ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
http://web.icf.ro/rrch/

Rev. Roum. Chim.,
2011, *56*(9), 875-882

POLYPHENOL OXIDASE IMMOBILIZATION ON FUNCTIONALIZED POLYPYRROLE

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Received March 2, 2011

Polypyrrole (PPY) was functionalized by reaction with glutardialdehyde using sulphuric acid as catalyst. The samples of functionalized polypyrrole were then used for the covalent immobilization of polyphenol oxidase. The surface elemental composition of this enzyme was determined by X-ray Photoelectron Spectroscopy. FTIR spectrometry, Raman spectroscopy, Scanning Electron Microscopy and X-Ray Diffraction were used to prove polypyrrole functionalization and the immobilization of this enzyme.

INTRODUCTION

In the last decade, electrically conducting polymers were considered as potential materials that will exhibit environmentally and thermally stable properties. Conducting polymers such as polythiophene, polyaniline and polypyrrole were used for the development of electro chromic devices, light-emitting diodes, gas sensors and organic transistors.[1]

Intrinsically conducting polymers (ICPs), such as polyanilines and polypyrroles, have been recognized as a class of organic materials which exhibits unique combination of optical, electrical and magnetic properties, consisting in the facile tailoring and reproducibly modulation of their doping level, via chemical and electrochemical processes. Devices fabricated from this class of materials are already known. These include biochemical and chemical sensors, field effect transistors, actuators, plastic optoelectronic liquid crystal display devices, electrostatic protective shields and electrodes from batteries. Among the ICPs, polypyrrole is one of the most investigated because of its good chemical/thermal stability, high conductivity and ease of preparation in a range of solvents. The in situ doping of pyrrole during synthesis produces a π-type semiconducting material whose electrical property can be traced back to the delocalized polaronic and/or bipolaronic species present in the structure heteroatomic rings.[2]

Natural enzymes have been extensively used in biochemistry, having significant practical applications in food and chemical industry, agriculture and medicine. Polyphenol oxidase is a dinuclear copper containing enzyme which is responsible for biosynthesis of melanin in animals and browning in plants, being involved in several physiological processes including plant defense against pathogens and insects and it is also known as phenol oxidase, phenolase, tyrosinase, *o*-diphenol oxidase depending on its substrate specificity. It is possible to synthesize its active site of in *met*-tyrosinase, *oxy*-tyrosinase and *deoxy*-tyrosinase form. It catalyzes the oxidation of phenol-derivatives in the presence of O_2, to respective orto-diphenol derivatives (monophenolase activity, Scheme 1) that

