Electrochemical Polymerization of (2-Dodecyl-4,7-di(thiophen-2-yl)-2H-benzo[d][1,2,3] triazole): A Novel Matrix for Biomolecule Immobilization[a]

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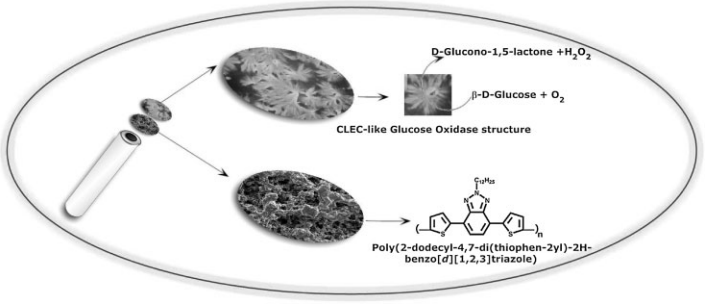
A recently synthesized conducting polymer [poly(2-dodecyl-4,7-di(thiophen-2-yl)-2H-benzo[d][1,2,3]triazole (PTBT)] was tested as a platform for biomolecule immobilization. After electrochemical polymerization of the monomer (TBT) on graphite electrodes, immobilization of glucose oxidase (GOx, β-D-glucose: oxygen-1-oxidoreductase, EC 1.1.3.4) was carried out. To improve the interactions between the enzyme and hydrophobic alkyl chain on the polymeric structure, GOx and isoleucine (Ile) amino acid were mixed in sodium phosphate buffer (pH 7.0) with a high ionic strength (250×10^{-3} M). The solution is then casted on the polymer film, and the amino groups in the protein structure were crosslinked using glutaraldehyde (GA) as the bifunctional agent. Finally, the surface was covered with a perm-selective membrane. Consequently, cross-linked enzyme crystal (CLEC) like assembles with regular shapes were observed after immobilization. Microscopic techniques such as scanning electron microscopy (SEM) and fluorescence microscopy were used to monitor the surface morphologies of both the polymer and the bioactive layer. Electrochemical responses of the enzyme electrodes were measured by monitoring O_2 consumption in the presence of glucose at –0.7 V. The optimized biosensor showed a very good linearity between 0.05 and 2.5×10^{-3} M with a 52 s response time and a detection limit (LOD) of 0.029×10^{-3} M to glucose. Also, kinetic parameters, operational and storage stabilities were determined. K_m and I_{max} values were found as 4.6×10^{-3} M and 2.49 μA, respectively. It was also shown that no activity was lost during operational and storage conditions. Finally, proposed system was applied for glucose biomonitoring during fermentation in yeast culture where HPLC was used as the reference method to verify the data obtained by the proposed biosensor.



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Introduction

Biosensors have attracted great interest among researchers as a worthy analytical tool for the detection of desired substances. They have had a considerable place in different technological areas such as clinical and food diagnostics and bioprocess and pollution monitoring due to their usability in rapid detection and monitoring.[1,2] Conducting polymers (CPs) offer an extensive possibility to modify the surface of conventional electrodes supplying fascinating properties.[3] Moreover, providing simplicity and high reproducibility in preparation, easiness in arranging the thickness of the film, compatibility with biological molecules, and possibility to produce at room temperature make electrochemically synthesized CPs charming in designing biosensors.[3,4] Immobilization of biomolecules onto electropolymerized films is acquiring importance in the fabrication of biosensors rendering as an enzyme immobilization platform.[5–8] Table 1 contains a number of results of the studies on glucose oxidase (GOx) biosensors and various CPs as a comparison.[9–15]

The monomer 2-dodecyl-4,7-di(thiophen-2-yl)-2H-benzo[d][1,2,3]triazole (TBT) was previously designed, synthesized, and polymerized both electrochemically and chemically. It was reported that the polymer reflects almost all impressive properties in a single polymer for optoelectronic applications.[16]

This paper reveals the use of PTBT to construct a polymeric matrix for biomolecule immobilization by taking advantage of ease of polymerization of the monomer. The ability of the polymer to last as coated onto the electrode together with excellent electrical conductivity were the driving forces to use it in the immobilization process. GOx which has been often used in immobilized forms for the determination of glucose[17–19] was used as the model enzyme to examine the possibility of its immobilization onto PTBT after electrochemical polymerization on the graphite electrode.

