Effect of the electrode surface on the tetrapolar impedance measurements of Hela Cells in suspension

S M Pinto, E F Pinzón, S P Corzo and D A Miranda
Universidad Industrial de Santander, Carrera 27 calle 9, Bucaramanga, Colombia.
E-mail: dalemir@uis.edu.co

Abstract. Electrical impedance spectroscopy (EIS) measurements with four electrodes is widely used in the study of electrical properties of cells in suspension. Under less than ideal conditions, this setup enables the removal of the surface polarization effects. However, in the case of HeLa cells in suspension, we noted that the electrical impedance spectra reproducibility is affected by the impurities and ionic species adsorbed on the electrode surface. We studied the influence of the electrode’s surface conditions on the EIS measurements of HeLa cells in suspension. EIS spectra were obtained before and after submitting the electrodes to a cleaning process. Chronopotentiometry and open circuit potential (OCP) measurements were carried out to verify the electrode’s surface conditions. The main effect of cleaning the electrodes was to decrease the standard deviation of the EIS data (On average, 3.42 Ω to 0.70 Ω for PBS and 17.22 Ω to 0.31 Ω for PBS with HeLa cells). OCP measurements evidenced surface differences between the cleaned and uncleaned electrodes. In addition, the chronopotentiometric curve obtained for the gold electrode/PBS system showed the adsorption of chloride and phosphate ions and other species existing in PBS on the electrode surface at 0.28 V and 0.53 V. Results suggest that the adsorbed species on the electrode surface led to an electrostatic build-up, which has a non-passive behavior.

1. Introduction
Cervical cancer is the fourth leading cause of female cancer deaths in the world [1]. This disease is related to the abnormal growth of cells on the cervix and can be prevented if detected in its early stages. However, the lack of symptoms until the cancer reaches advanced stages makes early detection a challenge. To ensure early detection, screening using several tests is promoted, with the most widely used being the Papanicolaou test. Notwithstanding the fact that this test has been successful in reducing the incidence and mortality rates (primarily in developed countries) [2], this technique has its limitations, especially because its specificity and sensibility may vary from values as low as 50% to values above 80% depending on the sampling conditions [2].

Electrical Impedance Spectroscopy (EIS) has been shown to be viable as a complementary technique to the conventional smear test, improving the screening sensibility and specificity and the test response time [3, 4]. Basically, this technique consists of measuring the response of tissue after applying an excitation signal. Recently, a methodology has been proposed to measure the electrical impedance spectrum of cells collected by Pap scraping [5], in which the cells are suspended in phosphate buffered saline (PBS) and deposited into a gold four electrode probe based on the Vander Pauw theorem, described by [5]. Findings suggested that this methodology
has the ability to discriminate between abnormal and normal cells.

Impurities can be adsorbed onto gold electrodes due to their exposure to a nonclean environment or by contact with samples measured previously. Although the tetrapolar arrangement minimizes the surface polarization effects [6], the adsorbed species could interfere with the impedance study leading to the loss of reproducibility. This situation can be avoided by implementing an appropriate gold cleaning protocol to guarantee more similar electrode surface conditions between different measurements [7].

The aim of this research was to study the impact of impurities adsorbed on the electrode surface on the reproducibility of EIS tetrapolar measurements. For this study, electrical impedance spectra of PBS and Hela cells suspended in PBS were measured using recently cleaned electrodes and contrasted with the same spectra measured with unclean electrodes.

2. Materials and Methods

2.1. Solutions and biological samples
Electrolyte solutions were prepared using Milli-Q water (18.2 MΩ). PBS was prepared by solving sodium chloride 138 mM, potassium chloride 3 mM, sodium hydrogen phosphate 8.1 mM and potassium dihydrogen phosphate 1.5 mM, adjusted at pH 7.4 with HCl. HeLa cells were cultured in Eagle’s Minimum Essential Medium (EMEN) supplemented with 7% fetal bovine serum and antibiotic (Gentamicin 50 mg L⁻¹). The cells were grown in plastic bottles and kept in an incubator with 5% CO₂ and at 37°C, for adhesion, growth and replication. A concentration of 121 * 10⁴ cells per mL was resuspended in 1 ml of PBS.

2.2. Electrode cleaning protocol
Electrodes made of gold-copper wire (75% Au, − 25% Cu) were chemically cleaned by immersion in a solution of H₂SO₄ and H₂O₂ (3 : 1) for 30 seconds. After this time, they were rinsed with an abundant amount of deionized water and electrochemically cleaned by cyclic voltammetry (CV) performing oxidation/reduction cycles in 0.5 M H₂SO₄ between -0.2 V to 1.4 V at a scan rate of 0.1 V s⁻¹ until the gold reduction peak stabilized.

Electrodes used in several measurements without undergoing the cleaning protocol are called uncleaned electrodes.

2.3. EIS measurements
EIS measurements were taken with an PGSTAT204 Autolab potentiostat/galvanostat with FRA module, 250 mL of PBS were deposited into the tetrapolar cell described by [5] (see Figure 1) and a disturbance current of 1x10⁻⁵ A in a frequency range between 0.1 KHz and 1 MHz was applied. A total of fifty data were recorded per spectrum.

The same procedure were carry out with 250 ml of the Hela cells suspension. Each measurement was done four times.

