

## GLUCOSE OXIDASE "WIRED" WITH POTASSIUM FERRICYANIDE POLYPYRROLE FILMS

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*S-a realizat funcționalizarea suprafeței unui film de polipirrol conductor electric (PPY) cu fericianură de potasiu (FC) pentru imobilizarea glucozoxidazei (GOD). FC a fost introdusă direct în soluția de polimerizare a pirolului. Rezultatele au fost comparate cu cele obținute în condiții similare fără fericianură. Compoziția filmului depus pe suprafață a fost stabilită prin spectroscopie fotoelectronică de rază X (XPS). S-a studiat răspunsul de voltametrie ciclică (CV) pentru substraturi de PPY funcționalizate cu GOD într-o soluție de tampon fosfat. Rezultatele CV susțin mecanismul în care FC acționează ca mediator pentru a transfera electroni între electrod și enzimă permițând regenerarea enzimei din reacția enzimatică cu glucoză.*

*The surface functionalization of an electrically conductive polypyrrole film (PPY) with potassium ferricyanide (FC) for the immobilization of glucose oxidase (GOD) has been carried out. FC was introduced directly in the pyrrole polymerization solution. The results were compared with those obtained in similar conditions without ferricyanide. The composition of the film on the surface was characterized by X-ray photoelectron spectroscopy (XPS). The cyclic voltammetric (CV) response of the GOD-functionalized PPY substrates was studied in a phosphate buffer solution. The CV results support the mechanism in which FC acts as mediator to transfer electrons between the electrode and enzyme affording enzyme regeneration in the enzymatic reaction with glucose.*

**Keywords:** Polypyrrole; Glucose oxidase; Potassium ferricyanide; Enzyme immobilization; Electrical wiring of enzyme

### 1. Introduction

Conducting organic polymers have emerged as potential candidates for electrochemical sensors. Due to their straightforward preparation methods, unique

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properties, and air stability, conducting polymers have been applied to energy storage, electrochemical devices, memory devices, chemical sensors, and electrocatalysis. Conducting polymers are also known to be compatible with biological molecules in neutral aqueous solutions. Consequently, they are extensively used in the fabrication of accurate, fast, and inexpensive biosensors and chemical sensors used in clinical laboratory and environmental monitoring. In these devices rapid detection, high sensitivity, small size, and specificity are achievable making them valuable for environmental monitoring and clinical diagnostics [1,2]. Moreover, the polymer itself can be used to immobilize biomolecules to an electrode [3].

During the last two decades, conducting polymers have emerged as interesting materials for the fabrication of electrochemical sensors [4]. The advantages of conducting polymer based sensors over other available techniques are improved response and sensitivity to small perturbations. Earlier inert polymers were being used only to provide mechanical strength to the membranes but conductive polymers improve the sensitivity of the sensors due to their redox properties.

Another advantage of conducting polymers is that electrochemical synthesis allows direct deposition of a polymer film onto the electrode surface followed by the immobilization of biomolecules. It is thus possible to control the spatial distribution of the immobilized enzymes, the film thickness, and to modulate the enzyme activity. Conducting polymers can act as electron promoters. Moreover, conducting polymers can be deposited over defined areas of electrodes. The unique properties of conducting polymers have been exploited for the fabrication of electrochemical sensors and biosensors. [5]

Nowadays, many biochemical compounds, such as salts, sugars, proteins, hormones, DNA, etc. are analyzed to assist in diagnosis and to assess disease. The monitoring of the biochemical compounds in the body fluids requires typical analytical methods for biochemical tests, experts to run the tests, and time to perform the clinical tests. Since the levels of various compounds in a body system are directly related to some diseases, continuous, fast, and sensitive monitoring of these species is required. In this context, an electrochemical biosensor is a promising analytical method for sensitive and selective detection of biomolecules [6].

Most biosensors described in literature use an enzyme as biological recognition element. Several amperometric enzymatic biosensors have been fabricated by covalent immobilization of oxidases on glassy carbon electrodes [7-17].

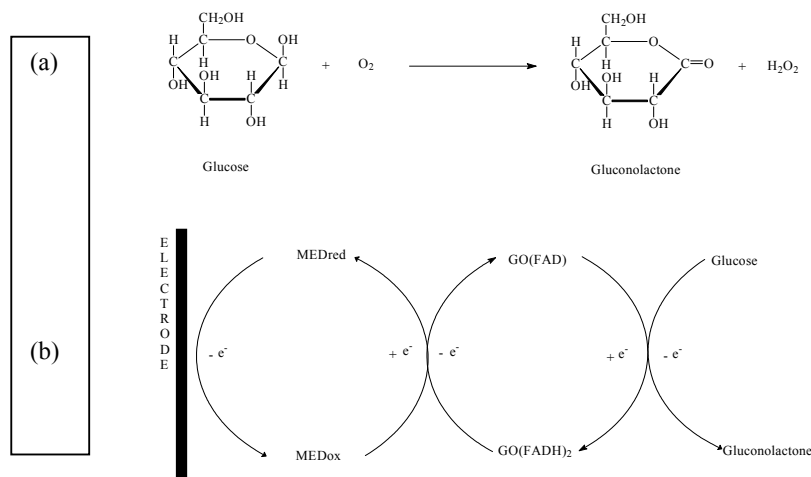


Fig. 1. (a) Reaction scheme for the oxidation of glucose in the presence of  $O_2$ ; (b) Reaction scheme for the enzymatic oxidation of glucose in the presence of a redox mediator

Among conducting polymers, polypyrrole (PPY) is well characterized and is probably one of the most suitable polymers for biosensor applications because it is stable and biocompatible. However, the mechanism by which enzyme molecules are trapped within the PPY network is uncertain [18], and the efficiency of the current response obtained from such electrodes, most of the time due to successful enzymatic oxidation of glucose in the presence of glucose oxidase (GOD), is lower than the expected values [19]. While the response of the enzyme electrode will increase with the amount of enzyme at the electrode, an increase of the film thickness can result in a decrease of the response. This is due to the fact that bulk analyte entering the film will react with the enzyme at the front surface of the film. As a result, the co-product is more easily lost to the bulk solution, instead of diffusing through the film to be detected at the underlying electrode [20].