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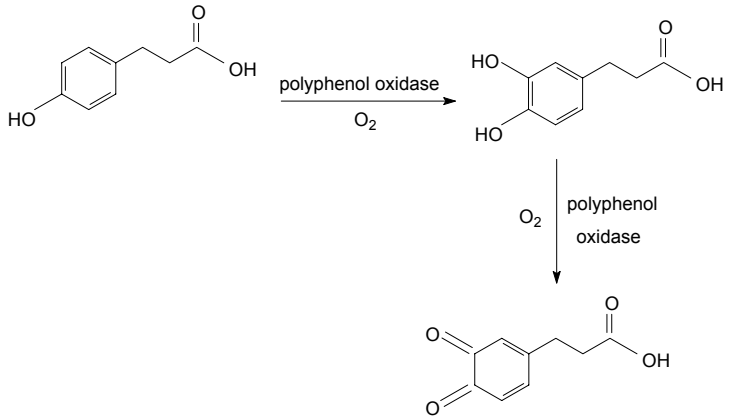
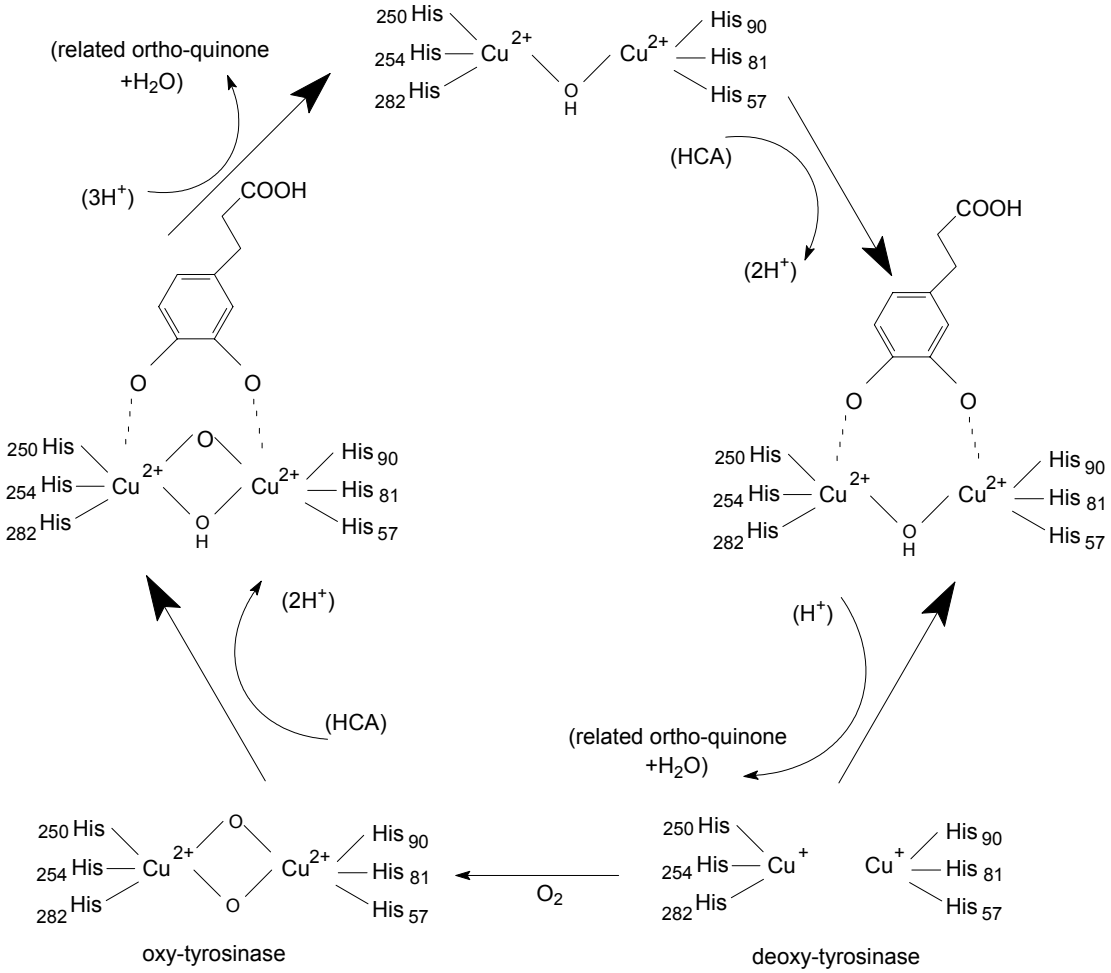
are further oxidized by enzyme to respective quinones (diphenolase activity, Schemes 1 and 2). This enzyme is formed of histidine (His, Scheme 3) residues. Histidine, an essential and proteinogenic amino acid, has a positively charged imidazole functional group. So, polyphenoloxidase exhibits amino and carboxyl groups.

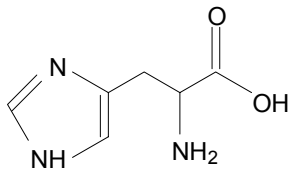
Polyphenol oxidases are believed to be ubiquitous in the plant kingdom, being detected in most known fruits and vegetables (tomatoes, Nicotiana tabacum, potatoes, wheat, tea, pears, mushrooms, wheat bran). Polypohenoloxidase oxidase from mushroom exhibits an isoelectric point value of 4.5 and takes a negative charge at pH>4.5 in aqueous solution.[3-8]



Scheme 1 – Monophenolase activity of polyphenol oxidase.[8]



Scheme 2 – Diphenolase activity of polyphenol oxidase.[8]



Scheme 3 – Histidine structure.[9]

Different kind of enzymatic composites were synthesized by immobilizing various enzymes on various polymers using different techniques. These composites were used either to obtain biosensors or to increase the enzyme stability.

Polyphenol oxidases which were isolated from various sources have been immobilized on different supports such as zeolite, bentonite and glass beads and used to control the pollution in water, to remove and transform toxic compounds.[10] Polyphenol oxidase immobilized on the SWCNTs (single-walled carbon nanotubes)/GC electrode is used to produce biosensors.[11] A novel amperometric biosensor based on horseradish peroxidase/polypyrrole deposited onto the surface of ferrocene carboxylic acid functionalized sol-gel derived composite carbon electrode for the detection of H_2O_2 has been reported.[8]

Sensors able to detect the phenolic compounds were obtained using the polyphenol oxidase entrapped in poly (ethylenoxide)/polypyrrole (PEO/PPY) and 3-methylthienyl methacrylate-co-*p*-vinylbenzyloxy polyethileneoxide)/polypyrrole (CP/PPY) [12] or in poly(methylmethacrylate-co-methyl thienyl methacrylate) and polypyrrole matrices,[13] poly 3,4-ethylene dioxythiophene (PEDT).[14] The sensor obtained using PEDT is able to detect herbicides also.

