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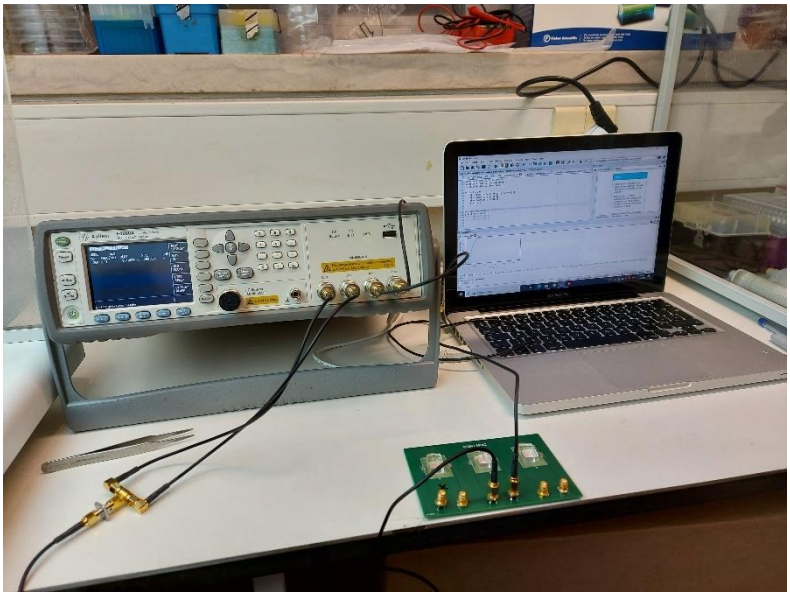

Impedance monitoring of cell cultures in real time in a biochip using integrated microelectrodes

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Thesis to obtain the Master's degree in

Biological Engineering

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Preface

The work presented in this thesis was performed at INESC MN (Lisbon, Portugal), during the period March-December 2020, under the supervision of Professor João Pedro Estrela Rodrigues Conde.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

First of all, I would like to start by thanking Prof. Dr. João Pedro Conde for the opportunity to work in this project, as well as the support and supervision through the development of this project at INESC-MN.

I would like to express my gratitude to all my colleagues from INESC-MN, for the support and help that they provided during my time working with them as well as maintaining a good and healthy work environment. I am especially grateful for the help of Cristiana Domingues, without whom none of the work would have possible as well as some good advice.

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Finally, I would like to thank my family, especially my parents and my brother and sister, for all the support that was given during all my academic journey as well as in the months of this project, that were particularly challenging.

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Abstract

The present work was focused on impedance measurements in cell cultures in microchips to better understand cell growth, cell proliferation and cell removal on a chip.

This was achieved using interdigitated microelectrodes in which cells were cultured and studied using impedance measurements.

The biochip was developed using microfabrication techniques and after which it was used for cancer cell seeding and culture with the aim of mimicking the conditions of cell growth in vivo. This was achieved and impedimetric measurements occurred.

To further understand the behaviour of the impedance of high concentration solutions and cell cultures, experiments using PBS, NaCl, E.-coli and IgG were conducted.

In this work it was possible to distinguish the impedance behaviour of solutions with low concentrations and higher concentration, such as the difference between DI Water and PBS.

It was also possible to grasp the impedimetric behaviour of suspensions of solid substances like E.-coli and IgG. However this is only possible in very low concentrations of solutions as the behaviour of the higher concentration solutions is the dominating factor in the impedance measurement.

It was possible to conduct the cell culture with the microfluidic device, however due to fact that the culture medium utilized for the cell culture has a high concentration of nutrients and other molecules it was not possible to detect the cells within the microchip as well as their growth, proliferation and removal.

As a result, the devices should be optimized for impedimetric measurements.

Resumo

O presente trabalho tem como foco medições de impedância em culturas de células em microchips para ter melhor conhecimento do crescimento de células, proliferação celular e remoção de células, num biochip.

Este objetivo foi atingido utilizando microeléctrodos interdigitados nos quais houve a cultura de células e estas foram estudadas usando medições impedimétricas.

O biochip foi desenvolvido utilizando técnicas de microfabricção e depois foi usado para seeding de células do cancro em cultura de modo a mimetizar as condições de crescimento celular in vivo. Isto foi atingido e procedeu-se a medições de impedância.

Para perceber melhor o comportamento the soluções de concentrações altas e de culturas celulares, foram feitas experiências com PBS, NaCl, E.-coli e IgG.

Neste trabalho foi possível distinguir o comportamento da impedância de soluções com menor concentração de soluções mais concentradas, como a diferença entre DI Water e PBS.

Também foi possível compreender o comportamento de soluções com suspensões como E.-coli e IgG. No entanto, isto só foi possível em soluções de concentrações muito baixas pois o comportamento da impedância de soluções mais concentradas é o fator dominante na medição impedimétrica.

Foi possível cultivar células no microchip, no entanto, devido ao facto de o meio de cultura utilizado ser de uma concentração relativamente alta de nutrientes e outras moléculas não foi possível detetar as células no biochip assim como monitorizar o seu crescimento, proliferação e remoção.

Desta forma, o microchip utilizado deverá ser otimizado com o objetivo de ser utilizado para medições de impedância.

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1. Introduction

1.1 Motivation

Cell cultures and their properties have been widely studied in regard to standard cell culture techniques, like Petri dishes and microwells. However, in cell biochips, cells are cultured in microfluidic chambers. The integration of the cells in microsystems has the advantage of allowing a very high level of geometrical, fluidic and chemical control and thus a high level of control of the cellular microenvironment. This could mean that the process could be faster, less complicated and cheaper.

Cancer research is a particularly important field and in high demand for drug screening and testing on a fast a big scale, as different patients respond to different drugs. As a result, Lab-on-chip could provide the means to achieve that by a being able to monitor cell growth, proliferation and death in a real-time scenario. This means that different drugs and concentrations can be tested faster and cheaper whilst being able to provide the patient with the necessary means of treatment.

This project is included in a larger scale on going work, the POINT4PAC - Precision Oncology by Innovative Therapies and Technologies, in which the microfluidic device for the cell culture and impedance measurement has been optimized. Has a result, if and when cell growth and proliferation can be detected then this has a larger implication for general cell chips and its importance.

1.2 Impedance Spectroscopy

Impedance is the characteristic of an electrical circuit that resists the flow of charges and is used in engineering to relate with the alternating current (AC). It is determined at a frequency ω as a function of time if an alternating voltage $u(t)$ with amplitude $\hat{u}$ is applied to the system, and the corresponding alternating current $i(t)$with the amplitude $\hat{\imath}$ has a phase shift φ_i. It is given by the ratio of the complex values of voltage and current, as seen in the equation 1 below:

$$Z(t) = \frac{u(t)}{i(t)} = \frac{\hat{u}\cos(\omega t+\varphi_u)+\hat{u}j\sin(\omega t+\varphi_u)}{\hat{\imath}\cos(\omega t+\varphi_i)+\hat{\imath}j\sin(\omega t+\varphi_i)} \quad (1)$$

From equation 1, impedance can be expressed either by its modulus and phase shift, ot its real part R and imaginary part X. Using the Euler formula 2, equation 1 can be written as:

$$e^{j\varphi} = \cos\varphi + j\sin\varphi \quad (2)$$

$$\underline{Z}(\omega) = \frac{\hat{u}}{\hat{\imath}}\frac{e^{j(\omega t+\varphi u)}}{e^{j(\omega t+\varphi i)}} = \frac{\hat{u}}{\hat{\imath}}e^{(j\omega t+j\varphi_u-j\omega t-j\varphi_i)} = \frac{\hat{u}}{\hat{\imath}}e^{j(\varphi_u-\varphi_i)} \quad (3)$$

By replacing $\frac{\hat{u}}{\hat{\imath}}$ with $|Z|$ and $\varphi_u - \varphi_i$ with ϕ, equation 3 can be simplified as:

$$|Z|e^{j\varphi} = |Z|(\cos\varphi + j\sin\varphi) \quad (4)$$

From this equation, the real part R is $|Z|\cos\varphi$ and the imaginary part X is $|Z|\sin\varphi$, and so the equation can be rewritten as:

$$\underline{Z} = R + jX \quad (5)$$

To calculate the alternating current networks within a time domain, it is necessary to use a Fourier transformation, that transforms the time domain to a frequency domain. So, the input signal is transformed using the equation:

$$F(j\omega) = \int_{-\infty}^{+\infty} f(t)\cdot e^{-j\omega t}\,dt \quad (6)$$

This allows to stimulate a linear time invariant (LTI) system with an oscillation, mostly with a sinusoid signal, of a certain frequency and measure the outcome, which is also an harmonic oscillation with a different amplitude $\underline{Y}(\omega)$and a phase shift. This amplitude can also be calculated using a multiplication of the complex amplitude of the input signal $\underline{X}(\omega)$ with the complex transfer factor $\underline{F}(\omega)$, as demonstrated in the equation below.

$$\underline{Y}(\omega) = \underline{X}(\omega)\cdot\underline{F}(\omega) \quad (7)$$

This is repeated for multiple frequencies and then the complex valued frequency response of the system is extracted, because for every frequency there is a corresponding complex transfer factor. The frequency response, $\underline{F}(j\omega)$,is the function of the frequency containing the transfer factors. It can be received through the Fourier transformation of the time domain, that originates

a continuous spectrum, or a calculation with the different amplitudes and building a function with it, that originates a discrete spectrum. [1]

The frequency response can be split into two parts: the amplitude response and the phase response. The former describes how the amplitudes of the harmonic oscillations at different frequencies are damped or amplified by the system. The latter, the phase response, describes the phase shift of the system and is important for the valuation of the duration of the vibration component though the system. They can be graphically depicted in a Bode plot, where the amplitude response is plotted on a log-log graph and the phase response is plotted on a log scale.[1]

The impedance spectrum can also be plotted in a Nyquist plot by its real and imaginary parts. Then, though Impedance Spectroscopy, the complex electrical resistance (impedance) of a system as a function of the frequency can be measure by exciting the system with a small amplitude sinusoid signal and varying the frequency. [2]

The Bode plot in then used to analyse the resistive, capacitive, and inductive behaviours of the system. Probing an impedance spectrum allows the characterisation of surfaces and layers by analysing the spectrum via an equivalent circuit, as well as characterisation of solution concentrations changes, in which selected frequencies are monitored to observe changes of one impedance element.[1]

1.2.1 Impedance Elements for Equivalent Circuits

An equivalent circuit that describes a system needs to be built by four elements. These elements can be connected in parallel, in series, or as a mixture of both in order to get the best approximation to the real system and should be made as simple as possible.[1]

When charges are being transported, the system will show a resistance behaviour, that can be identified in the Bode plot in the phase response. If there is no phase shift between the AC voltage and the AC current, the system's performance is solely resistant and the impedance value is not dependent on the frequency, and so the impedance is constant. For example, in a solution, resistance can be a significant factor, as ions are transported through the solution to the electrodes and this depends on the ionic concentration of the solution, the type and size of the ions, the temperature and the geometry of the area. Another resistant parameter that can occur is the charge transfer resistance caused by redox reactions when for example dissolving a metal electrode into the electrolyte and will depend on the temperature, concentration of the electrolyte and the potential applied to the system. The relating parameter is the charge transfer resistance R_{ct}.[3]

Capacitive behaviour indicates that charges are separated at interfaces, such as a double layer. An electrical double layer is the phase boundary between an electron conductor (electrode) and an ion conductor (electrolyte) and this phase boundary has an insulation space, due to the two opposite charged layers, similar to a capacitor. A solely capacitive behaviour results in a phase shift as the current is ahead of the voltage by 90° and a strong dependency on the frequency can be observed, much like the behaviour of an ideal capacitor in the electrical engineering. However, a real system is more complex, and the basic elements are not enough to describe the behaviour of the system. For instance, the double layer capacitance is more likely not behaving like an ideal capacitor due to surface roughness or non-uniform current distribution. An imperfect capacitor can be modelled with a constant phase element.[1]

