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Electrochemical sensors with interdigitated electrodes for counting T-cells

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Abstract. This paper presents the fabrication of an electrochemical sensor that is used for counting T-cells from blood, based on interdigitated electrodes integrated in a microfluidic system. The sensor is composed of two *working interdigitated electrodes* (WE), a *reference electrode* (RE) and a *counting electrode* (CE). Electrochemical impedance spectroscopy was used to study the cellular activities. Impedance spectra allows to analyse the changes and charge transfer resistance due to cell attachment on the interdigitated microelectrodes.

Key-words: Electrochemical sensors, interdigitated electrodes, impedance, T-cells.

1. Introduction

The cell was discovered by Robert Hooke and represents the functional unit of all known living organisms, being the smallest unit of life [1]. Nanotechnology is the engineering and manufacturing of technologies or materials at nanoscale. Due to the small size of the cells, it is the most important technology used to determine and count them. The number of cells in blood can be used for early diagnosis, prognosis evaluation and even treatment efficiency evaluation [2]. During recent decades, nanotechnology has captured a great attention in materials science, medicine and biomedical engineering [3].

The human lymphocytes are divided, according to their biological action and the presence of surface markers (from the surface of the cell membrane) into three major classes (populations): *Natural Killer Lymphocytes* (NK); B Lymphocytes and T Lymphocytes [4]. T lymphocytes have this name because they mature in the thymus [5]. Depending on the markers located on the

surface of T lymphocytes, they are subdivided into CD3+ cells; CD4+; and CD8+ cells. One of the most dangerous health problems that can be determined by the number of T cells is *Acquired Human Immunodeficiency Syndrome* (AIDS) [6]. Before the detection of the virus, we can see that it exists from the CD4/CD8 inverted report. The amount of CD4 and CD8 T cells from blood samples can give information about the immune system and HIV infection [7], [8]. There are different methods and devices used in detecting these types of cells [9-11].

The main application of lymphocyte T subclass determination is the screening, evaluation and monitoring of immune deficiencies, characterized by recurrent infections, which can be bacterial, viral and / or fungal depending on the nature of the deficiency. Cellular immune deficiencies can be numerical or functional, and lymphocytes may be involved. In the present, the total number of B, T and NK lymphocyte subsets is determining by flow cytometry, a very expensive equipment managed by overqualified personnel.

In this paper we describe an electrochemical sensor that can be used for detecting T–cells. Such sensors were discussed by Chen Ij. and White IM in [12], or by Varshney M and Li Y. [13]. The variation of resistance and capacitance with the number of attached, cell spreading, cell proliferation can be determined through modelling of the electrochemical data [14]. Detailed studies of the characteristics of *interdigitated microelectrodes array* (IDMEA) and its applications in electrochemistry were done by Ibrahim and co-workers in [15].

Testing with this microfluidic system, that incorporates the electrochemical sensors described in this paper, takes place within half an hour for each antigen. It is a shorter time than for any other device. Comparing with the other devices, our microfluidic system presents one big advantage: it avoids the cell congestion. This happens due the fact that the narrowed channel portion before the entrance in the selection and capture chamber prevents coagulation when the cells pass.

2. The sensors integrated in the microfluidic system

Microfluidics contributes significantly to cell biology, aided by the fact that it has the appropriate scale that matches the cell scale. Cell culture, fusion and apoptosis were successfully performed in microfluidics. We developed a microfluidic system which can count and capture T–cells from patient blood samples. In order to be able to do the cell separation, it is necessary to take into account: the separation principles, the separation markers, the resolution, the efficiency and the production capacity of these techniques [16]. The system has a lysis section, a lysis stopping and cell conservation region, two counting sensors and an antibody functionalized micro-structured capture chamber.

The lysis section is used to destroy erythrocytes, whose number is of millions per microliter of blood and can interfere with the count. For the separation of leukocytes from erythrocytes, redundant solutions for erythrocytes will be developed.

