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Diagnosis of Foot-and-Mouth Disease by Electrochemical Enzyme-Linked Immunoassay

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Abstract—The development of an inmunosensor for the point-of-care detection of the foot-and-mouth cattle disease is presented. The detector is based on an ELISA method with electrochemical detection. A non-structural protein, 3ABC, is used to selectively detect antibodies is used to selectively detect anti-3ABC antibodies produced after infection. The biological test is performed onto a screen printed electrodes. A dedicated small, portable potentiostat is employed for the control of the sensors, as well as data acquisition, processing, and storage.

I. INTRODUCTION

Biosensors are compact analytical devices which employ a biological element in order to detect a specific substance (i.e. the analyte). After the analyte has been detected by the biological recognition element, a signal of physical or chemical nature is produced which then is converted into an electrical signal by means of a transducer. In the case of electrochemical biosensors, a chemical signal generated by the interaction between the biological recognition element and the analyte is converted into an electrical current via an electrochemical reaction at an electrode surface [1]. In this paper, we present the method and instrumentation for an electrochemical enzyme-linked immunoassay for the diagnosis of the foot-and-mouth disease. This presentation covers all aspects of the development of the biosensor: the production of gold electrodes by thick film technology and electrochemical cells with a numeric control device, the chemical modification of the gold electrodes with the biological recognition elements, which are antigens of proteic nature, the electrochemical transducer via the action of a redox mediator (a chemical substance which acts as the electrical connection between the enzymes and the electrode), and finally the electronic instrumentation required to control the electrochemical system and process the resulting signal [2,3]. Results obtained for the diagnosis of the foot-and-mouth disease are presented.

II. EXPERIMENTAL DETAILS

A. *Electrodes design and manufacture*

A three-electrode cell was designed and manufactured. Ring-disk geometry was defined for the working and counter electrodes. Disks with a diameter of 1000 µm were employed as working electrodes. Counter electrodes were designed to have ten times as area as the working electrodes. The inner and outer diameters of the rings were 1600 µm and 3600 µm. The resulting gap between electrodes was 300 µm. Trace width was 300 µm and pad size was 2 mm × 2 mm for both electrodes. A 3 × two-electrode array was designed, so they could be evaluated together or individually (Fig. 1).

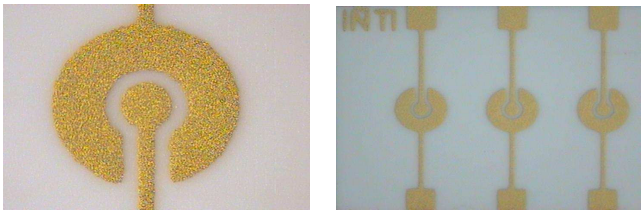

Fig. 1. a) Photograph of a thick film two-electrode configuration showing active circular area with 1000 µm diameter for the working electrode. b) Three pairs of electrodes as used in the electrochemical cells.

The two thick film electrodes were printed onto α-Al_2O_3 substrates by conventional screen printing technology. A wire of 1 mm diameter of Ag | AgCl was employed as a reference electrode. Commercial Au paste (Heraeus D5789) and 96 % α-Al_2O_3 substrates were employed. Electrode layout was transferred by means of photolithography to a stainless steel mesh (400 wires per inch) with a negative photosensitive film (Ulano CDF-2). Au ink printing was performed by an EKRA Microtronic-II printer, dried at 125ºC during 15 min and finally fired at 580°C.

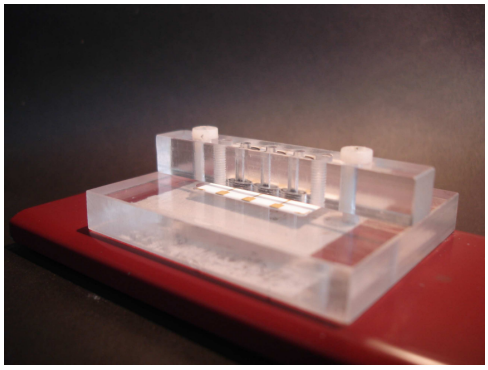

Fig. 2. Acrylic electrochemical cell with thick film gold electrodes.

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The electrodes were integrated in an electrochemical cell, constructed in PMMA using a numeric control device from a CAD layout. Syringes were employed for the supply of buffer solutions.

B. *Electronic instrumentation*

A portable potentiostat was developed to allow point-of-care measurements. The design can control the reference electrode in the -2.5 V to 2.5 V range, allowing its use for the present foot-and-mouth biosensor, as well as other electrochemical biosensors. The allowed working electrode current is in the -10 µA to 10 µA range. The circuit is shown in Figure 3.

The circuit uses a low offset operational amplifier (OpAmp) to control the counter electrode (OP07). To avoid loading the reference electrode an LT1056 OpAmp was selected, providing an input impedance of 10^{12} ohms. The current to voltage conversion, carried out in the working electrode circuitry, was also implemented using an LT1056 OpAmp. This OpAmp introduces a very low current error, typically in the range of 10 pA, and guarantied to be under 0.34 nA for the full operation range.