To enhance the electron transfer, redox mediators have afforded the coupling of enzymatic and electrochemical reactions. The mediator (MED) participates in the transfer of electrons between the electrode and the enzyme, between its reduced (MED<sub>red</sub>) and oxidized (MED<sub>ox</sub>) forms, thereby enabling the regeneration/recycling of the enzyme [12] (Fig. 1). The direct linkage of GOD via an electron mediator to the electrode is expected to increase the efficiency of electron transport, hence the sensitivity of the modified electrode. Our interest in using PPY is due to its high electrical conductivity, biocompatibility, and the possibility of fabricating small, lightweight, and flexible electrodes from this material.

It is known that the rate of the electron transfer decreases exponentially with distance [21]. The presence of enzyme and mediator in close proximity is a condition for building successful enzyme electrodes. A strategy toward achieving this, mentioned in the literature is immobilizing redox active centers (mediators) and enzyme in a polymeric matrix deposited on the electrode (“wiring”). [22]. This study aims to synthesize and characterize PPY films to be used for the immobilization of GOD together with potassium ferricyanide used as mediator.

## **2. Experimental**

### *2.1. Materials*

Pyrrole (Py) and potassium chloride (KCl) were obtained from Merck. Titrisol buffer pH 7 (PBS) from Merck was freshly prepared before use from a concentrate solution for 500mL buffer. The pyrrole was distilled before use and the freshly colorless distilled oil was kept at -20°C, in sealed vials. Preset quantities for experiments were extracted using a syringe, under argon pressure. Glucose (Sigma-Aldrich) stock solutions were prepared and allowed to mutarotate overnight.

Glucose oxidase (GOD, Type VII, 180200 units/G, lyophilized powder, from *Aspergillus niger*) was purchased from Sigma-Aldrich.

### *2.2. Apparata*

Electrochemical experiments have been carried out in a conventional three-electrode cell using an Autolab-PGSTAT12 potentiostat. Glassy carbon disks (3mm diameter) from Metrohm-Schmidt Ltd. served as working electrodes, while platinum wire gauze was used as counter electrode. Before each experiment the working electrode was polished with diamond paste (200µm) and subsequently washed with bidistilled water. An Metrohm Ag/AgCl electrode was used as reference electrode.

Modified electrode surface compositions were analyzed by X-ray photoelectron spectroscopy (XPS) using a ThermoScientific spectrometer K-alpha system with a monochromatized Al K X-ray source (1486.6 eV photons).

Scanning electron microscopy (SEM) imaging was performed using the SEM – QUANTA INSPECT F system with an electronic scanning microscope having a resolution of the field emission gun tunnel of 1.2 nm. The core-level signals were obtained at a photoelectron take-off angle of 90° (with respect to the sample surface). To compensate for the surface charging effect all core-level spectra were referred to the C 1s hydrocarbon peak at 284.6 eV. The peak area ratios for the various elements were corrected using experimentally determined instrumental sensitivity factors. The SEM apparatus was used coupled with an energy dispersive X-ray spectrometer (EDAX spectrometer). This analytical

technique (EDAX) is used for the elemental analysis or chemical characterization of a sample.

The UV-vis spectra were recorded using a JASCO V-670 spectrophotometer, in Suprasil quartz Hellma cuvettes, with an optical path length of 10.00 mm.

### 2.3. Procedure

#### 2.3.1. Modified electrode preparation

The modified electrodes were prepared by cyclic voltammetry (CV) scanning the potential between 0 and about 0.8 V or by controlled potential electrolysis (CPE). CPE has the advantage of deposition control (by charge). The synthesis solutions were first thoroughly degassed by bubbling pure argon. The electrochemical experiments were then carried out at room temperature, under an argon blanket.

PPY films were electrodeposited on the glassy carbon disks by electrochemical polymerization of pyrrole in an electrolyte solution of (about) 0.1 M pyrrole in 1:1 mixture (v/v) of 0.1 M KCl and PBS.

Four types of modified electrodes, (1), (2) and (3), were prepared in synthesis solutions, as follows:

(0) 2.5 mL PBS + 2.5 mL KCl 0.1 M + Py (100 mM)

(1) 2.5 mL PBS + 2.5 mL KCl 0.1 M + Py (100 mM) + FC 10 mM,

(2) 2.5 mL PBS + 2.5 mL KCl 0.1 M + Py (100 mM) + 3.5 mg GOD,

(3) 2.5 mL PBS + 2.5 mL KCl 0.1 M + Py (100 mM) + FC 10 mM + 3.5 mg GOD.

#### 2.3.2. Testing and characterization of modified electrodes

The modified electrodes were electrochemically characterized in a transfer solution containing a 1:1 mixture (v/v) of 0.1M KCl and PBS. Surface compositions were analyzed by XPS and SEM imaging.

