

Electrodeposited Biotinylated Polypyrrole as an Immobilization Method for Impedimetric Immunosensors

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Abstract—The potentialities of an electrodeposited biotinylated polypyrrole film as an immobilization matrix for the fabrication of impedimetric immunosensors are described. Biotinylated antibody (anti-human IgG), used as a model system, was attached to free biotin groups on the electrogenerated polypyrrole film using avidin as a coupling reagent. The resulting recognition interface consisted of a highly oriented monolayer immobilized onto the polymer surface. Cyclic voltammetry was used to characterize the polymer film. Additionally, scanning electron microscopy and atomic force microscopy were used to investigate the morphology of the immobilized material. This immobilization method allows the obtention of a highly reproducible and stable device. The resulting immunosensor has a linear dynamic range of 10–80 ng.ml⁻¹ of antigen and a detection limit of 10 pg.ml⁻¹. Furthermore, this immunosensor exhibited minor loss in response after two regeneration steps.

Index Terms—Biotinylated polypyrrole, electropolymerization, immunosensor, impedance spectroscopy.

I. INTRODUCTION

DURING the past few years, immunosensors [1], [2] have been the subject of increasing interest, because of their potentialities in several fields, such as clinical diagnostics and environmental control. Owing to their low cost, small size, possible use *in vitro* and *in vivo*, and also their short response time, these devices can compete efficiently with the standard clinical diagnostic methods based on classical immunoassays.

Typically, antibodies specifically directed against the desired analyte (antigen) are immobilized on the transducer surface as

a biofilm. Then, the sensor is exposed to the sample (blood or other biological fluid); if antigen is present, an antigen–antibody complex will be formed. This formation will induce some changes of physicochemical parameters (usually mass, optical, or electrical parameters) of biofilm on the transducer surface, and that change can be subsequently detected. Depending on the transducer technology used, immunosensors can be divided into three principal classes: optical [3], [4], piezoelectric [5], [6], and electrochemical [7], [8]. Regardless of the transduction principle, the coupling of the molecular recognition receptor to the transducer surface is of key importance for the control and the improvement of the characteristics of the derivative sensor.

Thus, in the last 40 years, considerable attention has been focused on conducting polymers because of their unusual electronic properties and their great potential for biomolecule immobilization. Actually, several immobilization procedures based on conducting polymers [9]–[11] were used to ensure the coupling of immuno species. They range from an entrapment within the polymer film [12], [13] to an electrostatic binding [14]. Many industrial applications of biosensors demand stable and defined binding of biomolecules to surfaces according easy and reproducible processes [15], [16]. Thus, some methods take advantage of specific interactions between avidin or streptavidin and biotin to immobilize biomolecules modified by methods of molecular biology in a highly oriented way [17].

Recently, in order to preserve their recognition properties, the immobilization of proteins on electrodeposited polymer film has been attempted using affinity system [18], [19] such as biotin–avidin.

Avidin, found in hen egg white, is a very fascinating protein because of its extraordinarily high binding affinity for biotin. In fact, avidin exhibits the highest known affinity constant K_a in nature between a protein and a ligand ($K_a \sim 10^{15} \text{ M}^{-1}$) [20]. Owing to its high affinity, the avidin–biotin system has been used for coupling proteins to biotinylated polymer films. Therefore, the attachment of proteins was achieved via the formation of an avidin–biotin bridge with biotin or avidin conjugated proteins. This method allowed a high control over the molecule orientation, which is an important parameter for the improvement of the immunosensor characteristics. This immobilization method was already used by other authors [21] to ensure the attachment of glucose oxidase to a glassy carbon electrode.

In this report, we have used the latter method to immobilize the antibodies on prefunctionalized gold electrodes. A

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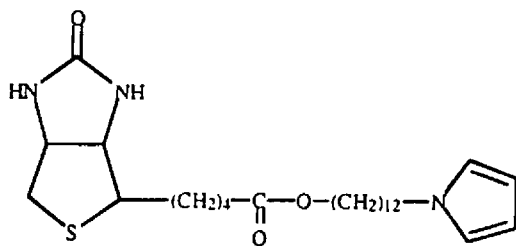


Fig. 1. Structure of the pyrrole biotin.

polypyrrole bearing biotin functions was electrodeposited on gold electrode and used for the subsequent antibody immobilization. The understanding of behavior and properties of the immunofunctionalized layers would allow a better approach of the key parameters involved for the optimization of the resulting immunosensor. In this aim, the properties of the polymeric layers including immunospecies have been studied in terms of permeability and electrochemical properties. Then, the performances of the impedimetric immunosensor based on biotinylated polypyrrole functionalized gold electrodes were studied.

