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Electrochemical Biosensors for Virus Detection

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1. Introduction

The rabies constitutes one of the most dangerous viruses causing many death cases every year. Each year approximately 55,000 people die of rabies, with high percentage of children [S et al., 2007; L et al., 2000; FX et al., 1994). High percentages (99%) of the registered cases were in Asia and Africa. In order to fight this dangerous disease, many techniques are usually used for diagnostic but are usually complex, time consuming, expensive, difficult to implement, and this is a necessity for developing new detection process [N et al., 1993; Crepin et al., 1998]. Avian Influenza Virus (AIV) infections are a major cause of mortality and rapid identification of the virus has important clinical, economical and epidemiological implications. The traditional methods for virus diagnostic are Enzyme Linked Immunosorbent Assay (ELISA) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) which are time consuming and expensive. Biosensors can play an important role in areas such as diagnostic of diseases, drug detection and food quality control. Biosensors are devices with qualities quoted as rapidity, sensitivity and specificity [G et al., 2002; D et al., 1999). Biacore based on Surface Plamson Resonance technique is effectively a successful biosensor used for antigen-antibody interaction [J et al., 1999]. Others commercialized biosensors such us glucometer was developed for self-monitoring of glucose in blood for diabetes care.

In terms of the transduction techniques used, the three main classes of biosensors are optical, electrochemical and piezoelectric. Out of the three, optical methods appear to be the most sensitive, with surface plasmon resonance and waveguide based devices being the technological spearhead. As for Electrochemical biosensors, they are cheaper than optical ones. They can be amperometric or impedimetric, depending on whether they monitor a current as a function of potential or the resulting sensor impedance as a function of frequency. The advantage of impedimetric methods is that, unlike amperometry, they do not need of enzymatic labels in order to detect. In this work, we use the high sensitive impedance spectroscopy technique for biosensors applications. This technique is very known to characterize the electrical properties of materials and their interfaces exposed to electronically conducting electrodes [A et al., 2004; S et al., 2006; A et al., 2006]. It may be used to investigate the dynamics of bound and mobile charges in the bulk or interfacial regions of any kind of solid or liquid material: ionic semiconducting, mixed electronic-ionic and dielectric. The biosensor is based on the immobilization of specific anti-rabies polyclonal antibodies and specific anti-H_7N_1 antibodies onto a functionalized gold electrode with micrometer size. The affinity interaction of the antibody with the specific antigen can

be measured with a good reproductibility with impedance spectroscopy [M et al., 2008; M et al., 2008]. The different steps of biosensor conception were characterized by Electrochemical Impedance Spectroscopy (EIS). The obtained limit detection was better than those obtained with the others traditional methods for clinical use. The non-specific interaction has been tested with the Newcastle antigen virus.

2. Experimental set-up

2.1 Specific rabies antibody preparation
Rabies immunoglobulins were produced by horse immunization. The immunization was carried out using human vaccine "RABIPUR" manufactured by "Chiron Behring Vaccines " in Ankleshwar (Gujarat), India. The horses were exposed to a series of injections to increase vaccine amounts. The immunization period lasted for 105 days (M et al., 2008).

2.2 Specific rabbit antibody (anti-H$_7$N$_1$) preparation
Three male rabbits were injected sub-cutaneously with different doses of NobilisTM, INFLUENZA H$_7$N$_1$ vaccine in different periods (15 days, 30 days, 45 days, 65 days). For each period, quantity of blood were analysed to study the kinetic of the rabbit vaccine immuno-response. Hyper immuno serums has been collected and specific rabbit-polyclonal antibodies (anti-H7N1) has been purified with affinity chromatography (M et al., 2008).

2.3 Antibody immobilization on gold electrode
The gold electrodes were cleaned with organic solvents (acetone and ethanol) and with piranha solution (1:3 H$_2$O$_2$ - concentrated H$_2$SO$_4$) for 1 min. After each treatment, the gold substrates were rinsed with ethanol and dried under nitrogen flow. The pretreated electrodes were immersed in 11-mercaptoundecanoic acid 1 mM in ethanol solution for 12 h in order to form a self-assembled monolayer (SAM). The substrates were then rinsed with ethanol in order to remove the unbonded thiols. To convert the terminal carboxylic groups to an active NHS ester, the thiol-modified electrodes were treated with 0.4 mM EDC-0.1 mM NHS for 1 h. After gold electrodes were rinsed with water and dryed under nitrogen, 20 µg/ml of Anti-Rabies IgG (respectively 5 µg/ml of Anti-H7N1) were dropped onto the surface at 37 °C for one hour. The excess antibodies were removed by rinsing with PBS. Then, the antibody-modified electrodes were treated with 0.1% BSA for 30 min, to block the unreacted and non-specific sites. After rinsing with PBS and water, the electrodes were dried under nitrogen (Figure.1).