GOx from *Aspergillus niger* catalyzes the oxidation of β-glucose to δ-gluconolactone in the presence of molecular oxygen which subsequently hydrolyzes spontaneously into gluconic acid and hydrogen peroxide. Homodimeric enzyme GOx is composed of two identical subunits with two molecules of flavin adenine dinucleotide (FAD).[20] According to earlier researches, native GOx does not show any remarkable affinity to bind the hydrophobic support.[21] Since TBT has hydrophobic alkyl group in its structure, it is incompatible with the enzyme due to the limited immobilization of the enzyme on polymer deposited electrode surface. To overcome this drawback, high ion concentrated buffer for immobilization, isoleucine (Ile) as an additive due to its hydrophobic nature and glutaraldehyde as the crosslinker were used. To obtain a stable immobilization, ''salting out'' like procedure was applied to

Table 1. Comparison of biosensor examples from the literature involving GOx and various CPs as immobilization matrix.

CP	Immob. tech.	K_m/V_{max} or I_{max}	Linear range	Sensitivity	Ref.
Polypyrrole/poly(methyl methacrylate (PMMA)-*co*-thienyl methacrylate (PMTM))	Entrapment	40.0×10^{-3} M/1.74 μmol·min^{-1}·cm^{-2}	NR	NR	[9]
Polypyrrole	Entrapment	NR	0–20×10^{-3} M	40 nA × 10^{-3} M^{-1}	[10]
Poly-*o*-phenylenediamine (POPD)	Entrapment	NR	8.0–14×10^{-3} M	0.2–0.7 μA × 10^{-3} M^{-1}·cm^{-2}	[11]
Polypyrrole	Entrapment	7.01×10^{-3} M/120 μA	0.5–10×10^{-3} M	7.4 mA·M^{-1}·cm^{-2}	[12]
Poly(*o*-anisidine) (POA)-dodecylbenzene sulfonic acid (DBS)	Covalent binding	3.0×10^{-3} M/6.5 μA	0–9.0×10^{-3} M	20 μA × ·10^{-3} M^{-1}	[13]
PANI nanofibers and nanocomposite of Au NPs	Covalent binding	NR	1.0×10^{-6}/ 8.0×10^{-4} mol·L^{-1}	20 mg·mL^{-1}	[14]
Poly(*N*-(4-(3-thienyl methylene)-oxycarbonyl phenyl) maleimide-*co*-pyrrole)	Entrapment	4.57×10^{-3} M/ 0.033 μmol (min-electrode)	NR	NR	[15]

NR, not reported.

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form more hydrophobic regions and dehydrated proteins were then cross-linked via glutaraldehyde. As a result of these subsequent treatments cross-linked enzyme crystal (CLEC)-like enzyme structures ingrained in their more favorable and active conformation[22] were obtained. Moreover, SEM and fluorescence microscopy were utilized to investigate the surface properties and morphology of the enzyme in tested conditions. Finally, GOx immobilized PTBT matrix was characterized as the electrochemical glucose biosensor and applied for bioprocess monitoring in yeast culture medium.

Experimental Part

Materials

Glucose oxidase (GOx, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4, 21 200 units/mg) from *A. niger*, Ile, D-glucose, and dialysis tubing cellulose membrane (25 mm × 16 mm) were purchased from Sigma (St. Louis, USA; www.sigmaaldrich.com). Acetonitrile, hydrochloric acid, sodium hydroxide were purchased from Merck (Darmstadt, Germany; www.merck.com). All chemicals needed for the synthesis of monomer and electropolymerization were purchased from Aldrich except tetrahydrofuran (THF) which was purchased from Acros (Geel, Belgium). All other chemicals were analytical grade.

Apparatus

For the chronoamperometric measurements and cyclic voltammograms, Palm Instrument (PalmSens, Houten, The Netherlands, www.palmsens.com) with three electrode configuration was used. A graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity), Ag/AgCl (3 M KCl saturated with AgCl as an internal solution) and a Pt electrode (Metrohm, Switzerland, www.metrohm.com) were used as the working, reference, and counter electrodes, respectively.

