Capter 172

Development of electrochemical biosensor: voltammetric analysis of lymphocytes and indication activation of the complement system

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ABSTRACT
This work reports the development of an electrochemical biosensor after immobilization of the lymphocytes to detect the reaction between antibodies and specific HLA antigens present in the serum samples. A clean homemade gold electrode with voltammetric polycrystalline characteristics was used. Lymphocytes were immobilized and tested with positive and negative human serum and complements on the gold electrode. The experiments were carried out in a cell with three electrodes: working - gold, and reference - Ag/AgCl/sat. KCl and auxiliary - platinum. The cyclic voltammetric analyses of immobilized lymphocytes on the gold surface presented an anodic current equal to 1.78 µA at c.a. 0.50 V vs. Ag/AgCl/sat. KCl. The electrochemical responses of the serum (positive and negative) and complement do not show signs of oxidation or reduction in the potential range used. The electrodes with cells and positive serum showed the amplified current signal in the oxidation potential of the cells. The electrode was developed to verify the antigen-antibody reaction, present lymphocyte cells, and human serum samples. The electrode was qualitatively efficient when compared to the methods of flow cytometric analysis and complement-dependent cytotoxicity, being able to be used with operational and economic advantages.

Keywords: lymphocytes, biosensor, blood cells

1 INTRODUCTION

Kidney transplantation is the most effective treatment for patients with end-stage renal disease due to chronic renal failure since there is a significant improvement in quality of life and long-term survival. However, allograft rejection is the major source of concern in renal transplant recipient therapy [1-3].

Analyzes for compatibility determination involve antigenic macromolecules which are common to all lymphocytes.[4]. The macromolecules are distributed on the surface of the lymphocytes allowing processes such as reactions, physicochemical interactions, and adsorption. [5] Macromolecules (proteins) contain amino acids such as Lisene, cysteine, histidene and arginine. The amino acids should be responsible for the adsorption process as a function of the NH_2 and SH groups. [6-9]

In organ donation surgery, the selection process of live donors requires human leukocyte antigen (HLA) typing of the recipient as well as possible donors to allow the selection of the best donor. Of great importance is the compatibility between Class II antigens, followed by compatibility between HLA-B antigens and Class I HLA-A antigens. Crossmatch testing, as proposed by Terasaki and McClelland in 1964 are employed to assess whether the recipient is sensitized to donor histocompatibility antigens (Ag) and should test negative. The analysis is performed by an in vitro assay in which a serum sample from the recipient is mixed with donor lymphocytes and incubated. After incubation, during which antigen-antibody complexes must form (if anti-HLA antibodies are present in the serum), a complement source is added to the system that should cause lymphocyte lysis recognition by the antibodies. In a positive reaction, once there are specific anti-donor antibodies in the circulation if a transplant is performed, it can quickly be rejected. [10]

Since 1964, crossmatch testing by complement-dependent cytotoxicity (CDC) has been the standard technique for assessing the presence of anti-HLA–specific antibodies against the donor before a transplant. Detected pre-formed alloantibodies can cause a hyperacute rejection and immediate graft loss. [11] The flow cytometry (FC) technique, used since 1983 by Garovoy, is considered the most sensitive for the detection of alloantibodies and the standard technique for the diagnosis of humoral activity through the detection of low titers of circulating alloantibodies. The crossmatch through this technique can detect anti-HLA antibodies in patients who tested negative for CDC crossmatching. [12] It can be 50 times more sensitive than CDC and up to 15 times more sensitive than CDC with anti-human globulin (AHG). [13-15]

A biopsy is used to verify the possibility of allograft rejection; however, it is invasive, subclinical, and often imprecise, which has aroused considerable interest in the development of non-invasive methods, and the rejection-predictive biomarkers appear as an alternative to estimate rejection in renal transplant patients. [16] The advantages of the development and application of biomarkers are those that are non-invasive and achieve disease activity monitoring, with considerable sensitivity, acceptable frequency, and low cost, hence reducing the number of episodes of early and late clinical rejection. [17] In this context, electrochemical sensors have been presented as adequate tools for these marker development proposals due to the increasing need for simple, fast, and inexpensive analytical responses. [18,19]

Considering the characteristics presented by the FC and CDC techniques discussed herein, the construction of an electrochemical sensor in a gold electrode is presented here. The voltammetry technique was used with gold electrodes after immobilization of the lymphocytes to detect the reaction between antibodies and specific HLA antigens present in the serum samples.