Polyphenoloxidase has also been immobilized on single walled carbon nanotubes (SWCNTs) by adsorption. Thus PPO/SWCNTs modified electrode was obtained and used for biological sensing and in bioelectrochemistry applications. The immobilized enzyme thus obtained proved to be a good catalyst.[10]

Enzymatic composites based on laccase, HRP and corresponding L-tyrosine enzyme co-polymers immobilized on calcium alginate beads cured with $CaCl_2$ were used for the discoloring of phenolic effluents.[15]

Another enzymatic composite was obtained by entrapment of glucose oxidase (GOD) covalently attached to polyethyleneglycol (PEG), called (PEG-GOD) within poly (3, 4-ethylene dioxythiophene) (PEDT). This composite was used to obtain a glucose sensor.[16]

At the same time cholesterol sensors were obtained by cholesterol oxidase entrapment within polypyrrole-polyvinylsulfonate (PPY-PVS) films.[17]

In order to increase its solvent stability and temperature resistance, horseradish peroxidase (HRP) was immobilized on polyaniline activated with glutardialdehyde.[18]

In the present paper, polyphenol oxidase (PPOx) was not immobilized by entrapment as in the above cited works,[12-15] but by covalent immobilization. At the same time, only one of this works has used polypyrrole as immobilization support.

Enzymatic composites based on other enzymes (HRP,[16, 19] GOD,[17] cholesterol oxidase[18]) have also been synthesized.

The immobilization method based on polymer activation using glutaradialdehyde was done, also, but using polyaniline as support and HRP as enzyme. In our work, this method was applied using polypyrrole as support and polyphenoloxidase as enzyme.

The aim of this work was to characterize some enzymatic composites, which were synthesized by covalent immobilization of PPOx on functionalized polypyrrole with glutaraldehyde. These composites will be used to obtain enzymatic biosensors able to detect the presence of nitrites and nitrates in water for human consumption. The possibility of using these composites to obtain biosensors will be checked later.

RESULTS AND DISCUSSION

In order to determine the pure enzyme surface elemental composition, XPS spectra were recorded. The elemental composition was determined in two points of sample surface (in Fig. 1 for one point), then it was made an average of the content for the two points (Table 1).

The enzyme proved to exhibit some calcium content due to the fact that it is extracted from a natural source (mushrooms). Copper was not detected, the amount being lower than 0.1 %.

In order to confirm PPY functionalization and covalent immobilization of PPOx on functionalized PPY, Raman spectra were recorded for thermal treated PPY reference, functionalized polypyrrole (PPY-F) and functionalized polypyrrole with immobilized enzyme (PPY-E) and for the enzyme itself (E).

From Fig. 2 one may notice that for the reference and PPY-F, two peaks at 1909 and 1384 cm^{-1} appear. These peaks are assigned to C=O bond from carbonyl and carboxyl groups which are formed through oxidative polymerization. These peaks are missing from PPY-E spectrum because they are consumed during enzyme immobilization, by reaction with COOH or NH_2 groups from the enzyme, according to Scheme 4.

The results mean that enzyme immobilization could be done directly on pure PPY, without previous functionalization.

Table 1

Elemental composition for PPOx (%)