JEOL JSM-6400 models SEM, Ambios Universal SPM model AFM and epifluorescence microscopy (Olympus, Tokyo, Japan) were used for surface imaging.

Synthesis of 2-Dodecyl-4,7-di(thiophen-2-yl)-2H-benzo[d][1,2,3]triazole (TBT) Monomer

Synthesis and characterization of the monomer, TBT were carried out according to a previously described method.[16]

Biomolecule Immobilization

Prior to the electropolymerization spectroscopic grade graphite rods were polished on emery paper and washed thoroughly with distilled water.

Electrochemical polymerization of TBT was potentiodynamically carried out between −0.5 and 1.4 V in 0.1 M ACN/$TBAPF_6$ solvent-electrolyte system. For immobilization of enzyme a proper amount of GOx solution [2.35 mg in 5 μL, 250×10^{-3} M sodium phosphate buffer (pH 7.0)] containing Ile (1 mg) was spread over the polymer coated electrode and glutaraldehyde (5 μL, 0.1%, in 250×10^{-3} M sodium phosphate buffer, pH 7.0) was then added and the electrode was allowed to stand at ambient conditions to dry for 1 h. Then, the layer was covered with a dialysis membrane which was pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring.

Measurements

All experiments were carried out at ambient conditions in a vessel containing 10 mL buffer solution. The electrode was washed with distilled water and kept in 50×10^{-3} M, pH 5.5 sodium acetate buffer for 5 min after each measurement. The enzyme electrodes were initially equilibrated in buffer and the substrate was added to the reaction cell. The biosensor responses were registered as the current signal (μA) via following the oxygen consumption at −0.7 V as a result of enzymatic activity. Buffer was refreshed after each measurement.

Sample Application

Glucose biosensors were tested in yeast culture by using the distillery strain of Saccharomyces cerevisiae H620 and the medium was directly injected to the reaction medium instead of glucose. Results were also compared with the ones obtained by high performance liquid chromatography (HPLC) as the reference method.

Yeast cultivation was carried out according to a procedure described in our previous study.[23] Cultures were incubated in an orbital shaker (New Brunswick Scientific, USA) at 30 °C and 200 rpm and samples were collected from fermentation broth during 8 h.

HPLC with a refractive index detector (RID) controlled by an HP-Chemstation from Agilent (Karlsruhe, Germany) was used for independent analysis of the glucose content. HPLC column (GL Sciences Inc. Inertsil NH_2 5.0 μm (4.6 id × 250 mm), Japan) was used for the chromatographic separation at 30 °C. Injection volume was 20 μL. The mobile phase was H_2SO_4 (5.0×10^{-3} M).[24] The flow rate was 0.6 mL · min^{-1}. Initially standard curve for glucose was plotted ($2.5–50 \times 10^{-3}$ M for glucose). After dilution with mobile phase and centrifugation, samples were applied to the column and glucose concentrations were calculated using the calibration plot.

Results and Discussion

Optimization Studies

Initially constituents of bioactive layer were optimized to improve the interactions between the enzyme and hydrophobic surface due to the presence of alkyl chain on the polymer. When enzyme in buffer solution (50×10^{-3} M, sodium phosphate buffer) was directly applied, it was not possible to spread it onto the surface due the incompatibility with the polymer. However, when proper amounts of

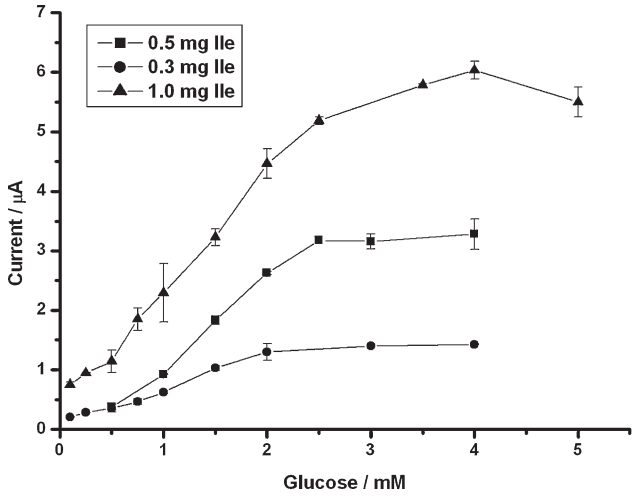

Figure 1. Effect of Ile amount in bioactive layer on the biosensor response (in sodium acetate buffer, 50×10^{-3} M, pH 4.0; 25 °C, –0.7 V). Error bars show SD.