2 MATERIALS AND METHODS

2.1 REAGENTS

All reagents were of analytical grade and were purchased from Sigma Aldrich. The water was purified with a purification system, Purelab Option Q (18.2 MΩ). The solutions were prepared in the

laboratory, phosphate-buffered saline (PBS) (pH 7.2); 0.5 mol L^{-1} H_2SO_4 solution; and saline solution (NaCl, 0,9%). Samples of mononuclear cells and their quantitation by volume, positive control serum, negative control serum, and complement were supplied by the Laboratory of Immunogenetics and Molecular Biology of the Federal University of Piaui- UFPI.

2.2 INSTRUMENTATION

All electrochemical measurements were performed on an Autolab Metrohm model 302 potentiostat/galvanostat controlled by NOVA 2.0.2 software using a conventional three-electrode configuration cell. The electrodes included: a gold homemade surface as a working electrode, a Ag/AgCl/sat. KCl as the reference electrode and a platinum plate as the auxiliary electrode.

2.3 CONSTRUCTION OF WORKING ELECTRODE

The working electrode was made by Au deposition on a polyester slide previously treated by ultraviolet light radiation. The gold deposition (99.99% purity) was performed using a metallizer (Edwards 306) under vacuum (10-6 torr) and progressive heating (up to 800°C). The gold layer obtained on the polyester sheet was 65–80 nm thick. This material was used to prepare the electrode as described elsewhere. [20]

The electrical contact of the electrode was through a copper wire and the sides were wrapped with Teflon tape to protect the electrode from possible infiltration when in contact with the solutions (Supplementary material, Figure S1).

2.4 CLEANING AND CONDITIONING OF THE WORKING ELECTRODE (E_{Au})

The gold surface was initially maintained at a potential of 0 V in the 10-s time interval. The electrode was then electrochemically cleaned with potential cycles in an acidic media (0.2 V/s sweep rate; in H_2SO_4, 0.5 mol L-1) within the potential range of -0.2 to 1.55 V vs. Ag/AgCl/sat. KCl. The oxidation and reduction processes of gold should be observed in a stable redox voltammogram typical of polycrystalline gold. All electrodes submitted to this stage were presented an by using an acrylic base as a reproducible template with a hole of 2 mm in diameter a geometric electrode surface area of 3.14 mm^2 was defined and the copper tape was incorporated for the electrical.

The electrochemical surface area of the gold working electrode was calculated from the charge associated with the gold oxides reduction peak obtained after the cleaning process, assuming that the reduction of the monolayer of gold oxide requires 386 μC cm^{-2}.[21] The calculated electrochemical surface area was 0.171 ± 0.017 cm^2 (n = 31), about five-fold the geometric one.

2.5 SEPARATION OF MONONUCLEAR CELLS

Cell purification was performed from whole human blood by density gradient separation in Ficoll-Paque solution following the protocol.[22] After separation using Ficoll reagent, the blood was diluted in PBS at a ratio of 1:3, and centrifugation was then performed (1500 rpm for 25 min at 22 °C).

Peripheral blood mononuclear cells form a ring in which lymphocytes predominate, whereby a total lymphocyte concentrate is obtained with approximately 8–10 × 10^6 cells in 1 mL, counted by a Neubauer chamber. These cells were centrifuged again (2000 rpm for 5 min at 4 °C) and the pellet containing the lymphocytes were homogenized for repeat centrifugation to obtain the cell solution for use in the biosensor formation.

2.6 CONSTRUCTION OF ELECTROCHEMICAL BIOSENSOR FROM CELL DEPOSITION

The first step, cell deposition on the electrode surface, involved the placement of 5 µL of the solution containing total lymphocytes on the physical area of the working electrode. The adsorption was tested at different times in the ranged from 15 to 60 min on the electrode surface.