2.4 Impedance spectroscopy
Many reports show that impedance spectroscopy is a useful tool to characterize self assembled monolayer on surfaces (A et al., 2004). A capacitor is formed between the conducting electrode and the electrolyte. The absolute impedance is related to the frequency by the equation:

$$|Z| = \frac{1}{2\pi f C} \tag{1}$$

where f is the frequency (in Hz) at which Z is measured.

The complex impedance can be presented as a combination of the real impedance (Z_{re}) and imaginary impedance (Z_{im}), Nyquist plot. To fit the measured spectra with the impedance spectra out of ideal elements, the ideal elements have been replaced with the constant phase elements (CPE):
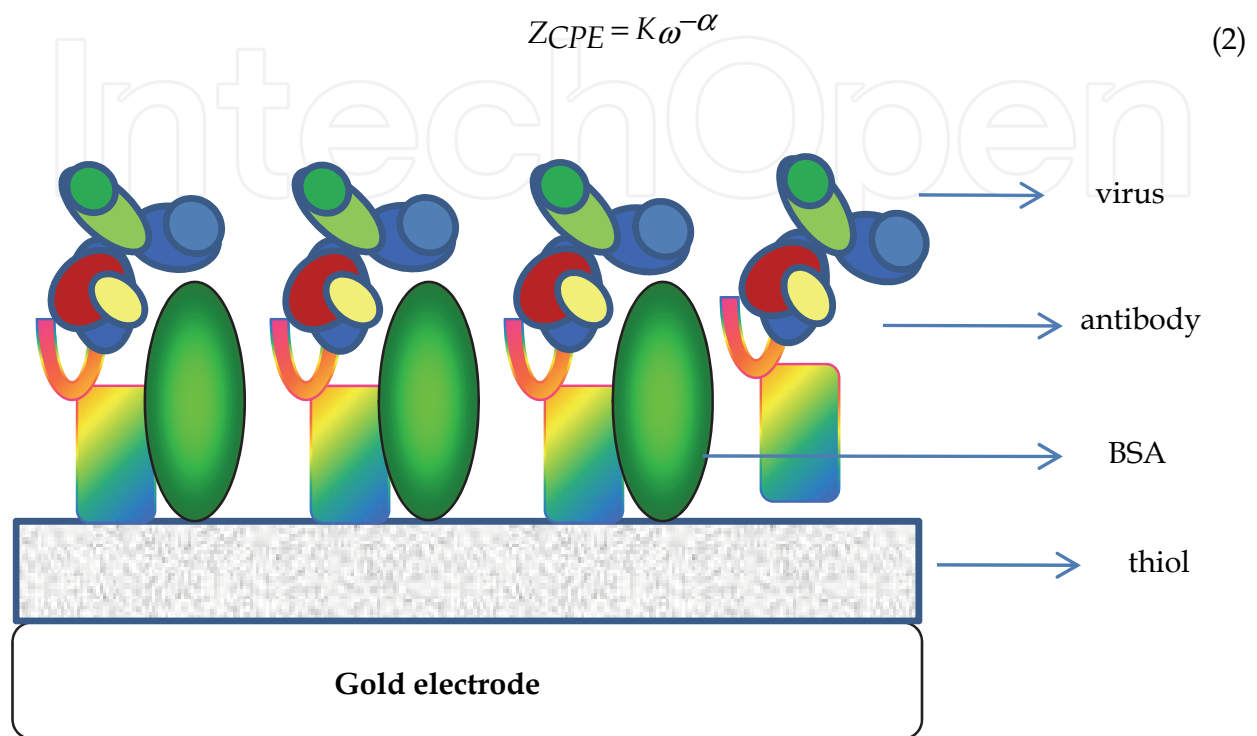
$$Z_{CPE} = K\omega^{-\alpha} \qquad (2)$$

