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Characterization and Potential Applications of Immobilized Glucose Oxidase and Polyphenol Oxidase

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Pyrrole functionalized polystyrene (PStPy) was copolymerized with pyrrole to obtain a conducting copolymer, P(PStPy-co-Py) which is used as the immobilization matrix. Glucose oxidase and polyphenol oxidase enzymes were immobilized via the entrapment method by electrochemical polymerization. Enzyme electrodes were prepared by electrolysis at a constant potential using sodium dodecyl sulfate (SDS) as the supporting electrolyte during the copolymerization of PStPy with pyrrole. Maximum reaction rates (V_{max}) and enzyme affinities (Michaelis-Menten constants, K_m) were determined for the enzyme entrapped both in polypyrrole (PPy) and P(PStPy-co-Py) matrices. Optimizations of enzyme electrodes were done by examining the effects of temperature and pH on enzymes' activities along with the shelf life and operational stability investigations. Glucose oxidase enzyme electrodes were used for human serum analysis and glucose determination in two brands of orange juices. Polyphenol oxidase enzyme electrodes were used for the determination of phenolics in red wines of Turkey.

Keywords: enzyme immobilization; biosensors; glucose oxidase; wine; tyrosinase

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1 Introduction

Enzymes are catalysts that speed up chemical reactions which take place in living organisms. They do this by lowering the activation energy of reactions without being unchanged. They differ from the inorganic catalysts by being specific to its substrate and operate in very mild circumstances. Since enzymes are proteins, they are all affected from the factors that denature proteins such as: pH, temperature and inhibitors. Immobilization not only prevents enzymes from those effects, but also decreases the cost since immobilized enzymes (biosensors) provide several operational advantages over free enzymes such as: reusability without loss of activity, enhanced stability, controlled product formation, ease of separation of enzyme from reaction products and high enzymatic activity in a small volume (1). Immobilization refers to the physical confinement or localization of enzyme molecules during a continuous catalytic process with retention of its catalytic activity leading to repetitive and continuous use (2).

Enzymes are immobilized by carrier binding (physical adsorption, ionic binding, covalent binding), by crosslinking, adsorption or entrapment methods (3). In this study, an entrapment type of immobilization was used for the preparation of enzyme electrodes due to the simplicity, cost, speed and reliability of the method. This physical entrapment within a polymeric matrix avoids the risk of denaturing the protein shell (4). Moreover, enzyme molecules can be entrapped during electropolymerization in one step and the polymer film uniformly coats the surface of the electrode of any shape or size (5). Control of the film thickness by regulating the amount of charge passed can be achieved (6). Several physical properties can be supplied to the polymer matrix using various supporting electrolytes or monomers.

Owing to some advantages over other methods including high selectivity and simplicity, biosensors provide solutions to some of the problems currently encountered in the measurement of metabolites for biomedical applications (7).

Glucose oxidase (GOD, EC: 1.1.3.4), which was discovered in 1928 by Müller in *Aspergillus niger* and *Penicillium glaucum*, is the enzyme that catalyzes the oxidation of ß-D-glucose to D-glucono 1,5-lactone and hydrogen peroxide in aerobic conditions. In the food-stuff industry, glucose oxidase is employed for removing oxygen from soft drinks and canned foods, as well as glucose from egg products. A major clinical laboratory use of glucose oxidase is in glucose detection or assay in urine and blood (8).

The major protein component of the blood plasma, which is also found in the interstitial fluid of the body tissues is serum

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albumin (9, 10). It was reported that some metals bind greatly to the blood components (11). There are various methods for the determination of glucose in human serum. Glucose determination by flow injection analysis (12) and automated analyzer (13) are the methods reported. The normal glucose level in human blood is 75–105 mg/dl.

Polyphenol oxidase (Tyrosinase, PPO, E.C: 1.14.18.1) is one of the most "versatile" enzymes in nature. It is a tetramer and weighs about 130,000 Daltons, where an active site contains two copper atoms. PPO is a bifunctional enzyme, which catalyzes ortho hydroxylation of monophenols (cresolase activity) and oxidation of catechols to the corresponding ortho-quinones (catecholase activity). Upon subsequent spontaneous polymerization reactions, these highly reactive quinones form melanin, which is the pigment of hair, eye, and skin. Besthorn's hydrazone method (14) was used for the determination of phenolic groups.