The lysis solution, which is a solution with acid pH, containing specific detergents, will be mixed with the blood sample and will cause the selective rupture of the erythrocyte membranes in a few seconds. Optimal erythrocyte lysis time is essential because it must be long enough to break all the red blood cell membranes, but short enough to keep the other cells intact. Then, a lysis stopping solution, a buffer solution, will be introduced so that the cells remain in a solution without impurities. These will reach the reaction chamber in which only the target cells, respectively CD3, CD4, or CD8, will bind to specific antibodies immobilized on the walls of the room or on the pillars. The last step is the quantification of the immune complexes formed, using the

electrochemical sensor.

The sensors, situated at the entrance and at the exit of the capture chamber, rely on Coulter Principle to count cells. For T-cells, the pillars from the capture room are functionalized with anti-CD4+, anti-CD3+ and anti-CD8+.

The microchannels are fabricated in SU-8 photoresist. Before passing over the sensors, the channel narrows at a 15μm dimension through which the cells pass in order to be counted. The pillars found inside the capture chamber have a distance of 12μm between them. SU-8 is used in photolithography and presents excellent chemical resistance, high transparency, it is biocompatible and shows strong adhesion [17], [18].

3. The sensors integrated in the microfluidic system

The sensor is a device that responds to a physical or chemical stimulus that converts it into an electrical signal. Each cell type responds to certain types of stimuli (mechanical, chemical, thermal, etc.), but all cells can be electrically stimulated.

The sensors within the microfluidic system were developed to be applied in determining the properties of T lymphocytes. Two identical sensors were used for counting both the cells that enter the selection and capture room as well as the cells that pass out this room. The system is based on electrochemical detection by using impedance spectroscopy, a non-destructive technique that can provide time dependent information about the material properties [19-21]. It can distinguish the dielectric and electric properties of individual contributions of components under investigation.

3.1. Design and modeling the sensors

The electrochemical sensors are built using three electrodes: the *working electrode* (WE) the *reference electrode* (RE) and the *counting electrode* (CE). Such a sensor has two pairs of *working electrodes* (WE) with interdigitated parallel microelectrodes. *Interdigitated Gold* (GIDs) Electrodes consist of two sets of comb-shaped metal electrodes, from which the name also appeared.

The main dimensional parameters of a pair of electrodes are:

1. The dimension of a digit = 20 μm.
2. The length of digits = 285μm.
3. The microelectrode finger width = 10μm.
4. The distance between digits = 5μ.

The width of the *reference electrode* (RE) is 200 μm and the counter electrode (CE) is 195 μm wide. *Interdigitated array electrodes* (IDAs) can be applied for detection and reaction analyzes of the test compounds. Both the generating and the collecting electrodes are found on the same plane. The small size of microelectrodes causes minimal disturbance to the sample, which can allow monitoring the events that occurred in a single cell. When the electrode finger width and spacing become small, the efficiency of molecule redox cycling between two bands of electrodes is increased, so that the electrochemical reaction current is improved. The interdigit can provide further current amplification, they have small charging currents, a small voltage drop and reach a steady state rapidly.

After establishing the dimensions, the design is made in a special program called CleWin5. After this step, the modeling was made in another special program: SEMulator3D (Fig. 1).

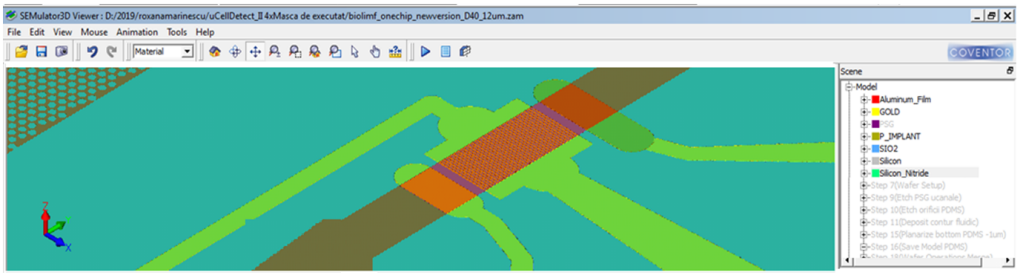

Fig. 1. SEMulator 3D view (color online).

3.2. Fabrication of Interdigitated Microelectrodes Array Electrochemical biosensor

The fabrication is made through a succession of deposits of specific materials. The basic fabrication process is presented in Fig. 2.