Fig. 1. Biosensor multilayer configuration

The frequency exponent is $\alpha = 1$ and $K = 1/C$ for an ideal capacitance, and $\alpha = 0$ and $K = R$ for an ideal resistance, respectively. The exponent α could be obtained, when the membrane capacitance (or layer capacitance) was replaced by a constant phase element Z_{CPE}. The deviation of the exponent α from the ideal values is attributed to the inhomogeneities of the analyzed layer, like defects or roughness. The measured spectra of the impedance were analyzed in terms of electrical equivalent circuits using a analysis program. The mathematical expressions of the equivalent circuit models were fitted to the data. The electric parameters of the system were calculated with the computer program and the fit error was kept under a maximum of 10%. The impedance analysis was performed with the Voltalab 40 impedance analyser in the frequency range 0.05 Hz - 100 kHz, using a modulation voltage of 10 mV. Three-electrode system was employed with a saturated calomel electrode (SCE), an immunosensor working electrode (0.19 cm²), and a platinum strip counter electrode (0.385 cm²). The impedance measurements were performed in the presence of a 5 mM $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ (1:1) mixture as redox probe in PBS. The measured spectra of the impedance and phase were analysed in terms of electrical equivalent circuit model using a Zview modelling programme (Scribrer and associates, Charlottesville, VA). All electrochemical measurements were carried out at room temperature and in a faraday cage. More details on electrochemical impedance spectroscopy can be found in reference (A et al., 2004; M et al., 2008).

3. Results and discussions

3.1 Avian influenza virus biosensor

First, we study the variation of the impedance spectra (the real part, it means the charge transfer resistance) of the functionalized gold electrode with different concentration of immobilised antibody. This allows us to know the saturation concentration of the antibody on our gold electrode, which will leads to the high sensitivity detection. Figure.2 shows the impedance spectra of the functionnalized gold electrode after the immobilisation of antibody with different concentration.
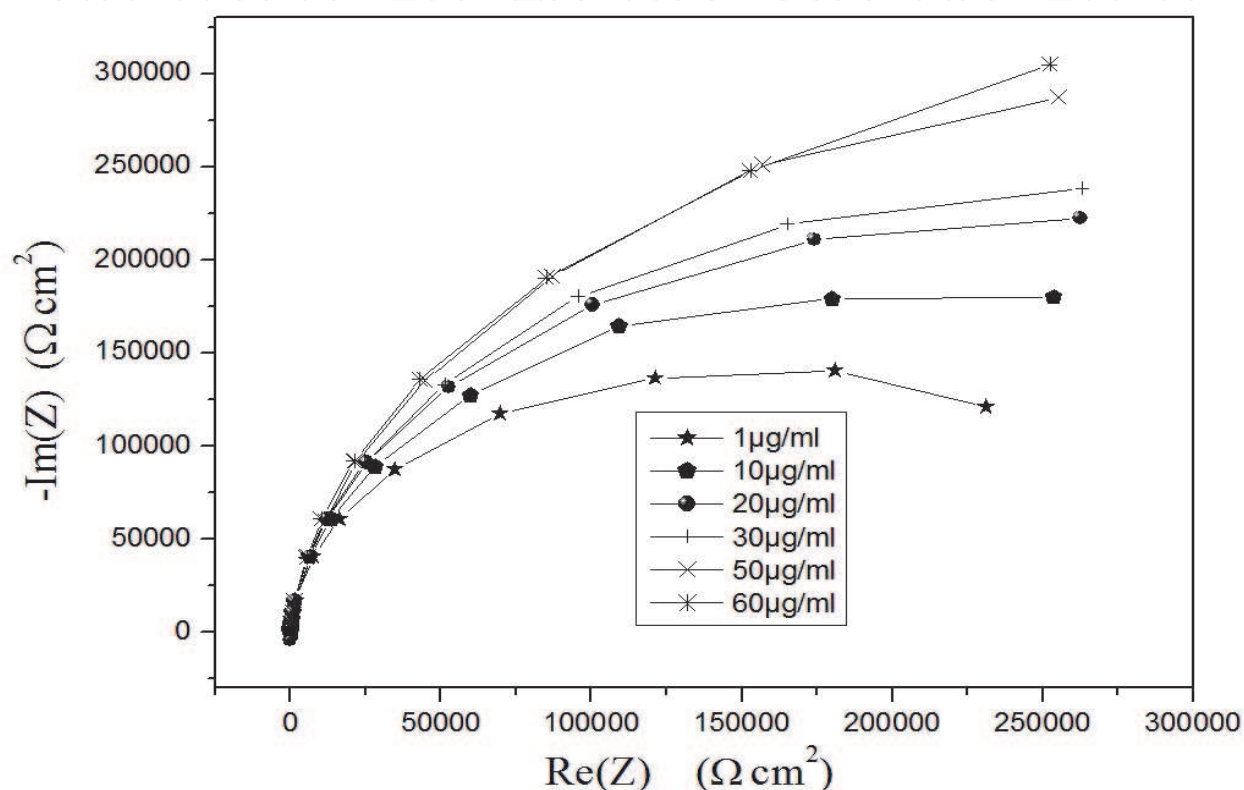
Fig. 2. Impedance spectra of the functionalized gold electrode after the immobilisation of different antibody concentration

