in Fig. 4 weakly depends on the field strength, since these are dielectrics, but a small variations in the conductivity of some media show the presence of conductive impurities namely in them, which are absent in the solvent (DAW). Fig. 4 shows that the best dielectric properties have DAW, the conductivity of which is (2.41±0.04) μS/cm at 20 ^{o}C and does not depend on the field strength.

The solution of 0.3 M sucrose was prepared on this water to measure the conductivity of cells. In comparison with DAW the Fig. 4 shows the plot of the conventional distillate

conductivity. Its conductivity is about 5 times higher than the DAW and, in addition, has small deviations, meaning the presence of some light ions in the water. Further, the mannitol and its 0.3 M solution in DAW (average 6.70±0.11) µS/cm are cleaner and better in dielectric properties than its 0.3 M solution in a conventional distillate (d) (average 18.45±0.32) µS/cm. It allows also comparing different chemicals from the same manufacturer on the dielectric properties of the medium. For example, according to the plot, 0.3 M solution of sucrose (average 7.69±0.13) µS/cm is preferable to measure the cell conductivity then glucose (average 10.53±0.18) µS/cm of the same firm.

The following methods and technologies were implemented based on the method and device of conductometry in PEFRS: measurement of PEF parameters for *in vitro* stimulation, electroporation, membrane electrical breakdown and activation of oocyte-cumulus complexes and oocytes of different animal species for use in cell engineering, reproductive biotechnology and cryobiology [14-21,43-45,49-51]; ecological studies of natural water quality and some foods [16,46-48].

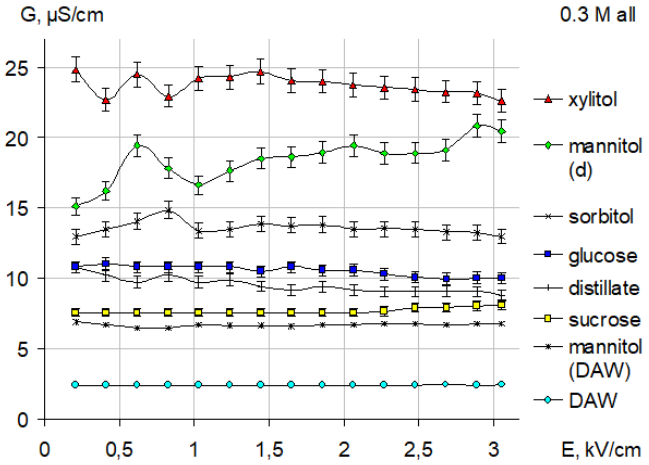

Fig. 4. Dependence the conductivity on the field strength of 0.3 M aqueous solutions: xylitol, mannitol (in distillate (d)), mannitol (in DAW), sorbitol, glucose, conventional distillate, sucrose, deionized apyrogenic water (DAW); pH 7.0, 20 ºC, pulse duration 50 µs, repetition period 5 s, ultimate output pulse voltage 30 V, distance between microelectrodes 95.0±2.5 µm

For mass measurements of cells conductivity of agricultural and other animals in various biotechnological processes, as well as to improve the accuracy of measurements the automated version of pulsed conductometer was developed [16,46]. This transformed device comprises an analog-digital converter (ADC) and a microchip. The microchip controls the GRPS and the ADC and is connected to PC via USB. The computer processes the raw measurement data for a given algorithm and the result is displayed on the monitor plot in conductivity-field strength

(*G-E*) coordinates. This device gives the principal possibility of transition to the robotic control of various biotechnological processes based on the conductometry of the cell in PEFRS.

4. CONCLUSION

The method and device to determine the conductivity of single cells and liquid media in the range 0.1-10^5 μS/cm (with an error about 3%) in the field strengths 0-10 kV/cm, pulse duration 50 μs, repetition period 5-10 s were developed. The graphical representation of the cell conductivity changes in PEFRS can detect and investigate all stages of membrane electroporation (reversible and irreversible electric breakdown) and purposefully select the desired mode of PEFRS action on the cell in accordance with electromanipulation tasks. The method and device of pulse conductometry in PEFRS can serve as an arbitration integral method for estimating the purity of the initial reagents and an objective express method for monitoring the purity of dielectric media, as well as quality control of other liquid media.

Acknowledgements

Author thanks to Dr. Alla Kolesnikova and Vadim Lisin for their help in working with cells and production of microelectrodes, respectively.

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