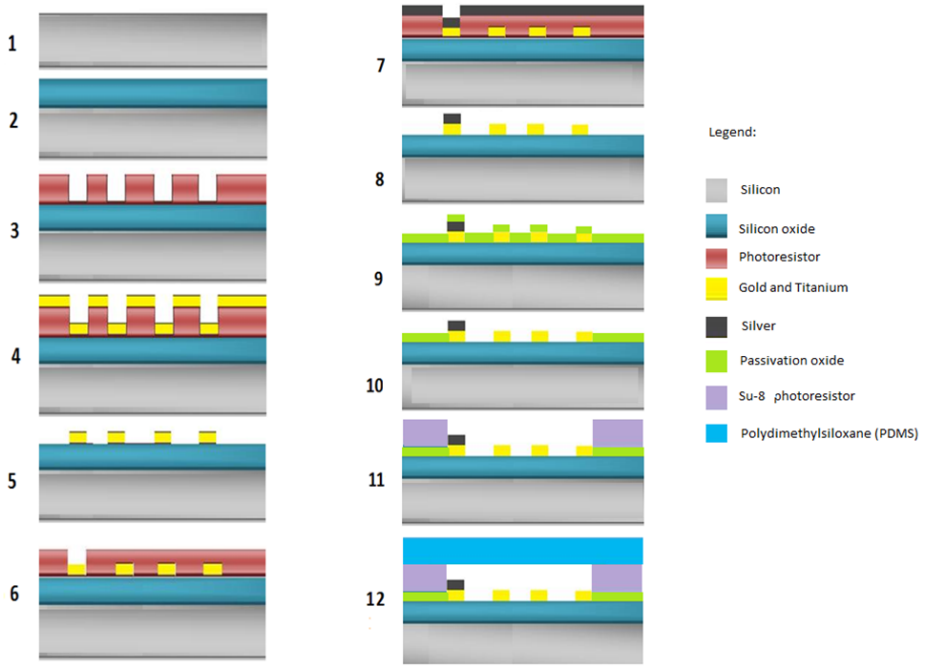

Fig. 2. Main steps of the microfabrication (color online).

The sensors are fabricated by photolithographic techniques, using four photolithographic masks and several steps (Fig.2). A 4-inch Silicon wafer is used as substrate for the fabrication (Step 1). A silicon dioxide layer was thermally grown (100 nm) in a dry ambient at 1100°C in Lindberg furnace (Step 2). The SiO_2 layer serves as an insulation layer between p-type silicon and the metal layers. The first mask was used to imprint the pattern on a thin layer of photoresist (Step 3) in order to deposit the Titanium/Gold (Ti/Au) substrate for the sensors (Step 4).

The reference and counter electrodes, along with the fingers of the working electrodes were defined with the first mask. The metal deposition was made by sputtering. A thin layer of Ti/Au (30nm/300nm) was deposited all over the substrate. Gold is used as material for electrodes and titanium as adhesion layer, because Ti is chemically inert with reagents used in the electrochemical detection of biological samples, and electrochemically inert under the experimental condition of detection. In case of Ti, titanium oxide forms on the surface at room temperature which is good since it acts as a passivation layer and a chemically inert material. The key limitation for the thickness of metal layer deposition depends on subsequent lift-off process. The unwanted material is removed by the lift-off process (Step 5). This process refers to the fact that the wafer is submerged in a recipient filled with acetone. The photoresist used as sacrificial material is detached and removed together with the material deposited on it. After the Gold-Titanium deposition, a second layer of photoresist was used (Step 6), for printing the image from the second mask. A deposition of 100 nm Silver (Ag) through electron-beam evaporation was made (Step 7). Another lift-off process was made after this deposition, so that the Silver remains deposited only on the reference electrode (Step 8). The chlorination of Ag was done using a strong oxidant, $FeCl_3$. After Ag deposition, the wafer was soaked in 1 % ferric chloride aqueous solution for 20 minutes.

$$Ag + FeCl_3 \longrightarrow AgCl + FeCl_2 \tag{1}$$

A third mask was used to cover the entire surface with a passivation oxide (Step 9), leaving only the portion of the sensor area exposed (Step 10) after etching. The fourth mask was used to design the microfluidic channels and the pillars of the selection and capture chamber through photolithography of a 50 μm SU-8 2050 photoresist film (Step 11). The microfluidic channel lets the cells pass one by one over the electrodes in order to be counted. Separately, a PDMS cap is prepared, which is placed over the microfluidic device. The PDMS must not cover the electrical contacts. Polymer bonding is done at a temperature of 90°C applying a uniform pressure of 20 Pa (Step 12). Using the optical microscope we can see the results obtained after each deposition, the process of fabricating the electrodes (Figures 3-6).