The impedance spectra can be fitted with computer simulated program using the electric circuit shown in figure 3. This equivalent circuit includes the ohmic resistance of the electrolyte solution R_0, the constant phase element Z_{CPE} and electron transfer resistance R_1. An excellent fitting between the simulated and experimental spectra was obtained for each antibody concentration. Figure4 shows the variation of the impedance versus the antibody concentration. We can see that the surface saturation can be obtained with 60µg/ml antibody concentration. For specific and non specific antigen detection, we will use this antibody concentration

3.1.1 Virus detection

Figure 5 show the impedance spectra of the functionalized gold electrode recorded at 0 V in PBS buffer at pH=7.2 in the range of 50 mHz to 100 KHz before and after addition of different H_7N_1 antigen concentration.
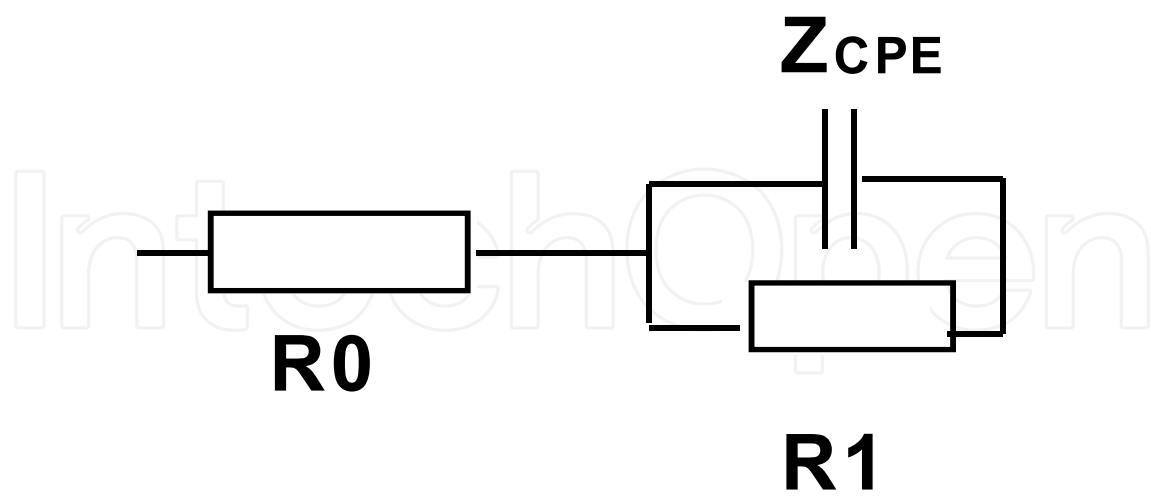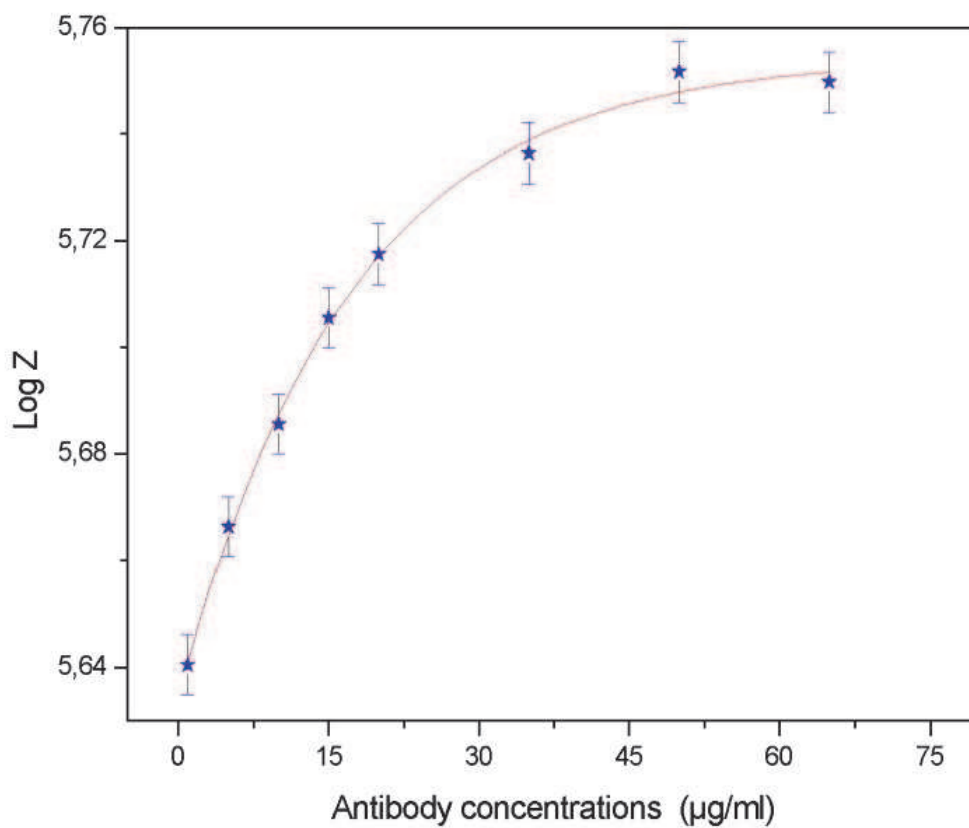
Fig. 3. Electric model