Polyphenols are the compounds which have beneficial effects on human health because of their antioxidant powers. They protect the body from the harmful effects of free radicals produced in the metabolism. Several studies show that polyphenols inhibit several type of cancers (15, 16). The sources of polyphenols are; some plants, fruits, vegetables, tea, and wine. In the literature, several analytical applications were studied for the detection of polyphenols such as liquid chromatography (17, 18), near-infrared reflectance spectroscopy, and micellar electrokinetic chromatography (19). Biosensors are an alternative to these methods, which are time consuming and extremely costly.

In this study, immobilizations of GOD and PPO were performed via entrapment within a conducting copolymer, P(PStPy-co-Py), which was synthesized by copolymerization of a pyrrole functionalized polystyrene with pyrrole. The copolymer was synthesized and characterized previously (20). The conducting copolymer was synthesized by performing constant potential electrolysis using sodium dodecylsulfate (SDS) as the supporting electrolyte and its characterization was achieved by FTIR spectroscopy and scanning electron microscopy (SEM). Temperature, pH optimizations were investigated for these enzyme electrodes along with operational and shelf life stabilities. Kinetic parameters (K_m and V_{max}) were also determined. These electrodes were used for real sample analyses.

2 Experimental

2.1 Materials and Methods

Glucose oxidase, Type II-S, (GOD, EC: 1.1.3.4), peroxidase, Type II, (POD, EC: 1.11.1.7), o-dianisidine, polyphenol oxidase (Tyrosinase, PPO, EC: 1.14.18.1), and sodium dodecyl sulfate (SDS) were purchased from Sigma. For the preparation of acetate buffer, sodium acetate (Sigma) and acetic acid were used as received. Glucose was purchased from Sigma. All glucose solutions were prepared in acetate buffer. Pyrrole was obtained from Merck and distilled before use. 3-Methyl-2-benzothiozolinone hydrazone (MBTH), acetone, and sulfuric acid were also obtained from Sigma. Catechol was purchased from Sigma. All catechol solutions were prepared in citrate buffer using citric acid (Sigma) and sodium citrate (Sigma). Bovine Serum Albumin (BSA) and Folin and Ciocalteu's Phenol Reagent were obtained from Sigma. Potentioscan Wenking POS-73 potentiostat and a Shimadzu UV-1600 model spectrophotometer were used. Varian 1000 FTIR was used for infrared analyses. Four probe conductivity measurements were also performed for the conductivity of the matrices.

2.2 Immobilization of Glucose Oxidase and Polyphenol Oxidase in PPy and P(PStPy-co-Py) Matrices

Synthesis and characterization of PStPy was described in an earlier study (20). Glucose oxidase and polyphenol oxidase enzymes were immobilized by electropolymerization of pyrrole on bare or PStPy (0.25% w/v in dichloromethane) coated platinum electrodes (1 cm × 1 cm) in a typical three electrode cell containing a platinum counter and working electrodes, and a Ag wire as a pseudo reference electrode at room temperature by constant potential electrolysis.

GOD enzyme electrodes were prepared in 10 ml acetate buffer (pH = 5.1) which contains 2 mg/ml GOD, 0.6 mg/ml SDS and 0.144 M pyrrole via applying a constant potential of +1.0 V for 30 minutes at room temperature.

PPO enzyme electrodes were obtained by constant potential electrolysis for 30 min in 10 ml citrate buffer (pH = 6.5) containing 0.4 mg/ml PPO, 1 mg/ml SDS and 0.072 M pyrrole.

As seen from Scheme 1, the reaction between pyrrole moieties of PStPy and pyrrole yields P(PStPy-co-Py) conducting copolymer, where GOD and PPO enzymes were entrapped. Enzyme electrodes were kept in buffer solutions at 4°C when not in use.