The second step was the dilution of the concentrated solution of cells to verify the smallest amount at which the electrode would present a satisfactory response. Considering that the lymphocyte cells are non-homogeneous in size and quantity in the blood, their distribution on the electrode's surface must be irregular and they can adsorb to form agglomerates or multiple layers.

The number of cells on the electrode depends on the amount of adsorption available. Thus, the calculation-based dilution process was performed to determine the number of cells available for adsorption on the electrode’s surface. To obtain the population of lymphocytes on the surface of the electrode, the theoretical values of sizes and quantities of lymphocytes already available in the literature was used with lymphocyte diameters (DL) of 7–20 µm.[23] Using these data, the geometric mean of the lymphocyte area was calculated. The minimum number of cells per electrode area was obtained using the correlation of areas. The mathematical correlation was electrode area (De = 2 mm)/mean geometric area of lymphocytes.

The electrochemical experiments were performed on the concentrated solution (5 µL) and in the dilutions obtained from 5 µL (42.450 cells) for the final volumes of 10, 50, 100, and 150 µL. Calculations of the number of cells for the dilutions were 28.300, 14.150, 2.830, and 1.415, respectively.

2.7 OBTAINING POSITIVE CONTROL HUMAN SERUM AND NEGATIVE CONTROL SERUM SAMPLES AND COMPLEMENT IN RABBIT CELLS

The negative control serum samples used were human serum, AB blood type (Sigma-Aldrich, São Paulo, Brazil). Serum samples for positive control obtained by the Laboratory - Lib were composed of grouped sera of patients hypersensitive to HLA molecules present in a specific population. The compliment was obtained from rabbits (Sigma-Aldrich, São Paulo, Brazil).

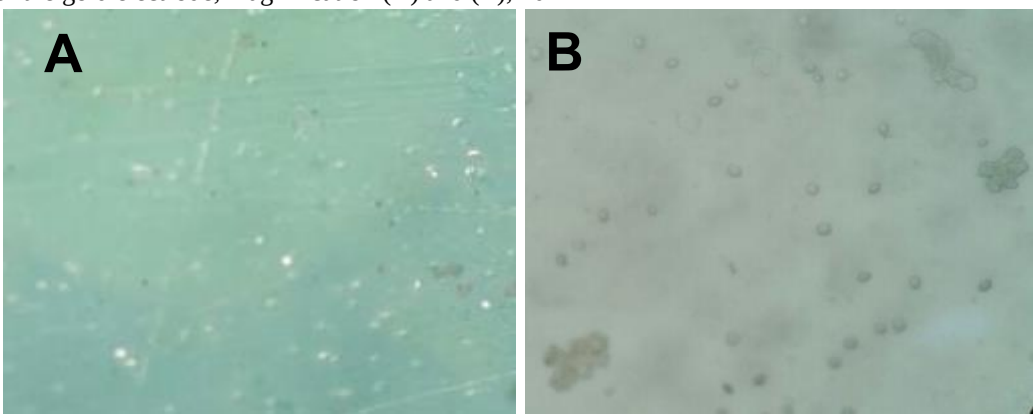
3 RESULTS AND DISCUSSION

The working electrode characterization was performed by cyclic voltammetry as a function of the reduction load (Supplementary Material, Figure S2). The voltammograms were obtained in a solution of 0.5 mol L^{-1} H_2SO_4, from -0.2 to 1.55 V vs. Ag/AgCl/sat. KCl [19] and they presented the same characteristics described in the literature for polycrystalline gold electrodes. [24] In the case of reverse sweeping, a well-defined reduction process was observed concerning the reduction of gold oxide on the transducer surface of the electrode free of impurities. [25]

3.1 CELL DEPOSITION ON GOLD SURFACE (E_{Au})

Using the various dilutions, in each case, 5 µL of the cell solution was deposited at each concentration mentioned previously. Figure 1 compares the E_{Au} surface and the adsorbed cells ($E_{Au+Cell}$).

