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# Characterisation by Impedance Spectroscopy and Capacitance-Voltage of an EMIS Sensor Functionalized by Catalase for Nitrite Detection

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**Abstract:** Impedance spectroscopy and capacitance-voltage (C-V) methods are a rapidly developing electrochemical technique for the characterization of biomaterial–functionalized electrodes and biocatalytic transformations on the electrodes surface, and specifically for the transduction of biosensing events at electrodes. Such techniques have been used in our work as a tool for the characterization of a new nitrite biosensor for environmental applications based on the immobilization of catalase on insulator-semiconductor (IS) systems (p-Si/$SiO_2$/$Si_3N_4$). The principle of the developed biosensor includes the following: Catalase catalyzed the breakdown of $H_2O_2$ into $H_2O$ and $O_2$. Nitrite was selected as an inhibitor of catalase. Under optimal conditions, i.e. buffer capacity corresponding to 3 mM phosphate buffer, the catalase enzyme insulator semiconductor sensors shows a high sensitivity to nitrite detection. In both cases, the responses of these biosensors based on nitrite additions are good with the detection limit around $10^{-11}$ M. It is expected that such an original and promising concept of inhibitor-based biosensors based on reactivation by inhibitive effects, will be useful for the development of environmental smart biosensors based on the integration of ENFET with the corresponding instrumentation in the same silicon chip. *Copyright © 2014 IFSA Publishing, S. L.*

**Keywords:** EIS structure, Biosensors, Catalase, Nitrite, EMIS, Electrochemical measurement.

## 1. Introduction

Ion-or analyte-sensitive capacitive EMIS sensors are often constructed from a pH-sensitive EIS structure by means of coupling the gate with different chemical or biological recognition elements using an additional organic layer of the linking molecules or by depositing an additional membrane, containing subsequent chemical or biological recognition elements [1-5]. An impedance effect of this additional membrane is, however, not always negligible resulting to unusual capacitance-voltage (C-V) curves and consequently, to possible inaccurate measurements [6, 7].

In the last few decades, the development of enzyme biosensor devices has been a topic of considerable interest due to their potential applications. Applications can touch a large variety of fields including medicine, drug discovery, environment, food and process industries [8]. Among a large number of enzymes used for biosensor construction, catalase takes a part in enzyme sensor development due to the large demand for nitrite determination [9]. Indeed nitrite is one of the well-



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known inorganic pollutants in environmental, food, industrial and physiological systems. The widespread nitrite pollutant becomes toxic in human body and Animals [10, 11]. So some Communities have established the maximum admissible levels of nitrite in drinking water at 0.1 mg/L, increasing the demand for sufficiently sensitive, accurate and uncomplicated analytical procedures for nitrite.

In this study, we have used silicon nitride ($Si_3N_4$) for its low drift, low permeability and low structural porosity. Our study has been carried out on Electrolyte-Insulator-Semiconductors. EIS devices are electronic devices that have been developed to measure pH [12]. To extend their affinity for other ions than $H^+$, it is necessary to functionalize these structures with sensitive membranes [13]. We propose to study the inhibition of the reaction catalase-nitrite by impedance spectroscopy and C-V method. We describe the possibility of obtaining a novel enzymatic sensor based on catalase deposited on $Si/SiO_2/Si_3N_4$ electrode sensitive to nitrite determination in the presence of oxygen, based on the catalase inhibition by this pollutant. The biolayer was deposited by a spin coating method on $Si/SiO_2/Si_3N_4$ electrode used as physical transducers. Therefore, the developed EMIS sensor has been characterized by means of impedance spectroscopy and capacitance-voltage (C-V) method, respectively. Electrochemical techniques have been proven to be a valuable tool for the study of catalase properties and applied to construction of an enzyme electrode. Direct electrochemistry of catalase provides a model for investigating mechanisms of redox transformations between enzyme molecules in biocatalysts and metabolic processes involving electron transportation in biological systems. From fitting impedance data to appropriate equivalent electrical circuit, one can get specific characteristics of working electrode, in particular, properties of electrode/electrolyte interface that can be changed due to the formation of enzyme–inhibitor complex [14, 15].