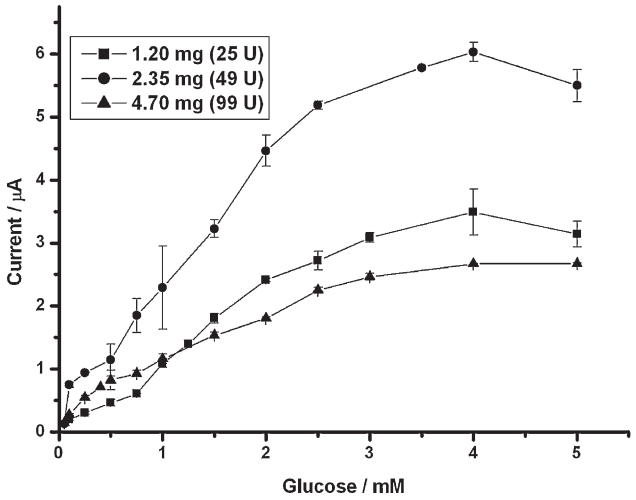

Figure 2. Effect of enzyme amount on the biosensor response (in sodium acetate buffer, 50×10^{-3} M, pH 4.0; 25 °C, –0.7 V). Error bars show SD.

Ile and buffer with higher ionic strength were mixed with the GOx solution, the mixture was easily dispersed on PTBT. Hence, in this part, Ile and GOx amounts, ionic strength of the buffer (sodium phosphate, pH 7.0) used in immobilization and quantity of glutaraldehyde were optimized accordingly.

For the optimization of the quantity of Ile, various electrodes containing different Ile amounts between 0.3 and 2 mg in bioactive layer were prepared and then sensors were calibrated for glucose (Figure 1). It was observed that in the absence of Ile, it was not possible to obtain a homogeneous dispersion on the polymeric surface. Addition of this hydrophobic amino acid up to 1 mg in the enzyme solution caused an increase in the response signals whereas at higher amounts insoluble mixtures were obtained, hence 1 mg was used as the optimum amount for further steps.

The amperometric response was examined as a function of the biomolecule amount. Three different electrodes were prepared with 1.2 mg (25 unit), 2.35 mg (49 unit), and 4.7 mg (99 unit) of GOx (in 5 μL sodium phosphate buffer (50×10^{-3} M, pH 7.0) containing 1 mg Ile). The enzyme was immobilized via glutaraldehyde on PTBT modified graphite electrodes. The amount of other components was kept constant. The highest signals were obtained by the biosensor with 2.35 mg (49 unit) GOx (Figure 2) in bioactive matrix while no increase in the higher loads were registered.

To obtain more compatible protein structure with the hydrophobic surface, "salting out" like conditions (the most common method used to precipitate a target protein[25]) were applied by increasing the ionic strength of sodium phosphate buffer in the bioactive layer containing the enzyme (2.35 mg which equals 49 unit) and Ile (1 mg). The increase in salt amount in the solution decreases the electrostatic interaction while increasing the hydrophobic ones.[21] It is expected that as the salt concentration of the solution is increased, more of the water becomes associated with the ions. Hence, less water is available to partake in the solvation layer around the protein, which exposes hydrophobic patches on the protein surface. The addition of ions to the enzyme solution generates an electron shielding effect which overrides some activity between water particles and the protein, lessening the solubility as the proteins bind with each other. Proteins may then exhibit improved hydrophobic interactions with the polymer structure and tend to aggregate on the surface. The effect of ionic strength on the current responses was shown in Figure 3.