The last element to build an equivalent circuit is usually the Warburg impedance. This is a diffusion circuit element, because at low frequencies the reactants have to diffuse farther which increases the impedance and inversely at high frequencies. The Warburg impedance is depicted in a Bode plot when the slope of $|\underline{Z}|$ is -1/2 and the phase is 45°. In figure 1 we have the expected for a Randles circuit. [1]

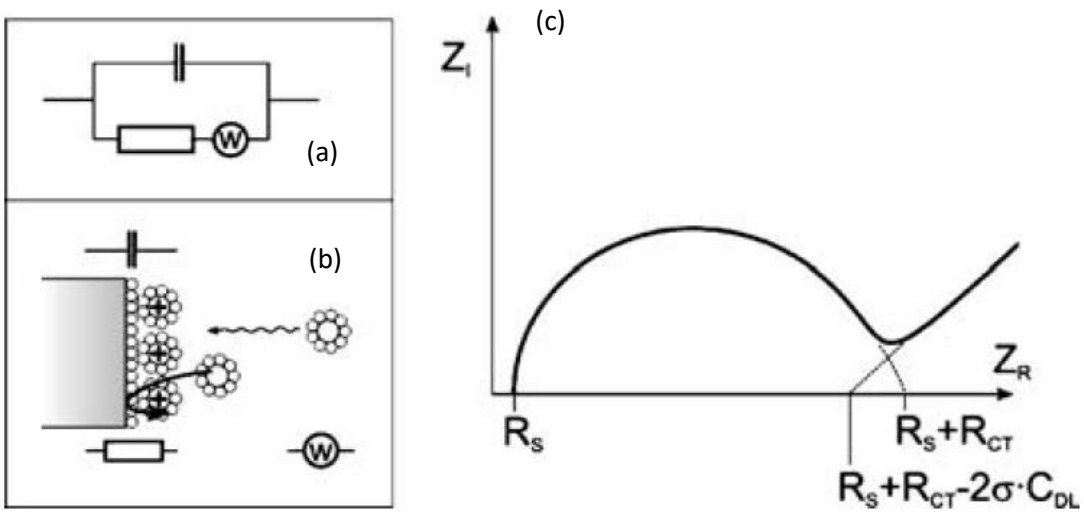


Figure 1 – (a) Randles equivalent circuit for an electrode in electrolyte contact, (b) Representation of the impedimentric elements on a interface, (c) Nyquist plot for a Randles circuit [1]

In the representation of the Randles equivalent circuit in an electrolyte, the capacitance of the double layer, that is formed in the interface of the electrode and electrolyte, is in parallel with the resistance of the charge transfer in series with the Warburg component, that represents the diffusion phenomenon that is the determining factor is impedance measures at low frequencies, and the resistance of the solution is in series with these impedance elements (figure 1 (a) and (b)).

The impedance measurements can then be plotted in the Nyquist plot where the values of the impedance elements can be extracted and studied. The values of the resistances and the capacitance can be obtained in the higher frequencies part of the graph (semi-circle curve) and the Warburg impedance values can be taken from the lower frequencies part of the graph (figure 1 (c)).

1.2.2 Impedimetric Monitoring of Adherent Cells

When monitoring cells, a consideration to be had is the type electrodes on the sensor. They can be single working electrodes or interdigitated electrodes for in plane impedance measurements and they can be designed in different materials and shapes. A single sensor electrode should determine the overall impedance. On the other hand, interdigitated electrodes consist of an array of finger like electrodes that are engaging but leaving a small non-conductive gap. These sensors allow system integration so that no external electrodes are necessary [1].

For the monitorisation of cells in a microfluidic chip with electrodes, the cell culture needs to be supplied with salts and nutrients, forming an electrolyte solution that has a solution resistance R_s. The double layer is formed between the electrodes and the surrounding electrolyte, which is the double layer capacitance C_{dl} and there is a charge transfer R_{ct} and diffusion layers Z_W. This is the easiest equivalent circuit referred to as Randles equivalent circuit for an electrode electrolyte contact. [1]

Impedance element	Definition	Phase angle	Frequency dependence
R	$Z = R$	0°	No
C	$Z_C = \frac{1}{j\cdot\omega\cdot C}$	90°	Yes
CPE	$Z_{CPE} = \frac{1}{A(j\cdot\omega)^{\alpha}}$	0–90°	Yes
W (infinite)	$Z_W = \frac{\sigma}{\sqrt{\omega}}(1-j)$	45°	Yes
	$\sigma = \frac{R\cdot T}{n^2\cdot F^2\cdot\sqrt{2}}\left(\frac{1}{\sqrt{D_O}\cdot c_O} + \frac{1}{\sqrt{D_R}\cdot c_R}\right)$		
W (finite)	$Z_W = R_0 \frac{\tanh l\cdot\sqrt{j\cdot\omega/D}}{l\cdot\sqrt{j\cdot\omega/D}}$	0–45°	Yes

ω	angular frequency
l	length of diffusion region
D	diffusion coefficient
R_0	diffusion resistance for $\omega = 0$
c_O,c_R	concentration of oxidized and reduced species

Figure 2 - Impedance definition, frequency dependence and phase shift of different impedance elements most often used for the description of bioeletrochemical systems [1]

However, this is a simplified version of the circuit, as there is also a biological component that the current has to go through (cells, solution and the electrode). If the cells are adherent, they

immobilise as a monolayer on the electrode and interact with the analyte. Binding events can be detected as a change in capacitance, or as a change in resistance as the double layer is thicker, or as the change of the dielectric constant due to the different surface. [1]

1.3 Microfluidics

Microfluidics is related to fluid-handling technologies, where the dimension of the typical elements is less than 1 mm and internal volumes are less than 100 pA.[11] It is directly linked to the fabrication of devices that allow the flow of fluids in microliter scale and permits the analysis of the behaviour of fluids in these devices [7]. This miniaturisation of fluid components allows the scaling down of already established processes and has revolutionised the field of Lab-On-Chip (LOC) devices. The LOC technology facilitates the analysis of biomolecules and chemicals by miniaturisation, parallelisation and diversification of laboratory processes that can this away become automated, portable, fast and cheap. [5] It also facilitates the mimicking of in vivo environments of cell cultures, so that the reproduction of real organs can be approximated to their real surroundings. [6].

Microfluidics brings several advantages related to scaling down, such as a reduction in reagent consumption and therefore less cost and waste. Moreover, at this reduced scale, flow is laminar ad mixing is promoted by diffusion, facilitating the handling of microfluidics devices. [8] Also, chambers and channels can be fabricated with the same size as the cells, making single-cell analysis possible. The temperature, nutrients concentrations, shear stress and other cell culture parameters can be controlled at a more precise level. [5,9].

For the fabrication of microfluidic devices, one of the most commonly used polymers is polydimethylsiloxane (PDMS), that is a silicon-based polymer, part of the siloxane family. It contains silicon, oxygen and alkanes [11]. This polymer is frequently used in soft lithography, a cheap moulding technology where photolitography is used to create a master mould which is then replicated with a polymer, like PDMS. This technique allows for layers to be stacked making this an attractive technology for cultivation of three-dimensional cell cultures to produce suitable structures for the medical and biomedical field. [10]

1.4 Cell Cultures and Microfluidic Devices

Conventional culture methods are well established processes, but difficulties related to static conditions, not accurate representation of real cell environments, unwanted concentration gradients and cost can be overcome with microfluidics. [12].

The implementation of continuous flow can supply cells with media continuously through pumping systems, which prevents gradients in pH and concentrations. On these devices, smaller volumes are required, and sensors can be included into the systems, reducing production and reagents costs and analysis time, as well as being user-friendly, sensitive, and providing reproducible results and conditions [12].

1.4.1 Microfluid Cell Culture

The first step to culture cells is the insertion and cell seeding. To this effect different approaches exist, such as the microfluidic sticker method, in which a resin that serves as a sticker is produced. This microstructured resin is then sealed to a substrate where cells are seeded [8].

Another procedure is the seeding of cells by modifying the surface of a substrate, that is already bonded to a microfluidic structure, with a matrix and then insert cells with syringe tubing and couplers and give them time to attach.

After solving the insertion and seeding the cell culture needs to be maintained. According to the final application of the cell culture a proper design should be considered cell chambers as well as the medium supply system. One example is a fully automated microfluidic cell culture system for parallel monitoring of different culture conditions. The microfluidic chip is monitored in real time on an automated microscope in order to obtain the cell culture status over time. [5]

1.4.2 Integration of impedance sensors in cell chips

In general impedance measurement in bio applications can be grouped into immune and DNA sensing, where for example a molecule is immobilised on the sensor surface and due to binding events the surface is more occupied, which leads to a change in resistance and capacitance that can be measured. Also immune reactions can be sensed using an impedance sensor when an antigen-antibody complex is immobilised on the electrode surface [1]. Another concept where electrochemical impedance spectroscopy is used within DNA biosensors is the immobilisation of DNA onto the electrode surface and a subsequent treatment with a chemical mutagen or drug. As a result, the DNA can recast, which can cause a difference in the resistance that is quantifiable [13].

The other group are impedimetric enzyme- and cell-based sensors. For enzyme turnover measurements it is sufficient to follow the charge transfer resistance as shown in blood sugar tests. Cell-based assays can be used to monitor cell adhesion on surfaces, response to potential drugs or metabolic activity in monitoring the complete complex system with capacitance and resistance. Cells can also be cultured directly on the electrode surface and therefore, change the resistance due to changed surface conditions [1].

1.4.3 Advantages and disadvantages of cell cultures in microfluidic devices

Like all devices, there are certain advantages of using microfluidic chips for cell cultures such as the responsiveness of the devices, which are faster than their normal sized counterparts, the lower consumption of reagents and other substances required, the ability to monitor several different conditions of study as well as the mimicking of *in vivo* cellular microenvironments that is fundamental to study and understand the cell cultures, such as the reaction to different nutrients, vascularization and interactions between cells. [14]

On the other hand, this strategy can also have its disadvantages, one of them being the fact that laminar flow, a rather slow process, is the limitation factor in diffusion of the nutrients to the cells. This results in a concentration gradient within the cell chip that is undesirable for the necessary growth conditions of the cells.

Moreover, while the microfluidic devices themselves might not be expensive to fabricate the production materials and equipment's as well as the specialized personnel increase the overall cost of these devices. [14]

1.4.4 Impedance Monitoring of Cell Cultures Integrated with Microfluidics

There has been made some progress in the field of impedance monitoring of cell cultures with microchips.

An example of this is the work developed by Noh and Kim [15], in which they developed a microfluidic device capable of in-air monitoring of in vitro monolayer cells under air-exposure utilizing electrochemical impedance spectroscopy. In this work, the fabricated in-air sensor successfully demonstrated *in situ* real-time measurement of cell populations in confluency and based on the measurement, it was concluded that the developed in-air EIS sensor platform could enable real-time monitoring of 'air-exposed' cells.

Another example is the work developed by Zang *et al* [16] in which it was possible to distinguish skin cancer cells and normal cells using electrical impedance spectroscopy The results indicate that these two cell types can be distinguished with EIS based on the differences in the values the resistances and capacitances during the proliferation process in a real-time and label-free manner.

2. Materials and Methods

In order to perform these experiments it was necessary to fabricate the microchip. The steps for the microfabrication are explained in this section and the preparation of the microchips was conducted in a class 100 cleanroom (100 000 particles over 1 µm per m^3).

2.1 Interdigitated Microelectrode Fabrication

The interdigitated electrodes were produced with a thin layer of Indium Titanium Oxide (ITO) deposited on a glass substrate, covered with a photoresist layer that was structured with exposure and development and the metal layer was structured using the resist as an etching mask (figure 3).