II. EXPERIMENTAL

A. Materials

The antibody used was a polyclonal anti-human IgG developed in goat (γ -chain specific), biotin conjugate, purchased from Sigma. The antigen was a human IgG reagent grade, from serum, purchased from Sigma. Bovine serum albumin (BSA, Fraction V, 96%), from Sigma, was used to avoid nonspecific adsorption phenomena. The pyrrole biotin (Fig. 1) used in this work was synthesized as previously described in [21].

The supporting electrolyte was a phosphate buffer solution (PBS), prepared by dissolving one tablet of phosphate buffer saline, purchased from Sigma, in 200 ml of desionized water. The obtained solution consisted of a 10-mM phosphate buffer, 2.7-mM potassium chloride, and 137-mM sodium chloride. The pH solution at 25 °C was 7.4.

A solution of 0.1-M glycine and 0.4-M NaCl was prepared by dissolution of the reagents in distilled water. The pH was adjusted to 2.3 by titration with HCl. This high ionic strength acidic solution was used systematically to dissociate the antigen-antibody complexes and so to regenerate the recognition surface of the sensor. The solution was stored in the range 0 to 5 °C.

B. Electrochemical Instrumentation and Procedure

The electrochemical experiments were performed with an EG&G Princeton Applied Research 273A potentiostat connected to a Kipp and Zonen BD90 XY|t recorder. The electropolymerization process was run in a conventional three-electrode cell under argon atmosphere at room temperature. A 10 mM Ag|Ag^+ in CH_3CN electrode, platinum foil, and a gold plate were used as reference electrode, auxiliary electrode, and working electrode respectively.

The impedimetric measurements were performed in a classic electrochemical cell, with three electrodes and a volume of 3.5 ml. The counter electrode was made of a platinum plate

with an active surface of 0.48 cm^2 , whereas the active surface of the working gold plate electrode, fixed by an O-ring seal, was 0.16 cm^2 . The polarization of the working electrode was fixed against a saturated calomel reference electrode (SCE), purchased from Radiometer Analytical S.A.

C. Modified Electrode Preparation

First, the gold plate electrodes were cleaned for about 10 min in an etching solution: 1/4(H_2O_2 35%) 3/4(H_2SO_4 96%). Immediately after, the pretreated electrodes were thoroughly rinsed with desionized water in order to remove any deposited impurities, and then dried under a stream of nitrogen. The electrogeneration of the biotinylated polypyrrole film was performed by electrooxidation of the monomer (2 mM) at 0.85 V/ Ag|Ag^+ in acetonitrile solution containing 0.1 M TBAP (Tetrabutylammonium perchlorate) as a supporting electrolyte. The specific binding of avidin to polymer biotin sites was achieved via the incubation of the subsequent functionalized gold electrodes in a 2 mg ml^{-1} avidin solution, for 30 min at 5 °C. The obtained electrodes were carefully rinsed with PBS.

The former electrodes were incubated in a solution of biotinylated antibody (anti-human IgG) at a concentration of 1 mg ml^{-1} during 12 h at 5 °C. These electrodes were then thoroughly rinsed with a PBS solution to remove weakly adsorbed antibodies. Finally, the resulting electrode was exposed to a known concentration of antigen.

D. Impedance Measurements

The various steps of the fabrication of the sensor were controlled by impedance spectroscopy. An impedance analyzer Voltlab 4 from Radiometer Analytical Instrument S.A., controlled by computer, was used to measure the total ac impedance of the electrochemical cell. These measurements were carried out by scanning the frequency from 10^{-2} to 10^5 Hz, acquiring five points per decade. An excitation voltage of 5 mV was over imposed [22], [23]. All measurements were performed in PBS containing 5% of BSA, at room temperature.

E. Atomic Force Microscopy (AFM)

AFM experiments were carried out using a Nanoscope III (Digital Instruments, Santa Barbara, CA) operating under a contact mode in air. We used triangular Si_3N_4 microcantilever (Nanoprobe, NP-S, Digital Instruments) with a spring constant of 0.06 Nm^{-1} .

III. RESULTS AND DISCUSSION

The electropolymerization of the biotinylated polypyrrole was achieved by controlled potential oxidation at 0.8 V to avoid the overoxidation of the polypyrrole and hence to preserve its conductivity [24]. The resulting electrode was transferred, after thoroughly rinsing, to a $\text{CH}_3\text{CN} + 0.1$ M TBAP solution free of monomer. Fig. 2 Shows a typical cyclic voltammogram obtained onto the polymer coated electrode. In the positive region, a reversible peak is recorded at 0.4 V, corresponding to the oxidation of the polypyrrole matrix. This value is in good agreement with the reported $E_{1/2}$ values for poly(N-alkylpyrroles) [25]. This indicates the formation of an

