Abstract

Electrical Impedance Spectroscopy (EIS) consists in the application of a small voltage or current to a material, varying its frequency in a wide range and measuring its response to the estimulation. In this sense, the spectral behavior of the observed substance is obtained, as for each discrete frequency point one value of impedance is measured. Specifically when applied to biological systems it is called Bio-Impedance Spectroscopy (BIS).

This research is about applying EIS to biological systems in order to characterize them. Specifically, the fundamental research question was to identify whether different cell types and tissues could be distinguished by means of EIS. The research was further narrowed by applying BIS to distinguish cancer cells and tumors from their healthy counterparts. The knowledge gap in this field is immense; the applications to medicine unforeseen. For as simple as the technique is, it is amazing how powerful it is. Applications of the technique researched during the five-year period of this PhD are:

- Distinction between metastatic and non-metastatic cancer cells
- Distinction between healthy tissues and cancer tumors based on their spectral response

During this research one physical characteristic was encountered which allows the distinction between metastatic and non-metastatic cancers: the cell surface charge. For all researched cancer groups (prostate, colorectal, breast and leukemia) the cell surface charge per cm^2 was found to be smaller for more metastatic cancers than for less metastatic ones. At the moment of the publishing of this Thesis, no other work in Literature is known to have identified so clearly such a physical parameter that distinguishes metastatic cancers from non-metastatic ones.

To be able to measure the impedance of biological cell suspensions and tissues up to very low frequencies (f < 1 kHz) a four-electrode-terminal measurement setup was used.

Therefore a new chamber which allowed for the measurements of the cells suspensions was developed. To measure the tissues an array of four-electrodes in a special configuration was used. The advantages of measuring cells and tissues at low frequencies is that in such frequencies a different physics is occurring therefore the amount of charge "perceived" is much larger than in higher frequencies. Nevertheless, due to strong artifacts caused by electrode polarization only with a four-electrode-terminal setup the experiments can be performed. Developing such geometry and system which allowed to eliminate all the artifacts in low frequencies was a quite challenging task. Not only to the geometry, but significant attention was also given to the materials of the chamber and the metals used to contact the biological tissues as the majority of materials are poisonous to the cells and kill them.

Throughout this research it was observed that healthy and cancerous cells and tissues have a different spectral behavior. This difference in the frequency behavior is closely correlated to the effects of the cancer in the cells and tissues. Through the signal processing of the experimental results considerable interesting information from the status of the cancers could be extracted. For physicians at the hospital, the technique may open a better understanding of cancers and may help in the gain of useful information in a clinical context to support the decision of the best therapies for patients.

Another topic discussed includes the proposal of the architecture of an implant to be placed inside solid tumors in vivo and monitor tumor response to treatment through BIS. Additionally, two different architectures of a sensor to measure the cancer metabolites or biomarkers concentration in vitro or in vivo were proposed.

Chapter 1

Introduction

Electrical Impedance Spectroscopy (EIS) also known as Bio-Impedance Spectroscopy (BIS) when applied to biological contexts is a simple and powerful technique to characterize systems. It consists in the application of a small amplitude voltage signal to a system and measuring its current response (potentiostatic EIS). If a current is applied and the voltage is measured it is called galvanostatic EIS. Current established applications of the technique are the characterization of battery systems, evaluation of painting quality in painting industry, estimation of body and fat composition in athletes, evaluation of food quality control and currently under development to distinguish healthy from diseased cells and tissues [1–5].

The fascinating about applying BIS to biological material is that biological systems present a special signature in frequency, similar to a fingerprint, which relates closely to their internal structure and status. Therefore interesting insights can be derived from the experimental results to characterize such systems. When comparing the impedance spectrum of cancer cells and tissues with healthy ones even more striking conclusions can be drawn. The spectrum of such tissues and cells is quite different from their healthy counterparts which is directly related to the effects of the cancer in the healthy systems. In the next chapter a short revision about the topic of BIS is presented and the following chapters will show more details on how the impedance spectrum of healthy and cancer cells and tissues can be analysed and correlated to their status. In this sense, BIS used in

clinical context has the promise of being an excellent tool to aid physicians in evaluating cancer status and the effectiveness of different therapies.

1.1 Purpose of the Work

The purpose of this research is to develop methods and tools to distinguish different cell types and tissues, with a special focus on distinguishing cancer cells and tissues from healthy ones. This project was financed by ProExzellenzia Program 4.0 and by the Research Center of Medical Technology Hamburg (FMTHH).

Applications of the results include the distinction of cancer and healthy tissues in a clinical context (Chapter 7), for instance, by defining tumor boundaries during surgery for tumor removal. Identification of the mechanisms which lead to metastasis, providing input to develop a new wave of less aggressive medicaments for cancer treatment based on the blocking of specific ionic channels present at the cell membrane (Chapter 5). Evaluation of cells response to chemotheraphy by identifying the most effective chemotheraphy agent for each cell type and the best concentration to give to patients (Chapter 6).