The potentiostat is controlled by a microcontroller connected to a PC using the Universal Serial Bus (USB). The microcontroller has a 10 bits A/D converter, used to measure the working electrode current, and a PWM output, used to control the reference voltage.

C. *Immobilization of enzymes and antigens onto the electrode surface*

3ABC, a non-structural protein from the foot-and-mouth disease virus, was immobilized on screen printed Au electrodes employing cysteine and a carbodiimide as molecular linkers between the gold electrode and the protein.

Electrodes were cleaned with a H_2SO_4 : H_2O_2 30% (2:1) solution and thoroughly rinsed with water (miliQ quality), immersed overnight in a solution containing 40 mM 3-mercaptopropionic acid and 75 % ethanol + 25% water.

The electrodes were treated during 30 min with 20 µl of a solution containing 0.1 M 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide and 25mM N-hydroxysuccinimide in 0.1 M PBS buffer of pH 7, and then 20 µl of 3ABC 0.22 µg/µl, also in a 0.1M PBS buffer of pH 7, were added onto each electrode. After 45 min, the electrodes were rinsed again with high quality water and immersed overnight in quenching buffer (0.262% glycine an 1% gelatine). Then the electrodes were rinsed again and ready to be used.

For the serologic tests, 3ABC coated electrodes were incubated with different sera during one hour and rinsed with 0.1% Tween 20 in PBS buffer by means of a controlled flux syringes. Then the electrodes were incubated with anti-Ig conjugates during an hour, rinsed with 0.1% Tween 20 in PBS buffer and finally the electrochemical measurements were carried out.

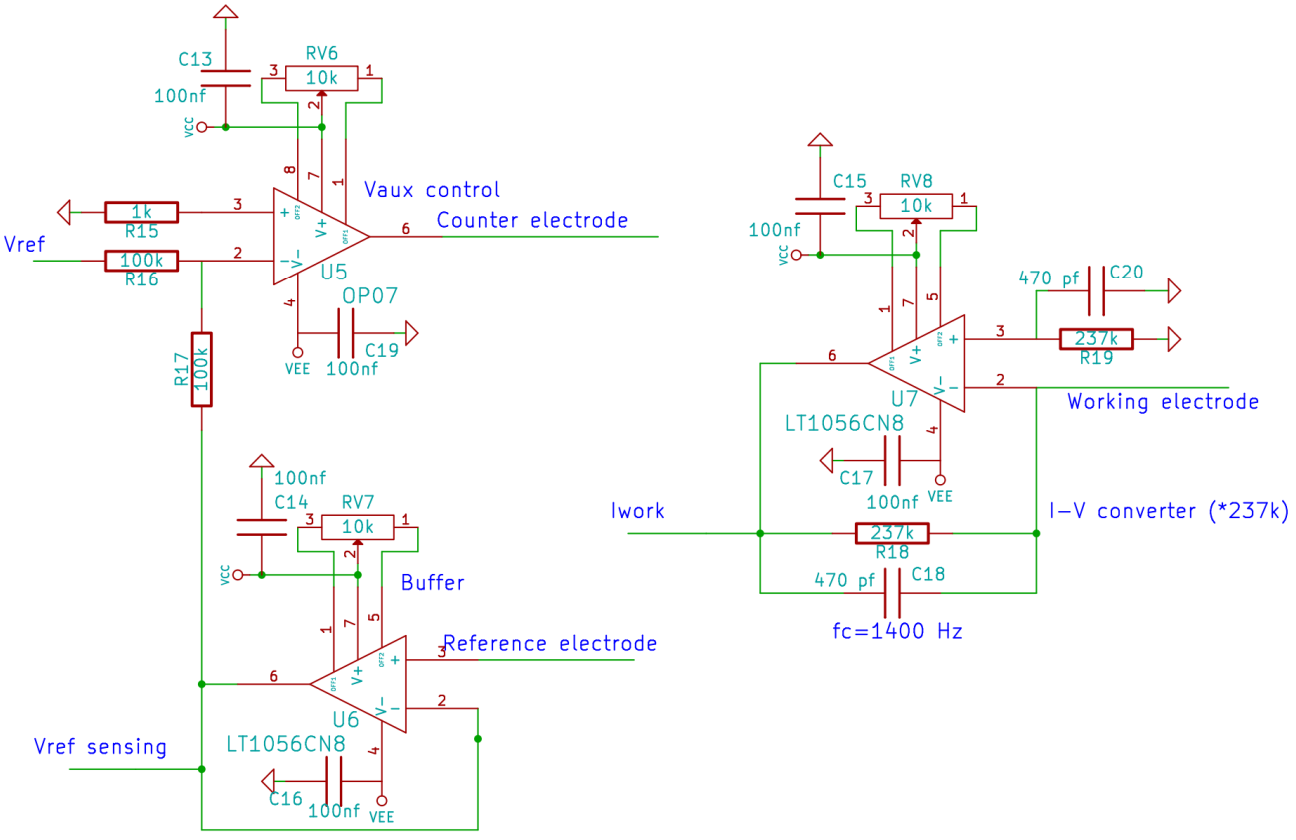

Fig. 3. Potentiostat circuit.