O	C	N	Ca
41.6	52.7	4.7	1.0

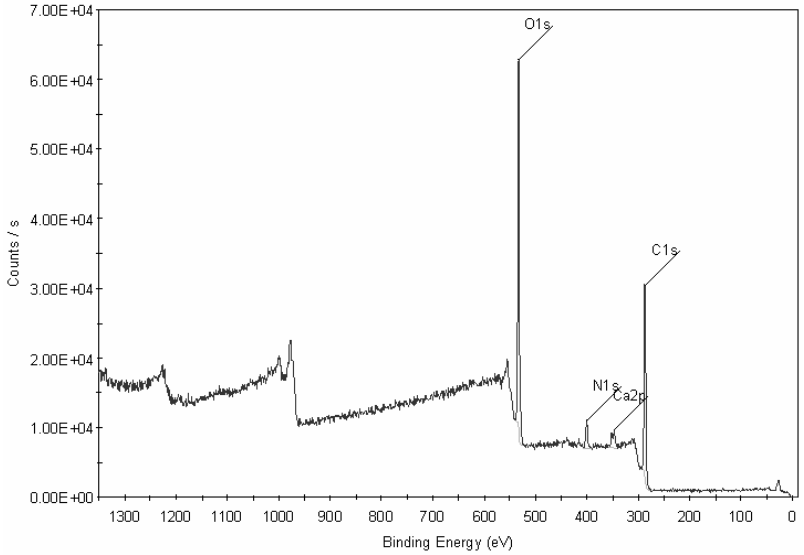


Fig. 1 – XPS spectrum for PPOx in one point.

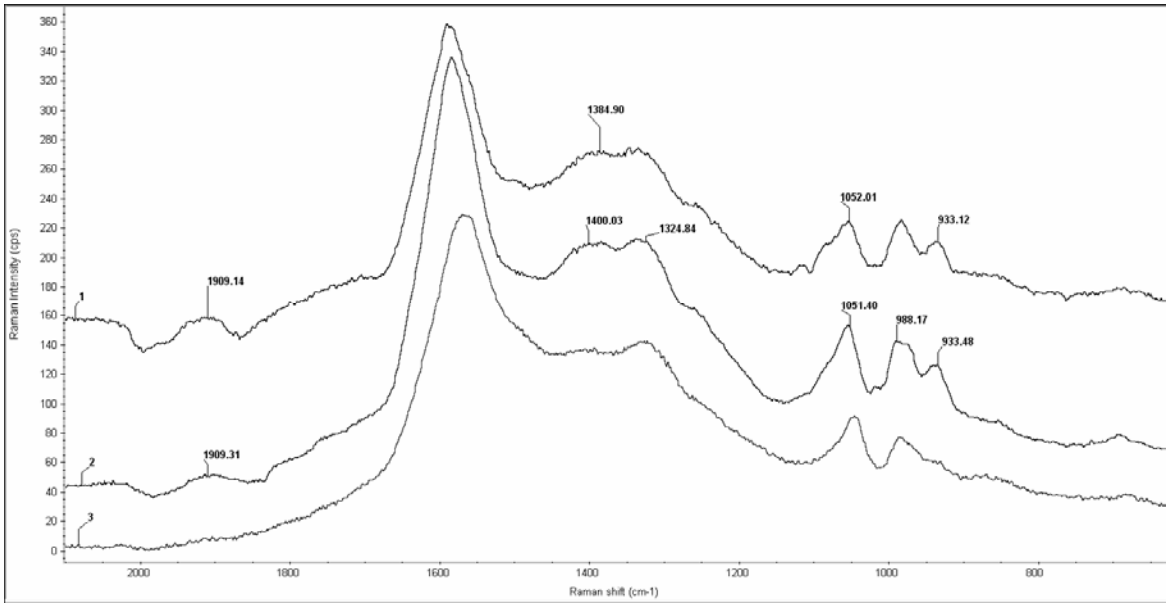
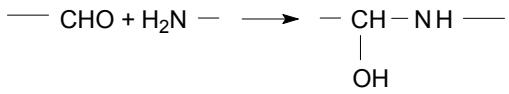


Fig. 2 – Raman spectra for reference (1), PPY-F (2) and PPY-E (3).



Scheme 4 – The chemical reactions for PPOx immobilization on PPY.

At the same time, the reference and PPY-F exhibit peaks at 933 cm^{-1}, which are missing from PPY-E spectrum. This peak is assigned to polypyrrole ring contortion frequency. After enzyme immobilization contortion does not take place any more. Therefore, Raman spectra prove enzyme immobilization. However the Raman spectra do not show significant differences between reference and PPY-F.

FTIR spectra (Fig. 3) were recorded in order to prove PPOx immobilization on functionalized PPY. Thus, the peak from 1643 cm^{-1} occurs in E and PPY-E samples spectra, but is absent in PPY-F spectrum. This peak is assigned to peptide groups from the enzyme. PPY-F does not contain peptide groups. Because PPY-E contains a peak which is characteristic to the enzyme, the immobilization is confirmed.

For PPY-F it appears a peak at 1471 cm^{-1} and for PPY-E at 1468 cm^{-1}. These peaks are assigned to C=C bond. The enzyme does not contain these bonds so it does not exhibit any signal in this region.

