Viviane S. Teixeira*, Jan-Patrick Kalckhoff, Wolfgang Krautschneider, and Dietmar Schroeder

Bioimpedance Analysis of L929 and HaCaT Cells in Low Frequency Range

https://doi.org/10.1515/cdbme-2018-0029

Abstract: In this work, Bioimpedance Spectroscopy (BIS) is used to study fluids a nd c ell s olutions. A n ew four-electrode-terminal (4T) chamber using 3D printing and stainless steel corrosion resistant V4A was designed to measure the impedance of live cell solutions at the frequency range 0.1Hz-1MHz. At f < 1kHz the double layer (DL) that builds at electrode's surface raises the impedance substantially preventing the observation of the real impedance of the cells. The new 4T design circumvents the DL, is more robust and cheap, and allows for the repeatability of the results. Experiments were performed in vitro with two cell lines, L929 (mouse fibroblasts) and HaCaT (human keratinocytes). Results show that it is possible to distinguish between the two cell types by means of its BIS measurements in the new setup. Also, a low-frequency dispersion (α-dispersion) was observed in HaCaT cells solution, but not in L929. Furthermore, a potentiostat circuit model was developed in LTSpice to simulate the hardware setup and two different circuit models were used to fit cell's data.

Keywords: Bioimpedance Spectroscopy (BIS), mouse fibroblasts L929, human keratinocytes HaCaT, four terminal impedance measurement.

1 Background

Bioimpedance spectroscopy (BIS) is a technique that can potentially be used to distinguish biological cell types based on the measurement of their electrical properties. It consists on the application of a low amplitude frequency variable voltage or current to a biological tissue or solution and measuring its impedance response. In [5] authors applied BIS to normal and cancer breast cell tissues in the frequency range 1kHz-3MHz and found significant variation in their electrical signature. In another study, O' Rourke et al. [6] measured the dielectric properties in vivo and ex-vivo of six different liver tissues extracted from patients affected by four types of liver cancer, cirrhotic and normal liver and found relevant differences in their wideband values.

While applying BIS at low frequencies is possible, implementing it experimentally can be challenging. In frequencies below 1kHz a double layer composed of oriented water dipoles and solvated ions (fig. 1) builds on top of electrodes surface [8]. This phenomenon known as electrode polarization causes a significant increase in the measured impedance, hindering the observation of the real impedance of the cells.

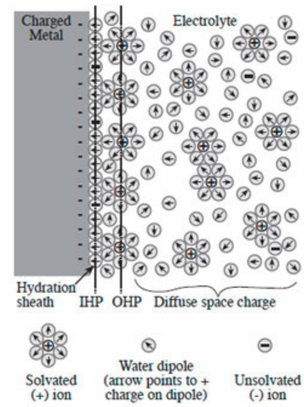

Fig. 1: Illustration of the double layer showing its composing parts Inner Helmholtz Plane (IHP), Outer Helmholtz Plane (OHP) and diffuse space charge [8].

Different authors try avoid the parasitics either going to higher frequencies (f >1kHz), or computing the additional capacities with equivalent circuit models (ECM) which are complicated and hardly fit the experimental data. Coating electrodes with noble metals or PEDOT also help to reduce DL effects. Although useful those techniques do not disappear with DL completely.

Besides, interesting cells activities can be seen at low frequencies. In [1] Schwann reports the existence of a low frequency α-dispersion observed in some cell types and tissues in frequencies ranging from 1Hz to a few kHz. In another study, Asami [9] reports its existence, but also highlights the challenge in measuring it. In the most common and simplest measuring method, a 2-electrode-terminal (2T) setup is implemented. Nevertheless, given electrodes polarization, the real impedance of the analyzed solution can not be observed.