2.3 Determination of GOD Activity

In order to determine the activity of GOD enzyme, a modified version of Sigma Bulletin was used (21). Glucose solutions of known concentrations were prepared and incubated at 25°C. For free enzyme activity, glucose solutions (0.1 mM–3 mM) were oxidized by the addition of 0.1 ml of GOD solution (2 mg/ml acetate buffer). They were incubated for specific times (2, 4, and 6 min) to achieve the reaction between enzyme and its substrate. At the end of a given time, 0.5 ml aliquots were drawn and 0.1 ml POD (60 U/ml) were added to catalyze the reaction of hydrogen peroxide and o-dianisidine (2.4 ml), the coloring reagent. Spectrophotometric measurements were done at 530 nm, after terminating the reaction with the addition of 0.5 ml (2.5 M) sulfuric acid. The activity determination of immobilized enzymes was performed with the same procedure described for free enzyme activity where enzyme electrodes were placed in glucose solutions (0.5 mM–80 mM). To define enzyme activity, a hydrogen peroxide standard calibration curve was used.

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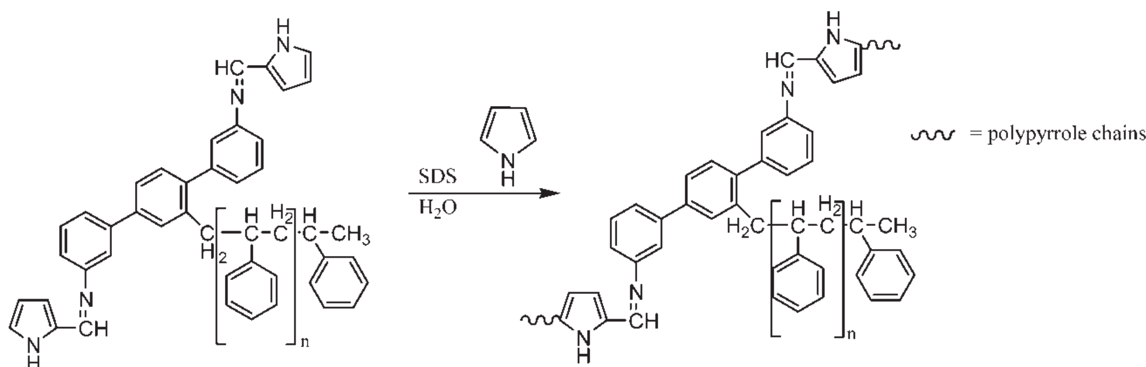

Sch. 1. Electrochemical synthesis of P(PStPy-co-Py).

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2.4 Determination of PPO Activity

Determination of PPO activities was achieved via the Besthorn Hydrazone method (22). Different concentrations of catechol were prepared for both free PPO activity determination (0.1 mM–2.5 mM) and for the immobilized one (0.1 mM–500 mM). Solutions were put in test tubes (3 ml) and incubated at 25°C. 1 ml of MBTH solution was added. After 1 min, a 0.2 ml enzyme solution (0.015 mg/ml citrate buffer) was added and specific reaction times were given. The reaction was terminated by adding 1 ml of 5% sulfuric acid. Resulting quinone reacts with MBTH to form a red color complex. 1 ml acetone was added for the dilution. After mixing, absorbances were measured at 495 nm. In the case of an immobilized enzyme, electrodes were put in different concentrations of catechol solutions. 1 min after the addition of MBTH and after specific times, they were taken out of the solution. Measurements were carried out in the same manner.

2.5 Determination of Optimum Temperature and pH

Determination of optimum temperature and pH for both enzyme electrodes were performed at constant substrate concentrations. Optimum temperatures and pH were determined by changing the incubation temperature between 10°C and 80°C. pH optimizations were done by changing the pH between 2 and 12 for GOD and between 2 and 11 for PPO.

2.6 Operational Stabilities and Shelf-Life

Operational stabilities of enzyme electrodes were investigated by 40 successive uses of electrodes in the substrate solutions. Shelf-life of enzyme electrodes was performed by determining the enzyme activities over 40 days.

2.7 Protein Determination

Protein determination measurements were performed by Lowry's method (23). Standard solutions of Bovine Serum Albumin (BSA) were prepared at different concentrations to obtain a calibration curve. 0.1 ml aliquots were taken from the electrolysis cell before and after the electrolysis and incubated at 25°C for 10 min after mixing. Then, 0.5 ml Floin-Chiocalteu's reagent (50% v/v in deionized water) was added to each tube and mixed. They were left for 30 min at 25°C for maximum color formation and absorbances were measured at 750 nm.