Fig. 4. Impedance spectra of the functionalized gold electrode after the immobilisation of different antibody concentration

The interface can be modelized with the electric model shown in figure3. An excellent fitting between the simulated and experimental spectra was obtained.
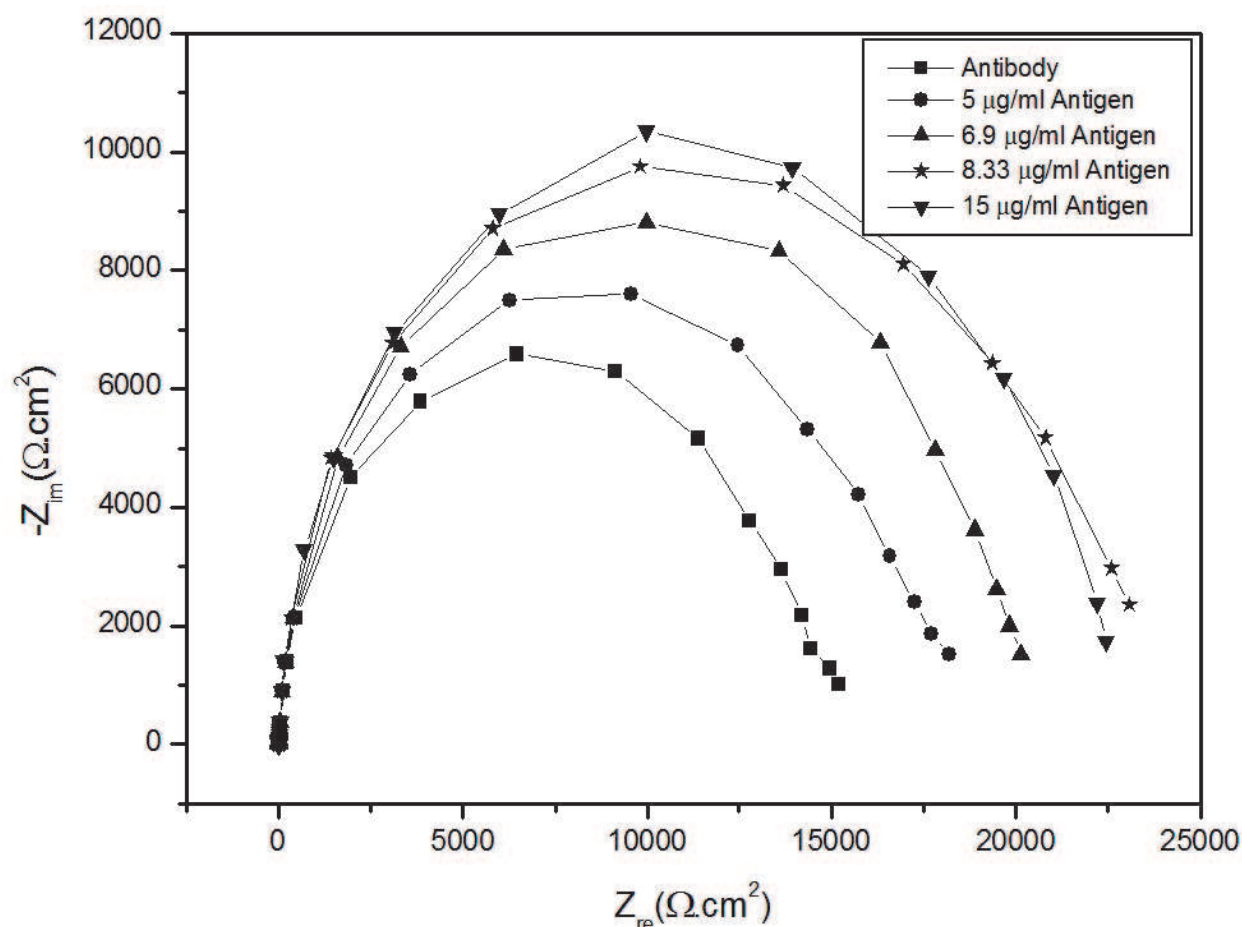

Fig. 5. Impedance spectra of the functionalized gold electrode before and after addition of different H_7N_1 antigen concentration.