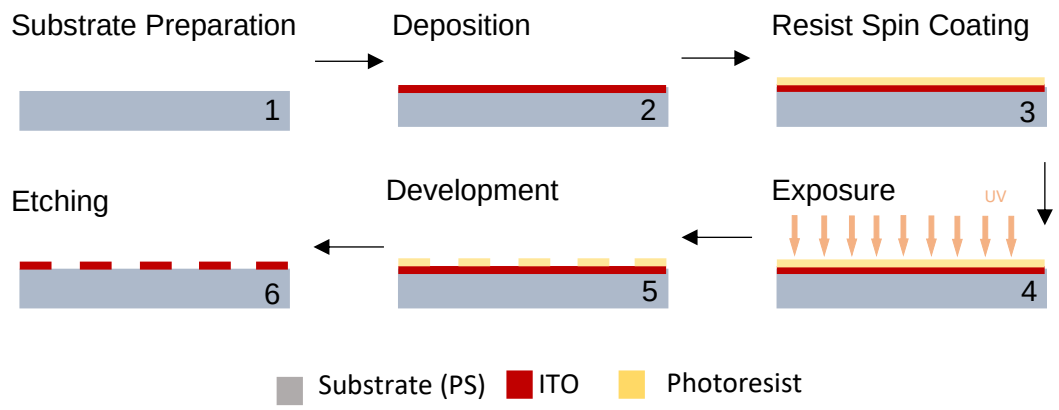


Figure 3 - Schematic overview of the fabrication of the interdigitated electrodes including substrate preparation, deposition, lithography, and etching.

The preparation of the substrate includes several steps such as its characterisation (i.e. roughness), surface treatment (i.e. adhesive properties) and cleaning to remove any and all contamination, with the latter being an ever-present step regardless of the use given to the substrate. For this glass substrate, the cleaning process was the following:

- Quick rinse with DI Water in order to remove most particles)
- Rinse with Isopropanol (IPA) for degrease the surface
- Rinse with DI Water to remove residues left behind by the fast evaporation of IPA
- Blow with Compressed Air until dry
- Treat the surface with Ultraviolet-Ozone (UVO) for 5 min to clean and activate the surface, improving the adhesive properties (removing organic residues and setting the surface into an energetic state to make the wetting possible, and the contaminations absorb the

energy of the ultraviolet and are decomposed or excited. The latter react with the oxygen radicals that are formed by the reaction of ozone with the UV light and later desorb from the surface. By bonding to the surface, the radicals form a polar surface) [17].

- Immersion of the substrate in an Alconox bath for 4h at 65ºC combined with ultrasounds to remove any particles left by the previous step.
- Rinse with DI Water and blow with Compressed Air until dry to remove any Alconox precipitates

The metal, in this case ITO, was deposited in a glass substrate (cleaned with the procedure explained earlier in this section) with magnetron sputtering in which the deposition rate is higher and the deposition is more uniform as a magnetic field is applied and brings the electrons on a spiral track and the mean free path is expanded originating a 140 nm ITO film deposited in the substrate.

To imprint the wanted structure onto the ITO film the technique used was photolithography and the design was made using AutoCad software (figure 4). The dimensions of the interdigitated electrodes and the design of the structures had previously been done and optimized. The finger of the electrodes is 8 μm wide and have 5,3 μm space between each other. The photolithography process had the following steps:

- The substrates were coated on the side with the deposited ITO with a 1,5 μm layer of positive photoresist with a spin coater
- Conduct a soft bake at 65º for 3 min to dry and harden the resist layer and to decrease the dark erosion [18]
- Expose the substrate with the ITO and resist using a direct wire laser (DWL 405 nm)
- Carry out a post exposure bake at 65ºC for 3 min to complete the chemical reaction of the photosensitive resist inhibited by light
- Cool down the substrate
- Develop using the SVG Resist developer track, in which the exposed parts of the photoresist got soluble when they are in contact with the developer and the desired pattern remained.

- Check for over and under development and contamination.

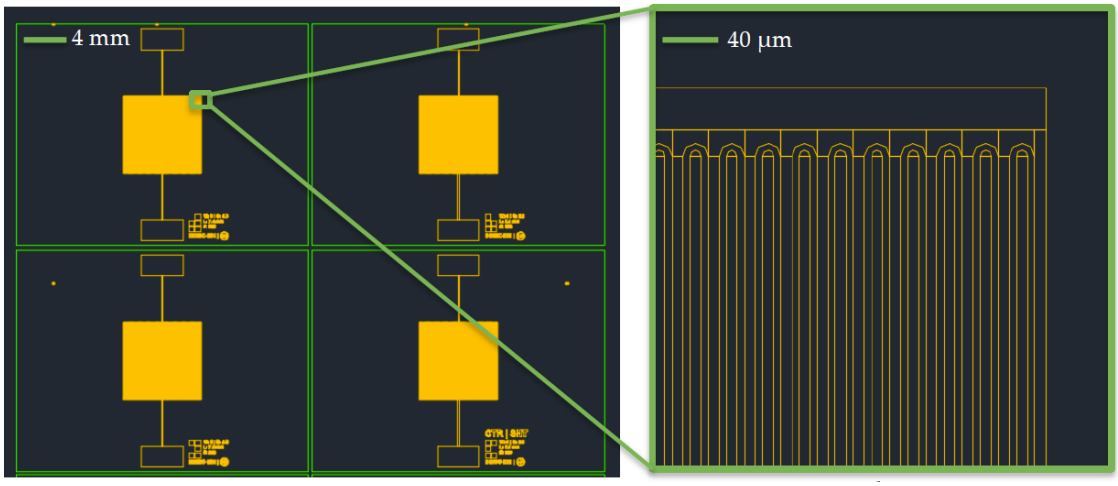


Figure 4 - AutoCAD 2D Design (right image) of the four interdigitated eletrodes with pads. Zoom in the pattern of the eletrodes (left image).

After the photolithography the result is a substrate with six interdigitated electrodes in a 7cmx7cm glass substrate, with one of the electrodes being designed shorted to serve as reference for measuring the resistance. This implies that dicing is required. To make sure the electrodes don't suffer any damage during the dicing, photoresist was spin coated on the substrate using the SVG resist coater track. It was then cut with a dicing saw and afterwards the resist was removed with microstrip.

The equipment, materials, and reagents for the fabrication of the interdigitated microelectrodes is detailed in table 1.

Table 1 - Equipment, materials and methods used in the Interdigitated Microelectrode fabrication

Substrate Preparation	**Equipment**	Dektak 3030 ST Profilometer, Veeco Instruments Inc. (Santa Barbara, CA/USA) Kerry ultrasonic cleaning bath, Guyson (Skipton, North Yorkshire, UK) UVO-Cleaner 144AX-220, Jelight company Inc. (Irvine, CA/USA)
	Materials	Glass substrate, Corning Inc, (Corning, NY/USA)
	Reagents	Alconox solution, Alconox Inc. (White Plains, NY/USA) Deionized (DI) water Isopropanol (IPA), (99,9%), LabChem Inc. (Zelienople, PA/USA)
Metal Deposition	**Equipment**	Thin film deposition system SCM 450, ALCATEL CIT (Paris, FR)

Photolithography	**Equipment**	AutoCAD software (Autodesk Inc., Mill Valley, CA/USA) _ DWL lithograph, Heidelberg Instruments (Heidelberg, DE) _ SVG resist coater and developer track, Silicon Valley Group Inc. (San Jose, _CA/USA)
	Materials	Glass substrate, Corning Inc, (Corning, NY/USA)
	Reagents	Photoresist PFR 7790G, JSR (Sunnyvale, CA/USA) AZ 726 MIF Developer, Merck KGaA (Darmstadt, DE)
Etching	**Materials**	Indium Tin Oxide Etchant TE-100, Transene Company Inc. (Danvers, MA/USA
Dicing	**Equipment**	Automatic dicing SAW DAD-321, Disco Corporation (Tokyo, JP) _ SVG resist coater and developer track, Silicon Valley Group Inc. (San Jose, _CA/USA)
	Materials	Photoresist PFR 7790G, JSR (Sunnyvale, CA/USA) Microstrip 3001, Fujifilm Electronic Materials Europe (Zwijndrecht, BEL)

2.2 Microfluidic Structure Fabrication

The fabrication of the microfluidic structure involves several steps such as the hard mask fabrication, the master mould fabrication and the production of the (Polydimethylsiloxane) PDMS structures. This concludes with the sealing of the electrodes.

The components of the hard mask of this structure are PDMS, which is a transparent substrate, and the previously structured ITO layer. The hard mask was fabricated using photolithography and etching (figure 5).

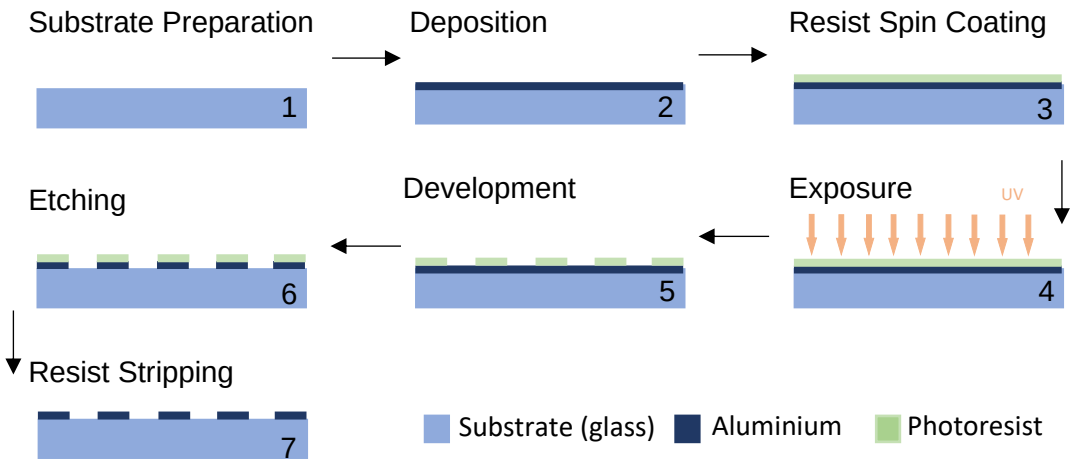


Figure 5 - Schematic overview of the fabrication of the hard mark including substrate preparation, deposition, lithography, etching, and resist removal.

Using the cleaned substrate the hard mask fabrication had the following steps:

- Substrate preparation (clean the substrate by rinsing with acetone followed by rinse with IPA, immersion in Alconox bath at 65º C for 30 min and then rinsing with DI Water and blowing with compressed air until dry)
- Deposition of a 200 nm layer of aluminium in the Nordiko 7000
- Resist Spin Coating of a 1,5 µm layer using the SVG track and soft bake for 60s at 85ºC
- Structure the resist using the DWL lithograph and then hard bake for 60 s at 110 ºC.
- Development for 60s
- Etching with an aluminium etchant so that the aluminium structures are dissolved
- Resist Stripping using acetone and IPA

The AutoCAD design of the microfluidic PDMS structure is shown in figure 6.

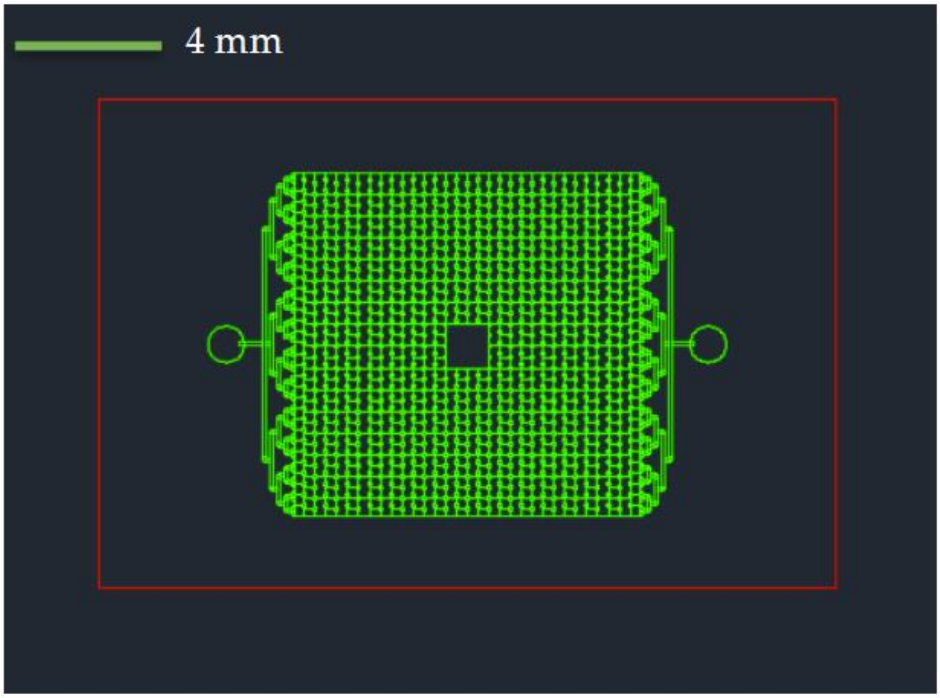


Figure 6 - CAD design of the microfluidic structure (green) and the border of the PDMS (red).