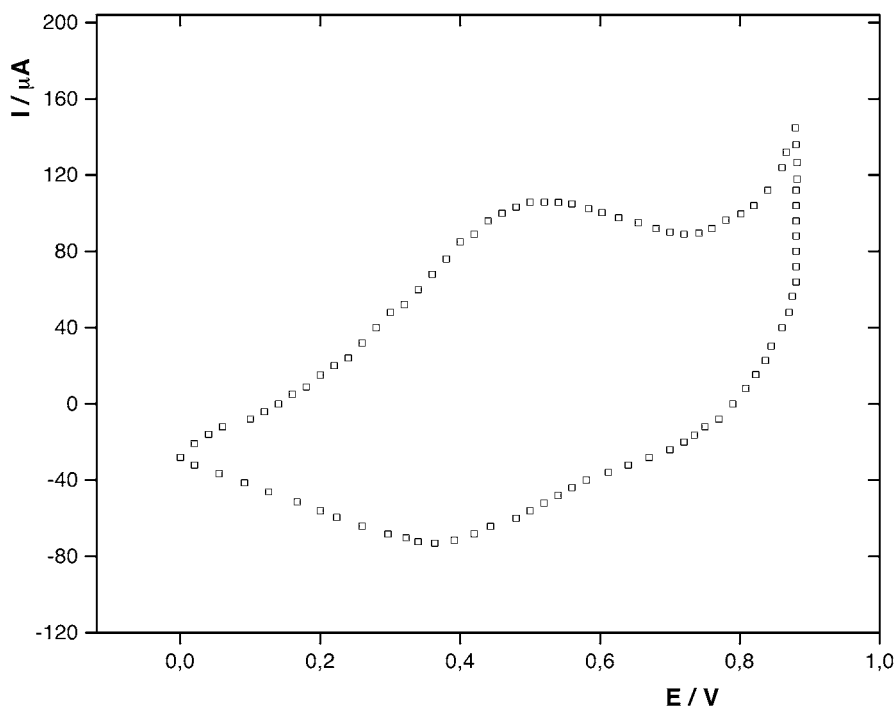


Fig. 2. Cyclic voltammogram of Au|poly(pyrrole biotin) electrode in $\text{CH}_3\text{CN} + 0.1 \text{ M TBAP}$, scan rate 50 mV s^{-1} .

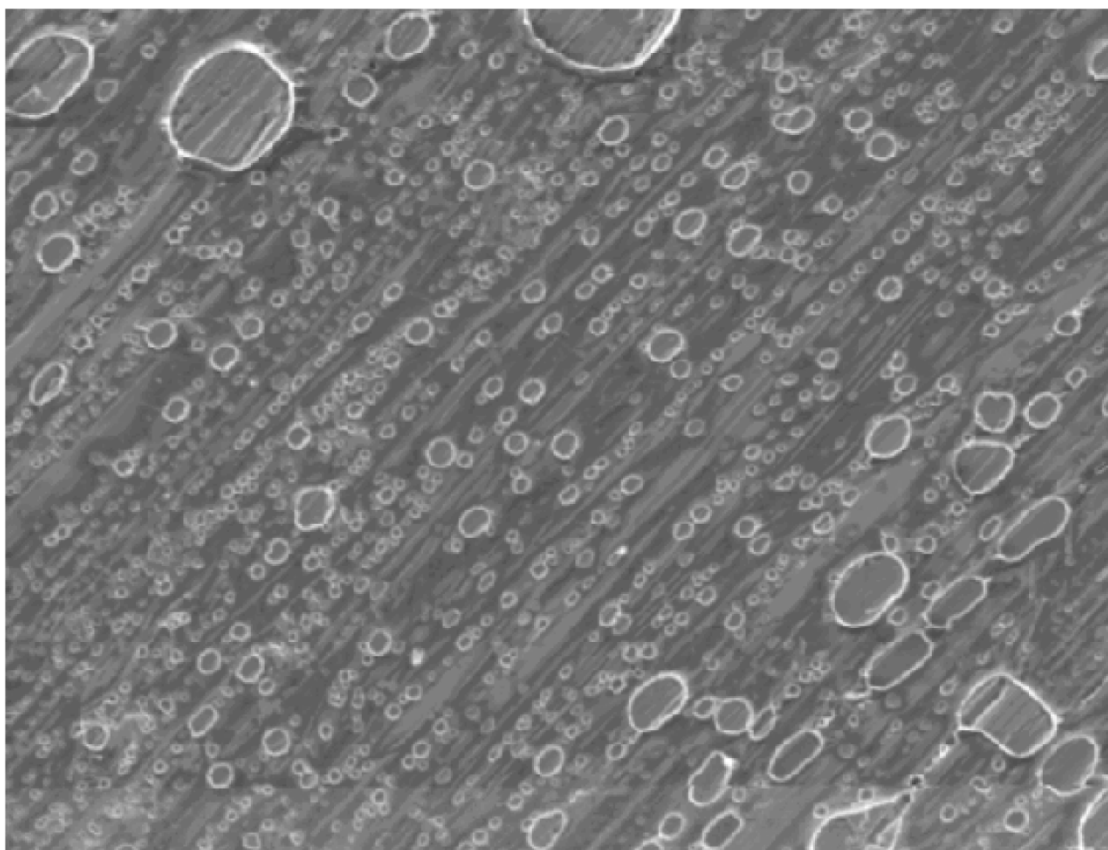


Fig. 3. SEM micrograph ($\times 600$) of poly(pyrrole biotin) electrodeposited on gold electrode.