#### 2.3.3. Assay of GOD activity

The GOD assay provided by Worthington Biochemical Company was used [23]. This procedure involved the determination of the reaction rate from the value of absorbance at 460 nm (A<sub>460</sub>) for the oxidation of o-dianisidine through a peroxidase coupled system. 2.5mL of a dianisidine-buffer pH 6.0 (PB6) mixture (prepared by diluting 0.1 ml of 1% o-dianisidine in 12 ml of 0.1 M potassium phosphate buffer pH 6.0), saturated with oxygen by magnetic stirring of the solution in air for 30minutes, 0.3 mL 18% glucose and 0.1mL peroxidase were added into the cuvette, in this order, to an appropriately diluted solution of enzyme in PB6. The increase in A<sub>460</sub> was recorded for 4 - 5minutes. The slope ( $\Delta A_{460}$ ) of the initial linear portion of the curve was then calculated. One unit of

enzyme activity causes the oxidation of one micromole o-dianisidine per minute, at 25°C and pH 6.0 under the specified conditions.

### 3. Results

#### 3.1. Electrochemical experiments

Some electrochemical experiments were performed in order to establish the best conditions for obtaining modified electrodes. First, a modified electrode type (0) was prepared by CPE. It is shown (Fig.2) that the film formed by CPE is conductive and has a current peak of approximately 15 $\mu$ A.

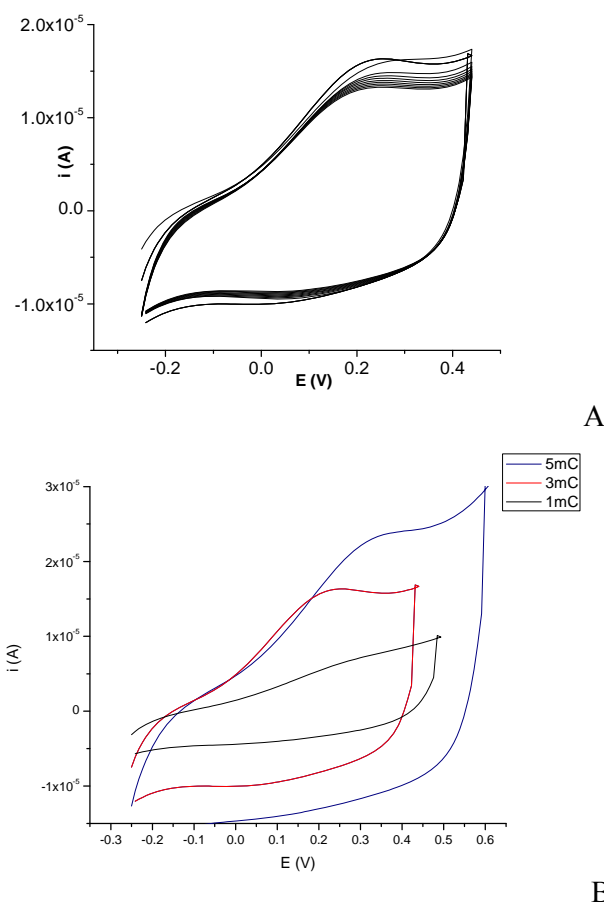


Fig. 2. CV curves in transfer solution for modified electrode type (0) obtained by CPE at 1.1 V, using charges of (A) 3 mC (successive cycles) and (B) 1, 3, 5 mC