Figure 1. Image of the electrode surface in an optical microscope (A) clean surface of the gold electrode and (B) cells deposited on the surface of the gold electrode; magnification (A) and (B), 40x



3.2 STUDY OF CELLULAR ADSORPTION TIME ON THE ELECTRODE (E_{Au})

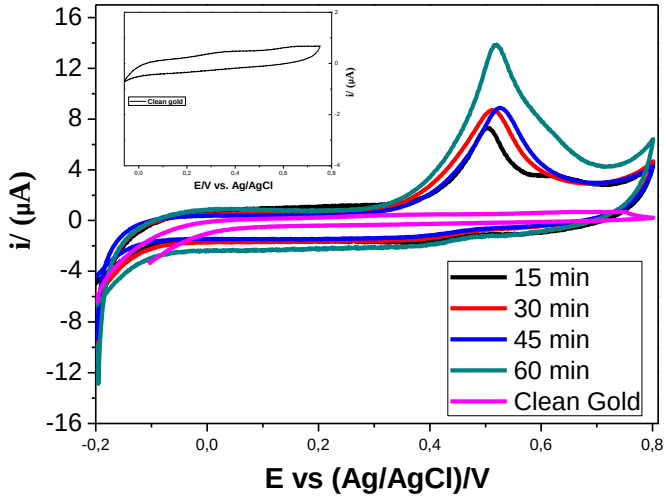
After the cellular adsorption process on the E_{Au} electrode, the cyclic voltammetry analysis was performed. The potential range chosen was in the region where the E_{Au} did not present reactions (-0.2 to 0.8 V vs. Ag/AgCl/sat. KCl). Initially, the voltammetry of the E_{Au} electrode was performed in the PBS solution to verify if they had electrochemical responses in the potential range. Figure 2 (A) (insert) shows faradaic processes that occur in this potential range, revealing that the E_{Au} electrode has no activity in the PBS solution.

The adsorption time study was performed by the addition of 5 µL of concentrated lymphocyte solution to the E_{Au} and varying the time. The voltammograms of $E_{Au+Cell}$ electrodes were obtained (Figure 2B) for the adsorption times of 15, 30, 45, and 60 minutes. According to Ahmed, 2017 [26], the adsorption of molecules such as proteins, DNA, and RNA in gold substrates is a highly reproducible process. In the literature, many studies have examined the adsorption of these different samples on gold surfaces.[27-32] Considering this principle, in a solution containing amino acid residues (nitrogen and/or sulfur atoms) in contact with a gold surface, covalent bonding is expected.[33]

The results were compared to the cyclic voltammetry of E_{Au} in PBS solution (Figure 2 (A)) in the potential range of -0.2 to 0.8 V. [34] In the first voltammetry performed after the 15-min cell deposition time (Figure 2B), the anodic current peak of 4.66 µA at a potential of approximately 0.50 V vs. Ag/AgCl/sat. KCl on the first voltammetry curve can be observed. The observed oxidation is irreversible. This process can be attributed to the oxidation of amino groups. In the surface of HLA cells NH_2 groups can easily be oxidized to NH• produces. Covalent bond (CN) is then formed by the free radical reaction of NH with groups of amino acids CO. [35] For the other adsorption times, an increase in the anodic peak current was observed, with an increase in the contact time of the cell solution with the surface of the E_{Au}, indicating an increase in cells on the surface at 30, 45, and 60 min.

Figure 2 shows that, at each voltammetry, an irreversible peak was observed at the potential of 0.50 + (0.01 or 0.02) V vs. Ag/AgCl/sat. KCl. The anodic peak currents of the voltammograms at 15, 30, 45, 60 min were Ipas 4.66 µA, 6.78 µA, 7.05 µA, and 11.09 µA, respectively. The Ipa values increased but showed a linear deviation. This result is attributable to the increased density and organization of adsorbed cells on the gold electrode's surface (Figure 1). Table 1 presents the values obtained for the area calculations of the anodic peaks in each of the voltammetry protocols at the analyzed time points.

Figure 2. (A) Voltammograms of E_{Au} in PBS solution; (B) $E_{Au+Cell}$ with cells adsorbed in the times of 15, 30, 45 and 60 min, in PBS solution, the scan speed of 250 $mV.s^{-1}$, in the range of potential from -0.2 to 0.8 V vs. Ag/AgCl/ sat. KCl.