electropolymerized film of poly(CEPy) on the electrode surface as a consequence of the pyrrole oxidation. The apparent surface coverage $\Gamma = 2.7 \times 10^{-8} \text{ mol cm}^{-2}$ was determined from the

charge recorded under the polypyrrole oxidation wave taking into account that the electrooxidation of the polypyrrole chain corresponds to $0.33 \text{ electron mol}^{-1}$ [26].

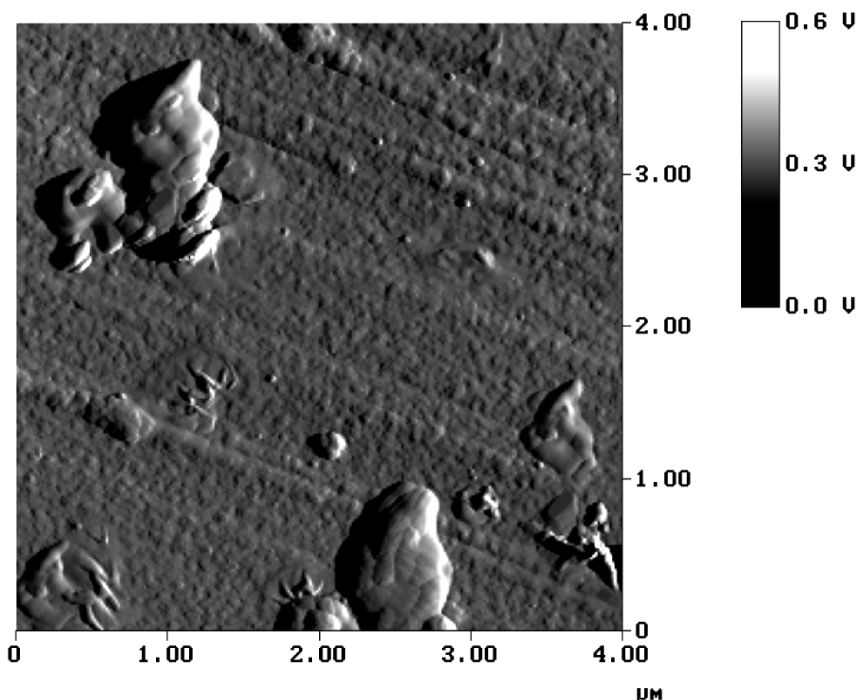


Fig. 4. AFM image in contact mode of poly(pyrrole biotin) film electrodeposited on gold electrode.

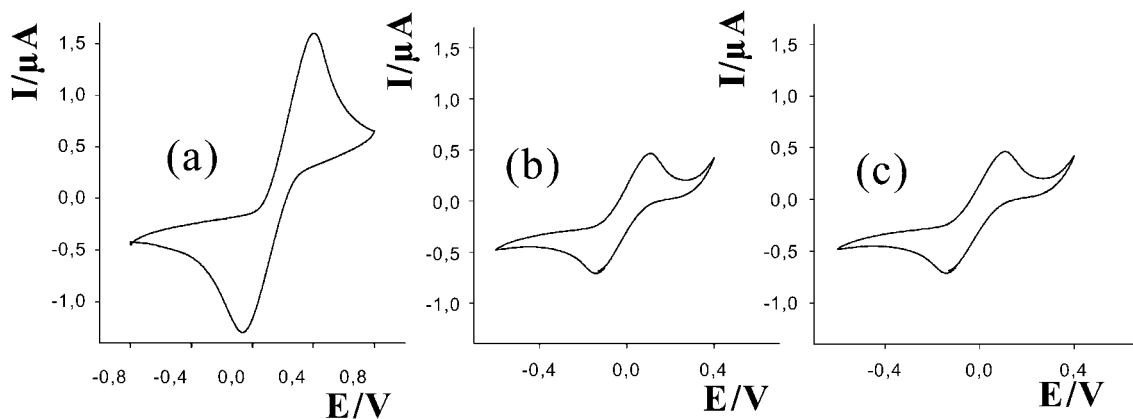


Fig. 5. Cyclic voltammograms of 10-mM ferrocyanide in PBS on (a) a bare gold electrode, (b) a poly(pyrrole biotin) electrode, and (c) a poly(pyrrole biotin) electrode with Avidin, scan rate 0.1 V s^{-1} .