The charge transfer resistance increases and reaches a new saturation value that can be determined with the fitting program. This increase could be attributed to a rearrangement in the structure of the antibody and a variation of the dielectric constant. The lowest detection limit that induces a signal variation is equal to 5 µg/ml . This value was lower than the limit detection obtained with ELISA technique.

3.1.2 Calibration and selectivity
In order to obtain the calibration data set, the values of log Z/Z_o versus the antigen H_7N_1 concentration were plotted in figure 6 , where Z is the value of the impedance resistance after antigen binding to antibody, Z_0 is the value of impedance as antibody immobilized on the electrode.

As we can be see in the figure6, the plot is linear and saturate at the higher concentration. To confirm that the above-observed impedance changes generated from the result of specific antibody-antigen interaction, the sensor was exposed to the solution of Newcastle antigen that are expected to bind non-specifically. Figure 6 shows the variation of the impedance

versus the non-specific antigen concentration. As we can see, the sensor was not subjected to the non-specific binding and applicable to the selective determination of H_7N_1.
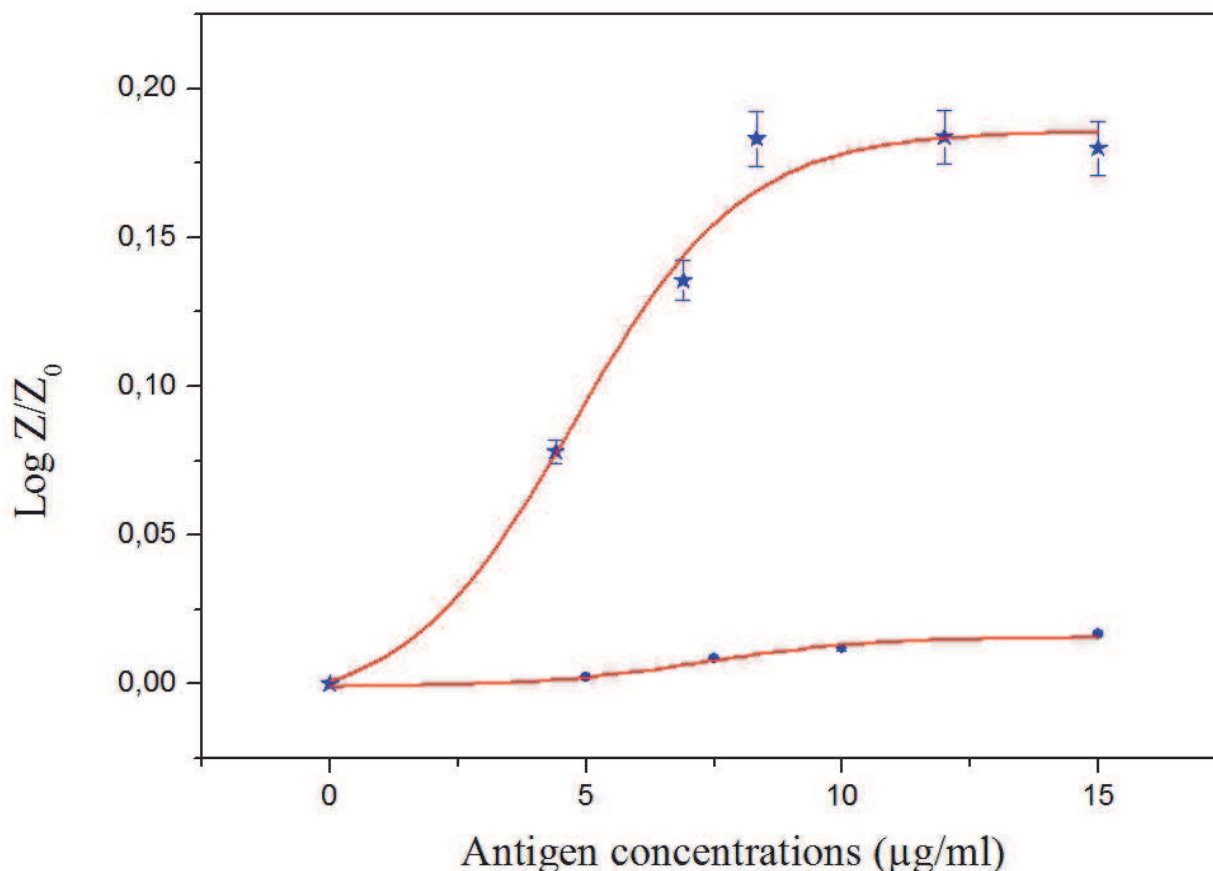

Fig. 6. Calibration curves and selectivity of the developed biosensor.

