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Four Versus Two-Electrode Measurement Strategies for Cell Growing and Differentiation Monitoring Using Electrical Impedance Spectroscopy

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Abstract— **The aim of this work is to provide optimization tools for cell and tissue engineering processes through continuous monitoring of cell cultures. Structural cell properties can be obtained from non-destructive electrical measurements by using electrical impedance spectroscopy (EIS).**

EIS measurements on monolayer animal cell cultures are usually performed using a two-electrode strategy. Because of this, the measurement is very sensitive to the electrode covering ratio and to the degree of adherence of cells. Of course, these parameters give useful information but can mask the behaviour of the cell layer above the electrodes.

In a previous work, preliminary measurements with commercial microelectrode structures were performed with simulated grow processes using the settlement of cell suspensions with two and four microlectrode strategies to validate the technique. In this work, real cell growths of Vero cells are described and the resulting EIS biomass density estimators are compared to cell counts. The four-electrode impedance spectra are fitted to the Cole-Cole impedance model and the time course of their parameters are extracted and studied.

I. Introduction

The work we are presenting is a part of a research project on myocardium regeneration using biocompatible scaffolds colonized by cardiac progenitors obtained from the differentiation of stem-cells.

The various techniques used for the maintenance, manipulation and monitoring of stem-cell cultures are characterized, from an instrumental point of view, by the following aspects:

- the need of the biological material characterization in each stage of the process.
- the use of techniques usually destructive to carry out this characterization.
- as any biological manipulation, they involve an intrinsic uncertainty in the results, so repeated experiments must be performed to obtain statistical parameters.

These facts drive to the need of a support system for the systematic performance of cell cultures. In this work, we present the initial stages of the use of macroscopic measurement techniques which should permit non-destructive on-line monitoring of passive and active electrical properties of monolayer and three-dimensional cultures. In this work, we present the passive electrical properties measurement set-up based on Electrical Impedance Spectroscopy (EIS) and the first quantitative validation results.

Electrical impedance measurements on monolayer animal cell cultures are usually performed with a two-wire strategy by using microelectrodes placed at the bottom of the culture plates [1], [2], even for this same application [3]. In this way, the resulting measured impedance is very sensitive to the microelectrode surface covered by cells and to the adherence of those cells. These features are of great interest by themselves [4] but introduce a source of indetermination if the goal was to obtain a quantitative measurement of the biomass growing in the layers above the microelectrode surface. An alternative is to use several electrode pairs, with varying interelectrode distance to achieve different penetration depth in the cell layers [5]. Measurements with cell suspensions usually employ four-electrode strategies in order to reduce the electrode-electrolyte interface impedance effect on the measurement, but this technique has not been implemented with microelectrode-based monolayer cell culture measurements.

In a previous work from our group [6], preliminary measurements performed with commercial microelectrode structures were presented. Double interdigitated microelectrode (IME) sets were used to implement both the two-electrode and the four-electrode strategies. Given that the use of real stem-cell experiments in the validation stage were too expensive and time consuming, we first used the settlement of cell suspensions (mesenchymal $CD34^-$ human blood cells) over the microelectrode surface as a quick

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model of the cell growing. After that phase, we also obtained qualitative monitoring of monolayer growing of condrocytes and vero cells. Preliminary monolayer growth were performed with the microelectrodes placed at the bottom of a Petri dish (3,5 cm ∅) and cell settlement experiments with microelectrodes placed at the bottom area of a 10 ml Falcon tube.

Electrode-interface effects clearly dominate at very low frequencies (<10Hz) but also include an additive impedance term at all frequencies which mask the electrical impedance relaxation which depend on the cell size and morphology and which becomes visible with four-electrode measurements.

Postprocessing of the preliminary results obtained with $CD34^-$ mesenchymal cells settling on the electrodes on a Falcon tube allow to obtain the time course of the biomass density estimator E_2 (1) [7], which is based on the relative variation of impedance magnitude at low frequency (below the relaxation frequency) and high frequency (above the relaxation frequency) and which is proportional to the cell volume fraction.

$$E_2 = \frac{|Z(LF)| - |Z(HF)|}{|Z(LF)|} \qquad (1)$$

Settling process should drive to an exponential rise of the biomass deposed on the electrode upper region. The four-electrode measurement clearly displays this behaviour, but two-electrode result shows two dynamic mechanisms, corresponding the first one to the electrode surface filling and the second one to the remaining settling process. Since the two-electrode measurement is highly dependent on the electrode impedance and, consequently, on the cell confluence and attachment properties, the cell density at high concentrations can be masked. In any case, switching between both measurement strategies could allow to separate both adhesion and growing mechanisms.