The master mould for this structure was fabricated using photolithography and followed the steps (figure 7):

- Substrate preparation by cleaning a silicon substrate first rinsing with IPA followed by rinsing with DI Water, then immersing in an Alconox bath at 65ºC for 15 min and afterward rinsing with DI Water and drying with compressed air after which the substrate undergoes UVO surface treatment.
- Resist spin coating with a 20 µm layer of SU-8 negative photoresist and soft bake of 4 min at 95º.
- Exposure for 30s using UV light and hard bake for 5 min at 95ºC.
- Development carried out between 2 and 5 min until all the non-polymerised resist was washed away.

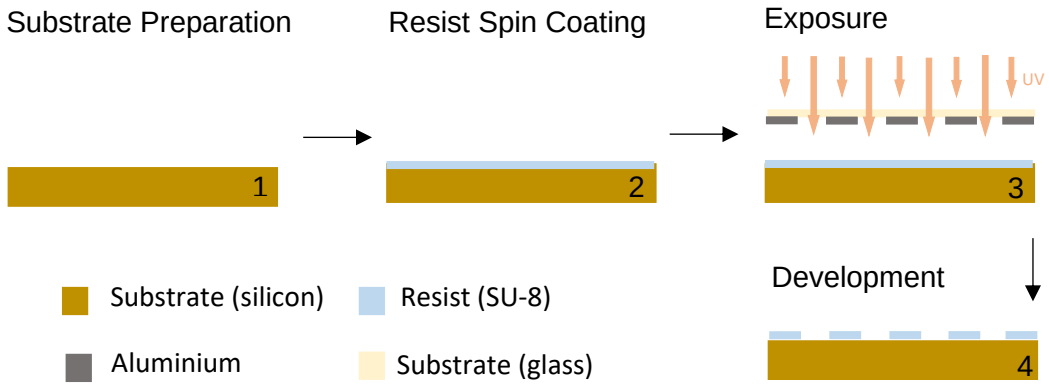


Figure 7 - Schematic of the master mould fabrication including substrate preparation and lithography.

The final microfabrication step is the PDMS structure fabrication. It is important that the microchamber structure is even in order to allow optimal cell growth and flow within the channels. The fabrication of the PDMS structures has the following steps:

Preparation of the PDMS, in which the curing agent and the PDMS were mixed with a 1:10 proportion and then put into the desiccator for 40 min.

The previously fabricated master mould is then placed in a frame and is fixed using Kapton tape. When the PDMS and curing agent mixture is degassed it is taken out of the desiccator an then poured into the mould until it overflows to make sure no bubbles are created when placing another frame on top, which is then kept in place with the help of some clamps.

The structures of the two frames goes into the oven for 90 min at 70ºC after which the frames are demounted and the PDMS is peeled off the master mould.

After the PDMS structure is made it is bonded to a glass structure. For this to occur both the PDMS structure and the glass are exposed to an oxygen plasma and a layer of hydroxyl groups are formed on the surface of the glass and silanol groups are exposed on the PDMS and when brought in contact permanent Si-O-Si bonds are formed.

The required equipment, materials and reagents used in the fabrication of the hard mask, the master mould and the PDMS structures is detailed in table 2.

Table 2 - Equipment, materials and methods used in the fabrication of the microfluidic structure

Hard Mask	**Equipment**	AutoCAD software, Autodesk Inc. (Mill Valley, CA/USA) Automatic dicing SAW DAD-321, Disco Corporatio (Tokyo, JP) DWL lithograph, Heidelberg Instruments (Heidelberg, DE) Kerry ultrasonic cleaning bath, Guyson (Skipton, North Yorkshire, UK) Nordiko 7000 magnetron sputtering system, Nordiko Technical Services Ltd (Havant, Hampshire, UK) SVG resist coater and developer track, Silicon Valley Group Inc. (San Jose, _CA/USA)
	Materials	Glass substrate, Corning Inc (Corning, NY/USA)
	Reagents	Acetone (99,6%), LabChem Inc. (Zelienople, PA/USA) Alconox solution, Alconox Inc. (White Plains, NY/USA) Aluminium etchant TechniEtch A180, Microchemicals (Ulm, DE) Deionized (DI) water Isopropanol (IPA), (99,9%), LabChem Inc. (Zelienople, PA/USA)

		Photoresist PFR 7790G, JSR (Sunnyvale, CA/USA)
Master Mould	**Equipment**	Hotplate, Stuart (Stafforshine, UK) Spinner, Laurel Corp. (North Wales, PA/USA) UV light (254 nm, 400 W), UV Light Technology Limited (Birmingham, UK) Vertical laminar airflow cabinet, FASTER-BSC-EN (Cornaredo, IT)
	Materials	Silicon wafer (150 mm diameter), University Wafer (South Boston, MA/USA)
	Reagents	Alconox solution, Alconox Inc. (White Plains, NY/USA) Deionized (DI) water Isopropanol (IPA) (99,9%), LabChem Inc. (Zelienople, PA/USA) Propylene glycol ether acetate (PGMEA) (99,5%), Sigma-Aldrich (St. Louis, _MO/USA) SU-8 2015 photoresist, Microchem Corp. (Newton, MA/USA)
PDMS Structures	**Equipment**	Analytical scale d=0.0001 g, Scientech (Bradford, MA/USA) Oven loading model 100-800, Memmert (Schwabach, DE) Vacuum desiccator, Bel-Art Products (South Wayre, NJ/USA)
	Materials	Moulding frames Tubing Plug SP22/12, Instech Laboratories Inc. (Plymouth Meeting, PA/USA)
	Reagents	Sylgard 184 PDMS and curing agent KIT, Dow Corning (Midland, MI/USA)
Sealing	**Equipment**	Expanded oxygen plasma cleaner PDC-002-CE, Harrick Plasma (Ithaca, NY/USA)

2.3 Integrated Systems

In order to be able to perform the impedance measurements the integrated microchip needs to be electrically connected to a measuring platform, In this case it was a printed circuit board (PCB) that was designed for the fabricated electrodes and taking into consideration the necessity of being able to fit into a microscope for scanning the whole microchip and to connect to the measuring instrument through SMA cables with SMA connector pins that were later added to the PCB. In most cases copper was added between the metal pins and the PCB to enhance the connection. The microelectrodes were wire bonded to the PCB and a multimeter was used to verify the connection the gold pads of the PBC that is connected to the electrodes and the connectors. (figure 8).

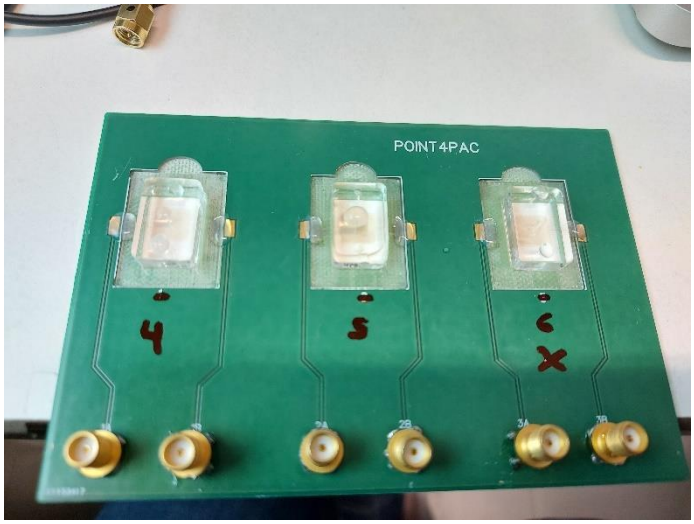


Figure 8 - Printed circuit board required for contacting the microchip electrically and contacting it to a measurement instrument.

The equipment and material used are detailed in table 3.

Table 3 - Equipment, materials and methods used in the fabrication of the integrated systems

Equipment	Wedge Bonder Model 4123, Kulicke and Soffa (Singapore)
Materials	Customised Printed Circuit Board SMA Cables and Connectors Soldering Iron set, Weller Tools GmbH (Besigheim, DE) Double Sided Tape, Scotch Brand (Maplewood, MN/USA) Microchip 30 µm Aluminium Wedge Bonding Wire, Heraeus Holding GmbH (Hanau, DE) Microfabricated microchip RTV-1 Silicone Rubber E41, Wacker Chemie Ag (Munchen, DE) Insulin single use syringe 1 mL U-100 Luer-Lock, CODAN Medizinische Gerate GmbH & Co KG (Lensahn, DE) Luer stubs blunt needles LS22K, Instech Laboratories, Inc. (Plymouth Meeting, PA/USA)

2.4 Experiments with Cells

The measurement of impedance of cells was conducted after cells were cultured in the fabricated microchips, in which they grew and multiplied and then injected with phages. The cells used were from colorectal carcinoma (CRC cells) from the HCT 116 cell line and are immortal and adherent.

Cryopreservation is used to maintain the cell lines. As a result, before the impedance measurement experiments can take place the cells must be passed periodically to be maintained viable. Aseptic techniques are then used to prepare the cells to be inserted in the microfluidic chamber, that is sterilized with UV light for 30 min prior to the cell's insertion.

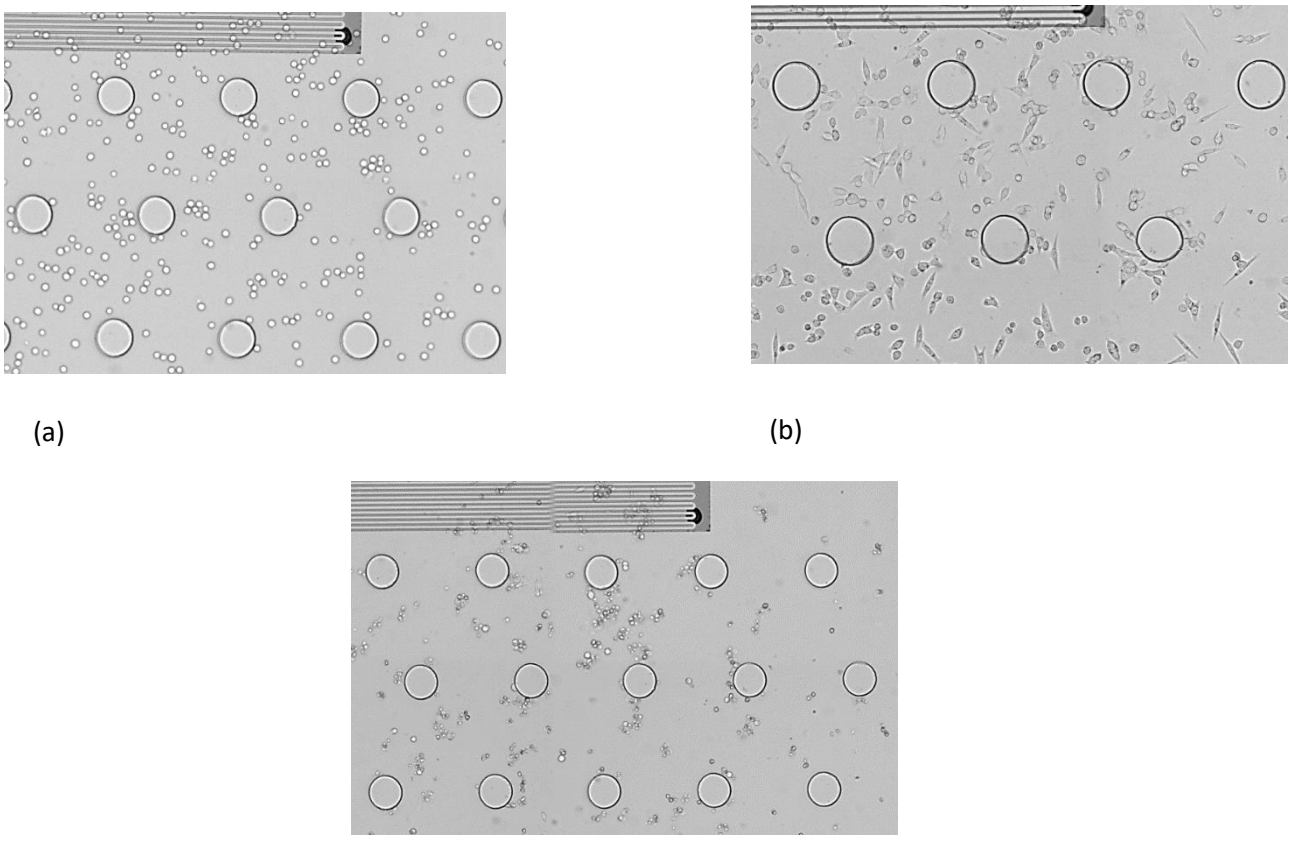
However, before the cells are inserted into the microchip, the following procedure happens:

- Inspection of the culture flasks with a microscope
- Removal of the serum that was supplemented to the cells with a pipettor (serum was composed of 10% heat-inactivated fetal bovine serum and 1% antibiotic/antimycotic solution)
- Wash the cells several times with the removed medium to remove unattached cells from the flask as well as 1mL of trypsin, with the latter being done in an incubator at 37ºC to activate the trypsin which is then inactivated when 3 mL of culture medium is added.
- Centrifuging of the trypsin, cell and media mixture at 300 xg for 5 min, with the cells settling at the bottom of the tube
-

When the cells are ready to be used the cell impedance measurement experiments abide by the following procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator. After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically (figure 9).

- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Injection of the phages for with a flow rate of 4 µL/min for 1h followed by the impedance measurement. During the injection of the phages, a fluorescence microscope captures pictures of the entire chip.
- Flow of the culture medium at 4 µL/min for 1h to remove the excess of phages followed by the impedance measurement. During the wash, a fluorescence microscope captures pictures of the entire chip.
- Flow of 10 µL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed
- Flow of culture medium with a flow rate of 10 µL/min for 5 min followed by the impedance measurement
- Flow of 10 µL/min for 5 min of DI Water followed by the impedance measurement



(a) (b)

(c)

Figure 9 - Cell culture in microelectrode at (a) 0h, (b) 24h, (c) 48h

The insertion of the solutions that were flown in the microfluidic channel as well as the cells were inserted using a syringe coupled with tubing and a pump that monitored the flow rate as well as the time. The syringe was placed carefully in the inlets of the microchamber.

The measurement of culture medium and DI Water after the removal of the cells serve as a means to monitor the behaviour the these solutions and the end of the experiment by comparing these measurements with the ones made before inserting the cells, as well as being indicators if the washing of the microchip, particularly the measurement of the DI Water at the begging and the end.

In two of the runs of these experiments the outflows of the medium that is continuously provided after the seeding period were collect (from different time periods, such as overnight and before the measurements at 24h and 48h) and later the impedance was measured. This happen to study the effects of cell growth reflected with the culture medium and its proteins produced during the cell growth.

The measurements of impedance are made with and LCR meter and using a python script previously developed. Before any measurement could take place, a correction was performed with a metal object and the LCR could not be turned off until the last measurement was made several days later to maintain the measurements relative to the same correction. The impedance measurements are run at 201 frequency points increasing from 20 Hz to $2x10^6$ Hz.

The equipment, materials and reagents used in the cell culture experiments is detailed in the table 4.

Table 4 - Equipment, materials and methods used in cell culture experiments

Cell culture	**Equipment**	Class II Type A2 Biological Safety Cabinet, Esco Micro Pte. Ltd. (Singapore) HERACELL 150i CO2 Incubator, Thermo Fisher Scientific (Waltham, MA/USA) Centrifuge 5810 R, Eppendorf AG (Hamburg, DE) Evos FL Auto 2 Brightfield Microscope, Invitrogen Thermo Fisher Scientific (Carlsbad, CA/USA)
	Materials	T-25 Culture Flask, Corning (Bedford, MA/USA) Centrifuge tube 50 mL and 15 mL, VWR International (Radnor, PA/USA)

		1,5 mL Eppendorf tube 3810X, Eppendorf AG (Hamburg, DE) Pipetman12 classic P1000, P100 and P20, Gilson Incorporated (Middleton, WI/USA) Macroman Pipette Controller, Gilson Incorporated (Middleton, WI/USA) 5 mL Serological Pipette Individually wrapped, Gilson Incorporated (Middleton,WI/USA) Haemocytometer Neubauer Improved Counting Chamber 0,0025 mm^2, BrandGmbH + Co Kg (Wertheim, DE) Cover glass 20x20 mm, Hirschmann Laborgerate (Eberstadt, DE) Cell lines HCT116, LGC Standards (Middlesex, UK)
	Reagents	Ethanol (99,9%), Merck KGaA (Darmstadt, DE) Heat-inactivated Fetal Bovine Serum, Gibco Thermo Fisher Scientific (Paisley , UK) Antibiotic Antimycotic Solution 100X, Corning (Bedford, MA/USA) Trypsin 0.25 % (1x), HyClone Laboratories (Logan, UT/USA) Trypan Blue solution, Sigma-Aldrich (St. Louis, MO/USA) McCoy’s 5A medium, Gibco Thermo Fisher Scientific (Paisley , UK)
Cell preparation	**Equipment**	Class II Type A2 Biological Safety Cabinet, Esco Micro Pte. Ltd. (Singapore) HERACELL 150i CO2 Incubator, Thermo Fisher Scientific (Waltham, MA/USA) Evos FL Auto 2 Brightfield Microscope, Invitrogen Thermo Fisher Scientific (Carlsbad, CA/USA)
	Materials	12 channel Syringe pump NE-1200, New Era Pump Systems, Inc. (Farmingdale, NY/USA) Polyethylene tubing BTPE-90, Instech Laboratories, Inc. (Plymouth Meeting, PA/USA)

		Tubing Coupler SC20/15, Instech Laboratories Inc. (Plymouth Meeting, PA/USA) Luer stubs blunt needles LS20K, Instech Laboratories, Inc. (Plymouth Meeting, PA/USA) Pipetman classic P1000, Gilson Incorporated (Middleton, WI/USA) Silicone adhesive film for PCR Plates, VWR International (Radnor, PA/USA)
	Reagents	Prepared cell suspension (4 million cells per 1 mL) Ethanol (99,9%), Merck KGaA (Darmstadt, DE) Insulin single use syringe 1 mL U-100 Luer-Lock, CODAN Medizinische Gerate GmbH & Co KG (Lensahn, DE) Phosphate buffered saline in tablet form: 137 mM NaCl + 2,7 mM KCl + 2 mM KH_2PO_4 (pH 7,4 @ 25 °C), Sigma-Aldrich (St. Louis, MO/USA) McCoy’s 5A medium, Gibco Thermo Fisher Scientific (Paisley , UK) Heat-inactivated Fetal Bovine Serum, Gibco Thermo Fisher Scientific (Paisley , UK) Antibiotic Antimycotic Solution 100X, Corning (Bedford, MA/USA) Collagen I, Rat Tail, 100mg, Corning (Bedford, MA/USA)
Measurement	**Equipment**	E4980A/AL Precision LCR Meter, Keysight Technologies (Kobe-shi, Hyogo/Japan) Python programming language, Python software foundation (Wilmington, Evos FL Auto 2 Brightfield Microscope, Invitrogen Thermo Fisher Scientific (Carlsbad, CA/USA)
	Materials	PCB with chip with cell culture Insulin single use syringe 1 mL U-100 Luer-Lock, CODAN Medizinische Gerate GmbH & Co KG (Lensahn, DE)

3. Results and Discussion

Before the experiments with cell could happen, it was necessary to understand the behaviour that more simple solutions have in regard to impedance as the main aspects of culturing HCT 116 on the microchip was already known from previous works. As a result, solutions with increasing complexity and concentration were studied.

3.1 NaCl and PBS

As a means to establish a baseline of impedance, the first solutions to be measured were DI Water as well as PBS and NaCl with increasing concentrations (figure 10).

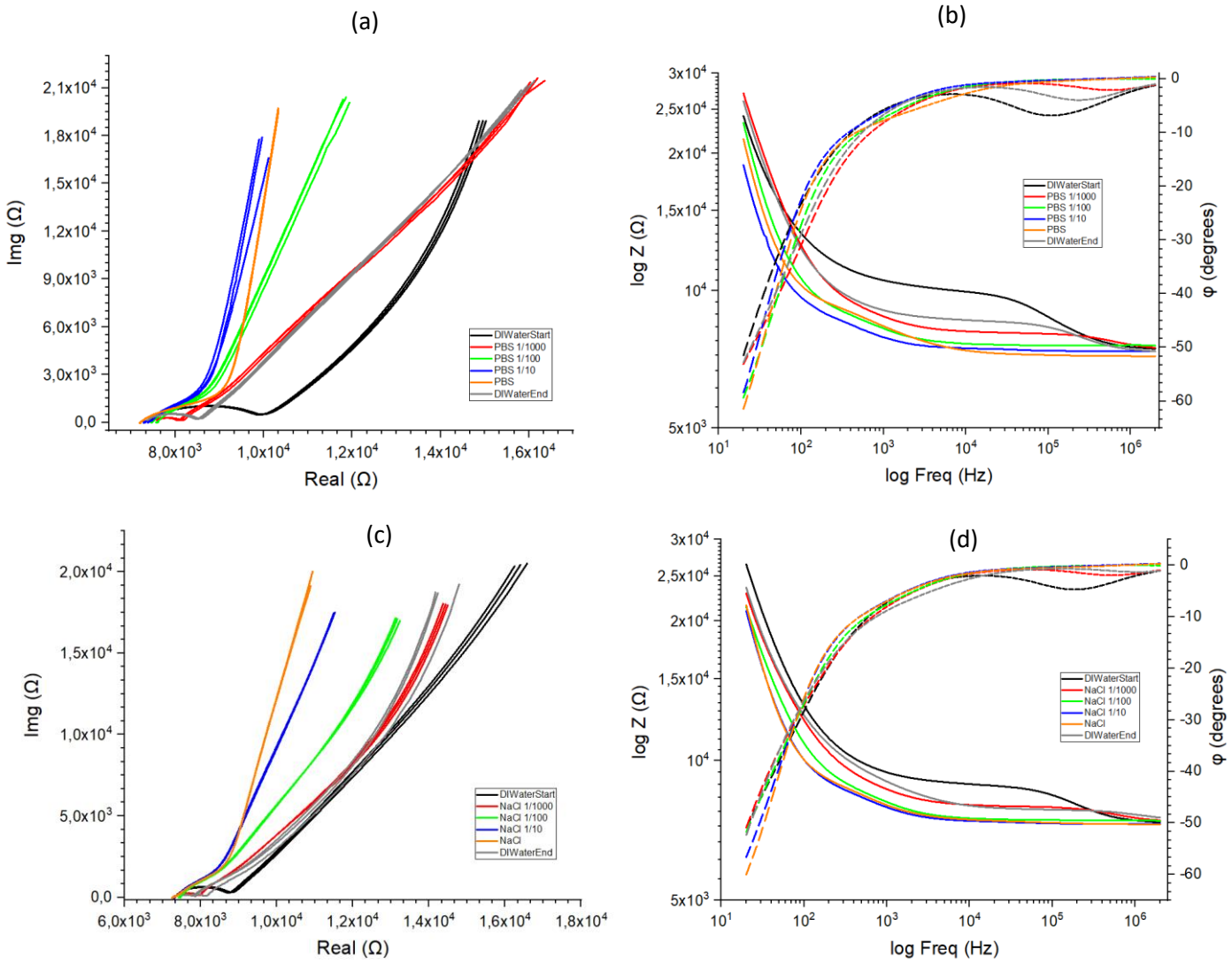


Figure 10 - (a) Nyquist plot for solutions of PBS, (b) Bode plots for solutions of PBS, (c) Nyquist plot for solutions of NaCl, (d) Bode plots for solutions of NaCl

Table 5 - Resistance of solution and charge transfer of solutions of PBS

	DIWaterStart	PBS 1/1000	PBS 1/100	PBS 1/10	PBS	DIWaterEnd
Rsol (Ω)	7449	7525	7448	7320	7208	7364
Rct (Ω)	2541	555	2152	2311	2946	1144

Table 6 - Resistance of solution and charge transfer of solutions of NaCl

	DIWaterStart	NaCl 1/1000	NaCl 1/100	NaCl 1/10	NaCl	DIWaterEnd
Rsol (Ω)	7364	7439	7440	7305	7266	7601
Rct (Ω)	1085	572	2086	2284	2492	357

By looking into the Nyquist plots, at first glance we are able to conclude that the resistances of both solutions is fairly similar and by looking into the Bode plots we are able to see that the phase and the modulus of the impedance have the same range in both the salt and the PBS.