2.8 Determination of Glucose in Orange Juices

GOD electrodes were used in the determination of glucose amounts of two brands of Turkish orange juice (Brand D and Brand M). The juices were filtered and diluted in the ratio of 1:50 with an acetate buffer. They were used as the substrates and activity assay described for glucose oxidase was applied.

2.9 Analysis of Human Serum

GOD electrodes were used in the determination of glucose amounts in human serum. Serum samples were obtained from Middle East Technical University (METU) Medical Center. These samples were obtained by centrifuging and a standard addition method was used. Different concentrations of glucose solutions (0.4 mM–1.6 mM) were prepared in an acetate buffer and 2.7 ml aliquots were added to 0.3 ml serum samples and placed into test tubes. A test tube which contains 0.3 ml serum and 2.7 ml acetate buffer was prepared. After incubation at 25°C for 10 min, GOD electrodes were immersed. After shaken for 10 min, 0.5 ml aliquots were drawn and 0.1 ml peroxidase and 2.4 ml o-dianisidine were added. Following the addition of 0.5 ml (2.5 M) sulfuric acid, absorbances were recorded at 530 nm.

2.10 Analysis of Polyphenols in Wines

PPO electrodes were used for the determination of phenolic compounds in two brands of Turkish red wines (Brand K and Brand D). Total phenolic compounds in Turkish wines were reported as 2000–3000 mg/l (24, 25). Red wines were diluted to a 1:3 volume with citrate buffer and polyphenol oxidase activity assay was applied.

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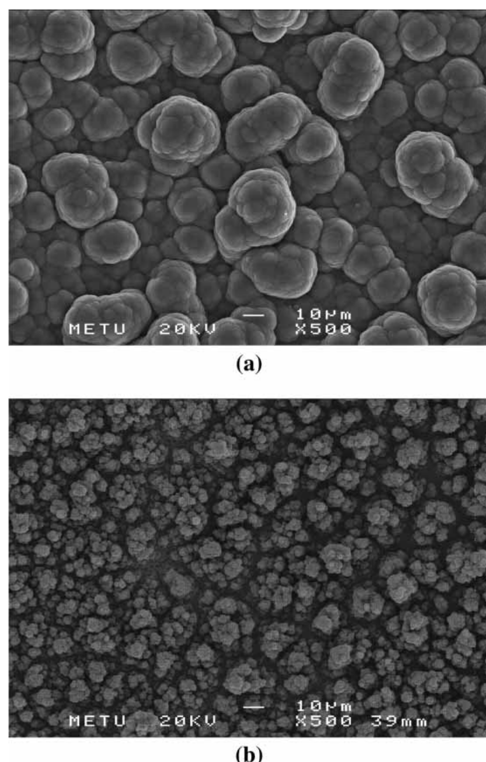

Fig. 1. SEM micrograps of (a) PPy film, (b) P(PStPy-co-Py) film.

3 Results and Discussion

3.1 Morphology of PPy and P(PStPy-co-Py) Matrices

The morphologies of PPy and P(PStPy-co-Py) were investigated via scanning electron microscopy (SEM) by JEOL JSM-6400. The solution sides of PPy matrix has a cauliflower-like structure (Figure 1a). However, for P(PStPy-co-Py) matrix, this structure was destroyed (Figure 1b) which proves the copolymerization formation. The structure of P(PStPy-co-Py) looks like popcorn.

3.2 FTIR Studies

In the FTIR spectrum of PStPy, an azomethane peak was observed at 1598 cm^{-1}, pyrrole at 3438 cm^{-1} and benzene peaks at the fingerprint region.

All characteristics peaks belonging to PStPy were also observed in the spectrum for P(PStPy-co-Py), together with peaks from the dopant anion of SDS at 1036 cm^{-1}, 1120 cm^{-1}, 1168 cm^{-1}. Hence, it was concluded that copolymerization was achieved.