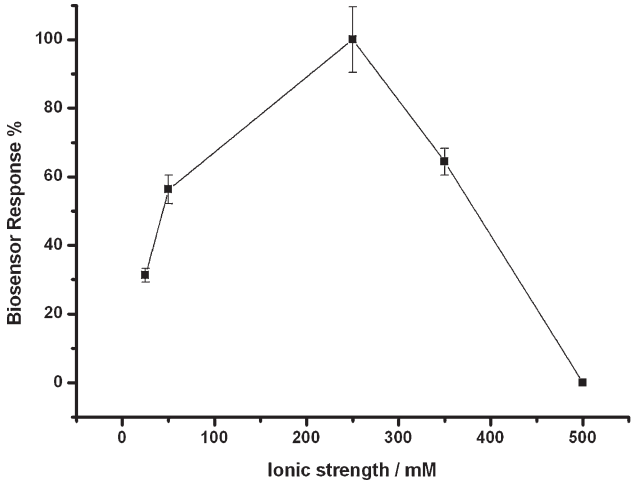

Figure 3. Effect of ionic strength in bioactive layer on the biosensor response (in sodium acetate buffer, pH 7.0; 25 °C, –0.7 V, [Glc]; 1×10^{-3} M). Error bars show SD.

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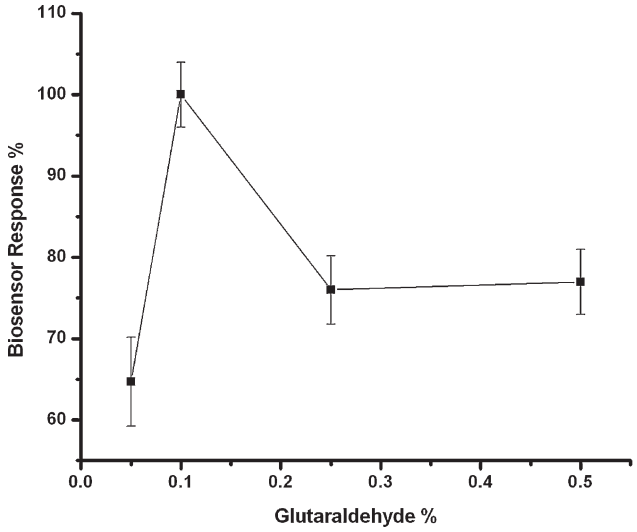


Figure 4. Effect of glutaraldehyde amount on the biosensor response (in sodium acetate buffer, 50×10^{-3} M, pH 4.0; 25 °C, −0.7 V, [Glc]; 1×10^{-3} M). Error bars show SD.

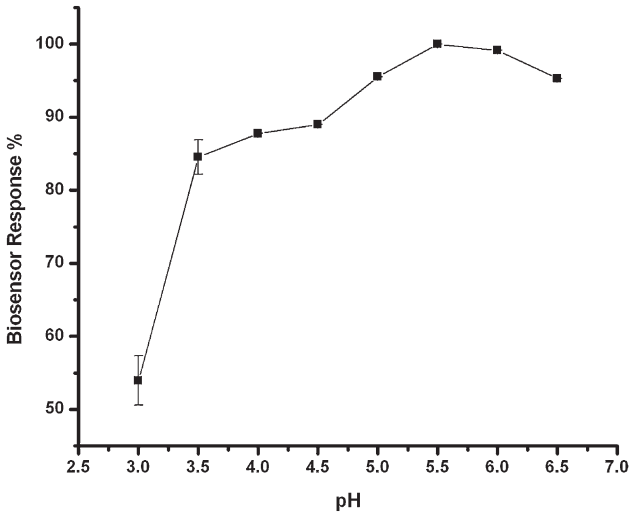


Figure 5. Effect of pH (sodium citrate buffer at pH 3.0; 3.5, sodium acetate buffer at pH 4.0; 4.5; 5.0; 5.5 and sodium phosphate buffer at pH 6.0; 6.5, 25 °C, −0.7 V, [Glc]; 1×10^{-3} M). Error bars show SD.