3.2 Rabbies virus biosensor

We start to characterise the insulating properties of the thiol monolayer on gold surface. The Nyquist diagram of bare gold (fig.7) presents a half-circle, characteristic of a resistance in parallel with a capacity and a linear part which appears at low frequencies and which is assigned to diffusion phenomenon [17]. However, after acid thiol treatment, the diameter of the half-circle of Nyquist diagram increases clearly and the Warburg impedance was not observed. This is shows the high insulating properties of the acid thiol (M et al., 2008).

After, impedance measurements were performed after gold surface activation with EDC/NHS, antibody immobilization and BSA blocking step. After each step, acquisitions of impedance data in PBS were carried out over 5 decades of frequency. Figure 8 presents the Nyquist diagram for the various steps of the biosensor development: SAM layer, antibody layer, blocking with BSA and antigen injection.

We observe that each layer deposition on the gold surface generates an impedance increase. This increase is due to the change of the electric properties of the gold/electrolyte interface.

3.2.1 Calibration and selectivity

For rabbies detection, Figure 9 presents the calibration of the developed biosensors for specific and non-specific detection. It shows a dynamic range between 0.1 µg/ml and 4

µg/ml and a saturation reached at 4 µg/ml. This behaviour can be explained: when the antigen concentration increases in the electrochemical cell, the number of immobilized antibodies, but not complexed, decreases and reaches zero when concentration of antigen is higher than 4 µg/ml. The limit detection of this sensor is about 0.5 µg/ml. This limit detection is better than the limit detection obtained with the others traditional methods for clinical use. In order to prove sensor selectivity, the immunosensor was exposed to a solution containing the Newcastle antigen viruses.
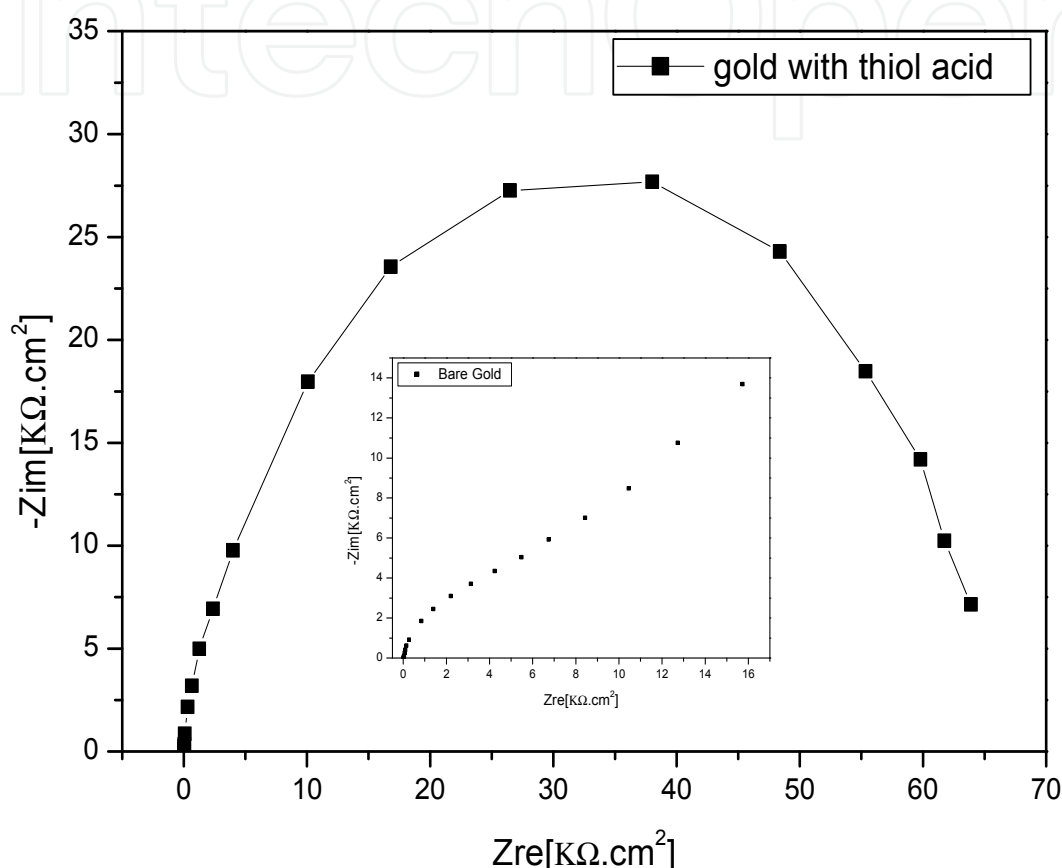
Fig. 7. Nyquist plot for bare gold (small curve) and gold with thiol acid (big curve) in PBS solution with 1 mM redox couple

As shown in figure 9, there is a little variation of impedance after no-specific antigen addition. This little non specific variation can be explained by the use of polyclonal antibodies. In order to avoid non specificity, the use of monoclonal immunoglobulins G (IgGs) is necessary.