In addition, the morphology of the polymer-coated electrode was monitored by both atomic force microscopy (AFM) and scanning electron microscopy (SEM). Fig. 3. presents the texture of the polymer film, carried out by SEM. So, the SEM micrograph of the polypyrrole biotin deposited on gold electrode, exhibits a randomly distribution of polymer clusters with different sizes (Fig. 3). This character is evenly highlighted on the AFM image shown in Fig. 4, which illustrates that the coverage of electrode surface is not homogeneous.

Additionally, further characterization, using permeation measurements, was carried out to evaluate the effectiveness of each step in the fabrication of the immunosensor. For this purpose, we have proceeded by recording cyclic voltammograms of ferrocyanide used as permeant electroactive probe. Thus, the cyclic voltammetry experiments were carried out in a PBS solution containing 10 mM of both $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6]$. Fig. 5 presents the cyclic voltammograms rela-

tive to the different modification steps. As it clearly observed in Fig. 5, the difference between the gold bare electrode and the polymer-coated electrode voltammograms is obvious. Hence, the electrodeposited polymer covers the surface of the electrode and the access of the redox couple is hindered. Nevertheless, the faradaic process of oxidation and reduction of the redox couple $[\text{Fe}(\text{CN})_6]^{4-/3-}$ is only partially inhibited after the deposition of the polymer film. This suggests the existence of pinholes in the dense polymer film, through which the redox couple can pass toward the gold surface. The clusters observed by SEM and AFM are formed on this dense polymer film. In contrast, the immobilization of avidin does not cause any significant additional variation in the voltammogram spectra.

It is well established that the polymer state (conducting or insulator) is quite dependent on the potential value [27]. In addition, the diffusion phenomena, taking place at low frequency,

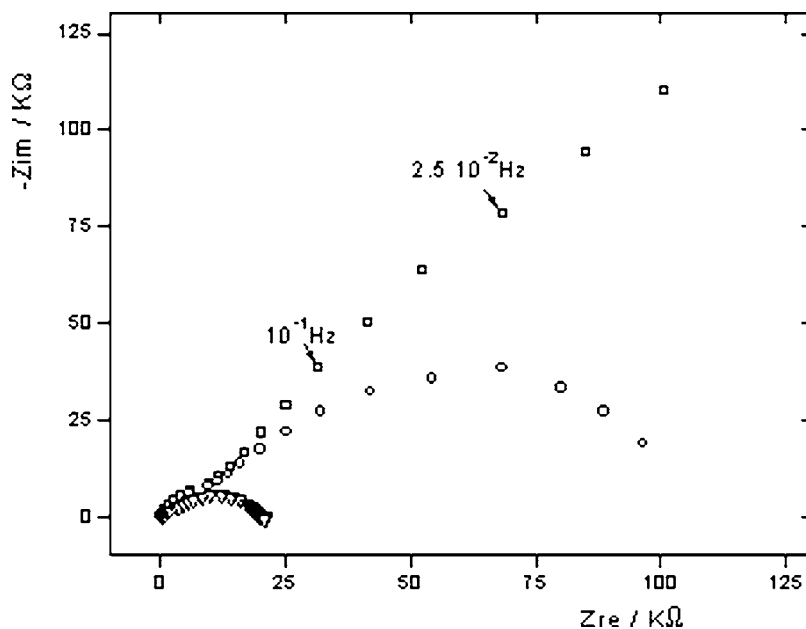


Fig. 6. Complex plane impedance spectra obtained for gold electrode functionalized by electropolymerization of the pyrrole biotin at three different potentials. \square : 0 V. \circ : -1.3 V. ∇ : -1.4 V.

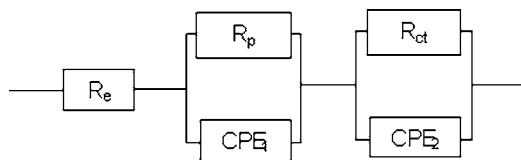


Fig. 7. Equivalent electrical circuit used in the theoretical fit of the impedance diagram of the polymer-coated electrode at a bias potential -1.4 V.

could mask the specific antibody–antigen interaction, which also takes place at the same frequency range. So, it will be necessary to minimize the former phenomena in order to allow the latter phenomenon to appear. For this purpose, the impedance of the polymer-coated electrode was recorded at different bias voltage (Fig. 6). The impedance measurements show typical curves for polypyrrole films deposited on metal [28]. For all potentials, a semicircle is present at high frequency. Whereas, in the low-frequency region, next to the first semicircle, a straight line having a slope at an angle higher than 45° to the real axis, reflecting both diffusion phenomena and/or the roughness of the electrode surface. This behavior is attenuated with the decrease of the bias potential and vanished quasitotally at a potential of -1.4 V. Therefore, this latter potential was chosen for the following studies.