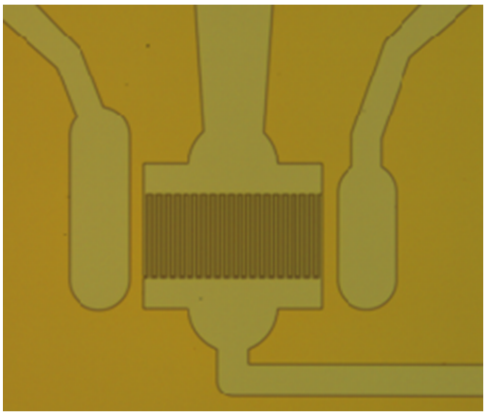

Fig. 3. Gold-Titanium deposition (color online).

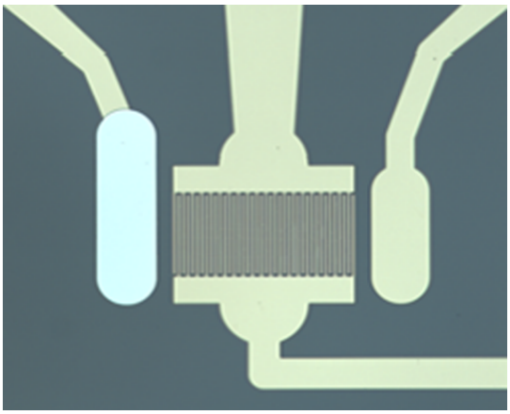

Fig. 4. Silver deposition (color online).

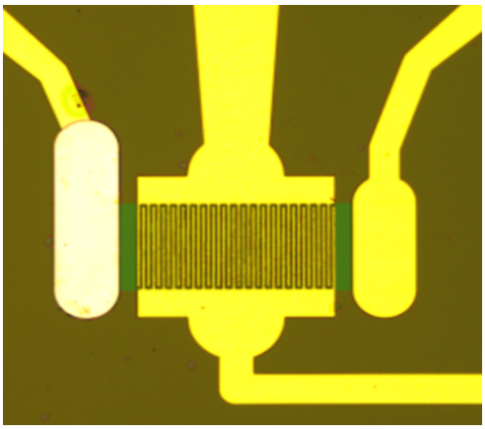

Fig. 5. Oxide deposition (color online).

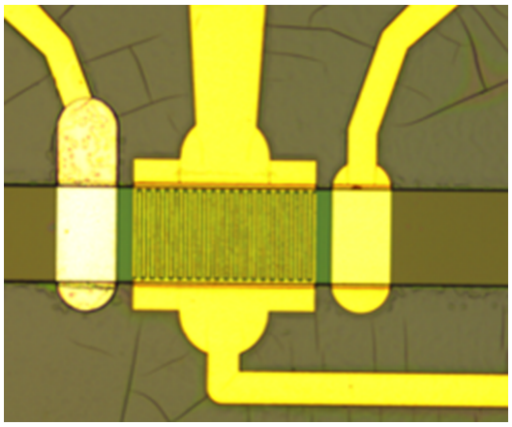

Fig. 6. SU-8 deposition (color online).