PPY-F exhibits a peak at 1290 cm^{-1} and PPY-E at 1283 cm^{-1}. These signals are assigned to C-O groups. The enzyme (curve 3) does not exhibit peaks in this region. For PPY-E the peak is less intense then in PPY-F, because these groups are consumed during immobilization. This fact confirms again the immobilization.

X-Ray diffractograms for PPY, reference, PPY-F and PPY-E are shown in Fig. 4. The three samples diffractograms profile prove the formation of a partially crystalline material with a preponderant amorphous phase. The associations of diffraction bands for each sample obtained for diffractograms are presented in Table 2. As it can be noticed from Fig. 4, the XRD curves of PPY, reference and PPY-F exhibit three large diffraction bands which are indicated in Table 2. Nogami and coworkers presented for the first time an interpretation of polypyrrole diffractogram.[19] Thus, the first band from low angles is assigned to concentration of ions which are present in the polymer amorphous structure.

Based on this model the second large diffraction band from PPY, reference and PPY-F diffractograms is assigned to polymer amorphous regions and the third diffraction band is assigned to PPY rings superposition. The last band intensity increased by thermal treatment of PPY, and decreased a little by functionalization, showing decreasing in polypyrrole rings superposition during the functionalization. For polypyrrole with immobilized enzyme (curve 4) the diffraction spectrum is much modified in comparison with the previous 3 samples. This fact is probably mainly due to the enzyme immobilization on polymer chain.

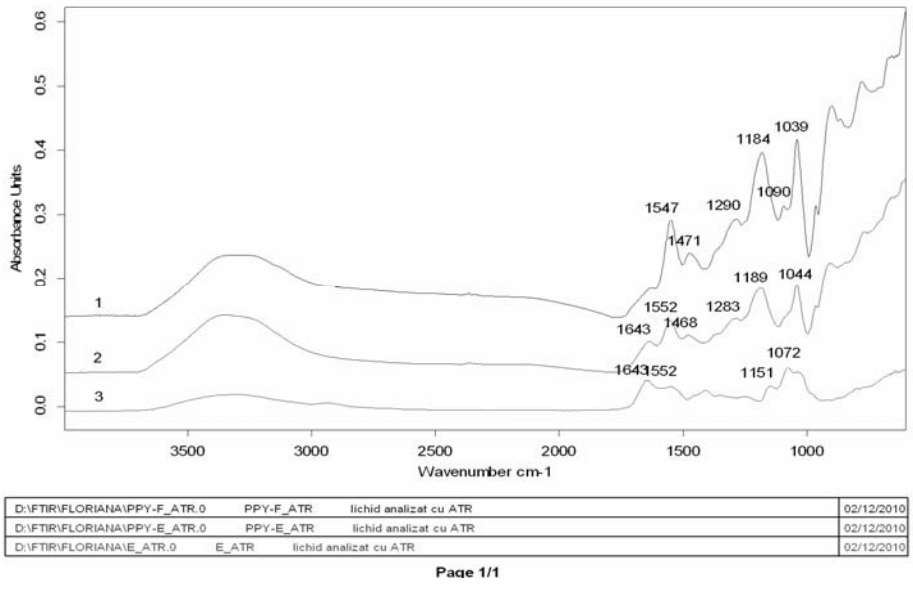


Fig. 3 – FTIR Spectra for PPY-F (1), PPY-E(2)and E(3).

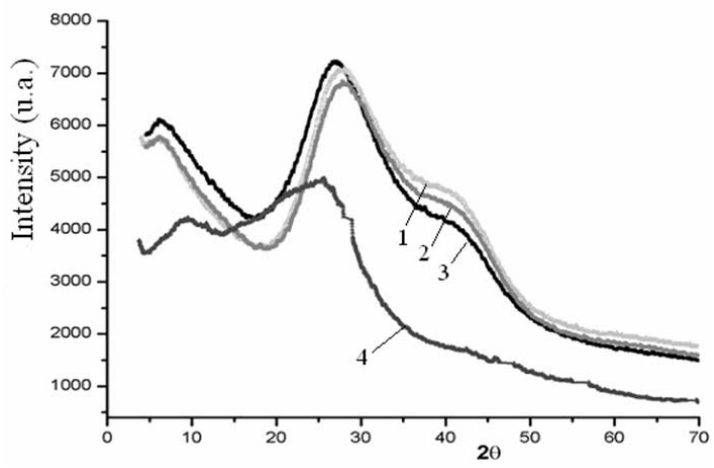


Fig. 4 – X-Ray Diffractograms for PPY (3), reference (1), PPY-F (2) and PPY-E (4) samples.