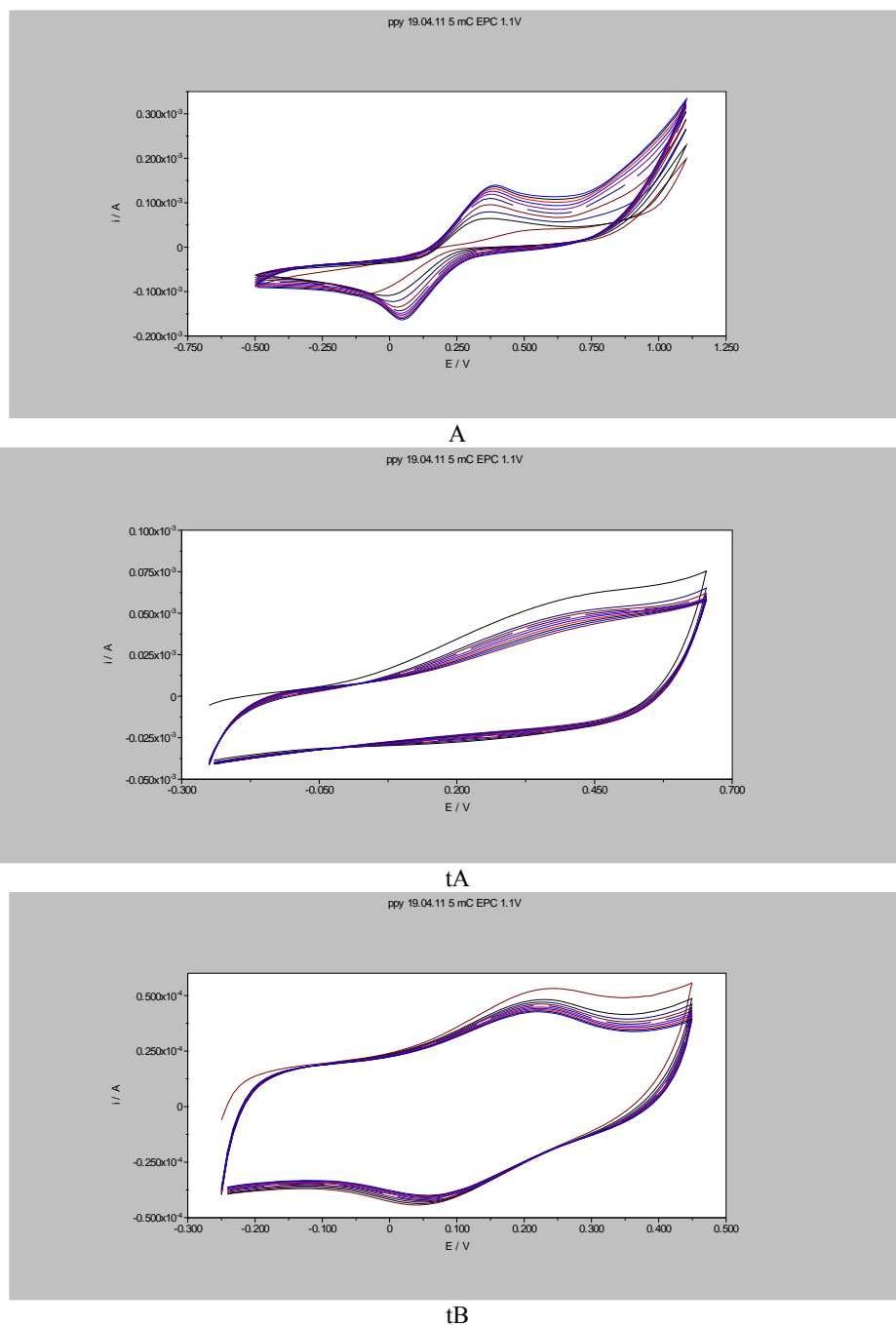


Fig. 3. Successive CV curves recorded during the synthesis (A) and in the transfer solution for films obtained by CV (tA) and by CPE at 1.1 V, 5 mC (tB), in the case of modified electrodes type (1)

From Fig. 2B it is notable that a better film is obtained for the highest used charge ( $26 \mu\text{A} (5\text{mC}) > 16 \mu\text{A} (3\text{mC}) > 5 \mu\text{A} (1\text{mC})$ ).

For the preparation of the modified electrode type (1), FC was added to the synthesis solution. Better films were obtained than in the previous case, both by CV and CPE, respectively (Fig. 3). However, CV proved to be a better synthesis method than CPE because more active films were formed as shown by the bigger currents of the PPy signal in Fig. 3tB compared to those in Fig. 3tA.

For the preparation of modified electrodes type (2), GOD was added to the synthesis solution. The film formed shows a higher response than that obtained for type (0), but lower than that obtained for type (1). (Fig. 4B).

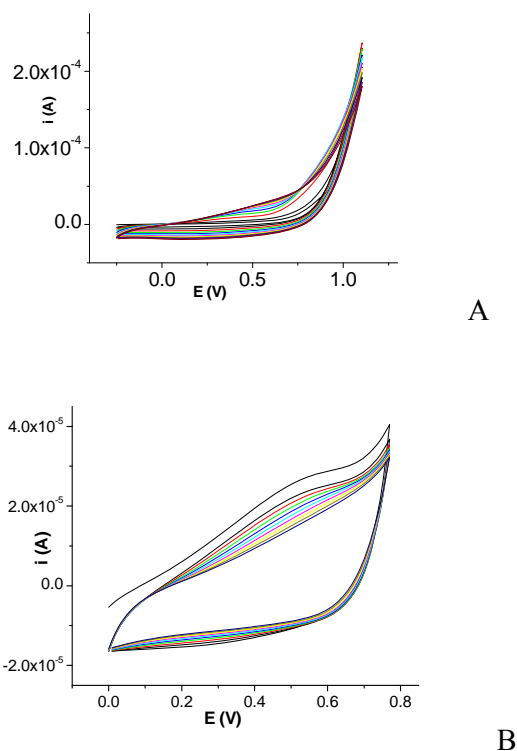


Fig. 4. Successive CV curves recorded during the synthesis (A) and in the transfer solution (B) for the modified electrode type (2)

### 3.2. SEM Analysis

Fig. 5 shows the SEM images for the three types of modified electrodes (1), (2), and (3). The classic "cauliflower" structure [24] is put into evidence for



type (1), and, partially, for type (3). For type (2) and also, partially, for type (3) a granular structure was observed.