With further analysis of the Nyquist plots, we are able to obtain the values of the resistance of solution as well as the resistance of the charge transfer. In both cases, the resistance of the solution decreases with the increase of concentration whereas in the case of the resistance of the charge transfer the opposite happens, as the value increases as the concentration does so as well. This happens because with higher concentration of the same solution we have more free charges and therefore more resistance due to the increase of the number of molecule complexes that are formed. On the other hand, the more charges mean that the solution is less resistant (tables 5 and 6).

3.2 E.-coli and IgG

After having an understanding of the behaviour of the impedance of PBS and NaCl, the next step was to measure the effects of solid substances in solutions of which the behaviour of impedance was already known, DI Water, and in the culture medium that would be used for the cell cultures in the experiments with cells. For this to happen, solutions of bacterial cells, E.-coli, and proteins, IgG, were prepared with different concentrations.

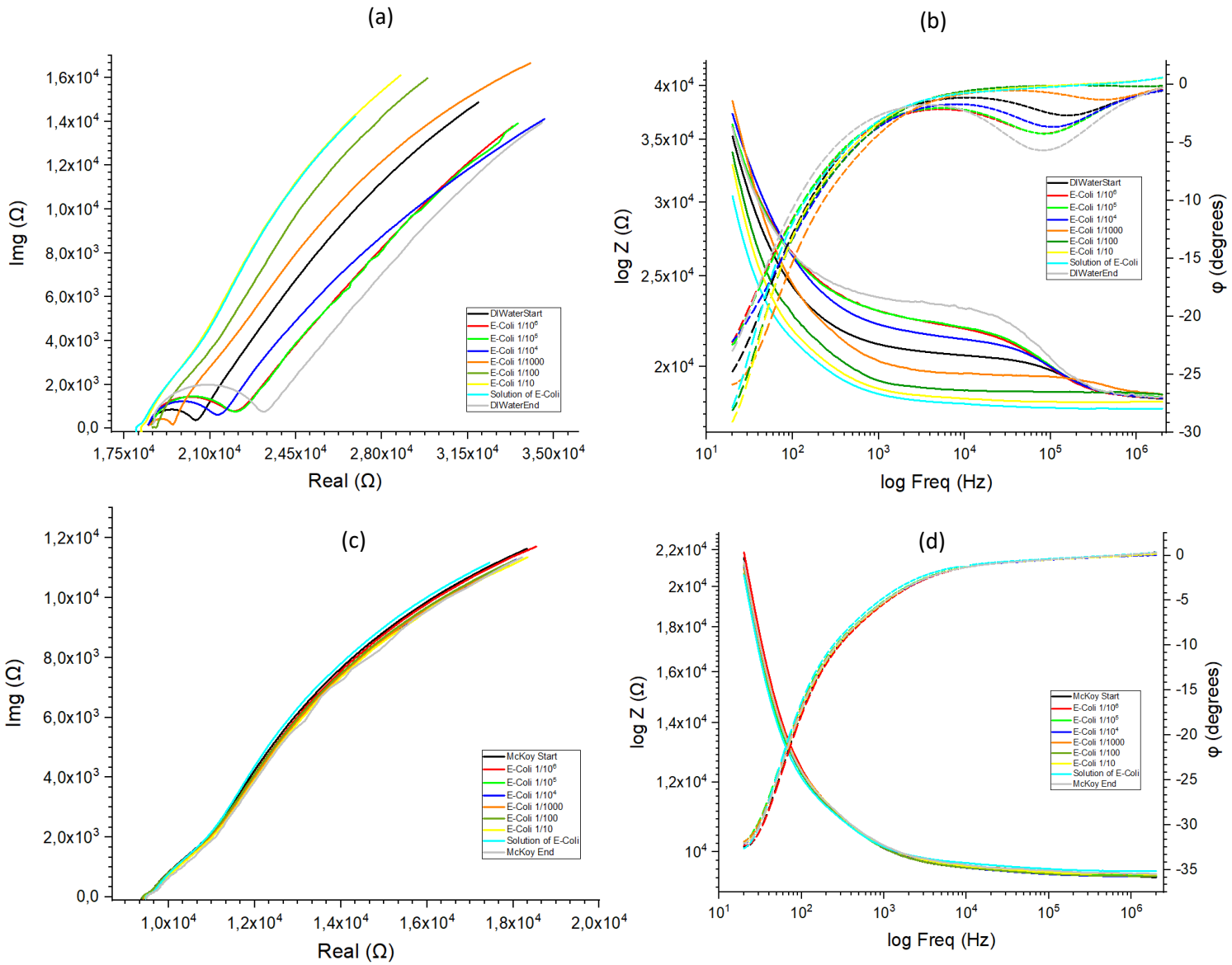


Figure 11 - (a) Nyquist plot for solutions of E.-coli in DI Water, (b) Bode plots for solutions of E.-coli in DI Water, (c) Nyquist plot for solutions of E.-coli in culture medium, (d) Bode plots for solutions of E.-coli in culture medium

From figure 11 both from the Nyquist plot and the Bode plot, a clear distinction of the different concentration of E.-coli can be had in the lower concentrations. However with solutions of higher concentration, such as the solution of 1:10 ratio of E.-coli solution and DI Water and the stock solution of E.-coli itself there is little difference in behaviour and values of resistance. This is even more evident in figure, that shows the Nyquist and Bode plots for the experiments made with different concentrations of E.-coli and culture medium. This happens because the solution of culture medium itself is more prevalent than the cells in the solution, and as a result is the substance of which the impedance is being measured.

By further analysing both the Nyquist plot and the Bode plots for the solution of E.-coli in culture medium, the values a few impedance parameters are found such as the resistance of the solution and charge transfer, as well as the capacitance of the double layer and both the transition zone and the semi-infinite diffusion Warburg impedances (Appendix A). However, no distinct pattern emerges in any of these parameters, despite the fact that in the solutions of E.-coli in DI Water a pattern emerged in some of the parameters such as the resistance of charge transfer. This then resulted in the decision to look into different parameters that could simulate this pattern, like the value of the modulus of impedance at 5000 Hz (by analysing the Bode plot) and the value of the real component of impedance when the imaginary component is fixed at 6000 Ohm (by analysing the Nyquist plot).These, however, proved just as inefficient at characterising the E.-coli solutions in culture medium as the other parameters did.

As mentioned before, this experiment was also conducted with IgG with different concentrations in solutions of DI Water and culture medium, and had the same results as the experiments with E.-coli and even more so due to the fact that the relative size of each protein is smaller than that of a cell and therefore had even less impact on the measurements made.

3.3 CRC Cells

As reference in section 2.2 of this work, the experiments with cells occurred in a duration of roughly 48h and several impedance measurements were made before, during and after cell culture. Five runs of experiments were conducted to varying degrees of success and slight alterations to the protocol. However all were conclusive in the fact that cell growth monitoring and detection are not detectable using these microelectrodes and culture conditions (Appendix B).

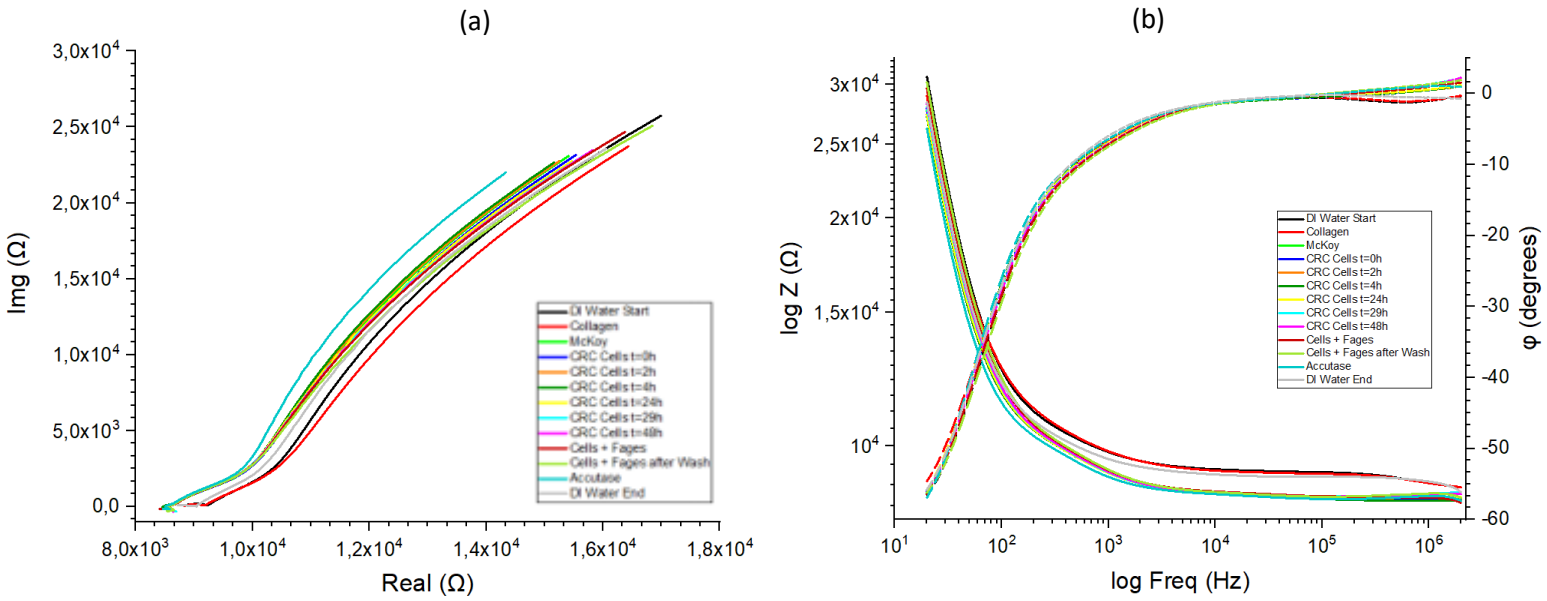


Figure 12 - (a) Nyquist plot of full cell culture experiments, (b) Bode plots of full cell culture experiments

In figure 12 are the results of an experiment in with the cell culture was monitored at 2h during seeding, at 4h after seeding, at 24h after insertion of the cells in the microchamber, at 29h of cell culture and at 48h, at the end of the cell culture.