The values of the areas of the anodic peaks cannot be represented in a linear graph, indicating the possible influence on the adsorption due to the cellular sizes and types; besides, the ions were present in the solution. On the other hand, since the response at 15 min has an area value in the same decade (10^{-7} C) as the other times and due to the necessity of the rapid test responses, this adsorption time was the one used in the dilution experiments.

3.3 STUDY OF CELL DILUTION AND VOLTAMMETRY ON THE ELECTRODE (E_{Au})

After the adsorption time analysis, the cell dilution was performed concerning adsorption on the electrode. The dilution was made to evaluate the adequate proportion of cells that could be adsorbed on the surface of the gold electrode.

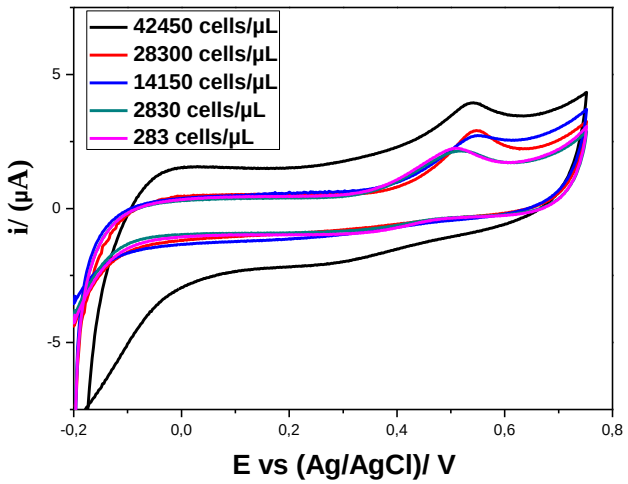
The cell samples received showed in their label the concentration of cells per milliliter. Dilutions were performed using this value.

Observation of the electrochemical responses of $E_{Au+Cell}$ in the various dilutions revealed that the number of cells is not the only factor to determine the adsorption. Physicochemical parameters of the substances exposed on the surface of the cells and ions present in the solution are involved.

Figure 3 shows the voltammograms for the dilutions made from 5 µL of the cell sample initial volume in PBS buffer to obtain volumes of 10, 50, 100, and 150 µL. These dilutions from the initial sample allowed the calculation of the number of cells present in each volume of 28.300, 14.150, 2.830, and 283 cells, respectively.

According to the data obtained from the cyclic voltammetry, oxidation peaks were observed at the potentials of 0.54, 0.54, 0.50, and 0.50 V vs. Ag/AgCl/sat. KCl , respectively, for each dilution, revealing that decreasing the number of cells on the electrode surface shifts the current signal I_{pa} to a less positive potential. Dilution of 5 µL of the initial sample to 100 or 150 µL showed very similar voltammograms, allowing the performance of all subsequent experiments at a dilution of 150 µL.

Figure 3. Voltammograms for each dilution between 282 and 42450 cells/µL, with adsorption time of 15 min. $E_{Au+Cells}$, in PBS solution, the scan speed of 250 mV.s-1, in the range of potential from -0.2 to 0.8 V vs. Ag/AgCl/sat. KCl



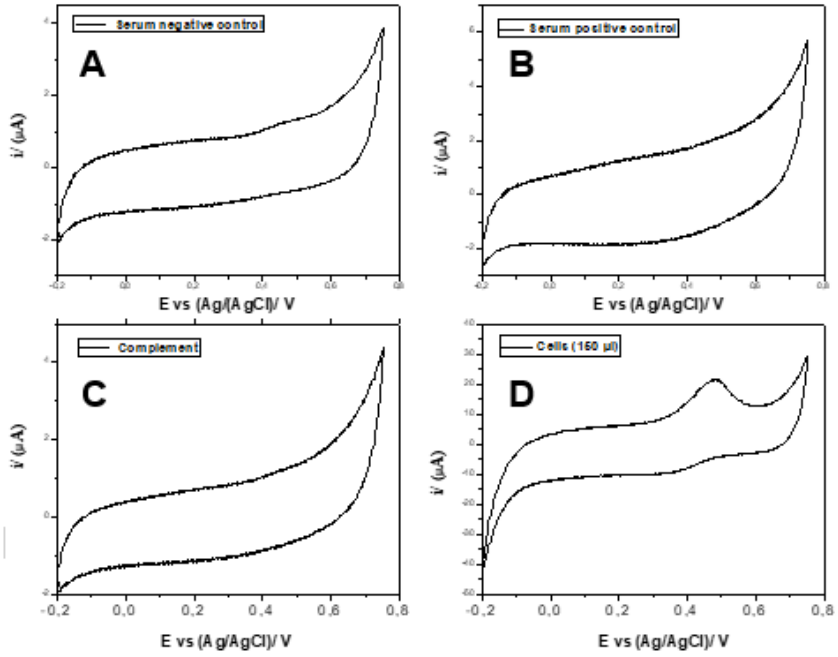
3.4 CYCLIC VOLTAMMETRY MEASUREMENTS OF THE POSITIVE AND NEGATIVE SERA SAMPLES AND COMPLEMENT