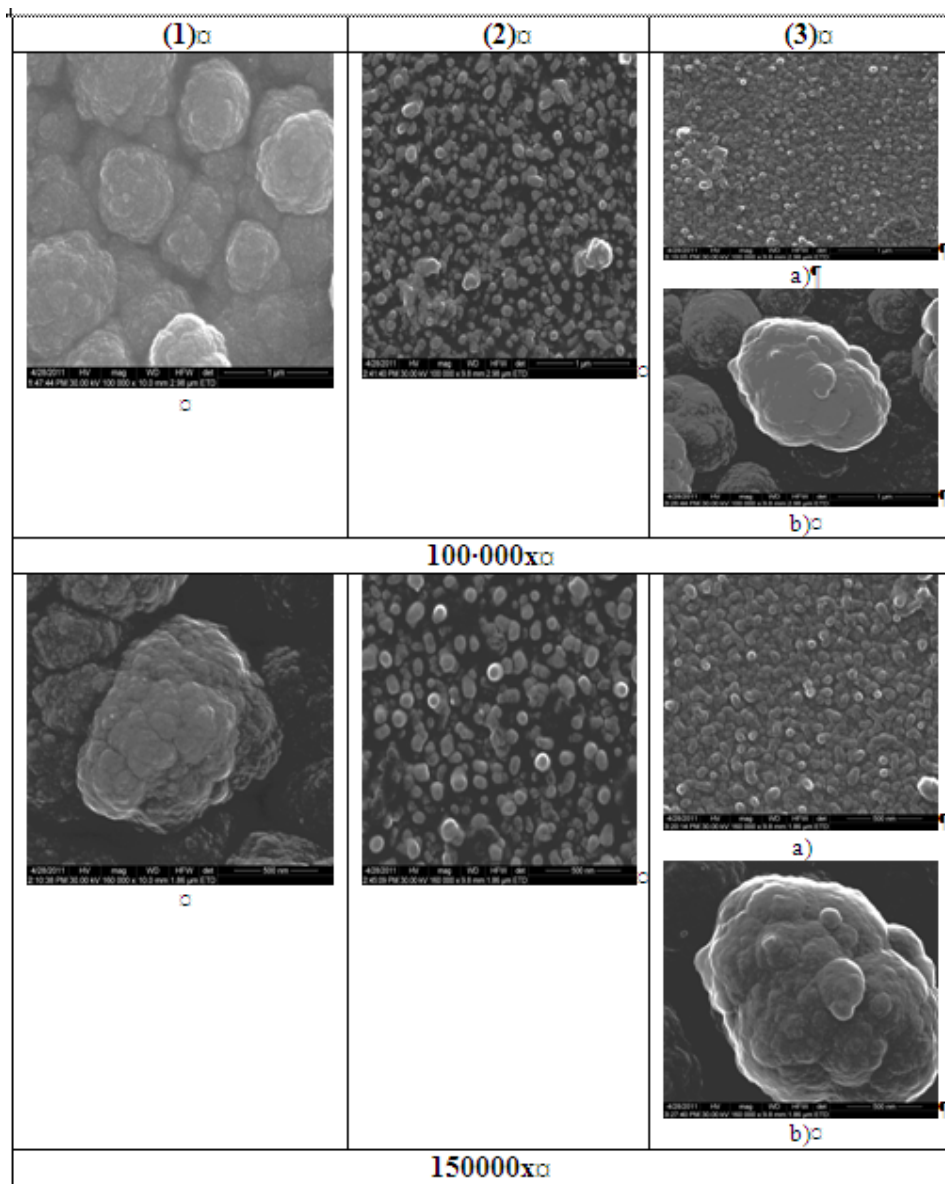


Fig. 5. SEM images for the modified electrodes types (1), (2), and (3), in two different zones a) and b)

Fig. 6 shows the EDAX spectra related to three different zones of the modified electrode type (1). Its homogenous film has been tested on three different (randomly chosen) areas. Area 1 presents a C signal coupled with the one for N, in a ratio of almost 1:1. This could be mainly due to the presence of pyrrole cycles. The presence of phosphorus, sodium, chlorine (most probably from KCl traces in the polymeric structure) was also proved. The presence of iron (from potassium ferricyanide,  $K_3[Fe(CN)_6]$ ), which remains incorporated in some quantity inside the film) can be seen, too. Oxygen can also be observed in the film, probably due to the incomplete degassing of the synthesis solution. Its presence is very important for assessing film properties such as adherence, porosity, etc. which are greatly influenced by this component.

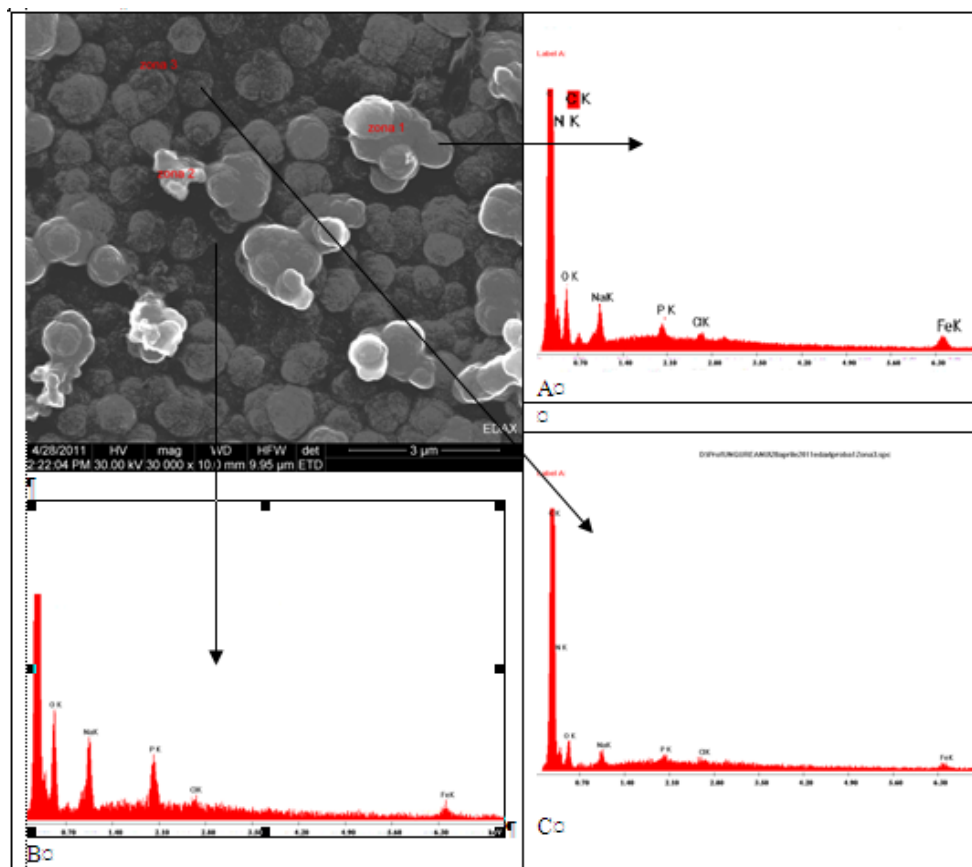


Fig. 6. SEM image for modified electrode type (1) and EDAX spectra in three different areas (A, B, and C, as shown); CK, NK, OK, NaK, PK, OK and FeK peaks are marked

The type (2) film has been tested on two different areas (Fig. 7). They were selected as to enable a better characterization of the coated film composition

in this position. The P and O contents in area A are high and the C content is very low (Fig. 7A). The existence of phosphate salts traces (originating probably from the buffer solution in which the electrode was kept) are the probable source. The presence of sodium is also shown in A (from the buffer, as well), but also of potassium and chlorine (from the synthesis solution). In area B the characteristic elements of the formed film, without buffer spots, are put into evidence (Fig. 7B): C, N and a small quantity of O.