***Corresponding author: Viviane S. Teixeira,** Institute of Medical and Nanoelectronics, Technical University of Hamburg, Eißendorfer Str. 38, D-21073 Hamburg, Germany, e-mail: viviane.silva.teixeira@tuhh.de
Jan-Patrick Kalckhoff, Wolfgang Krautschneider, Dietmar Schroeder, Institute of Medical and Nanoelectronics, Technical University of Hamburg, Eißendorfer Str. 38, D-21073 Hamburg, Germany, e-mail: jan.kalckhoff@tuhh.de, krautschneider@tuhh.de, d-schroeder@tuhh.de

Those restrictions were the motivation to develop a new 4-electrode-terminal (4T) chamber using 3D printing. Special attention was put on choosing the resin to print the chamber and electrode's material as bio-compatibles. From our experience and as reported by other authors, biological cells are especially sensitive to materials which they come into contact. The chamber enables the investigation of electrolytes and cell solutions in the spectrum 0.1Hz to 1MHz without noise correction. To test the performance of the new design the impedance of two cell lines, L929 and HaCaT, was measured and compared.

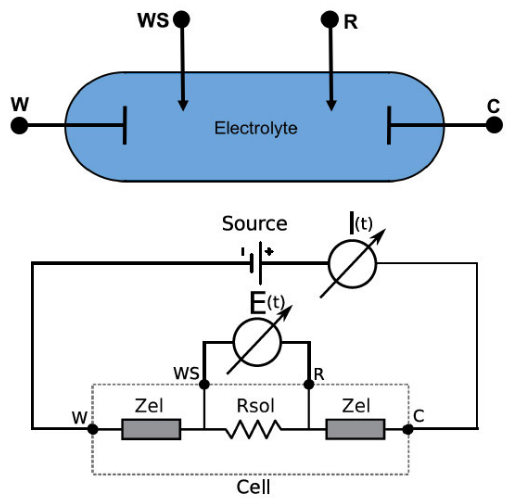

Fig. 2: Schematic and simplified circuit model of a 4T setup. The four probes are: Working (W), Counter (C), Reference (R) and Working Sense (WS). W and C carry the current while WS and R are sensing probes (for measuring the potential). Observe how 4T circumvents the DL by dislocating the sensing probes (WS and R) far from electrodes surfaces. In the 2T setup, WS and W are shorted at point W; R and C are shorted at C. In this case electrodes impedances Z_{el} are also measured.

2 Materials and Methods

AutoCAD 2017 for Mac was used to create the schematic and 3D sketches of the new chamber. The design was built as a 3D model, including the shape of the electrodes and a plug for closing it. File format .stl was exported to be used by the 3D printers. Prototypes in different dimensions were produced.

Electrodes were made of stainless steel V4A, due its robustness against corrosion, damaging and scratching. This allowed for several times use, leading to the reproducibility of the experiments. Electrodes quality was monitored each experimental day through the measurement of its cell constant; a maximum of 1.23% deviation was observed. Electrodes thickness was 0.5mm, width 16mm and height 17mm. The chamber was created with slide-ins to fit their geometry exactly.

After experiments, Matlab was mostly used to post-process the experimental data and display it. By last, *LTSpice* software was used to develop and simulate a working 4T potentiostat circuit and two different circuits models [7] to simulate cell's solution.

2.1 Experimental setup

Experiments were prepared and conducted at the laboratories of the Institue of Biomechanics and the Institute of Bioprocess and Biosystems Engineering (IBBE) at the Hamburg University of Technology (TUHH). To guarantee a stable temperature the heating cabinet WB 120[1] was used. Temperature was constantly set to 25°C with a pre-heating period of 30min. For a steady installation a screw clamp with a suction cup foot was used.

Experiments started with the impedance measurement of a commercial conductivity solution for verification of the chamber's cell constant.[2,3] Following, the impedance of the buffer solution (phosphate buffered saline - PBS) used as medium to grow and feed the cells was measured.[4]

The next step consisted on the biological cells solution measurement. Cells were provided by the Cell Service Center from IBBE. L929 cells were measured in the densities 0.5mio cells/ml[5], 0.9 mio cells/ml, 1.4 mio cells/ml and 7.4 mio cells/ml. HaCaT cells at the density 0.9 mio cells/ml were available.

Cells were first unfrozen, then cultivated and harvested weekly. They were grown in a culture medium which was removed in order to re-suspend them in PBS solution. Afterwards, impedance measurements could be performed.