As observed by the Nyquist plot and the Bode plots, there is very little difference between any of the measurements during the cell culture, regardless of the time, and the behaviour of the culture medium. This notion is further enhanced when looking into the parameters of impedance established before. However, for the cell experiments, another parameter was had in consideration.

$$Normalized\ Impedance = \frac{|Z|_{Cells} - |Z|_{McKoy}}{|Z|_{McKoy}} \quad (8)$$

With this parameter of normalized impedance, a further look into the impedance of cells was gained as the interference of the culture medium was taken away (figure 13).

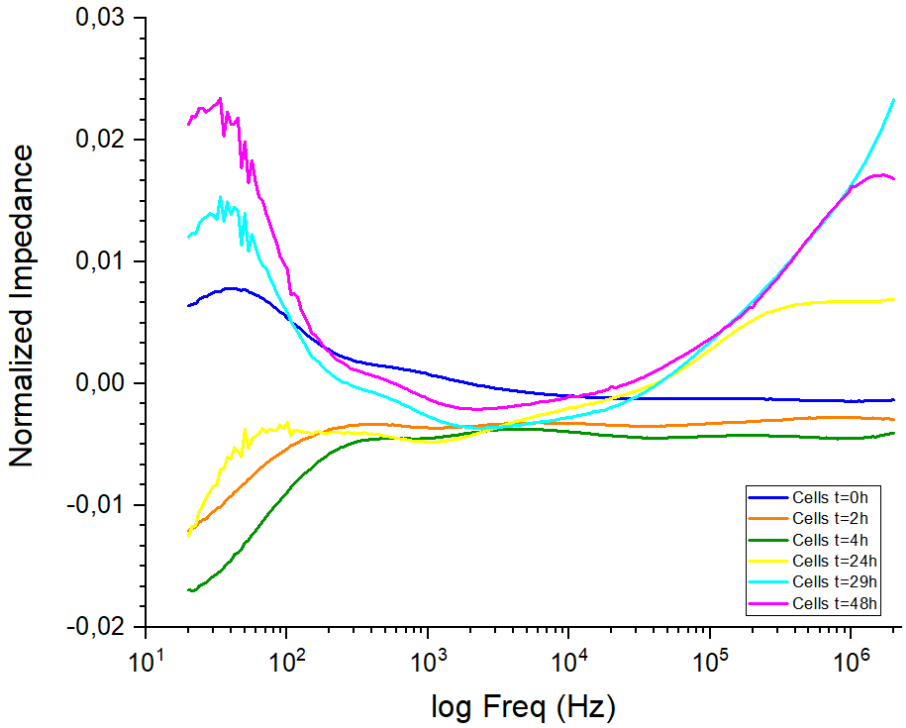


Figure 13 - Normalized Impedance [16]

As a result, when more experiments were conducted, the outflows of the culture medium that was being fed to the cell chips was collected during different time periods, such as overnight during the 4h after insertion of the cells in the microchamber and 22h, from 22h to 24h, overnight during the period between 24h and 46h and the last two hours of cell culture between 46h to 48h. (figure 14) These outflows later had their impedance measured, and as in any experiment Di Water and culture medium served as reference, before and after. In between measurements of outflows, DI water was flown into the channel to remove any interference from previous solutions.

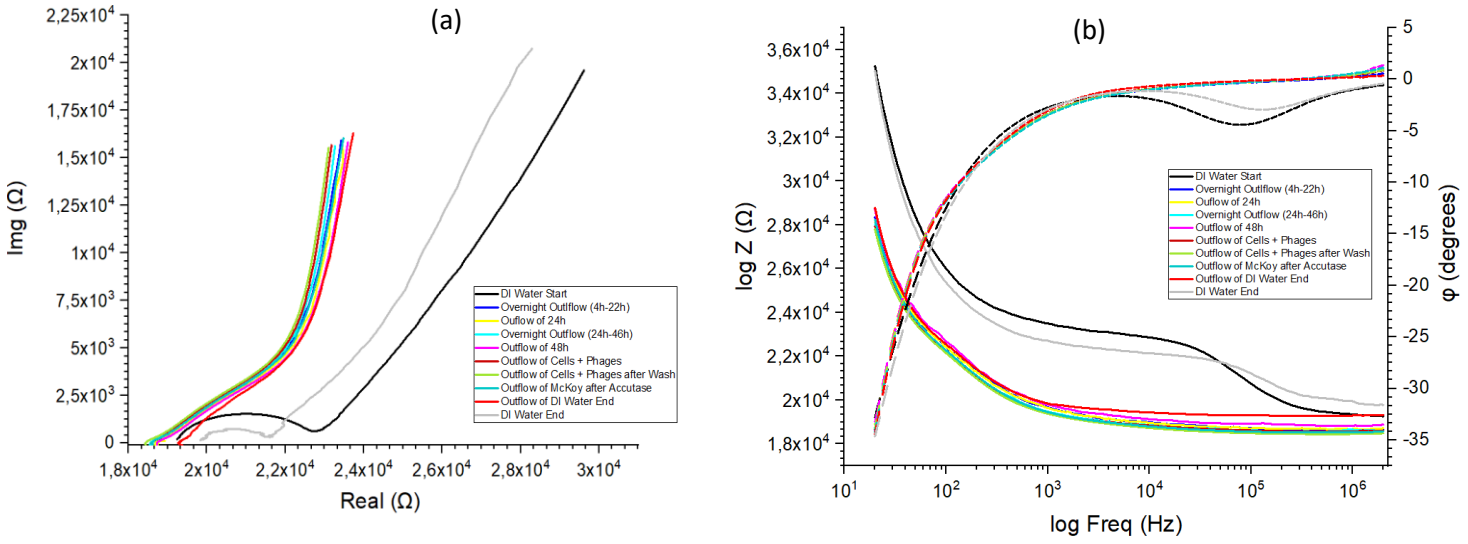


Figure 14 - (a) Nyquist plot outflows of cell experiments, (b) Bode plots outflows of cell experiments

Has can be observed in figure 13, there is very little difference in the behaviour of the impedance of the outflows, regardless of the time and duration of the outflow.

As mentioned before, the protocol of these experiments varied slightly from run to run, with sometimes more time points during cell culture being measured or increasing the concentration of cells or even not flowing accutase or injecting phages into the microchip (Appendix C). However all the results were inconclusive as to monitorization of cell growth, proliferation, and detection.

4. Conclusions and Future Prospects

4.1 Results

With this work, the focus was to be able to detect cell growth and proliferation and detection with impedance spectroscopy.

For this purpose, an interdigitated ITO microelectrode was used for the cell culture and subsequent impedance measurements.

In order to achieve this, the first step was to study and monitor the impedance behaviour of less complex and concentrated solutions. By monitoring DI Water and solutions of PBS and NaCl we were able to grasp the effects that different concentrations and solutions had when the impedimetric measurement was taken.

Also by studying the impedance measurements of solutions such as solutions of E.-coli and IgG we were able to ascertain how the presence of solids affected the impedance of the previously measured solutions, as a means to understand how the cell culture experiments would behave.

Cell culture inside the microchip and cell removal were possible. However, the microelectrodes that were fabricated and had been used previously and achieved good results for cell culture did not have the initial aim of impedance spectroscopy experiments and therefore the microfluidic device was not the most optimised for this end.

4.2 Outlook

As previously stated, the end goal of this project was not achieved as initially foreseen.

Factors that may have contributed to this situation and that can be further studied are, for example:

- The size of the chamber, that in this electrodes was rather square and may not have allowed for the uniform distribution of the cells inside as they were inserted in as inlet in the middle of the chamber and created preferential paths to adhere, so a straightened chamber may eliminate some of the uniformity in cell distribution;
- The material of the electrode is probably a contributing factor as to the fact that the range of impedance measured is somewhat low, as it allows to have reading of low concentration solution however when this values increases or solid substances are in the solution (i.e. cells) the results plateaux to a certain value, and to improve this a more conductive metal may be chosen for future experiments;
- The fact that the inlets and outlets of the microchamber and the posts of the microfluidic device interfere with the electrode.
- Another factor that may improve the results is a gelling of the 3D culture as to have more electrical stability.

When the cell culture experiments are successful in identifying the several stages of cell proliferation and growth as well as removal, only certain frequencies should be monitored as to be able to administer different types of drugs and successfully identify each and every one by their impedance measurements thus creating and effective drug screening tool.

5. References

[1] O. Pänke, T. Balkenhohl, J. Kafka, D. Schäfer, and F. Lisdat, “Impedance spectroscopy and biosensing,” Adv. Biochem. Eng. Biotechnol., vol. 109, pp. 195–237, Nov. 2007.

[2] P. Boškoski, A. Debenjak, and B. Mileva Boshkoska, “Fast Electrochemical Impedance Spectroscopy,” in SpringerBriefs in Applied Sciences and Technology, no. 9783319533896, 2017, pp. 9–22.

[3] “Basics of EIS: Electrochemical Research-Impedance.” [Online]. Available: https://www.gamry.com/application-notes/EIS/basics-of-electrochemical-impedance spectroscopy/.

[4] H. Andersson and A. Van den Berg, “Microfluidic devices for cellomics: A review,” Sensors Actuators, B Chem., vol. 92, no. 3, pp. 315–325, 2003

[5] R. Gómez-Sjöberg, A. A. Leyrat, D. M. Pirone, C. S. Chen, and S. R. Quake, “Versatile, fully automated, microfluidic cell culture system,” Anal. Chem., vol. 79, no. 22, pp. 8557–8563, 2007

[6] “Microfluidics? A general overview.” [Online]. Available: https://www.elveflow.com/microfluidic-tutorials/microfluidic-reviews-and-tutorials/microfluidics/.

[7] G. J. Sommer et al., “Introduction to microfluidics,” in Microfluidics for Biological Applications, 2009, pp. 1–34

[8] Y. Xiao, B. Zhang, A. Hsieh, N. Thavandiran, C. Martin, and M. Radisic, “Microfluidic Cell Culture Techniques,” in Microfluidic Cell Culture Systems, Elsevier Inc., 2012, pp. 303–321.

[9] B. K. Gale et al., “A review of current methods in microfluidic device fabrication and future commercialization prospects,” Inventions, vol. 3, no. 3, 2018

[10] “PDMS: A review - Elveflow.” [Online]. Available: https://www.elveflow.com/microfluidic-tutorials/microfluidic-reviews-and-tutorials/the-poly-di-methyl-siloxane-pdms-and-microfluidics/.

[11] Picó, Yolanda, ed. Chemical analysis of food: Techniques and applications. Academic Press, 2012, chapter 7

[12] M. L. Coluccio et al., “Microfluidic platforms for cell cultures and investigations,” Microelectron. Eng., vol. 208, pp. 14–28, 2019.

[13] I. O. K'Owino and O. A. Sadik, "Impedance spectroscopy: A powerful tool for rapid biomolecular screening and cell culture monitoring," Electroanalysis, vol. 17, no. 23. pp. 2101–2113, Dec-2005.