4. Testing the sensors

The Coulter principle assumes that the cell will block the passage of current into the microchannel. Thus, the electrical current in the microchannel will be disrupted and will generate a distinct impedance change, recorded by a specific impulse. The amplitude and the width of the impulse will be directly proportional to the size of the cell and the speed of passage through the electrodes electrolyte solution;

4.1. Materials and methods

There are 3 types of cells in a blood sample: Leukocytes, Erythrocytes and Platelets. The last category is insignifiant. In order to remain only with the Leukocytes category, a solution of: 0.05% acetic acid, 0.15% formic acid in water for lysing the erythrocytes will be introduced. Due to the fact that we don't want to destroy the leukocytes, after a few seconds will be applied the lysing stopping solution: 3.13 sodium sulphate, 1.45% NaCl, 0.6% anhydrous sodium carbonate. Stopping is done after 6 seconds by adding 265μL of stopping solution

The tests were made individually for each cell type, using specific antibodies for each type of cell subpopulation prior immobilized onto interdigitated gold electrodes (GIDs). The antibodies were immobilized with the following steps:

1. Cleaning GIDs with successively immersion for 10 minutes in: 2 isopropanol, ethanol, acetone and finally for 1 minute in Piranha (70:30 H_2SO_4:H_2O_2), rinse with water and use N_2 for dry.

2. A mixed self-assembled (mSAMs) monolayer was formed by immersing GIDs in a mixture of two thiols in different ratios for each immobilized antibody: for anti-CD4, 1mM 11mercaptoundecanol acid (11-MUA) :7mM 3 –mercaptopropionic acid (3MPOH), for anti-CD3 in 3mM 11 MUA: 7 mM 3MPOH and for anti-CD8 in 1mM 11 MUA: 9 mM 3MPOH;

3. Blocking nonspecific binding with 1% bovine serum albumin (BSA) in phosphate buffer PBS, pH= 7,1;

4. Rinse with water and dry;

5. Carboxyl activation of each GIDs with 75mMEDC and 15mMNHS for 30 minutes, rinse, dry and use immediately for next step;

6. 100 μL Protein G (PG) conc. 100 pg / mL in PBS 7.4 are placed on each GIDs surface and incubated for 18 h at room temperature; rinse and use for antibodies immobilization;

7. Add 40 μL Ab with anti CD4, CD4, and CD8 with conc. 5 μg/ml and incubate each GIDs for 18 h at 4°C.

To verify the antibodies immobilization and for antigen quantification the *impedance spectroscopy* (EIS) was used by recording Nyquist Plots (-Zi vs Zr) for GIDs electrodes in 5mM Fe $(CN)_6{}^{4-}$ and 5mM de Fe $(CN)_6{}^{3-}$ solution. The GIDs electrodes were working GIDs of the configured electrochemical cell on cip made by: chlorinated silver electrode (as a reference electrode) and gold as counter electrode. The EIS measurement was carried out at equilibrium potential (open circuit potential generated between electrodes dipped in electrolyte), without external biasing in the frequency range of 100 mHz–100 kHz, with 10 mV amplitude using VoltaLab PGZ 100 Potentiostat/Galvanostat (Germany). The Auto.R1R2C Fitting method is used to fit the curve to a circle while running the sequence.

4.2. The EIS measurements

After the functionalization stage with SAMs, Nyquist diagrams showed an increase in the semicircle for all GID electrodes (Figure 9), which pointed out that the surface is covered with thiols, more or less depending on the ratio used. Thus, percentages of the degree of coverage were obtained as it can be seen in Table1:

Table 1. Percentages

mSAMs ratio 11-MUA:3MPOH	$\theta = (1 - Rct_i/Rct_0)^*100$
1mM :7mM	$80\% \pm 12\%$
3mM:7mM	$92\% \pm 5\%$
1mM:7mM	$99.8\% \pm 0.1\%$

where:
θ = degree of coverage;
Rct_i = the signal recorded at each concentration;
Rct_0 = the noise;

The semicircle increase in all functionalization stages for all GIDs. In Figures 7-9 are presented the Nyquist Plots after Protein G immobilisation, antibody immobilization and after reaction with the first concentration of the antigens. Antibody grafting was not performed covalently but the high affinity of the G protein for anti-CD antibodies in the Fc portion results in targeted antibody immobilization. After the addition of G protein, the last step was to incubate for a sufficient time (optimized as 18 hours at 4∘C) with specific anti-CD antibodies. The functionalized electrodes, after rinsing with PBS are ready for uptake of CD antigens. All the GIDs electrodes responded by increasing semicircle when incubate for 20 minutes with first concentration of antigen.