## 2. Experimental

### 2.1. Reagents

Peroxidase from horseradish (HRP, EC1.11.1.7, 222 U mg−1) and catalase (EC1.11.1.6, from bovine liver, 1600 U mg−1 solid) were obtained from Sigma-Aldrich. Glutaraldehyde (grade II, 25 % aqueous solution) was purchased from Sigma-Aldrich. All other reagents were of analytical grade and used without further purification.

### 2.2. Sensor Insulator-Semiconductor (IS) Structure

The studied $Si/SiO_2/Si_3N_4$ structures were provided by LAAS–CNRS (Toulouse, France). They were based on a p-type silicon substrate, 400 μm thickness, with 10 Ωcm resistivity, covered with a 50 nm layer of thermally-grown silicon dioxide and a 100 nm layer of silicon nitride prepared by low-pressure chemical vapour deposition (LPCVD) technique at 750 °C. The ohmic contact was obtained by deposition of indium/gallium alloy on the silicon unpolished face.

### 2.3. Enzyme Immobilization

Before use, $Si/SiO2/Si_3N_4$ electrodes were sonicated for 10 min in acetone, and then dried under nitrogen flow. After that, the $Si_3N_4$ surface was cleaned for 1 min with a freshly prepared “piranha” mixture ($H_2O_2$: $H_2SO_4$, 3:7, v/v) and rinsed carefully with large amounts of deionized water. Then plates were washed with ethanol, rinsed with water and finally dried under a nitrogen flow. The biocoatings were elaborated by spreading 20 μL of phosphate buffer (20 mM, pH 7.5) containing a mixture of 6 % of BSA, 10 % of glycerol and either 4% of catalase After deposition of the protein solutions, the sensors were placed for 25 min in saturated glutaraldehyde vapour inducing a cross-linking process and dried at room temperature.

### 2.4. Measuring System

The measurement set-up used an electrochemical cell with three electrodes: auxiliary platinum electrode, saturated calomel electrode (SCE) and the bio-membrane Si3N4/SiO2/Si structure as a working electrode (contact surface: 0.3 $cm^2$). Capacitance measurements were performed at a frequency of 10 kHz and with signal amplitude of 10 mV using an impedance analyzer (Voltalab 40 from Radiometer Analytical, SA, France) and a PGZ 301 as a potentiostat. The capacitance voltage measurements were carried out at DC voltage that was swept from 0 to 2 V. The whole system was computer-controlled. The experiments were performed in a dark and grounded metal box to eliminate electrical interferences. Solutions for testing the ion-sensitivity were PBS (Phosphate Buffer Solution) in the concentration 5 mM at pH<7.3.

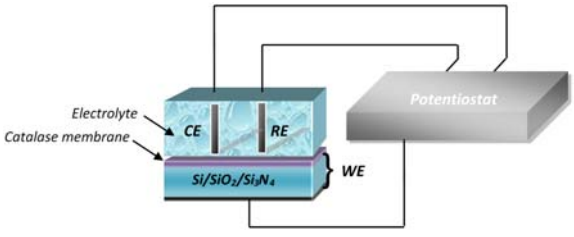


**Fig. 1.** Schematic representation of the Electrolyte-Insulator-Silicon (EIS) structure with a work electrode (WE), counter electrode (CE), and a reference electrode (RE).

## 3. Results and Discussion

### 3.1. Sensor Characterization

The $Si/SiO_2/Si_3N_4$/catalase heterostructures were characterized using impedance spectroscopy and capacitance vs. applied voltage (C-V) measurements (Fig. 1). Experiments were made using an electrochemical cell biased through the classical potentiostatic three-electrode method [16-17].

Capacitance measurements were performed in 5 mM PBS at pH 7.3. The measurements have been performed at a working frequency of 10 kHz [18]. Fig. 2 shows a typical set of C–V curves of the nitrite-sensitive EMIS sensor as a function of the concentration of nitrite. As expected, due to the change of the potential at the electrolyte/membrane interface, the C–V curves shift along the voltage axis depending on the concentration of nitrite, towards the negative potential when the concentration increases.