All measurements were carried out three times in a row to avoid abnormalities and verify the reproducibility. Most of them were also repeated in different days. After each experiment, the chamber was washed with demineralized water and ethanol and dried with cosmetic tissues.

A low amplitude AC voltage (10mV) was applied to the solutions to keep the linearity of the system. Frequency range was set from 1MHz to 100mHz. A zero DC voltage in relation to open circuit potential (Eoc) was applied.

The potentiostat *Interface 1000E* from Gamry Instruments was used to perform all BIS experiments. It operates as a high precision 4T impedance analyzer in a frequency range 10μHz - 1MHz. Since the device is lightweight it can be easily transported to different laboratory locations. Connection cable is shielded against external eletromagnetic interference.

---

1 From the company mytron Bio- und Solartechnik GmbH.
2 VWR International GmbH, standard solution KCl 1413μS/cm at 25°C.
3 A stable cell constant of the chamber is an indicator of electrodes quality and experiment reliabilty.
4 Gibco PBS Tablets. Composed of 0.01M phosphate buffer, 0.0027M KCl and 0.137M NaCl, pH 7.4 at 25°C.
5 Million cells per mililiter.

3 Results and Discussion

Figure 3 shows the impedance measurements of 2T vs. 4T setups for the standard conductivity solution KCl 1413μS/cm at 25°C. Observe how 4T improves the quality of the results by eliminating electrodes polarization in f < 1kHz. The difference in impedance magnitude between the two setups for f > 1kHz comes from the different locations of the sensing electrodes.[6] Results of 2T experiments correspond to the resistivity of the solution along the whole chamber geometry, while in 4T setup the sensing leads are dislocated further from W and C electrodes. Therefore the measured volume and hence the resistivity of the solution is smaller.

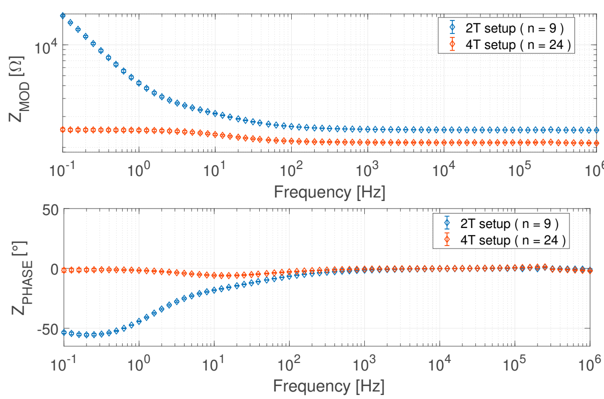

Fig. 3: Bode plot of magnitude (top) and phase (bottom) comparing 2T and 4T setups. Observe how the 4T setup eliminates electrodes polarization at low frequencies.

Following, impedance of the four cell densities was measured. Figure 4 shows the results.[7] Observe that the buffer solution (pre-PBS) has the lowest impedance. When L929 cells are added, impedance raises as cell density raises. At approximately 100Hz, impedance curves show a pronounced decay up to 10kHz when they reach a plateau. An explanation for such behavior is that as frequency increases the conductivity of the solution formed by solvated ions plus cells immersed in the buffer increases leading to a consequent decrease of the impedance. As frequency increases further, particles reach a speed limit where electric field F_{el} and frictional forces F_{fric} are equal and opposite to each other [2]. At this point particles move at a constant speed, therefore leading to a constant conductivity.

Contrary to L929 cells, HaCaT are easily distinguishable from buffer solution (figures 5 and 6). When HaCaT cells are added to pre-PBS in the concentration 0.9 mio cells/ml the impedance of the whole solution raises significantly. One possible reason for such behavior comes from the different sizes and geometries of both cell types. While L929 cells average diameter is between 5μm - 10μm [3], HaCaT cells diameter varies from 20μm - 25μm [4].

6 $Z_{mod_{2T}}(1MHz) = 1474\Omega$ and $Z_{mod_{4T}}(1MHz) = 1108\Omega$.
7 Error bars were omitted from the plots to make them readable.