The electrochemical behavior of the polymer-coated electrode, under a bias potential of -1.4 , was simulated by the electrical equivalent circuit of Fig. 7, which was found to give the best fit results. The two R and CPE (constant phase element) combinations correspond to the two depressed semicircles. Where R_e is the electrolyte resistance, R_p and CPE_1 are the parameters which characterize the polymer film, CPE_2 is a constant phase element which substitutes the well-known double layer capacitance (C_{dl}) and which takes into account the phenomena related to the roughness of the electrode surface

and diffusion processes [29], and R_{ct} is the charge transfer resistance. Curve fitting of the experimental result was performed with the program “EQUICRVT” [30] (Fig. 8).

The ability of the polymer film for the immobilization of biotinylated antibodies via the formation of avidin-biotin bridge has been tested by impedance spectroscopy. For this purpose, the impedance of the polymer layer, for the successive bindings of the avidin and antibody (anti-human IgG) has been recorded (Fig. 9). The diameter of the second semicircle is shown to increase after immobilization of avidin, as well as after immobilization of the antibody. This clearly indicates the binding of avidin to biotin groups of the polymer film and then the binding of biotinylated antibodies to avidin.

In order to decrease nonspecific effects, 5% of BSA was permanently present in the buffer solution. The antibody-functionalized electrode was exposed to different concentrations of antigen (human IgG reagent). As shown in Fig. 10, the second semicircle diameter in the Nyquist plot seems to increase with increasing the antigen concentration. Another possible representation of the impedance spectra is the so-called Bode plot. In this representation, it appears that the major changes in the impedance character occurred at low frequency (Fig. 11). This means that the most significant change of the electrochemical properties of the biofilm takes place at low frequency. Therefore a frequency of 40 mHz appears to be suitable for concentration-dependent impedance measurements.

In order to obtain a calibration curve for the sensor, the variation of $\text{Log}(|Z|/|Z_0|)$ was plotted versus the values of the antigen concentration at frequency of 0.04 Hz, where $|Z_0|$ is the modulus of the impedance of the antibody modified electrode before the injection of antigens and $|Z|$ is that of the same electrode after each antigen addition. A typical calibration curve for antigen is shown in Fig. 12.

This calibration curve was quasilinear with antigen concentration from 10 to 80 ng, and then curved gradually at higher

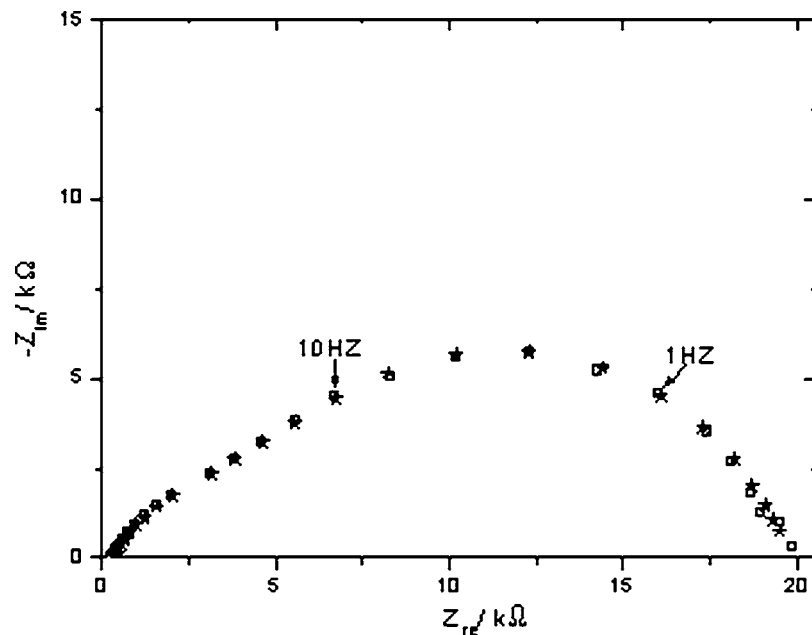


Fig. 8. (O) Experimental and (*) calculated impedance plots obtained for a polymer-coated electrode at bias potential -1.4 V.