In this work, the measurement set-up has been improved and an experimental protocol which includes an alternative method to determine the cell density has been used. Two and four-electrode measurements of Vero cells growing have been performed, the relaxation spectra has been fitted to the Cole-Cole impedance model and the time course of their parameters has been extracted and studied.

II. Materials and Methods

A. Microelectrodes

Double sets of Interdigitated Microsensor Electrodes (IME) from Abtech [8] were chosen. They are made with deposed Pt on borosilicate glass (Figure 1). The interelectrode distance and the track width is 15 μm. The length of each set is 5 mm. According to the manufacturer information, they are intended to perform differential electrochemical measurements (including EIS) after placing electroactive layers on the IME surface.

The measured electrode impedance in physiologic saline solution presents a high frequency value around 50 Ω with a corner frequency around 300 Hz.

B. EIS measurement set-up:

The measurement system was based on an HP4192A impedance analyser. The system was controlled by a custom LabWindows based software which performed a frequency sweep between 10 kHz and 1 MHz at 10 frequency points per decade every 15 s. A custom front-end stage allowed different electrode configuration measurements (2-4 wire).

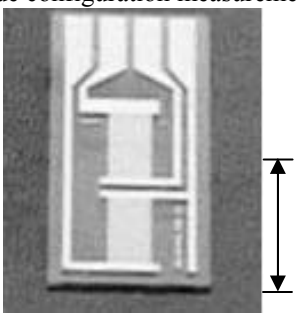

Fig. 1. Differential IME set composed by two interdigitated electrode pairs (Abtech). Scale bar is 1 cm long.

Two-wire measurements were performed with the two extreme electrode pair. In four-wire measurements, current was injected through outer electrodes and voltage was measured in the inner electrodes. The front-end includes a current injector, a transimpedance amplifier and a wide-bandwidth differential amplifier coupled in AC and with very high input impedance. The amplifier presents a CMRR of 95 dB at LF and 60 dB at 1 MHz without any adjustment.

C. Measurement Cells

Vero cell growths were performed in 10 ml minibioreactors (Monoscreen™, Hexascreen Culture Technologies SL), with the microelectrodes placed at the bottom (Figure 2). The contacts cross the wall through an aperture which is sealed with a biocompatible silicone compound (Sylgard 184, Dow-Corning).

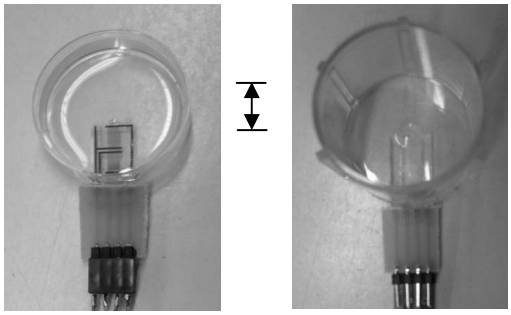

Fig. 2. Electrodes in a Petri dish and in a Monoscreen™ minibioreactor. Scale bar is 1 cm long.

Before starting the culture, and after inserting the IMEs in the bottom of the dishes, a cleaning protocol must be

performed in order to guarantee that cell growing on dishes with electrodes and adhesives would be comparable with growing in control Petri dishes. This protocol consists in pouring into the dish 3-4 ml of distilled water and removing it after five minutes (repeating three times). Afterwards, adding 4 ml of distilled water and incubate over night. The day after, remove the water and clean with 3-4 ml of medium three times again. Afterwards, the dish with the IME is ready for culture. Sterilization is performed using gamma radiation exposure.

The biological model used for the monitoring of cultures were vero cells, a cell line derived from green monkey liver. Vero cells were chosen by their adherence, robustness, short duplication time and wide use in biotechnology [9]. The medium used was GMEM (Glasgow's Modified Eagle Medium), supplied with 10% FBS (Fetal Bobine Serum). Vero cells were incubated in a 5% CO_2 atmosphere at a temperature of 37 ºC. Figure 3 shows vero cells attached on the electrode surface.