The materials, serum, and complement used in the determination of interactive activities between cells, serum, and complement were tested by cyclic voltammetry to determine which electrochemical responses could be present on E_{Au}. Figure 4 shows the electrochemical response 4 (A) of the negative

control serum, 4 (B) positive control serum, and 4 (C) of complement. The voltammogram obtained for the negative control serum, positive control serum, and complement did not show any faradaic (oxidation/reduction) currents, indicating the absence of an interaction between the serum and complement with the electrode.

Conversely, it can be observed that the double layer in the voltammograms presents different capacitive areas for negative serum (Figure 4A) and positive serum (Figure 4B). The latter presents a greater voltammogram area than the negative serum, where one can attribute the presence of substances (antibodies) that undergo polarization effect in the potential range.

Figure 4. (A) sample - 5 µL of serum positive control in the clean working electrode of Au, (B) sample - 5 µL of serum negative control in the clean working electrode of Au, (C) sample of complement - 5 µL in clean working electrode of Au and (D) diluted solution of cells (283 cells).



The voltammetry responses of the positive control serum, negative control serum, and complement were compared with the dilution voltammetry for the lowest number of cells (dilution 5–150 µL) and the adsorption time of 15 min. The cell samples (Figure 4D) show an oxidation peak defined at 0.50 V vs. Ag/AgCl/sat. KCl , while for the other samples, positive control serum, negative control serum, and complement, no peak was observed.

3.5 VOLTAMMETRY ANALYSIS FOR CROSSING OF DIFFERENT SAMPLES

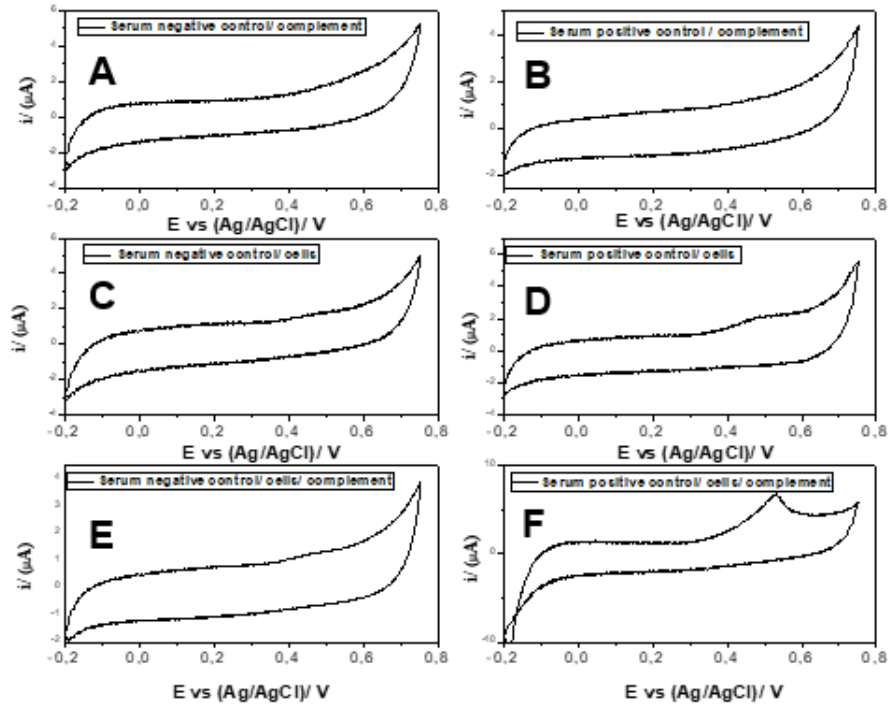
From the voltammetry obtained for the control (positive and negative) sera and complement, tests equivalent to a crossmatch were performed, that is, the samples were mixed and submitted to polarization in a cycle of voltammetry with $E_{Au+Cell}$. Figure 5C and 5D show cyclic voltammetry performed on samples containing: (a) negative control serum and cells; and (b) positive control serum and cells.