D. *Electrochemical measurements*

Electrode potential was changed from 0 to –300 mV applying 50 mV steps in a 0.1 M phosphate buffer of pH 7 + 0.1 M KCl. 4 mM hydroquinone was employed as a redox mediator and the H_2O_2 concentration was increased from 0 to 1.5 mM. Ag | AgCl was employed as a reference electrode (a wire of 1 mm diameter).

III. RESULTS

In the case of electrochemical immunodetectors, an antigen (3ABC proteins in this case) is bonded to the electrode surface. If this electrode is placed in contact with a serum containing 3ABC antibodies (i.e. a "positive serum"), antigen-antibody complexes are formed, which can further be recognized by a second antibody. The second antibody is labeled with an HRP. Thus, an antigen-antibody-(HRP-labeled antiantibody) complex is formed on the electrode. The activity of the HRP can be electrochemically detected after addition of hydrogen peroxidase and a suitable redox mediator, in a similar fashion as in the case of enzymatic electrodes [4]. This process is schematically shown in Fig. 4.

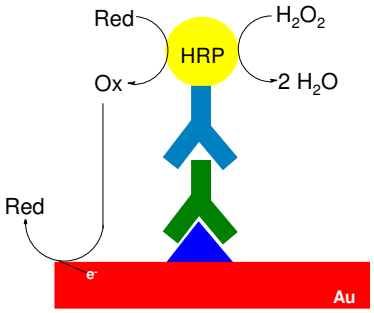

Fig. 4. Schematic representation of enzyme-linked immuno assay with electrochemical detection.

On the other hand, if a 3ABC coated electrode is placed into contact with a serum without the presence of 3ABC antibodies (a "negative serum"), no antigen-antibody complexes are formed and, consequently, no HRP activity is electrochemically detected. It is important to bear in mind that 3ABC is non structural protein, while vaccines for the foot-and-mouth disease are based on structural proteins, without 3ABC. Therefore, infected cattle produce antibodies against 3ABC, while vaccinated cattle do not produce anti-3ABC antibodies. This fact allows a differentiation between vaccinated cattle and infected cattle, as the former give a positive result with this test while the second give a negative one.

Figure 5 shows the current-potential curves obtained for a positive and a negative serum. The difference in current values is high enough as to permit the discrimination of infected and non-infected specimens from this electrochemical enzyme-linked immunoassay.

Figure 6 shows the measurement results for different sera. Abscissa axis represents each serum with its code identification, while y-axis represents current (nA) results, normalized to the serum number 325.

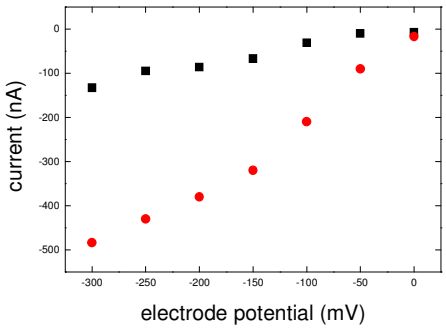

Fig. 5. Current.potential curves obtained under steady state conditions for an enzyme-linked immuno assay for a negative serum (squares) and a positive serum (circles). Hydrogen peroxide concentration was 1.5 mM.

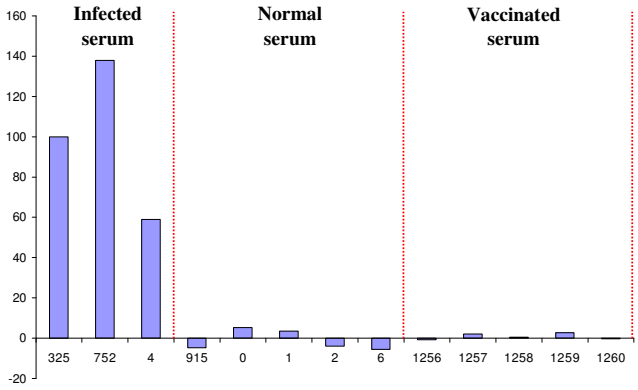

Fig. 6. Results for positive (infected) serum, negative (normal) and vaccinated serum, normalized to #325 serum.

IV. CONCLUSIONS

The development of an inmunosensor for the point-of-care detection of the foot-and-mouth disease was presented. The detection method, based on an electrochemical ELISA test, proved to be as sensible as the standard fluorimetric method. The serum of infected cattle was clearly differentiated from those of vaccinated and non-infected cattle. The detection system is small, portable and specially suited for the detection of the disease in remote and harsh environments.

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