Table 1. Conductivities of the films

Matrix	Conductivity (S/cm)
PPy	7.4×10^{-3}
P(PStPy-co-Py)	9.0×10^{-6}

Table 2. Kinetic parameters of glucose oxidase

	V_{max}	K_m (mM)
Free	0.68 (μmol/min · ml)	3.0
Ppy	0.04 (μmol/min · electrode)	11.8
P(PStPy-co-Py)	0.11 (μmol/min · electrode)	5.1

3.3 Conductivities of the Films

Conductivities of PPy and P(PStPy-co-Py) films were measured by a four-probe technique and given in Table 1. Since an insulating polymer was used for the copolymerization, the conductivity of the resulting material is lower than that of PPy.

3.4 Kinetic Parameters

Maximum reaction rates, V_{max}, and Michaelis-Menten constants, K_m, were calculated using Lineweaver-Burk plots (26). V_{max} and K_m values for free and immobilized enzymes were determined at constant pH and temperature while varying substrate concentrations.

Kinetic parameters of GOD were tabulated in Table 2. As expected, K_m values of immobilized enzyme increased, while V_{max} values decreased compared to that of a free enzyme. This difference was significant for the PPy matrix. However, GOD immobilized in P(PStPy-co-Py) matrix had similar affinity to glucose as for the free enzyme. This indicates that the P(PStPy-co-Py) matrix did not cause a handicap for GOD in doing its job. This argument was also proven by the higher reaction rate observed for P(PStPy-co-Py) matrix (Table 3).

As to the PPO, K_m parameters are different in these matrices, although V_{max} values are approximately the same. For P(PStPy-co-Py) matrix, the affinity of the enzyme towards catechol is lower than the one for PPy matrix. This means that the enzyme and substrate leave each other rather quickly, but the separation is productive.

3.5 Protein Determination

Amounts of entrapped enzymes in both matrices were given in Table 4. Results were consistent with the V_{max} values for immobilized enzymes. The amount of GOD entrapped in P(PStPy-co-Py) is 2.6 times higher than the one for PPy matrix. The amount of PPO enzyme entrapped in both matrices was the same. Same reaction rates observed for PPO immobilized in both matrices may be due to the equal amounts of enzyme immobilized.

Table 3. Kinetic parameters of polyphenol oxidase

	V_{max}	K_m (mM)
Free	0.10 (μmol/min · ml)	0.14
PPy	0.0064 (μmol/min · electrode)	17.0
P(PStPy-co-Py)	0.0064 (μmol/min · electrode)	23.0

Table 4. Amount of entrapped enzyme

	GOD (mg)	PPO (mg)
PPy	9.5×10^{-5}	2.4×10^{-4}
P(PStPy-co-Py)	2.5×10^{-4}	2.4×10^{-4}

3.6 *Effect of Incubation Temperature on Enzyme Activity*

The temperature effect on enzyme activities were investigated for GOD and PPO enzymes immobilized in PPy and P(PStPy-co-Py) matrix. The optimum temperature for free glucose oxidase was reported as 30°C (26). As seen in Figure 2, the temperature of maximum activity for PPy/GOD electrode is 40°C and for P(PStPy-co-Py)/GOD electrode between 30°C and 40°C. Further increase of temperature above 40°C, resulted in a decrease of GOD activity due to the altered enzyme conformation.

Polyphenol oxidase activity was also investigated with respect to a temperature change between 20°C and 80°C. PPO entrapped in the PPy matrix exhibited maximum activity at 60°C, whereas in P(PStPy-co-Py) at 70°C. Data show that P(PStPy-co-Py) matrix protects both enzymes against high temperatures.

3.7 *Effect of pH on Enzyme Activity*

Maximum activity for GOD was observed at pH 6 for PPy matrix as illustrated in Figure 3. On the other hand, enzyme immobilized in P(PStPy-co-Py) matrix exhibited