In Figure 5C and 5E, voltammograms of $E_{Au+Cell}$ with negative serum and $E_{Au+Cell}$ with negative serum and complement showed very little or no oxidative processes compared to E_{Au+Cel} alone. This behavior was attributed to the removal of E_{Au} cells by chloride ions present in serum. [36] The negative serum was tested for the presence of chlorides (Supplemental Material, Figure 3). The antibodies were non-reactive, which facilitated the action of the chlorides on the HLA cells.

In Figure 5D, a voltammogram of $E_{Au+Cell}$ with positive serum can be observed as an electrochemical response at the approximate potential of 0.50 V vs. Ag/AgCl/sat. KCl, with the area of the anodic peak of 7.8 × 10-7 cm^2, an approximate value to that found for $E_{Au+Cell}$. Figure 5F shows the voltammogram obtained for the crossing of samples of positive serum control/compliment in $E_{Au+Cell}$. In cyclic voltammetry, the oxidation peak at potential 0.52 V vs. Ag/AgCl/sat. KCl, with an area of 9.19×10^{-7} cm^2 and the increase of all double-layer region of the voltammetry curve differ from the voltammogram obtained for $E_{Au+Cell}$ with negative serum control/compliment.

These results observed in Figure 5C–5F show a distinction between $E_{Au+Cell}$ analyses with samples from positive serum/cells and from negative serum/cells as the current signal in the positive serum is amplified. The results of the electrochemical analysis of negative and positive serum samples and complement using $E_{Au+Cell}$ are qualitative indicators of the reactions between the antibodies and specific HLA antigens present on the cell surface [4], and can be used for the qualitative analyses of FC and CDC. Nevertheless, the voltammetry tests are faster and can be measured qualitative.

Figure 5. Voltammograms cyclic of the solutions (A) solution of 5 uL of negative control serum and supplement on the clean gold electrode , (B) solution of 5uL of positive control serum and complement (C) solution of 5 µL of negative serum and cells, (D) solution of 5 µL of positive serum and cells, (E) solution of 5 µL of diluted cells, negative control serum and complement (5:1 µL:4 µL) and (F) sample of 5 µL of cells, positive control serum and complement (5:1 µL:4 µL) on the clean gold electrode potential between 0.2 and 1.55 V, 3 scans at 250 mV.s-1.



4 CONCLUSIONS

The assembly of the electrodes using polystyrene sheet covered with gold using the sputtering technique and copper films serving as a contact proved to be a system of easy execution and low cost. The cells were attached to the gold electrodes by adsorption, allowing the evaluation of the surface oxidation signal. The process involved in the cellular adsorption on the electrode is probably due to the interactions of groups of nitrogen and/or oxygen exposed on the cell surface that can be removed by the presence of chloride ion. The adsorbed cells on the surface of the gold electrode presented an oxidation peak, in an irreversible process, at approximately 0.50 V vs. Ag/AgCl/sat. KCl, when the potential sweep was performed between -0.2 and 0.8 V vs. Ag/AgCl/sat. KCl. Electrical measurements (by voltammetry) showed that the sera (positive and negative) and the complement did not show oxidation or reduction signals in the measured potential range (-0.2 to 0.8 V vs. Ag/AgCl/sat. KCl. When the electrodes with cells and positive serum were subjected to potential sweeping in the range of -0.2 to 0.8 V vs. Ag/AgCl/ sat. KCl, there was an amplified current signal in the oxidation potential of cells (0.50 V), probably due to the interaction of substances (antibodies) of the positive serum with the cellular elements.

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