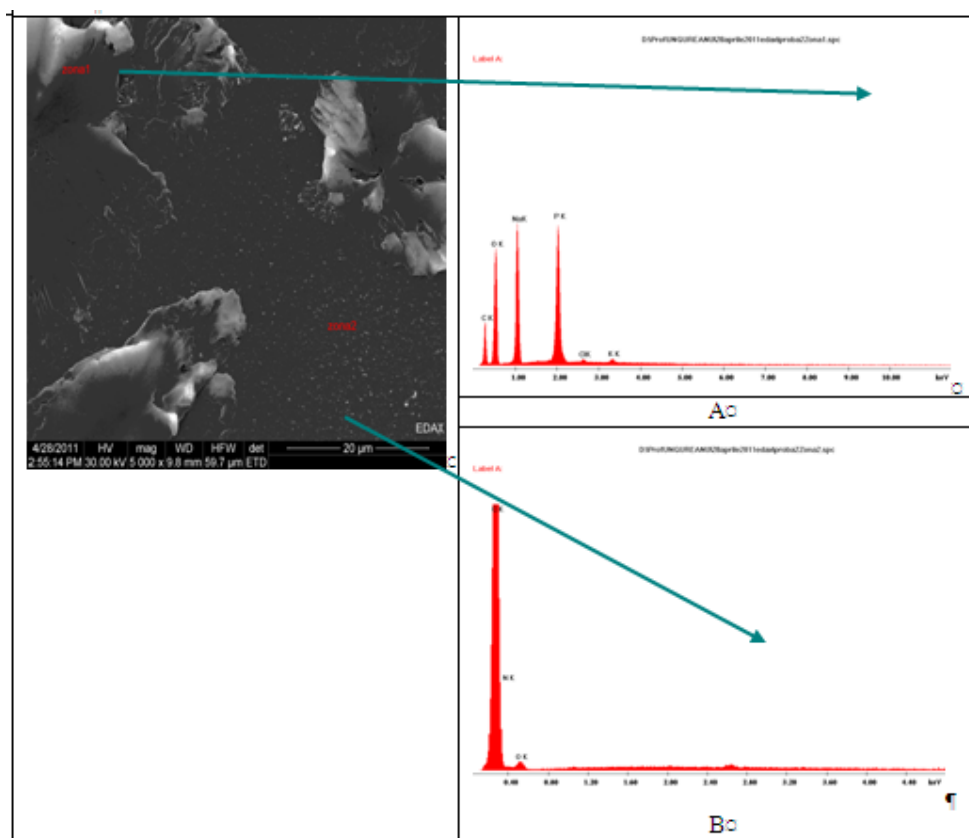


Fig. 7. SEM image for modified electrode type (2) and EDAX spectra in two different areas (A and B)

The exact structure of the deposit cannot be evaluated from the EDAX data only. However, the presence of a PPY deposit is certain. The incorporation of some enzyme in this structure is likely to occur. The ratio N:C of approximately 1:3 (in Fig. 7B), different from the ratio of about 1:1 observed in Fig. 6A, indicates a different structure of the films in these cases, for the modified electrode type (2). The intense peak at about 0.4 keV corresponding to the pyrrole

nitrogen (-NH-) clearly indicates that the polypyrrole chains represent the main part of the deposit [25]. The value of the N:C ratio shows that GOD macromolecules are distributed in the PPY layer, in the proximity of FC, generating what is called a “wired” enzyme. FC is the “wire” in this case. It is necessary to further analyze the detailed structure of the film deposited onto the electrode in order to clarify how the enzyme was immobilized.

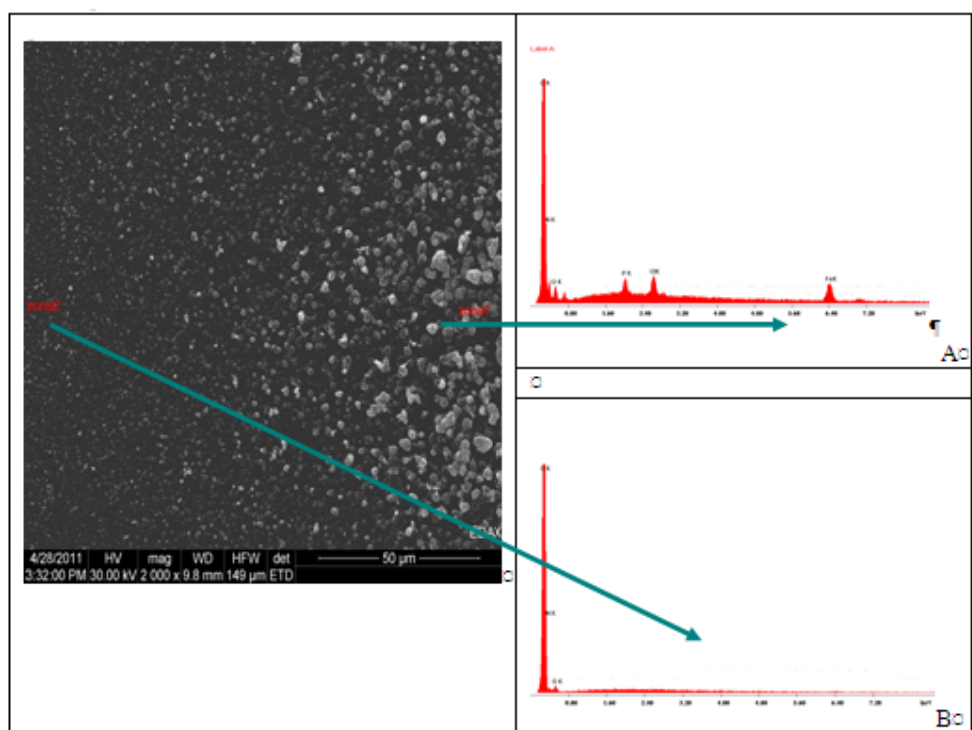


Fig. 8. SEM image for modified electrode type (3) and EDAX spectra in two different areas ((A and B))