[14] M. L. Coluccio et al, "Microfluidic platforms for cell cultures and investigations", Microelectronic Engineering, no. 208, pp 14-28, Jan-2019

[15] S. Noh and H. Kim, "In-air EIS sensor for in situ and real-time monitoring of in vitro epithelial cells under air exposure", Royal Society of Chemistry, Mar-2020

[16] F. Zhang, et al," Distinguishing skin cancer cells and normal cells using electrical impedance spectroscopy". Journal of Electroanalytical Chemistry, Jun-2018

[17] D. A. Hook, J. A. Olhausen, J. Krim, and M. T. Dugger, "Evaluation of oxygen plasma and UV ozone methods for cleaning of occluded areas in MEMS devices," J. Microelectromechanical Syst., vol. 19, no. 6, pp. 1292–1298, 2010

[18] MicroChemicals, "Baking Steps in Photoresists Processing," 2010. [Online]. Available: www.microchemicals.com/downloads/application_notes.html

Appendix

Appendix A

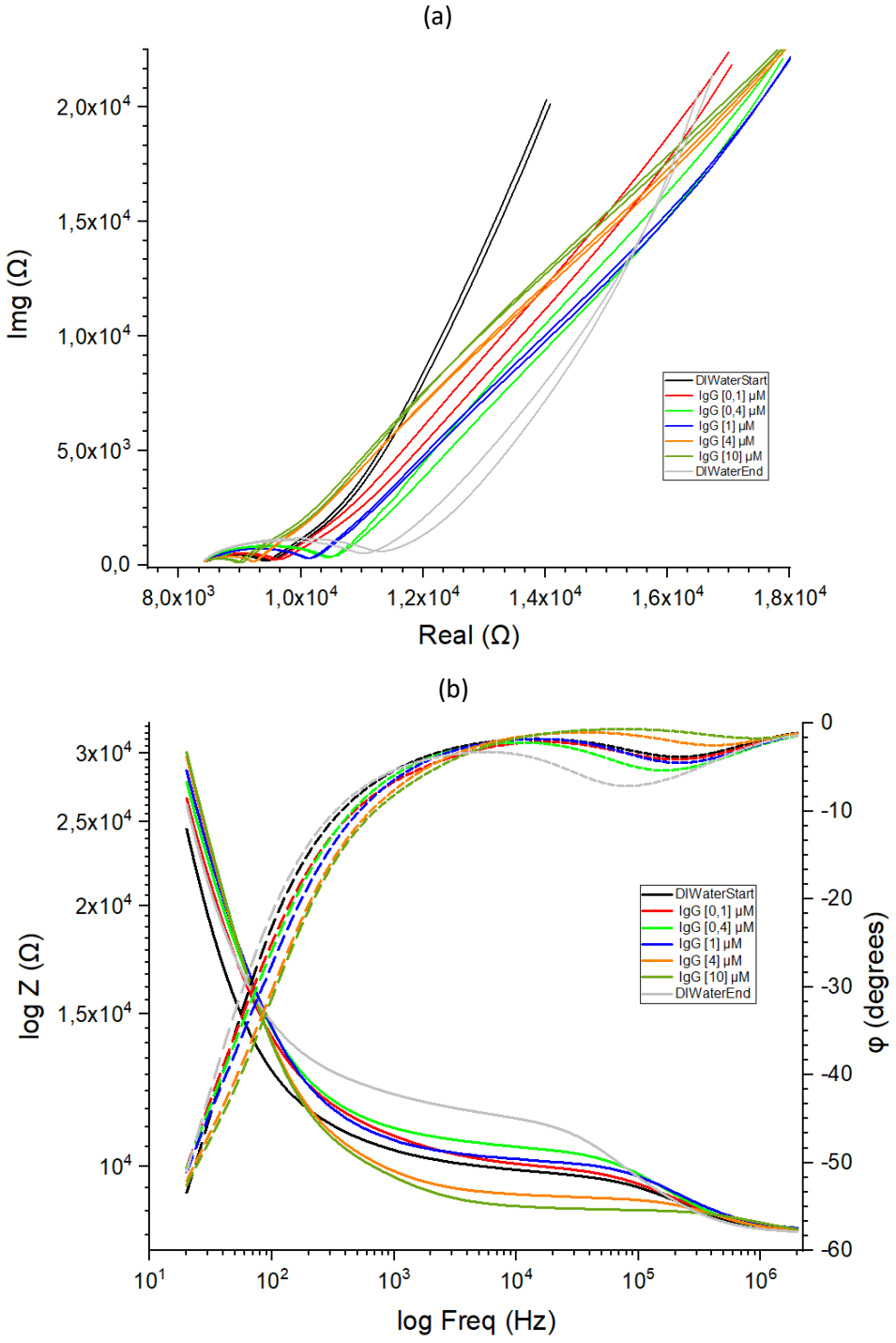


Figure 15 - (a) Nyquist plot of solutions of IgG, (b) Bode plots of solutions of IgG

Appendix B

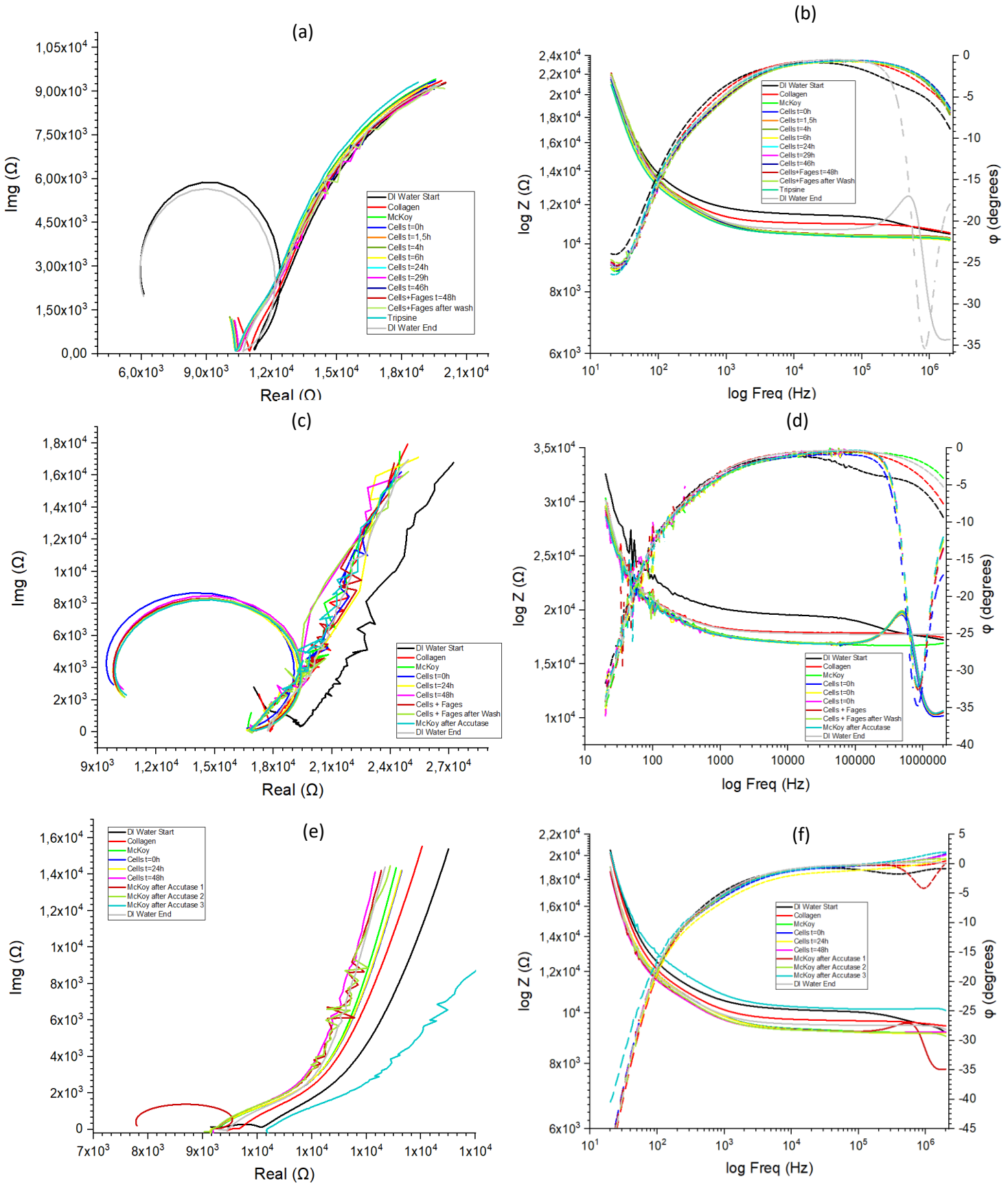


Figure 16 - (a) and (b) Nyqust and Bode plots, respectively of 1st run of experiments, (c) and (d) Nyquist and Bode plots from 3rd expeirments, (e) and (f) Nyquist and Bode plots from 4th experiments in wich the chip was washed three times with culture medium and had the iimpedance measured each time

Appendix C

The 1st Experiments with cells followed the procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator and an impedance measurement occurs after 2h and at the end of the seeding process (4h after the cell insertion). After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically.
- Monitoring of the cells at the microscope 6h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 29h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 46h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Injection of the phages for with a flow rate of 4 μL/min for 1h followed by the impedance measurement. During the injection of the phages, a fluorescence microscope captures pictures of the entire chip.
- Flow of the culture medium at 4 μL/min for 1h to remove the excess of phages followed by the impedance measurement. During the wash, a fluorescence microscope captures pictures of the entire chip.
- Flow of 10 μL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed

- Flow of culture medium with a flow rate of 10 µL/min for 5 min followed by the impedance measurement
- Flow of 10 µL/min for 5 min of DI Water followed by the impedance measurement

The 2nd Experiments with cells followed the procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator and an impedance measurement occurs after 2h and at the end of the seeding process (4h after the cell insertion). After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically
- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 29h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Injection of the phages for with a flow rate of 4 μL/min for 1h followed by the impedance measurement. During the injection of the phages, a fluorescence microscope captures pictures of the entire chip.
- Flow of the culture medium at 4 μL/min for 1h to remove the excess of phages followed by the impedance measurement. During the wash, a fluorescence microscope captures pictures of the entire chip.
- Flow of 10 μL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed
- Flow of culture medium with a flow rate of 10 μL/min for 5 min followed by the impedance measurement
- Flow of 10 μL/min for 5 min of DI Water followed by the impedance measurement

The 3rd Experiments with cells followed the procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator. After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically.
- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Injection of the phages for with a flow rate of 4 μL/min for 1h followed by the impedance measurement. During the injection of the phages, a fluorescence microscope captures pictures of the entire chip.
- Flow of the culture medium at 4 μL/min for 1h to remove the excess of phages followed by the impedance measurement. During the wash, a fluorescence microscope captures pictures of the entire chip.
- Flow of 10 μL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed
- Flow of culture medium with a flow rate of 10 μL/min for 5 min followed by the impedance measurement
- Flow of 10 μL/min for 5 min of DI Water followed by the impedance measurement

The 4th Experiments with cells followed the procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator. After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically.
- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Flow of 10 μL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed. This occur only in half of the electrodes to observe the effects of the existence of cells in the channel.
- Flow of 10 μL/min for 5 min of culture medium followed by the impedance measurement. This procedure is repeated twice, once with the same flow rate and another with a flow of 40 μL/min for 5 min of culture medium.
- Flow of 10 μL/min for 5 min of DI Water followed by the impedance measurement

The 5th Experiments with cells followed the procedure:

- UV sterilization of the microchips
- Flow of 4 μL/min for 2 min ethanol to prevent the creation of bubbles within the channel of the microchip
- Flow of 4 μL/min for 5 min of DI Water followed by the measurement of impedance
- Flow of 4 μL/min for 2 min of collagen (0,3 mg/mL)
- Flow of 4 μL/min for 2 min of culture medium
- Insertion of the cells with a flow rate of 4 μL/min for 2 min proceeded by monitorization of the cells with a microscope which is followed by the impedance measurement. After the cells are inserted in the microchip, a period of seeding occurs and lasts for about 4 hours. During this stage the PCB with the microchips is put into an incubator. After the seeding period, medium is continuously fed through the inlet chambers of the microchip (at a flow rate of 0,2 μL/min) during 48h to provide the cells with the required nutrients and during this time, monitorization of the cell culture occurs periodically (figure 9).
- Monitoring of the cells at the microscope 24h after the insertion of the cells followed by the impedance measurement
- Monitoring of the cells at the microscope 48h after the insertion of the cells followed by the impedance measurement
- Flow of 10 μL/min for 5 min of accutase, a solution that contains collagenase and trypsin to break the collagen and peptide bonds formed by the cells. This step occurs inside the incubator with periodic monitoring of the microchip at the microscope until all cells have been removed. This occur only in half of the electrodes to observe the effects of the existence of cells in the channel.
- Flow of 10 μL/min for 2 min of culture medium followed by the impedance measurement. This procedure is repeated twice, once with the same flow rate and another with a flow of 40 μL/min for 2 min of culture medium.
- Flow of 10 μL/min for 5 min of DI Water followed by the impedance measurement