1.2 Thesis Outline

The structure of this doctoral thesis is organized as follows:

Chapter 2 presents a literature review of several topics important for the thesis, for instance, the structure of biological cells and their composition, Electrical Impedance Spectroscopy, graphical representation and mathematical formulation of impedance data, among others.

In chapter 3 a detailed description of the four-electrodes-terminal (4T) system developed is given. The development of such a system was essential to perform the electrochemical experiments in cells suspensions up to frequencies below 1 kHz.

In chapter 4 a large set of experimental results from the impedance measurements of several cancer cell lines using the 4T chamber described in Chapter 3 is presented and compared. Also the interpretation and correlation with physical parameters of the system is mentioned. To complete the chapter useful recommendations to perform future experiments are given.

In chapter 5 the experimetal results of cells suspensions are processed to extract the cell's external surface charge and this charge is correlated with the metastatic potential of the cancer cells.

In chapter 6 adherent cell cultures are measured using an interdigited electrode system (IDE) to evaluate their growth and response to two different types of chemotheraphy medication.

In chapter 7 the impedance spectrum of several healthy organs and tumors from mice is measured and compared. Interesting insights could be obtained from this chapter to distinguish cancer from healthy tissues. Chapter **??** concludes the thesis and gives recommendations for future research.

The topic of cancer is huge and very complex, as cancer is a single name for a large set of diseases with some common characteristics (uncontrolled cell proliferation, ability to form distant metastasis) nevertheless with lots of differences among the different types of cancers. Therefore finding characteristics which are common to the majority of cancer types is essential and this doctoral thesis has made an important step towards this direction. As cancer is already of major medical concern and with population aging it will evolve into a leading cause of death worldwide[1], any new research which supports physicians in understanding cancer mechanisms and providing better treatments to patients is a valuable contribution to humanity.

[1] It is estimated that with population aging, during the next 20 years up to 25% of European and North American populations will die of cancer [6].

1.3 Contributions

The first contribution to the state of the art of this doctoral thesis was the development of a cheap, easy to produce and scalable four-eletrode-terminal chamber system (4T) using 3D printing techniques. Since the concept up to the first prototype two years were necessary and throughout the last five years several improved versions were produced. The last and smallest version is presented in chapter 3. It belonged to this task finding the best geometry, the size, how to produce it efficiently in a cheap way, the chamber material and specially important was the identification of a proper metal (Pt) to interface directly with the cancer cells inside the electrochemical chamber without killing them and providing an stable open circuit potential for the experiments in low frequencies. The challenge on performing impedance experiments in liquids for frequencies below 1 kHz lies on the double layer effects which hide the real response from the biological systems. Therefore, finding a geometry which does not distort the electric field, which eliminates or reduces the double layer effects to a minimum and producing it in a cheap and fast way was a great contribution.

A second and important contribution to the state of the art was to find one characteristic specific from the cells, the external cell surface charge, and correlate it with their metastatic potential. The ability to separate charge between the extracellular and intracellular medium is affected by the cancer, i.e., metastatic cancer cells in general show a lower capacity to separate charge between the two media. In other words, in general, the cell membrane of more metastatic cancers will be leakier than less metastatic cancers. This will translate into a higher (towards positive values) resting membrane potential, namely, a depolarized cell membrane potential. As the depolarization of cell membrane potential functions as a signaling for the cells to start their cycle of division [7], this result is of extreme importance for Medicine. Translating into other words, possibly the development of new drugs which block specific ionic channels in specific types of cancers can reduce the aggressivenes of the cancer by causing the shifting of the resting potential of the cancer cells towards more negative values (hyperpolarization) and reducing the speed of cancer proliferation. Important to highlight is that given the complexity of the metastatic process this is certainly not the only mechanism leading to it. Additionally, for each

type of cancer it has to be researched which specific channels should be blocked in order not to affect the surrounding healthy cells blocking their channels too.

A third contribution to the state of the art was to perform a large number of experiments with a broad set of cancer cell types and repeating the experiments with different cell seeds to give more confidence to the results. Additionally the identification of some of the mechanisms that affect cancer cells impedance spectral behavior (conductivity of the supernatant, temperature, volume fraction of suspendend cells, frequency) and the suggestion of new experimental practices to reduce the experimental spread. The majority of the results published in literature refer to experiments which were not repeated with different cell seeds, therefore being hard to generalize such results.

A fourth contribution was to find two common characteristics that distinguish most types of cancers from healthy tissues and proving them by the repetition of the experiments, namely, the higher tumor conductivity and lower impedance magnitude when compared to healthy tissues. There are a few studies in literature [3, 8] which also reported the observation of a higher conductivity in tumors, nevertheless here the study was extended through a large set of experiments, a large set of repetitions, a large number of healthy tissues compared to the tumor ones and the measurement of two different types of tumors.