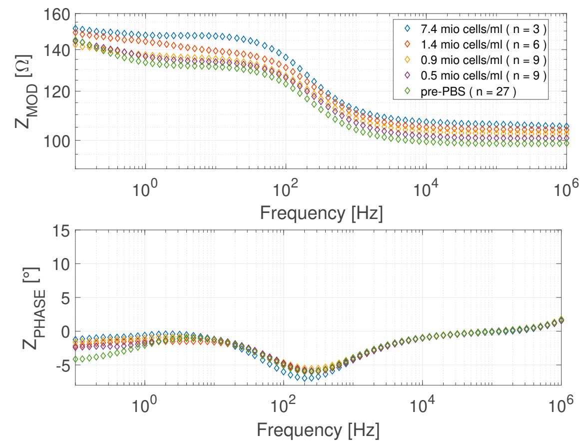

Fig. 4: Bode plot of magnitude (a) and phase (b) of L929 cells at different densities.

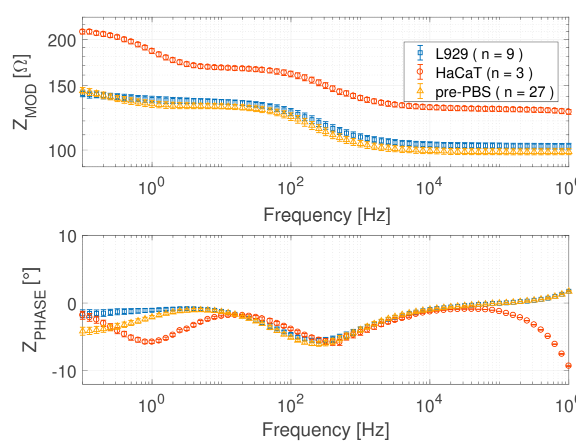

Fig. 5: Bode plot of buffer solution, L929 and HaCaTcells.

Worth noting is that L929 solutions showed one dispersion in the analyzed frequency range, while HaCaT showed two clearly indicated by the two semicircles in the Nyquist plot of figure 6. The first is as explained previously while the second is attributed to a double layer of ions forming an ionic atmosphere surrounding cell's membrane, also called α-dispersion [1, 9].

Our last exercise consisted of extracting cell's parameters by fitting and using them to build and simulate a circuit model in LTSpice. A potentiostat circuit was developed to represent *Gamry Interface 1000E*. Two circuit models were used to simulate cell's solution: Voigt's and single shell model [7] with additional components (C_d, R_d and R_s) to represent the buffer solution. Figure 7 shows the two simulated circuits and figure 8 shows the results of the LTSpice simulation together

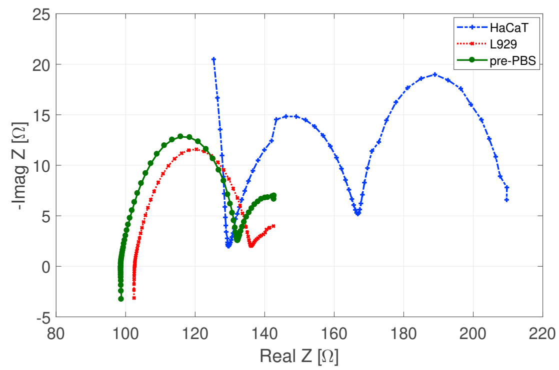

Fig. 6: Nyquist plot of HaCaT and L929 cells in the concentration 0.9 mio cells/ml together with buffer solution (pre-PBS). Frequency increases in counter-clockwise direction.

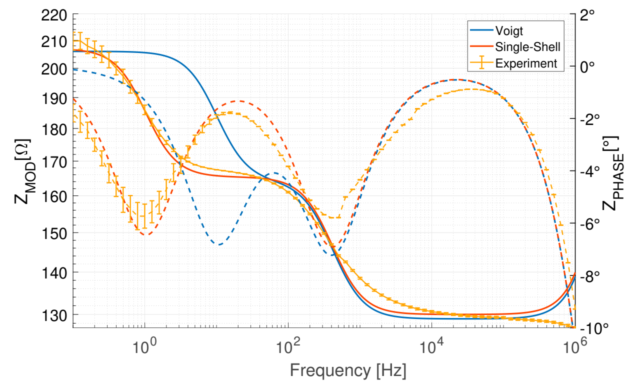

Fig. 8: LTSpice simulation vs. measured HaCaT cells in concentration 0.9 mio/ml. Filled lines represent the impedance module and dashed lines represent the phase. Experimentally measured impedance is plotted with error bars.

with the experimentally measured impedance. Single shell circuit model gives a better fit to experimental data then Voigt's model. Additionally, a dispersion around 1Hz and another at approximately 300Hz can also be seen in figure 8.