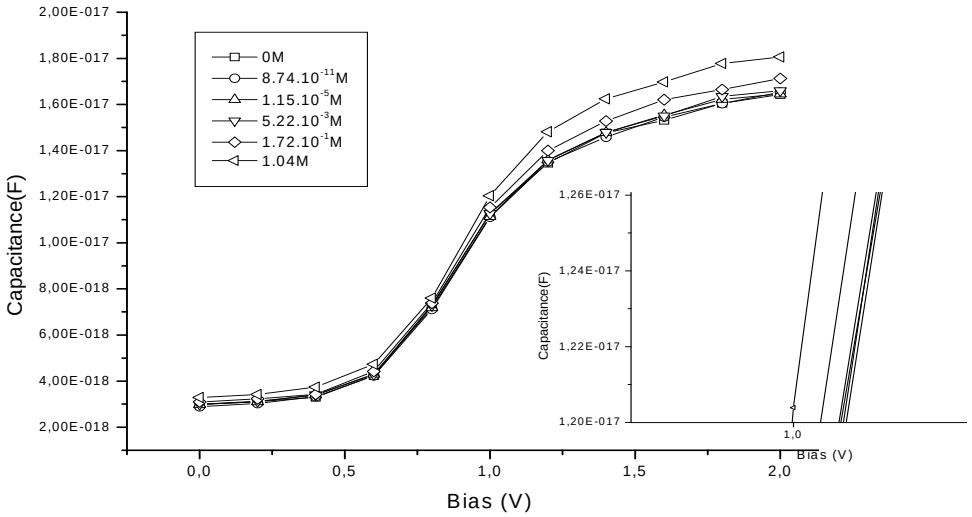


**Fig. 2.** Variation of capacitance versus potential for $Si/SiO_2/Si_3N_4$/catalase biomembrane for nitrite concentration.

When charges are absorbed at pH-sensitive insulator-semiconductor (IS) transducer surface, the C(V) curves shift along the potential axis. This shift is due to the accumulation of ions produced during the enzymatic transformation of nitrite at the bio-functionalized transducer surface. The flat-band potential, $V_{FB}$, varies with nitrite concentration in a bulk solution. The sensor sensitivity is determined by the slope of the curve giving $\Delta V_{FB}$ as a function of the nitrite concentration. On the nitrite concentration increase, the capacitance in accumulation mode increase, which means that the changes in the dielectric constant and in thickness of the bio-recognition membrane. In depletion region, field effect and C(V) curves shift along the potential axis takes place, which involves variation of the flat-band potential towards the increase of nitrite concentration. In order to study the sensitivity of bio-functionalized $Si/SiO_2/Si_3N_4$ structures, the concentration of nitrite was varied from $10^{-11}$ to 1 M.

The sensor sensitivity is determined by the slope of the curve giving $ДV_{FB}$ as a function of the nitrite concentration. The corresponding sensitivity plot, in which the flat band voltage shift E is plotted versus $pNO_2^-$ (Fig. 3). The observed response is linear with a slope of 69 mV per decade, it is quasi nernstian for monovalent anion for concentrations between $10^{-5}$ to 1 M. The detection limit found is $10^{-11}$ M.

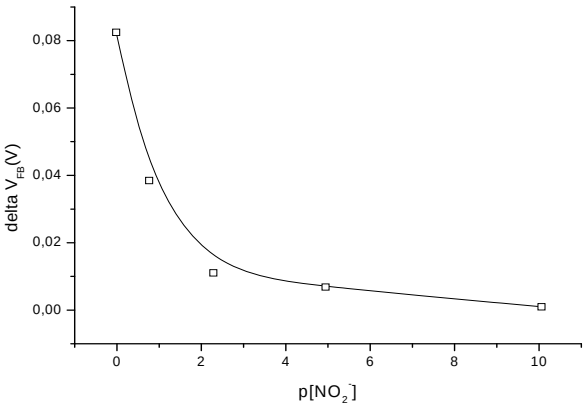


**Fig. 3.** Variation in flat-band potential versus nitrite concentration.

The electrochemical impedance spectroscopy measurements were presented in the complex plan as a plot of $-Z_{im}$ vs. $Z_{re}$ which represents the imaginary and real part of the Z, respectively. Fig. 4 shows the Nyquist plots of the bare IS electrode and of the IS/catalase biomembrane performed in the frequency range from 100 kHz to 1 Hz. The impedance of $Si/SiO_2/Si_3N_4$/catalase structure was measured in the accumulation range. The interface membrane-electrolyte was blocking, it is characterized by an impedance spectrum as a straight line in Nyquist

representation. We observe an increase of impedance, correlated to $R_p$ augmentation, occurred after nitrite addition. The increase of $R_p$ parameter may be attributed to the modification of enzyme conformation following nitrite binding process, inducing charge redistribution and/or conductance changes in the enzyme membrane. Nitrites form a complex with the iron of the heme of the enzymes catalase, thereby reducing catalytic activity of the latter.