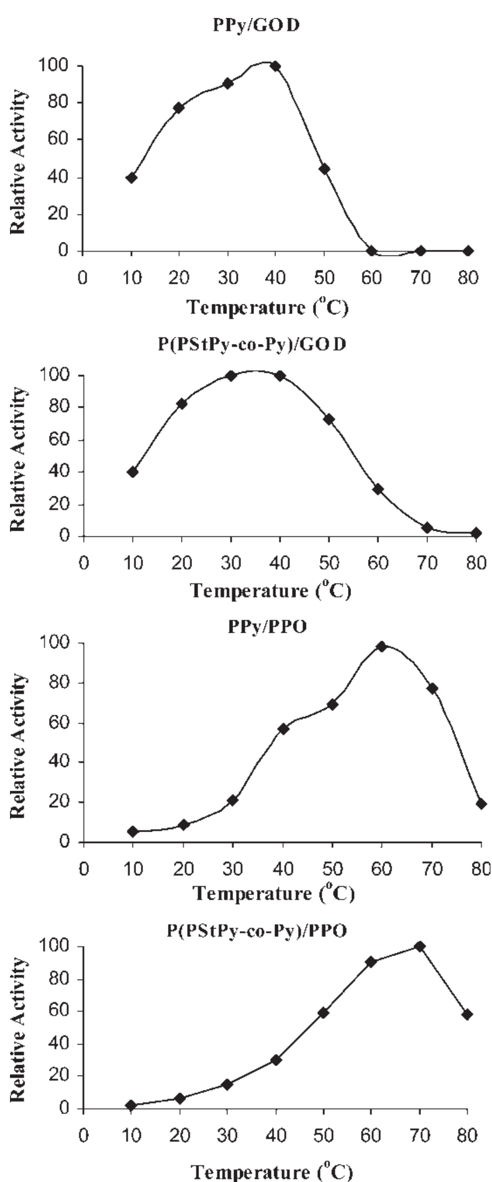

Fig. 2. Temperature stabilities of enzyme electrodes.

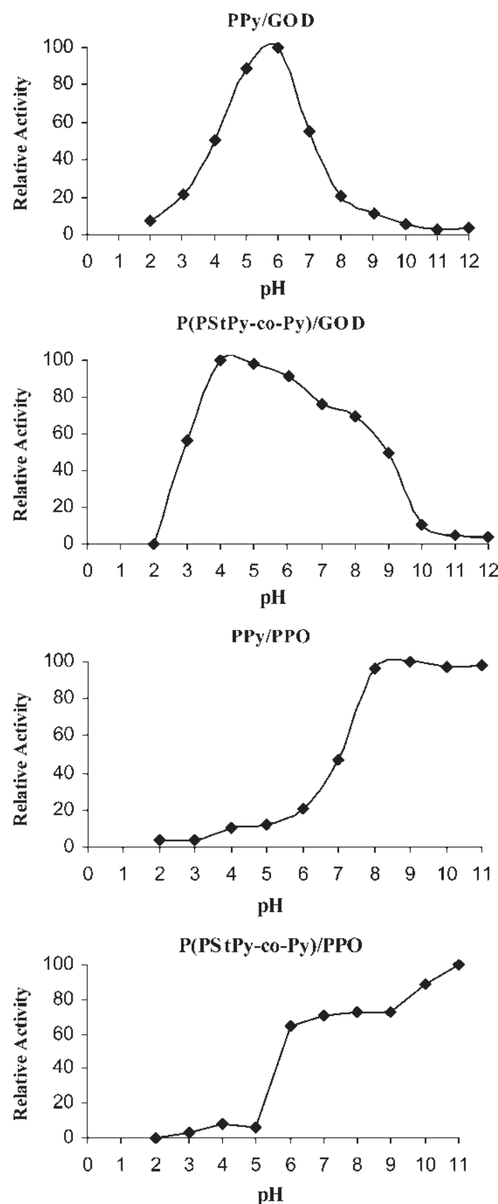

Fig. 3. pH stabilities of enzyme electrodes.

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higher activities over a broad pH range in the alkaline side. This might be explained by partitioning of protons (22, 27). Protons that are attached to the negatively charged groups of matrix provide an environment to the enzyme which has lower pH than the bulk where the measurements were done. High stability in a broad pH range provides the possibility to analyze the glucose amount in human serum. We also investigated the pH profiles of the PPO electrodes (Figure 3). The PPy/PPO electrode has higher activities between pH 8 and pH 11. P(PStPy-co-Py)/PPO electrode has higher activities in a broader pH range (pH 6–pH 11). Thus, the matrix protects the enzyme against high pH. As a result, this enzyme electrode can be used effectively in a wide range of pH for different applications.