Fig. 8 shows the SEM image of the modified electrodes type (3). It was shown that the film has two different morphologies deposited on the same electrode. Those two films are similar with type (1) film and type (2) film, respectively. The elemental analysis spectrum for area A (Fig. 8A) is similar with the spectrum for the type (1) film (Fig. 6 A, B, C). Fig. 8A also shows the presence of small amounts of P, Cl and Fe (from potassium ferricyanide). In Fig. 8B oxygen is present in a lower amount compared to Fig. 8A. However, the spectrum presented in Fig. 8A shows a different N:C ratio than in Fig. 8B.

In Fig. 8A and 8B, the N:C ratio is 1:3, same as for the type (2) electrode (modified with polypyrrole film and enzyme). This ratio confirms the non-

covalent retention of GOD in the film. This could mean that the enzyme has been incorporated in both areas. The difference in morphology for areas A and B means there is a preferential formation of the PPY film in the presence of FC compared to the synthesis of PPY in the absence of FC. The EDAX spectrum for the area B in Fig. 8B is almost identical with the spectrum in Fig. 7B, corresponding to the PPY-FC film.

### 3.3. XPS Analysis

XPS spectra were recorded for each electrode and deconvolutions were made for the corresponding peaks C1s and N1s. Peak tables 1-3 summarize the start binding energy (BE), the peak binding energy, the final binding energy, the peak height and atomic percent for C1s, N1s and O1s.

#### Modified electrode type (1)

The XPS spectrum for type (1) film gives almost the same information as the EDAX analysis (Fig. 6C). A high ratio N:C can be observed. A high content of O (19,42%) it also present. A closer analysis showed the existence of C-N, C-C and mainly C-H bonds (approximately 60%), which is normal for a PPY film. The high content of O-C=O and C-O bonds indicates the presence of some areas with oxidized film (overoxidized). This could be due to film degradation during the analysis, but also during the preparation (too high synthesis potential), and/or storage.

It is possible to use an oxidized polypyrrole film as it does not change the recognition properties of a potential sensor. However, it can influence the immobilization of the enzyme due to the potential semi-permeability and selectivity for some oxygenated functional groups (e.g., carboxylic, carbonilic and hydroxilic) [26]. While the unoxidized polypyrrole is a good conductor with a compact structure, the oxidized polypyrrole is a porous insulator, which could function as a good matrix for the immobilization.

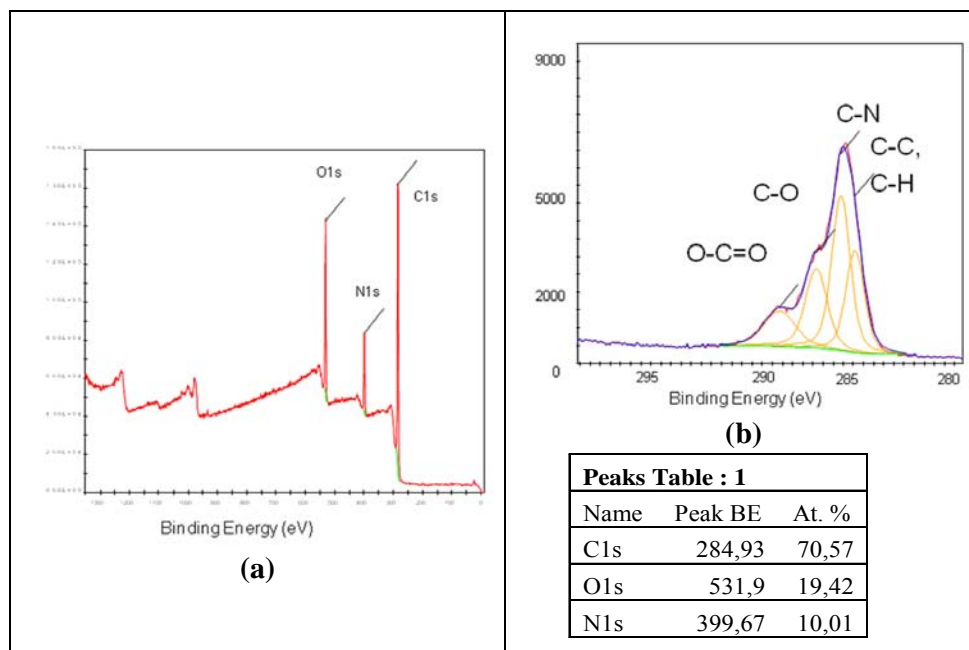


Fig. 9. (a) XPS spectrum for a type (1) film. (b) Detail for peak C1s and its Peaks Table

All these results are in good agreement with the results presented in the literature [19, 20].

### Modified electrode type (2)

For the type (2) film, the XPS spectrum (Fig. 10) indicates, in contrast to the type (1) film, a lower content of N. The ratio between the signals for N and C is approximately equal to the ratio obtained from EDAX analysis for the same film. The presence of amidic bonds proves the existence of enzyme macromolecules. The oxygen content is almost the same as in the previous case.