2.4. Electrochemical measurements
Chronopotentiometries and Open Circuite Potential measurements were carried out at room temperature with a PGSTAT302N Autolab potentiostat/galvanostat and a conventional three-electrode cell. The gold-copper electrodes acted as a working electrode and, a high purity graphite rod (99.9995%) as a counter electrode with respect to a Ag/AgCl (3 MKCl) reference. The support electrolyte was PBS saturated with high purity nitrogen.

Chronopotentiometries were carried out with clean electrodes, aplying constant current pulses of 10 μA, 1 μA and 0.1 μA for 100 s. OCP measurements were taken, both with clean and uncleaned electrodes (exposed to the lab environment and to previously measured samples).
3. Results and discussion

To study the influence of the surface conditions of the electrodes on the EIS tetrapolar screening methodology, electrical impedance spectra were measured using both clean and uncleaned electrodes. Differences on the electrodes surface conditions before and after the cleaning protocol can be evaluated by the OCP measurement. Figure 2 shows that, for uncleaned electrodes, OCP did not stabilize within a period of 300 min, while, after cleaning the electrodes, the stabilization time was 70 min. This behavior evidences the presence of chemical species on the uncleaned surface of the electrodes which were removed by the cleaning process [8].

Due to the ionic nature of the support electrolyte, PBS, many ions are adsorbed on the electrodes surface during each measurement [9].
The PBS and PBS + Hela cells spectra module averages are shown in Figures 3(a) and 3(b) respectively. It can be seen that, for the frequency range studied, the impedance module and the standard deviation are lower for the measurements performed with clean electrodes than those with unclean electrodes. Nevertheless, the spectrum shape is the same in both cases.

In the four electrode technique the alternating current signal is fed by two electrodes (excitation electrodes) and the electrical potential difference is measured by the other two (i.e. measurement electrodes)[10]. The cleaning methodology performed guarantees a clean surface, free of environmental contaminants and chemical species [7]. Once the cleaned electrodes are immersed into PBS, the electrical double layer begins to take shape at the gold electrode/PBS interface. The latter being due to the reorganization of charge carriers on both sides [11]. The excitation current signal varies with the frequency scan and time, thus, the electrical potential registered in each measuring electrode also varies. This leads to its polarization at different potential values. If these potentials correspond to the absorption potentials of an ionic specie on gold, this specie will be adsorbed on the electrode, randomly occupying the surface sites [12].

![Figure 3. Average EIS $|Z|$ of (a) PBS and (b) PBS with HeLa cells in suspension.](image)

In summary, as the electrical potential on the measuring electrode changes, different ionic species are adsorbed forming an ionic adlayer and altering the electrode surface conditions. This can be described as a charge accumulation phenomenon in the electrode/electrolyte interface.
The electrical impedance spectra showed a contribution from the charge accumulation to the electric potential value, registered by the measuring electrode. This suggests that, in this particular case, the ionic adlayer cannot be considered a passive element (capacitor) but in contrast, it behaves like an active element, (e.g. a voltage source [14]).

On the other hand, for the measurements performed using the uncleaned electrodes, the environmental contamination and ions adsorbed during previous measurements must be taken into consideration since, in this case, the initial conditions of the system are not reproducible and could alter the interfacial electrical charge reorganization and therefore the electrical potential registered in each electrode.

Chronopotentiometry enables the recognition of processes or reaction on the electrode [15]. Each process has a characteristic transition time which can be identified by the maximums of the derivative of the potential-time curve [16]. Figure 4 shows the potential-time curve obtained for a gold electrode in PBS by applying 0.1 µA, The curve displays two transition times $\tau_1$ and $\tau_2$ which correspond to 0.281 V and 0.53 V for the studied system.

PBS contains a high percentage of chloride and phosphate anions, which have high adsorption affinity on gold [17] and are expected to be adsorbed with greater probability than other species.

According to Cuesta and Kolb [12], the cyclic voltammetry (CV) of gold in 0.1 M $H_2SO_4$ + 1 mM $HCl$ presents two oxidation peaks around 0.15 V and 0.7 V, limiting the voltage range in which the chloride ion adsorption process occurs. In a similar way, Schlaup and Horch reported anodic peaks in 0.4 V and 0.95 V associated with the adsorption of phosphate ions in CVs of gold in a phosphate buffer solution.

Schlaup and Horch reported values are shifted 200 mV respect to those found by other authors for the same ion in acid media [12]. This is due to the pH difference, however, the voltage range remains the same.

The studies mentioned above suggest that the adsorption process of both chloride and phosphate ions occurs within a potential window of 5.5 V. It is worth noting that this value matches with the corresponding potential to $\tau_2$ in Figure 4. Therefore it is reasonable to infer that this transition time is associated with phosphate and chloride ions while $\tau_1$ can be related to the adsorption of other species present in the PBS.

![Figure 4. Potential-time curve for gold in PBS. Current applied: 0.1 µA.](image)
4. Conclusions
We evaluated the effect of the initial conditions of electrodes’ surface on the electrical impedance spectra of HeLa cells, measured with the tetrapolar method. The exposure of gold electrodes to PBS and HeLa cells in suspension leads to the adsorption of phosphate and chloride ions (among other species). Thus, an ionic adlayer is formed on the electrode surface.

We found that this ionic adlayer behaves like an active element which plays an important role in the tetrapolar EIS measurement, mainly affecting the spectra reproducibility. Therefore, by applying an appropriate cleaning method on the gold electrodes before each measurement, the standard deviation of the measurement decreases significantly, improving its reproducibility.

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5. References
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