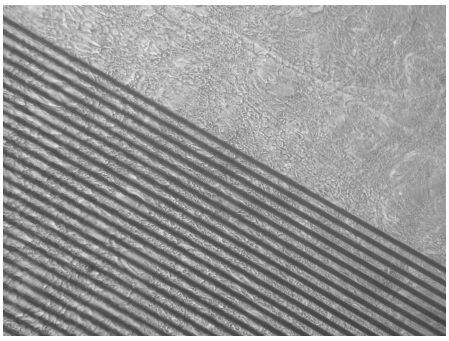

Fig. 3. Vero cells attached to the electrode surface

In addition of the monitored minibioreactor culture, parallel cultures were performed with the same conditions in order to validate the results obtained, one culture for each cell concentration measurement. The cultures were inoculated at the concentration of 5000 cells/cm^2, and an off-line measurement was carried every day. Finally, after cell confluence was achieved, the cell concentration of the minibioreactor was also measured.

To proceed to the cell concentration measurement, the cells of the parallel cultures were detached by replacing the culture medium with a trypsin (Sigma) 0.25% solution at a temperature of 37 ºC for about 5 minutes. Afterwards, GMEM supplied with 10% FBS was added to inactivate the detaching effect of the trypsin. A haemocytometer was used to determine the final cell concentration.

In the previous experiments, the full IME surface was exposed to the cell growth. Because of this, current flowing through wide Pt tracks contributed to the voltage drop in a different way in two and four measurement strategies. In the actual measurements, only the interdigitated microelectrode areas are exposed, being the remaining device surface covered with Sylgard 184 silicone compound.

III. Results and Discussion

Figure 4 shows the E_2 biomass density estimator time courses of two Vero cell growths measured with two and four electrodes respectively.

The estimator is compared with cell counting performed in parallel cultures and with final cell counting in the monitored bioreactors. Initial offset has been corrected. We can see how both methods allow grow monitoring, but four-electrode measurement shows higher sensitivity $\Delta E_2 = 62\%$ in comparison with $\Delta E_2 = 38\%$ for the two-electrode case.

The main advantage of the four electrode strategy is not, however, the sensitivity increase but its ability to incorporate morphological information that could be extracted from the impedance spectrum. Two-electrode spectrums are highly influenced by the electrode interface, then making difficult to extract specific information.

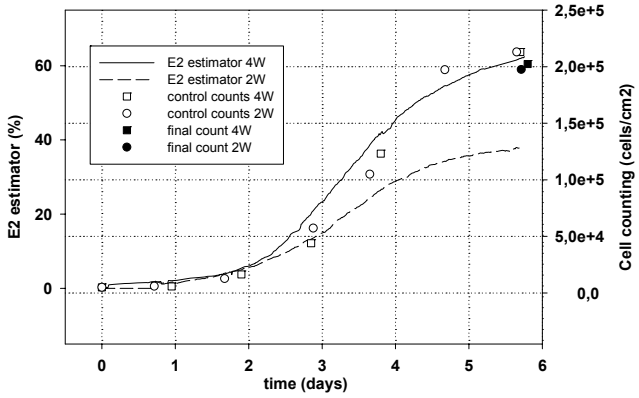

Fig. 4. Two (segmented) and four (full line) electrode measurements biomass estimator time courses of two vero cell growths. Empty circles (two electrodes) and squares (4 electrodes) show cell counting in parallel control cultures and the full symbols display the final cell counting in the minibioreactors. Initial offset has been corrected.

Spectra obtained from four electrode measurements allow model fitting with the impedance Cole-Cole model, whose parameters are related with cell size and with cell size and shape dispersion. Given that the final aim of that work is to obtain not only growing but differentiation information, this feature is of grand interest.

Figure 5 shows the magnitude and phase angle impedance spectra of the four electrode measurement series between 10 kHz and 1 MHz. Measurements were taken each 15 min but in the graphs are represented one spectrum each 150 min. Data was filtered, frequency by frequency by a moving average filter with n=10. The spectra were calibrated with respect to the first measurement, which was assumed to have a very low cell concentration. The rise in the impedance relaxation can be clearly appreciated both in magnitude and in phase angle, where, in addition, a decrease in the central relaxation frequency can be appreciated.