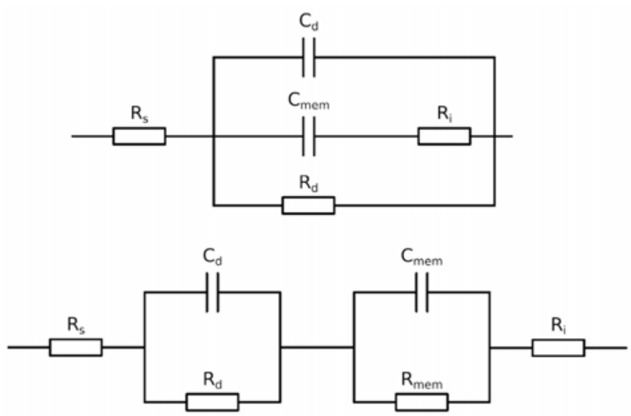

Fig. 7: Single shell (top) and Voigt's (bottom) circuit models [7]. C_d and R_d stands for diffusion capacitance and resistance; R_s solution resistance; C_{mem} and R_{mem} for cell membrane capacitance and resistance; R_i stands for cytoplasmatic resistance.

4 Conclusion

In this study BIS was used to distinguish L929 from HaCaT cells by means of its impedance measurement. The new 4T designed chamber was useful in avoiding DL at electrodes surface. Electrodes material showed to be biocompatible, not killing the cells or oxidanting during experiments. The new chamber enables the reproducibility of the results and also to see the impedance differences between the cell types in 0.1Hz-1MHz frequency range. Therefore, the BIS spectrum of the cells can possible be used as its fingerprint.

Acknowledgment: We thank Dr. Christiane Goepfert and Prof. Dr. Ralf Pörtner from IBBE Institute from TUHH for providing us with cell material and the lab to perfom the experiments. We also thank Mr. Ranjan, Mr. Mendoza and Mr. Montero from our Institute for the useful insights in developing the potentiotast circuit.

Author Statement

Research funding: Authors state no funding involved. Conflict of interest: Authors state no conflict of interest. Informed consent: Informed consent is not applicable. Ethical approval: The conducted research is not related to either human or animals use.

References

[1] Schwan HP. Electrical properties of tissues and cell suspensions: Mechanisms and cell models. Phys. Med. Biol. 1994.
[2] Teixeira VS, Kalckhoff J-P, Krautschneider W. Impedance Spectroscopy Measurement of Ionic Solutions in order to Distinguish between Different Ions. ICT Open; 2017.
[3] Higuchi A, Tsukamoto Y. Cell separation of hepatocytes and fibroblasts through surface-modified polyurethane membranes. In: J. Biom. Mat. Research Part A 2014.
[4] Vedula SRK, et al. Mechanics of epithelial closure over nonadhrent environments. In: Nature Communications 6; 2015.
[5] Qiao G, *et al.* Bioimpedance Analysis for the Characterization of Breast Cancer Cells in Suspension. IEEE Trans Biom. Eng. Vol 59. No 8. August 2012.
[6] O'Rouke AP, *et al.* Dielectric properties of human normal, malignant and cirrhotic liver tissue. Phys. Med. Biol. 52 (2007).
[7] Lvovich VF. Impedance Spectroscopy: Applications to Electrochemical and Dielectric Phenomena. John Wiley & Sons Inc; 2012.
[8] Borkholder D. A. Cell based biosensors using microelectrodes. Dissertation, 1998, Stanford University.
[9] Asami K. Characterization of biological cells by dielectric spectroscopy. J Non-Cryst Solids, 2002.