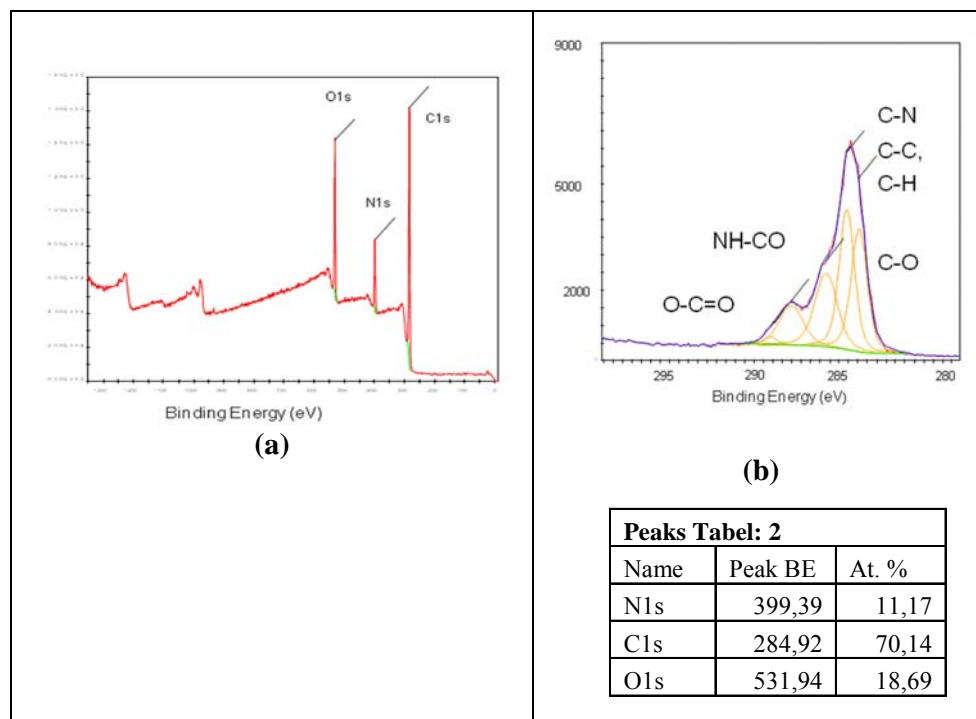


Fig. 10. (a) XPS spectrum for a type (2) film. (b) Detail for peak C1s and its Peaks Table

### Modified electrode type (3)

For the type (3) film, the XPS spectrum (Fig. 11) shows a lower N content compared to the type (1) film. The ratio between the N and C signal heights is approximately the same as the one obtained from the EDAX analysis (for the same film). The detail for area C1s shows the existence of amidic bonds ( $\text{N-C=O}$ ) which confirms the enzyme immobilization. In this case the oxygen content is lower than in the other two cases. The lower percent of amidic bonds (9,99%) compared to the percent for type (2) film (13, 86 %) indicates a reduced incorporation of enzymes in this film.

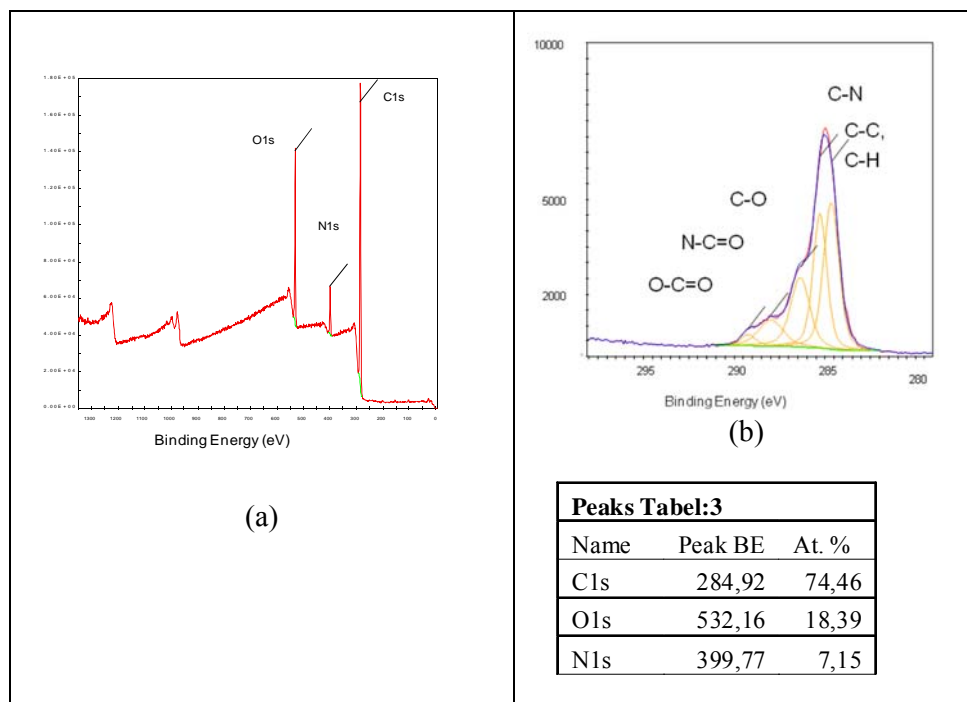


Fig. 11. (a) XPS spectrum for a type (3) film. (b) Detail for peak C1s and its Peaks Table

#### 4. Conclusion

The noncovalent incorporation of the enzyme into the growing conducting polymer film during electropolymerization is a convenient and simple method of immobilization. Pyrrole polymerization is influenced by the presence of FC and/or GOD. This non-invasive method affords an immobilized (“wired”) enzyme with minimal loss in 3-D structure which has a good potential for use in biosensors for glucose analysis.

SEM imaging and EDAX analysis offered information about the structure of the films deposited on electrodes. Potassium ferricyanide induces the formation of a film with an increased porosity that can be a promising feature for a glucose oxidase selective electrode. The XPS analysis shows differences only in the C1s spectra. This is a confirmation of the better enzyme immobilization in the presence of potassium ferricyanide which could act as mediator for the electron transfer between the electrode and the enzyme.



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