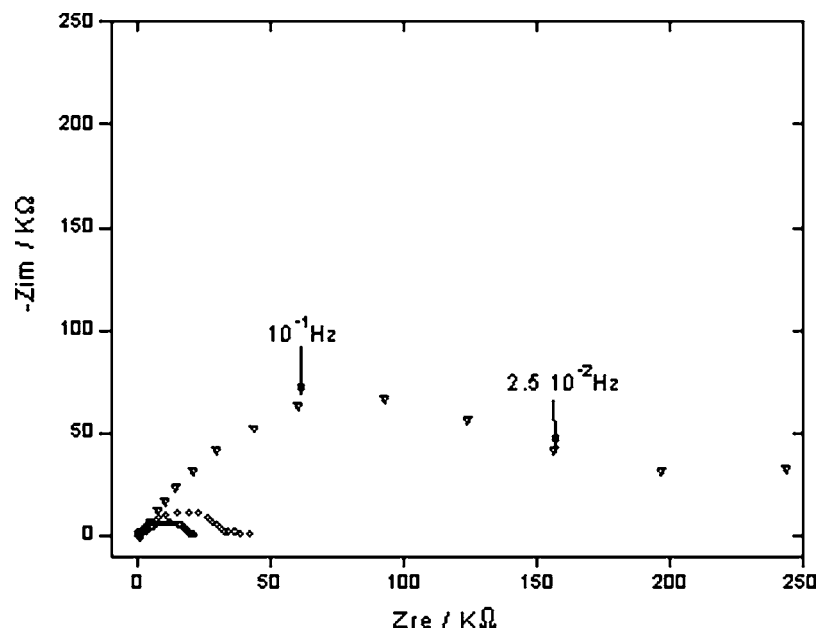


Fig. 9. Complex plane impedance plots for a bare gold electrode after different modification steps. □: After electrodeposition of the poly(pyrrole biotin) film. O: after immobilization of avidin. Δ : After immobilization of the antibody.

concentrations. Therefore, the immunosensor had a linear dynamic range from 10 to 80 ng ml^{-1} and a detection limit of about 10 pg ml^{-1} . The former limited dynamic range can be explained by the poor electropolymerization ability of the biotin pyrrole derivative. So, only few biotin sites could be generated. To overcome this drawback, copolymerization of pyrrole biotin with a pyrrole ammonium have been attempted, and layers with different thickness have been obtained. Unfortunately, the derivative electrodeposited film had poor stability in the solution used for immunosensing tests.

Since the antigen-antibody binding is mainly determined by the high degree of complement in steric structures between antigen epitopes and antibody paratopes, solutions such as

acid, base, or salts of high ionic strength [31]–[33] could be used to split the immune complexes. Furthermore, it should be noted that the effect of an elution reagent depends on the elution time. Therefore, in order to split the antibody-antigen complex and, hence, to regenerate the recognition surface of the sensor, glycine buffer (pH 2.3) was applied for 3 min. Such protocol was, already, shown to be efficient by other authors [34]. Next, the electrode was washed with PBS. As unexpected, the sensitivity of the sensor, given as the slope of the linear part of the calibration curve, increased after the first regeneration (Fig. 12). Such a surprising result was already found by other authors using classical immunosensing tests (ELISA) [35]. This unexpected phenomenon can be explained, in part, by

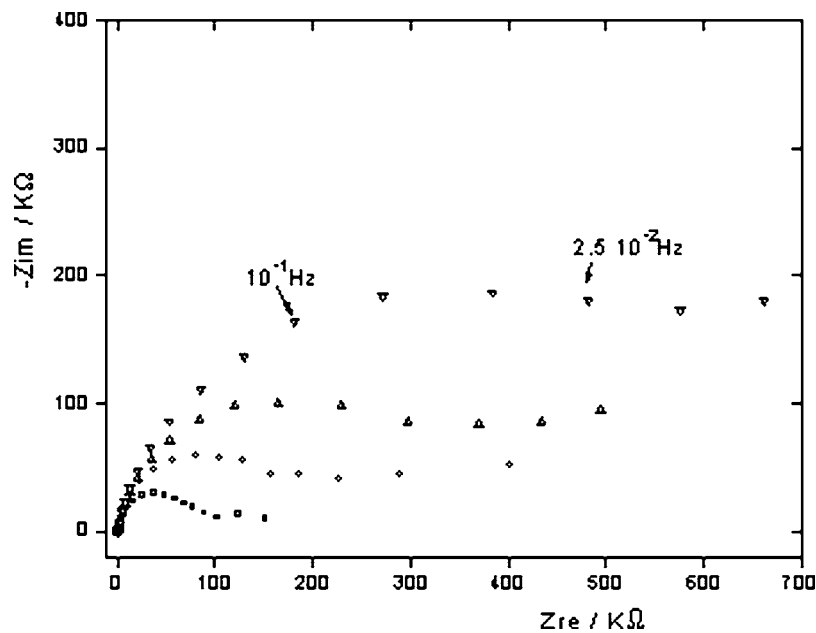


Fig. 10. Complex plane impedance plots obtained for an antibody modified electrode at a potential of -1.4 V versus SCE and ac signal of 5 mV, in PBS. \square : Without antigen injection; after antigen injection at different concentrations. \circ : 10 ng ml^{-1} . Δ : 50 ng ml^{-1} . ∇ : 90 ng ml^{-1} .