4. Conclusion

In this study, we reported the development of biosensor for rabies and for H_7N_1 antigen detection. The affinity interaction of the antibody with specific antigen can be measured with detection limit of 0.5 and 5 µg/ml respectively. The impedance spectroscopy technique is a higher sensitive technique which can be used to measure the high insulating properties of biomembranes. The different steps of biosensor conception were characterized by
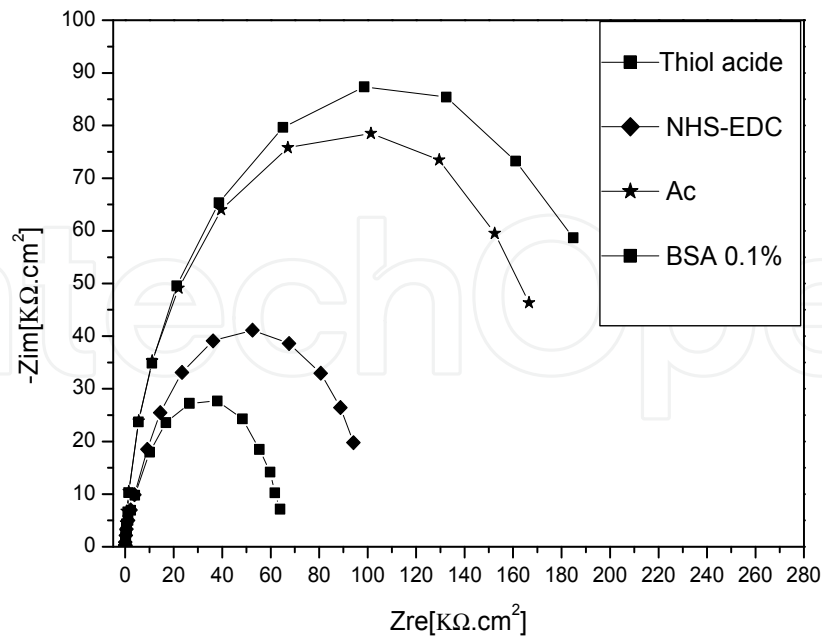
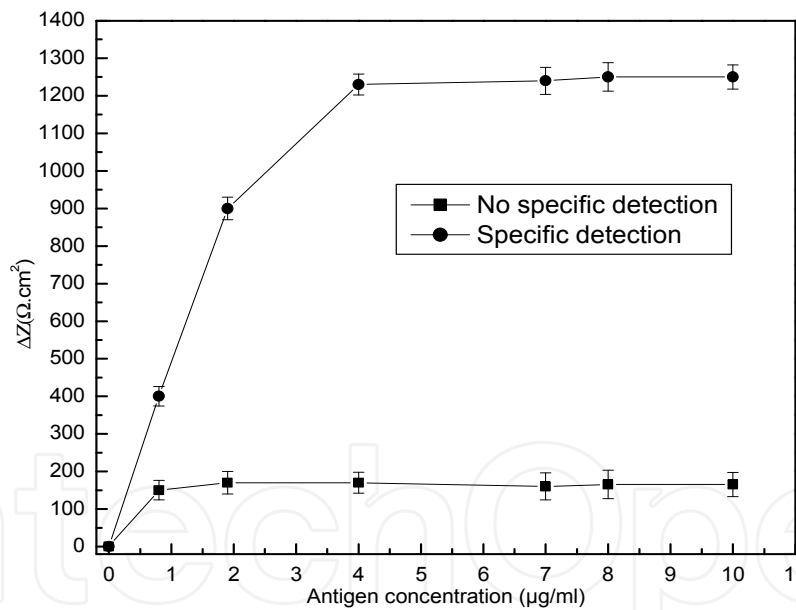Fig. 8. Nyquist plot of the different layers of the rabies biosensors

Fig. 9. Selectivity of the developed biosensor.

electrochemical impedance spectroscopy. The sensitivity of miniaturized biosensor prototype will be improved by using interdigitated gold microelectrodes with microfluidic cell for industrial development and clinical use.

5. Acknowledgements

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 16 different countries. The book consists of 24 chapters written by 76 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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