3.8 Operational Stability and Shelf Life of the Enzyme Electrodes

Stabilizing the enzyme for a biosensor to be used repeatedly over a long time is an important aspect of enzyme immobilization. The change in the activities of enzyme electrodes upon repetitive uses were investigated. In order to determine the stability of enzyme electrodes in terms of repetitive uses, 40 successive measurements were done at 25°C in one day. As seen from Figure 4, both GOD electrodes exhibit very high operational stabilities.

We also determined the operational stabilities of PPO enzyme electrodes. Although PPy/PPO enzyme electrode lost its 49% of activity after the 40th use, P(PStPy-co-Py)/PPO electrode retained 85% of its activity.

In order to determine the loss in the activity of enzyme electrodes shelf life, experiments were done. The activities of the enzyme electrodes were first measured for five consecutive days, then once in five days throughout 50 days. While the P(PStPy-co-Py)/GOD enzyme electrode retained 90% of its activity at the 25th day, the PPy/GOD enzyme electrode lost 70% of its activity at the same day (Figure 5). Shelf life studies of entrapped PPO enzyme were also extremely sufficient for biosensing applications.

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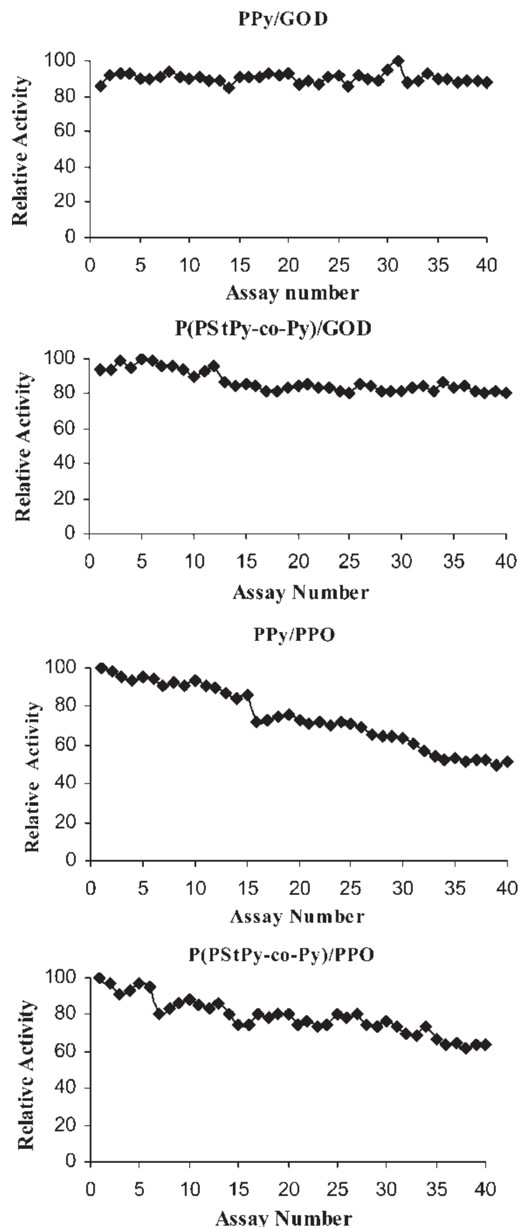

Fig. 4. Operational stabilities of enzyme electrodes.

3.9 Glucose Determination in Human Serum with GOD Enzyme Electrodes

The results of pH studies of GOD enzyme electrodes showed that glucose determination in human blood could be performed. We analyzed human serum using PPy/GOD and P(PStPy-co-Py)/GOD electrodes. The standard addition method was used for glucose determination. Different concentrations of glucose standards were added into the same amounts of serum samples and the same procedure was performed as explained in GOD activity determination.

Serum samples were obtained from the METU Medical Center. Samples were also analyzed at the Medical Center with Beckman Coulter Synchron CX 7-9 ALX. The glucose amount detected by PPy/GOD electrode was very close to the value obtained from the Medical Center. However, the P(PStPy-co-Py)/GOD electrode exhibited low values, meaning that inhibitors can also reach the entrapped enzyme. This causes the enzyme to be affected by the inhibitory properties of metal ions naturally found in serum (11).