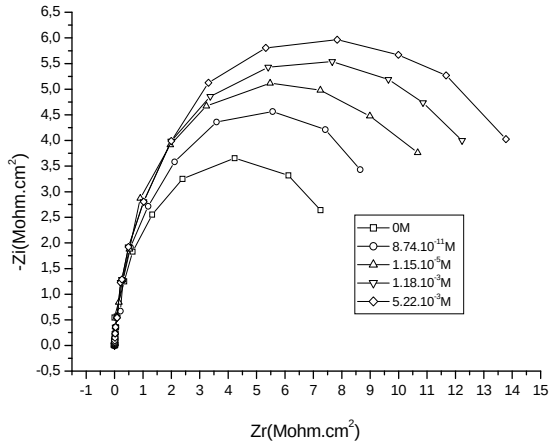


**Fig. 4.** Nyquist diagram ($-Z_{im}$ vs. $Z_r$) at +1.2 V vs. SCE in PBS solution pH 7.3 obtained for the impedance measurements on the catalase biosensor under various concentrations of Nitrite concentrations. Spectra were obtained between 1 MHz and 100 kHz. Amplitude of alternative voltage: 10 mV.

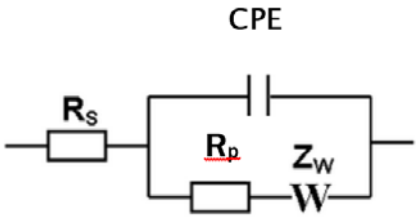


**Fig. 5.** Randles equivalent circuit of the biosensor device.

Rs, $R_{ct}$ and CPE values, obtained by fitting experimental data with the Randles model (Fig. 5), remained quite constant over the whole range of nitrite concentration. The absence of variation for CPE parameter may be explained by the small size and mass of nitrite compared to that of catalase biomembrane thickness is therefore not significantly modified by nitrite binding. Benilova et al. and Bouyahia et al. reported similar results for glycoalkaloids binding to butylcholinesterase and catalase bending to cyanide [15, 19].

To obtain the calibration curve of the $Si/SiO_2/Si_3N_4$/catalase sensor, we have plotted the variation of log Z vs. the nitrite concentration. As seen in Fig. 6, the response of catalase biomembrane shows a large linear range. Moreover, $R_{tc}$–$R_{tc0}$ increased linearly with nitrite concentration from $4.5\times10^{-5}$ to $3\times10^{-2}$ M. The detection limit is determined to be ~ $10^{-11}$ M of nitrite.

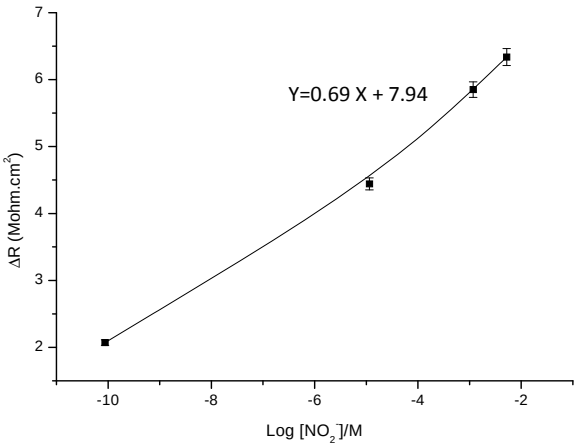


**Fig. 6.** Response of $Si/SiO_2/Si_3N_4$/catalase sensor in the presence of different concentrations of nitrite in PBS solution pH 7.3.

## 4. Conclusion

It has been shows that catalase biomembrane deposed on pH-sensitive $Si/SiO_2/Si_3N_4$ structures can be used for the development of capacitance sensor sensitive to nitrite. A nernstian sensitivity and a low detection limit of about $10^{-11}$ M were obtained. These results can be considered as promising concept of inhibitor-based biosensors will be of interest for other enzymatic systems.

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