The last contribution to science was the extension of the experimental results of epsilon and sigma for a large number of tissues up to very low frequencies. Literature is scarce or inexistent for several organs in frequencies below 1 kHz and this study makes a significant contribution in this direction by using a four electrode terminal setup. The values of dieletric constant and sigma for the tissues are used in dosimetry studies, namely, to evaluate the effects of electromagnetic fields in human tissues interacting with such fields and to stablish safe limits for such interaction.

Minor contributions include:

- Proposal of the architecture of the implant (appendix A).
- Proposal of an improved version of interdigitated electrodes system to measure adherent cells and observe their growth and response to chemotheraphy (chapter 6).

- Proposal of a method to measure the cell to cell junctions resistance strength and correlate it with the metastatic potential of cancer cells (chapter 6).

The architecture of the implant was developed in LTSpice with discrete macro components and is described in figure A.1. Of course when developing the miniaturized version of such circuit instead of using discrete components, a couple of transistors should replace the function of those macro components, for instance one can think of replacing the operational amplifiers in figure A.2 to a simplified version of a differential amplifier with five transistors. The architecture of a four electrode terminal potentiostat is proposed in figure A.2 and is a combination of three differential amplifiers with five buffers. Observe that for measuring the impedance of cancer tumors up to frequencies below 1 kHz necessarily a four-electrode-terminal (4T) architecture must be used. The lock-in detection described in Appendix A is used to separate the real and imaginary parts of the signal. Alternative archiectures are proposed in literature, for instance using an integrator instead of a frequency mixer, nevertheless such archicture requires a long integration time in order to have a reasonable signal to noise ratio. Given the low frequencies used in such 4T experiments, the lock-in detection using frequency mixing was preferred for being faster.

1.4 List of Publications

Here there is a list of international and national conference publications and presentations, both as a first author or co-author, produced during the PhD (1-8) and the master thesis (9-10) which preceeded the PhD as a preparation for it. The publications are listed in chronological order.

1. V. S. Teixeira, V. Labitzky, U. Schumacher, W. Krautschneider. Use of Electrical Impedance Spectroscopy to Distinguish Cancer from Normal Tissues with a Four Electrode Terminal Setup. In: 54th DGBMT Annual Conference, 29 Sept – 1st October, 2020, Leipizig, Germany.

2. V.S. Teixeira, T. Barth, V. Labitkzy, U. Schumacher, W. Krautschneider. Electrical Impedance Spectroscopy for Characterization of Prostate PC-3 and DU 145 Cancer

Cells. 41st Annual International Conference of IEEE Engineering in Medicine and Biology Society (EMBC), 23-27 July 2019, Berlin, Germany.

3. T. Barth, V. S. Teixeira, W. Krautschneider. Designing electrodes for electrical impedance spectroscopy in a four terminal setup. Additive Manufacturing Meets Medicine 2019, Lübeck, Germany.

4. T. Barth, V. S. Teixeira, W. Krautschneider. Chamber miniaturization for four terminal electrical impedance spectroscopy. Additive Manufacturing Meets Medicine 2019, Lübeck, Germany.

5. V. S. Teixeira, J. J. Montero-Rodríguez, W. Krautschneider. Bioimpedance Spectroscopy for Characterization of Healthy and Cancerous Tissues. IEEE International Conference on Electrical Engineering and Photonics EExPolytech-2018, 22-23 October 2018, Saint Petersburg, Russia.

6. V. S. Teixeira, J-P Kalckhoff, W. Krautschneider, D. Schröder. Bioimpedance Analysis of L929 and HaCaT Cells in Low Frequency Range. Current Directions in Biomedical Engineering 2018; 4(1):1-4. In: 52nd DGBMT Annual Conference, 26-28 September, 2018, Aachen, Germany.

7. V. S. Teixeira, J-P Kalckhoff, W. Krautschneider, D. Schröder. Electrical Impedance Spectroscopy Measurement of in Vitro Cells Solutions in order to Distinguish Different Cells Types. SAFE Workshop – annual conference on Microsystems, Materials, Technology and RF Devices, Twente University, Enschede, The Netherlands, June, 2018 (Poster and Presentation)

8. V.S. Teixeira, J-P Kalckhoff, W. Krautschneider. Impedance Spectroscopy Measurement of Ionic Solutions in Order to Distinguish between the Different Ions. In: ICT Open 2017 Conference Proceedings der NWO, 2017, p. 14-17 Amersfoort, The Netherlands, March 21-22, 2017.

9. V. S. Teixeira, D. L. Villarreal, D. Schöder, W. Krautschneider. Circuit Modelling of Action Potential Generation and Propagation in Damaged Nerve Cells. ICT Open Conference, March 2015, Amersfoort, The Netherlands. (Poster & Presentation)

10. V. S. Teixeira, D. L. Villarreal, D. Schöder, W. Krautschneider. Concise simulator for neuromuscular stimulation. 11. Hamburger Studententagung für Innovative Medizin und Biotechnologie, May 14, 2014, Hamburg, Germany.