3.10 Determination of Glucose in Orange Juices

Two brands of orange juice in Turkey were used for the determination of glucose amount. Lane-Eynon analyses of glucose amount for both brands were also performed (27). The results were given in Table 5. The amount of glucose detected with P(PStPy-co-Py)/GOD electrode was not even close to the one determined by the Lane-Eynon method.

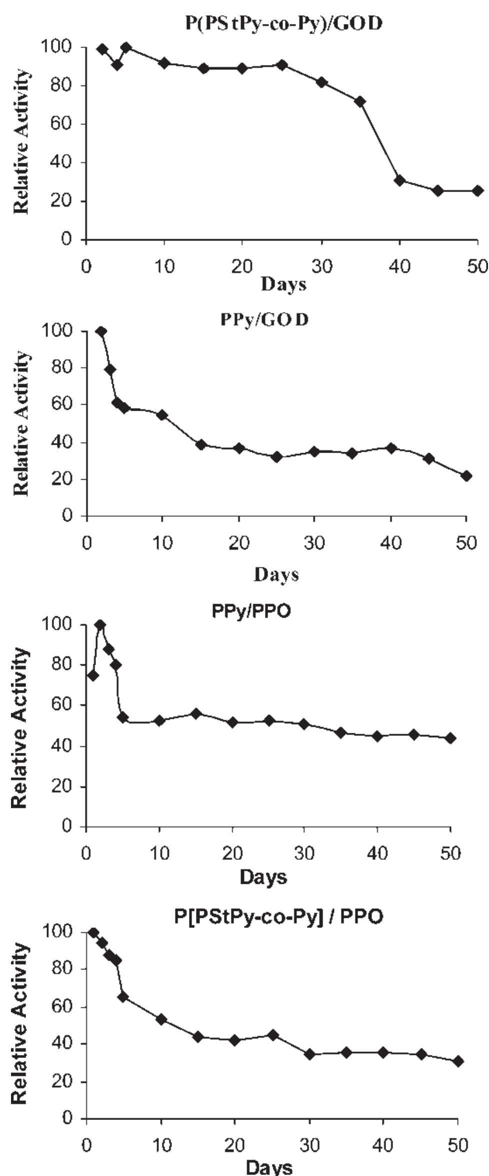

Fig. 5. Shelf-life stabilities of enzyme electrodes.

3.11 Determination of Polyphenols in Wine

The results of phenolic compounds determined for the two brands of Turkish wines were given in Table 6. The amount of phenolics present in Brand K is double, compared to brand D. This was also confirmed by enzyme electrodes. However, the values determined by P(PStPy-co-Py)/PPO electrode was low. It may be concluded that copolymer matrix cannot protect the enzyme from inhibitors as much as the PPy matrix does (Table 7).

Table 5. Glucose amount in human serum

	Glucose amount (mM)
Medical center	6.32
PPy	6.15
P(PStPy-co-Py)	2.65

Table 6. Glucose amount in two brands of orange juice

	Brand D (g/100 ml)	Brand M (g/100 ml)
Lane-Eynon Method[a]	1.50	2.15
PPy[a]	1.45	1.44
P(PStPy-co-Py)	ND	1.30

[a][27]; ND: Not detected.

Table 7. Phenolic compounds in Turkish red wines

	Brand K	Brand D
Free PPO[a]	220 mg/l	270 mg/l
PPy/PPO[a]	4000 mg/l	2200 mg/l
P(PStPy-co-Py)/PPO	1954 mg/l	1026 mg/l

[a][22].

4 Conclusions

In this study immobilization of glucose oxidase and polyphenol oxidase enzymes in PPy and P(PStPy-co-Py) matrices were achieved. Enzyme electrodes were characterized against temperature, pH, operational and shelf-life stabilities. The enzyme electrodes were used in real samples. GOD electrodes were used for the determination of glucose in orange juice and in human serum. The PPy/GOD electrode yielded good results compared to the P(PStPy-co-Py)/GOD electrode. Moreover, the the PPO enzyme electrode was used for the determination of phenolics in two brands of Turkish wines. The P(PStPy-co-Py)/PPO enzyme electrode functions better compared to free enzyme, but not as good as the PPy/PPO enzyme electrode.

5 Acknowledgements

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