Table 2

Diffraction bands indexation for PPY, Reference, PPY-F and PPY-E samples

Polymer	Band 1 (2θ)	Band 2 (2θ)	Band 3 (2θ)
PPY	6.2	26.8	37.6
BLANK	6.2	27.4	38.5
PPY-F	6.4	27.6	38.5
PPY-E	10.4	25.0	-

Scanning Electron Microscopy (SEM)

SEM images for the four samples (PPY, reference, PPY-F and PPY-E) are shown in Figs. 5 and 6. All three samples (PPY, reference and PPY-F) in Fig. 5 exhibit a uniform texture with a granular morphology, with non-regular shape particles, without important differences for the three samples. In PPY case these particles are agglomerated exhibiting cluster morphology. The SEM image for the reference shows that by heating polypyrrole with distilled water a slight reduction of the agglomeration of PPY particles was noticed.

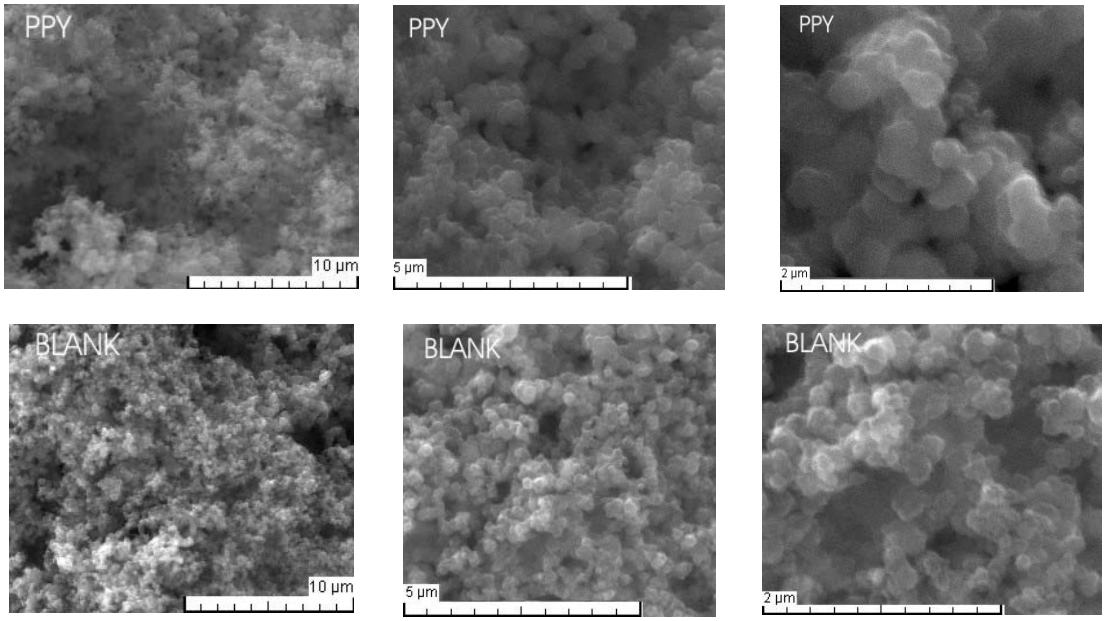


Fig. 5 – SEM images for PPY, REFERENCE and PPY-F.

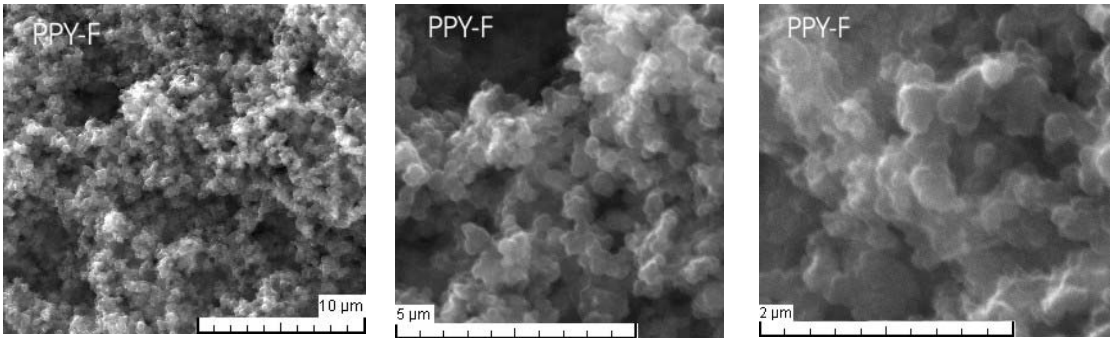


Fig. 5 (continued) – SEM images for PPY, REFERENCE and PPY-F.