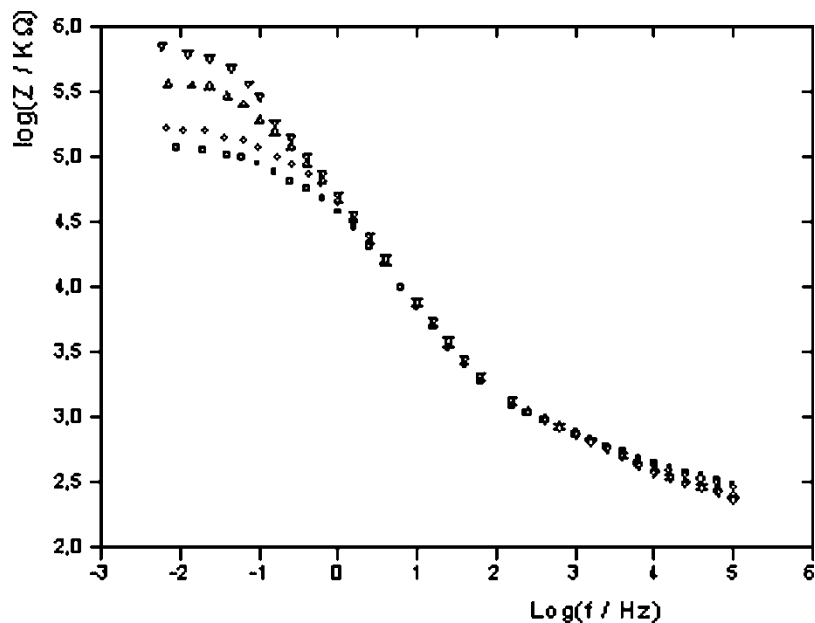


Fig. 11. Bode plane impedance spectra obtained for an antibody modified electrode at a potential of -1.425 V versus SCE and ac signal of 5 mV, in PBS. \square : without antigen injection; after antigen injection at different concentration. \circ : 10 ng ml^{-1} . Δ : 50 ng ml^{-1} . ∇ : 90 ng ml^{-1} .

the improvement of the accessibility of the binding sites of the antibody after short-time acidic washing, whereas repeated acidic washing of the sensing layer may reduce the activity of the antibody, which can explain the decrease of the sensitivity of the sensor after the second regeneration.

IV. CONCLUSION

A biotin-avidin system has been used to immobilize biotinylated antibodies on gold electrode-functionalized by

electropolymerized biotinylated polypyrrole in order to conceive an impedimetric immunosensor. Commercial anti-human IgG, biotin conjugated, was used as a model system. The sensor, exhibits a dynamic range 10 – 80 ng ml^{-1} and a detection limit of 10 pg ml^{-1} . Furthermore, the regeneration of the sensing biolayer was successfully reached using an acidic solution. In future work, different ways will be tested in order to enlarge the dynamic range of this sensor: increasing density of biotin groups on the electropolymerized pyrrole film, piling up of several biotinylated antibody layers with successive avidin treatments.

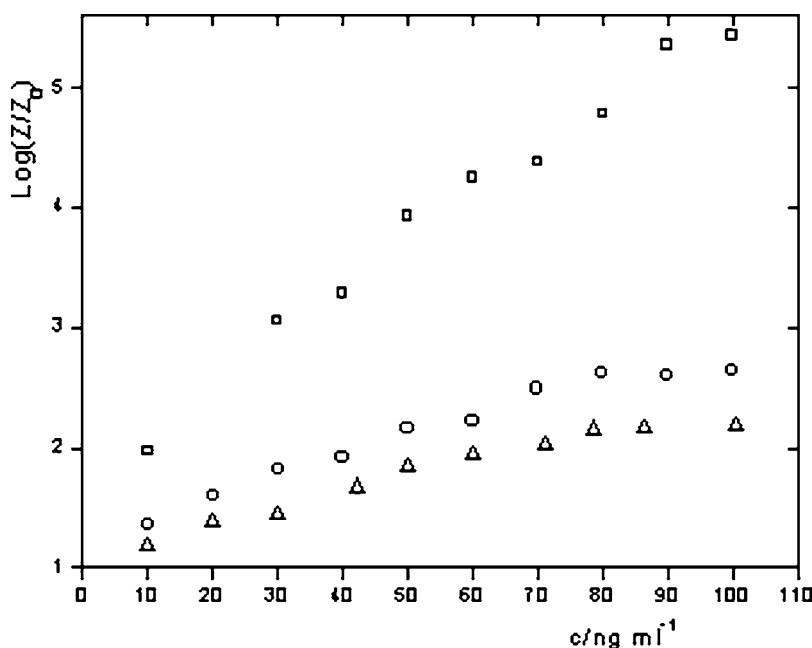


Fig. 12. Response curve of the sensor, applied potential -1.4 V versus SCE, frequency 0.04 Hz, and 0.01 M PBS (pH 7.2). O: First usage. □: after the first regeneration. △: After a second regeneration.

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