Figure 6 shows the Real(Z) vs. –Imag(Z) impedance arcs. When fitted to the Cole-Cole impedance model (2), the zero

and infinite resistance could be extracted and indirect biomass density estimators can also be derived [7].

$$Z(f) = R_{\infty} + \frac{R_0 - R_{\infty}}{1 + \left(j \frac{f}{f_c} \right)^{1-\alpha}} \quad (2)$$

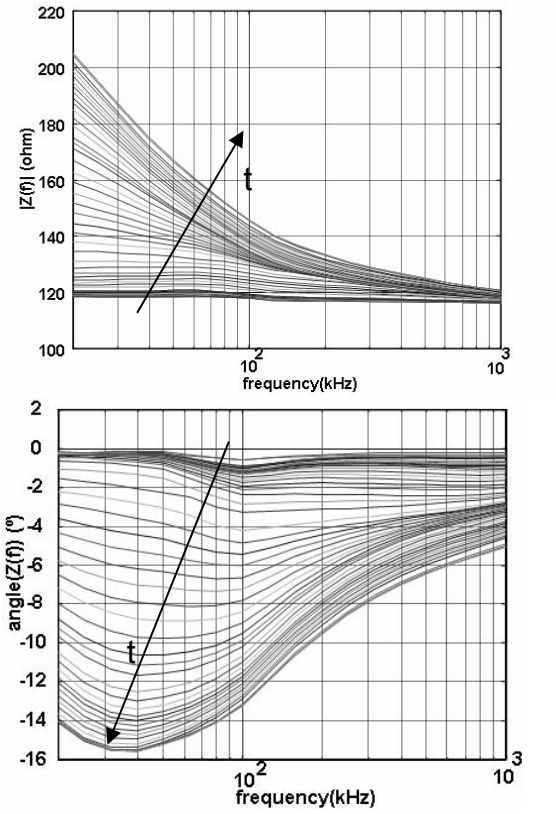

Fig. 5. Magnitude (upper) and phase angle (lower) Impedance spectra of the four-electrode measurement series between 10 kHz and 1 MHz represented in 150 min intervals.

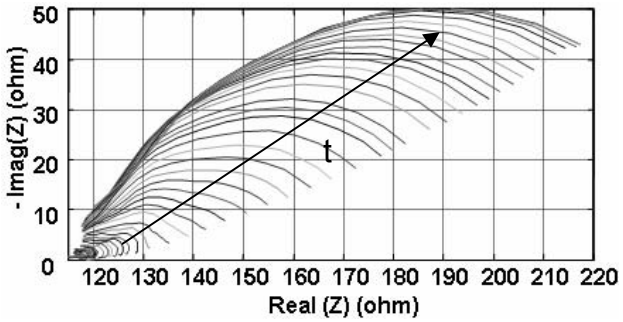

Fig. 6. Cole-Cole impedance plot of the four-electrode impedance series, represented in 150 min intervals.

Central relaxation frequency displays a decrease from around 150 kHz at the culture beginning to 50 kHz, which become stable in the last third of the culture time. This behaviour can be associated with the gradual cell size growing when confluence is being completed and attached cells become apparently bigger, then stopping the cell growing process.

IV. Conclusion

The double pairs of interdigitated microelectrodes, whose initial function was to perform differential measurements between two biosensors allow the realisation of both two and four electrode impedance measurement, giving characteristics of the electrode interface and the cell layer.

Four-electrode measurements show higher sensitivity to biomass density than two-electrode measurements while two-electrode measurements present a first stage in which the ratio of electrode area covered by cells is the dominant effect. After cell confluence, the sensitivity loss displayed by two-electrode measurements can be due to a masking effect of the first cell layer to electrode impedance. Consequently, four electrode measurements could provide more accurate cell density estimators and also morphological information. The combined use of two and four electrode measurements can provide structural information about the cell culture in a continuous and non-destructive way along the growth and differentiation processes in stem-cell experiments.

Current work deals with the design of an experiment in which changes in cell adhesion are produced without biomass loss, in order to separate both effects. The final goal is to determine changes produced in differentiation processes.

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