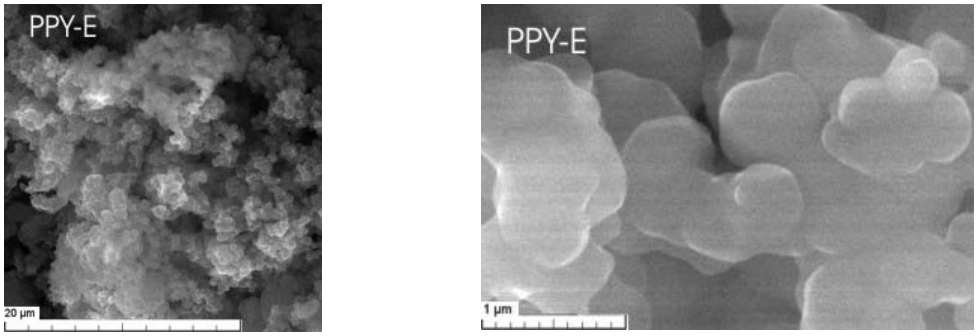


Fig. 6 – SEM images for PPY-E.

After PPY functionalization with glutaraldehyde a cluster compactation also takes place which could be explained by aldehyde groups attachment on PPY chains or by PPY chains crosslinking. At the same time PPY granules agglomeration was observed, the morphology being similar to PPY. The SEM images from Fig. 6 show a sharp change of PPY-E sample aspect in comparison to the samples in Fig. 5, which can be assigned to PPOx immobilization.

EXPERIMENTAL

Materials

Pyrrole (PY) was supplied by Merck and distilled for further purification and then polymerized using ammonium persulphate as initiator in order to obtain polypyrrole (PPY). Ammonium persulphate (APS) was provided by Scharlau and used as received. Glutardialdehyde (GLA), aqueous solution 50% and sulphuric acid were received from Merck and used without further purification. Polyphenol oxidase was supplied by Worthington and used as received.

Sample synthesis

The PPY was synthesized by oxidative polymerization with ammonium persulphate, using modified method reported in literature.[20] The modified procedure for PPY synthesis was described in our previous paper: [21] 5mL pyrrole were dissolved in 250mL water; then, 16.4g APS were added stepwise during two hours under continuous stirring, at 5°C. After that, the obtained slurry was filtered, washed and dried in oven at 50-60^{0}C for 6 h.

Enzyme immobilization

Before covalent immobilization of PPOx many stages were accomplished. Firstly, PPY was heated with distilled water at 70^{0}C for 30 minutes. Thus, the REFERENCE was prepared in order to check direct immobilization of PPOx on pure PPY. The reference was then functionalized with GLA (Table 3). Thus PPY-F sample was prepared. The last step was the covalent immobilization of PPOx on PPY-F, leading to PPY-E sample. In order to covalently immobilize polyphenol oxidase, this enzyme was solved in phosphate buffer solution. Then, the enzyme solution was put in contact with PPY-F sample for two hours, at room temperature.

Table 3

Sample preparation conditions

Solid/liquid ratio	**Reference** g	**H_2O** mL	**GLA** mL	**Concentrated H_2SO_4** mL	**Reaction temperature** °C	**Reaction time** min
1:100	0.1000	9.98	0.02	0.136	70	30

Sample characterization

X-Ray Photoelectron Spectroscopy (XPS). The XPS spectra were recorded on a Thermo Scientific K-Alpha equipment, fully integrated, with an aluminum anode monochromatic source (1486.6 eV). The charging effects were compensated by an argon flood gun.

Fourier Transform Infrared Spectrometry (FTIR). The FTIR spectra were registered on a BRUKER VERTEX 70 equipment using 32 scans and 4 cm^{-1} resolution in 400-4000 cm^{-1} region. The samples were analyzed using ATR unit.

Raman Spectroscopy. The Raman spectra were recorded on a DXR model Raman microscope, equipped with Omnic 8 software from Thermo Fisher Scientific USA (Madison, Wis.), with signal to noise ratio 100. The excitation laser wavelength was 532 nm, using a laser power level of about 10 mW. The Raman shift was in range of 50 to 3550 cm^{-1}.