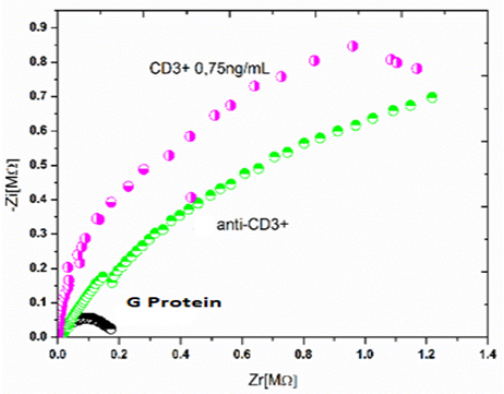

Fig. 7. Nyquist Plot (-Zi vs Zr) of GIDs electrode for CD3+: after incubation with G protein, anti-CD3 and after adding 0,75 ng/mL of CD3+antigens.

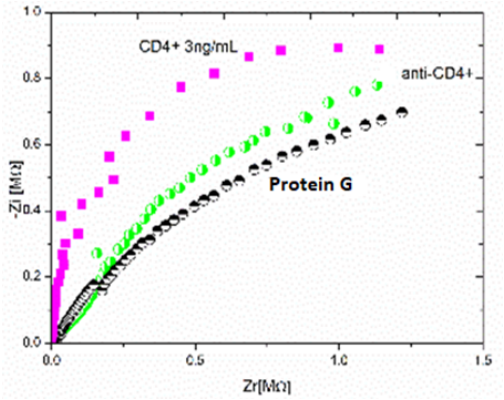

Fig. 8. Nyquist Plot (-Zi vs Zr) of GIDs electrode for CD4+: after incubation with G protein, anti-CD4 and after added of 3 ng/mL of CD3+antigens.

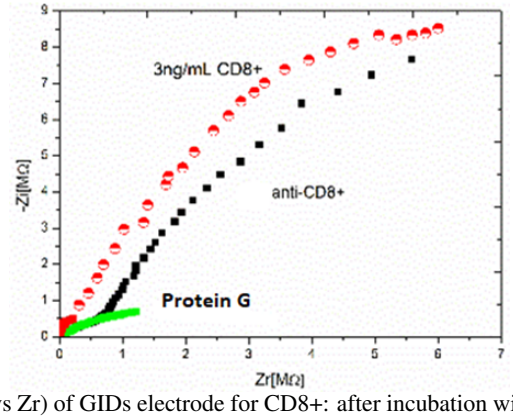

Fig. 9. Nyquist Plot (-Zi vs Zr) of GIDs electrode for CD8+: after incubation with G protein, anti-CD8 and after added of 3 ng/mL of CD8+antigens.

5. Conclusions

The Coulter principle is used for counting the leukocytes entering the capture chamber and those coming out the capture chamber using interdigitated electrodes and it separates the cells after the electric permittivity. It is a device that has small sizes, a short time of response and it is easy to use.

Nyquist plots analyze the response of the electrode to the application of an alternating current of small amplitude.

Identifying and counting the different subpopulations in human blood, in addition to being of clinical importance, is also an important technological challenge. The presented devices can bring many benefits that come in improving a quick diagnosis. Electrochemical sensors using interdigitated electrodes for CD antigen detection can become such a device after rigorous validation and calibration.

The most common and dangerous disease that can be detected with the help of T cells is the human immunodeficiency virus (HIV). In [9] Ishii H. et al show the importance of $CD8^+$ T cells due to the fact that these cells can control HIV infection. A potent receptor from $CD8^+$ T cell can combate a receptor from AIDS. So, by cloning these cells, the AIDS progression can be delayed. HIV replication can be controlled by potent $CD8^+$ T-cell responses.

Noor A.M. et al [10] developed a microfluidic system for counting CD3+ T cells. They developed a very simple device, a PDMS microfluidic chip which captures these cells using a pillar based filtration technique. Bystryak S. et al [11] enumerate only CD4+ T cells for staging HIV disease. They use the prototype FTCA cassette, digital camera and image analysis software. Compared with these two researches, our microfluidic system can determine all the T cells: CD3+, CD4+ and CD8+.

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