X-Ray Diffraction (XRD). The X-ray Diffraction (XRD) tests were done on X' Pert PRO MDP (PANalytical, Holland), with rapid acquisition mode, low angles measurement equipment and 40 samples auto sampler. The diffractometer has a 2Θ Bragg-Bentano geometry, using a CuK_{α} (λ=1,54056Å).

Scanning Electron Microscopy (SEM). SEM images were obtained on VEGA II XMU microscope at an accelerating voltage of 30 kV.

CONCLUSIONS

PPOx is an enzyme with low calcium content, due to the fact that it is extracted from a natural source. Polypyrrole is able to directly immobilize enzymes covalently as it exhibits COOH and CHO groups, formed through oxidative polymerization. Raman and FTIR spectrometry confirm PPOx immobilization on functionalized PPY. XRD curves and SEM images also confirm immobilization, because visible changes after immobilization are noticed.

PPOx immobilized on PPY will be used to obtain enzymatic sensors.

Acknowledgements: The work has been funded by the Sectoral Operational Programme Human Resources Development 2007-2013 of the Roumanian Ministry of Labour, Family and Social Protection through the Financial Agreement POSDRU/88/1.5/S/61178. This work was also supported by the Roumanian Research Project PNII 52 159/2008.

REFERENCES

1. B. Yigitsoy, S. Varis, C. Tanyeli, I. M. Akhmedov and L. Toppare, *Thin Solid Films,* **2007**, *515*, 3898-3904.
2. R. C. Yu King, M. Boussoualem and F. Roussel, *Polymer*, **2007**, *48*, 4047-4054.
3. C. Y. Shi, Dai, B. Xia, X. Xu, Y. Xie and Q. Liu, *Plant. Mol. Biol. Rep.*, **2001**, *19*, 381 a-381 h.
4. Y. Yamasaki, H. Konno and K. Noda, *Acta Biochim. Pol.*, **2008**, *55*, 325-328.
5. D.Y. Kwon and W.Y. Kim, *J. Biochem. Mol. Biol.*, **1996**, *29*, 163-168.
6. P. Thipyapong, M. J. Stout and J. Attarusit, *Molecules*, **2007**, *12*, 1569-1595.
7. C. Queiroz, M. L. Mendes Lopes, E. Fialho and V. L. Valente-Mesquita, *Food Rev. Int.,* **2008**, *24*, 361-375.
8. A. Mohammadi, A. B. Moghaddam, R. Dinarvand and S. Rezaei-Zarchi, *Int. J. Electrochem Sci.*, **2009**, *4*, 895-905.
9. C. D. Nenițescu, "Chimie Organică", *2*, 6th Edition, Editura Didactică şi Pedagogică, Bucureşti, 1968, p. 341.
10. H. Yagar and A. Sagiroglu, *Acta Chim. Slov.*, **2002**, *49*, 893-902.
11. A. Rahman, P. Kumar, D. S. Park and Y. Bo-Shim, *Sensors*, **2008**, *8*, 118-141.
12. H. B. Yildiz, S. Kiralp, L. Toppare and Y. Yagci, *React. Funct. Polym.*, **2005**, *63*, 155-161.
13. H. B. Yildiz, L. Toppare, Y. H. Gursel and Y. Yagci, *Enzyme Microb. Tech.*, **2006**, *39*, 945-948.
14. C. Vedrine, S. Fabiano and C. Tran-Minh, *Talanta*, **2003**, *59*, 535-544.
15. S. Davis and R. G. Burns, *Appl. Microbiol. Biot.*, **1990**, *32*, 721-726.
16. B. Piro, L. A. Dang, M. C. Pham, S. Fabiano and C. T Minh, *Electroanal Chem.*, **2001,** *512,* 101-109.
17. F. Yildirimoglu, F. Aslan, S. Cete and A. Yasar, *Sensors*, **2009**, *9*, 6435-6445.
18. K. F. Fernandes, C. S. Lima, F. M. Lopes and C. H. Collins, *Process Biochem.*, **2004**, *39*, 957-962.
19. Y. Nogami, J.-P. Pouget and T. Ishiguro, *Synthetic Met.*, **1994**, *62*, 257-263.
20. J. Honey, M. T. Rinku, J. Joe, J. Rani and K. T. Mathew, *Microw. Opt. Techn. Let.*, **2006**, *48*, 1324-1326.
21. T. Sandu, A. Sârbu, S. A. Gârea and H. Iovu, *UPB Sci Bull; Series B*, **2011**, *73*, 123-132.