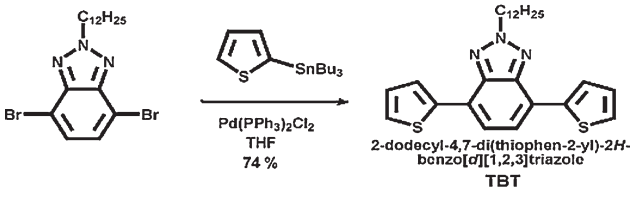
After optimizing GOx/Ile mixture in 250×10^{-3} M sodium phosphate buffer (pH 7.0), different amounts of glutaraldehyde were then added to obtain crosslink between the amino groups in the protein structure. While studying the influence of the amount of cross-linker, optimum glutaraldehyde amount was found as 0.1% (in phosphate buffer, pH 7.0, Figure 4) and used in further experimental steps. For higher amounts of GA, enzyme cannot be in contact with the modified surface due to the excess cross linkages, thus, cannot diffuse enough into the polymer network and fix its proper position. It was also demonstrated that the decrease in enzyme activity with the increase in glutaraldehyde amount showed that the excess linkages between the enzymes are destroying tertiary structure so do the activity.[26]

The pH dependence of the responses was investigated over a pH range of 3.5–6.5 with sodium acetate and sodium phosphate buffers (50×10^{-3} M) in the presence of 1×10^{-3} M glucose. The biosensor revealed the best result at pH 5.5 as given in Figure 5. It was also reported that the native enzyme is acidic (with $pI = 4.2$) and shows activity over a wide range of pH values (pH 3.0–8.0).[27] For the further experiments pH 5.5 sodium acetate buffer was used as the buffer solution.

Characterization

Synthesis (Scheme 1) and potentiodynamic polymerization of TBT on graphite electrodes were carried out as described earlier.[16] Film formation on the electrode surface, regardless of its shape or size, can be performed with large electrode surfaces with a control of thickness. Moreover, the film thickness can easily be followed by measuring of the total charge during the deposition of the CP.[28] On the other hand, electropolymerization time is quite effective both on the rate of growth and the quality of the CP films.[29] According to the experiments 63 min of polymerization time (referring to 100 cycles) was chosen and the charge involved in the film formation was measured as 8.37×10^{-4} C. Repeated potential-scan electropolymerization of TBT in 0.1 M ACN/$TBAPF_6$ solvent-electrolyte system at a scan rate of 100 $mV \cdot s^{-1}$ on graphite up to ten cycles are shown in Figure 6.

Morphology of PTBT was shown in Figure 7(A, B) and 8(A, B). SEM technique is used to monitor the surface characteristics. Figure 7(A) shows the PTBT as a result of 100 cycles of electropolymerization on graphite. After immobilization, hydrophobic polymer surface was fully covered by the bioactive layer at the optimized conditions [Figure 7(B)]. Apart from SEM, PTBT deposition was also monitored by fluorescence microscopy after obtaining it on the ITO glass.[16] Figure 8(A) shows the bare matrix prior to immobilization and enzyme molecules on PTBT is shown in Figure 8(B). Since GOx itself exhibits the fluorescent property due to the FAD center, it could also be possible



Scheme 1. Synthesis of TBT.

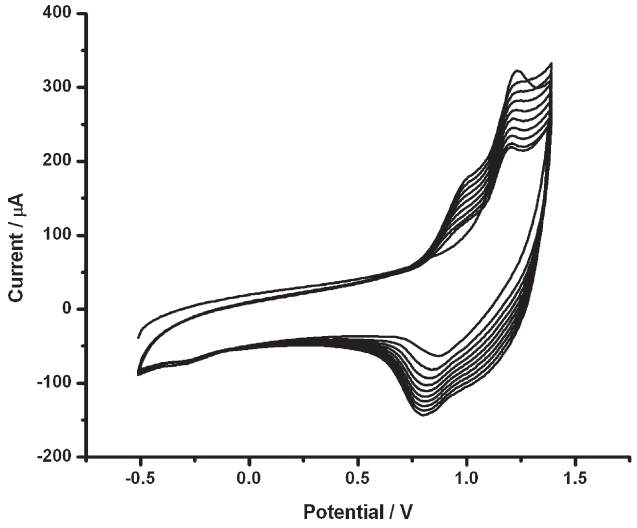


Figure 6. Repeated potential-scan electropolymerization of TBT in 0.1 M ACN/TBAPF$_6$ solvent-electrolyte system at a scan rate of 100 mV · s^{-1} on graphite (up to ten cycles).

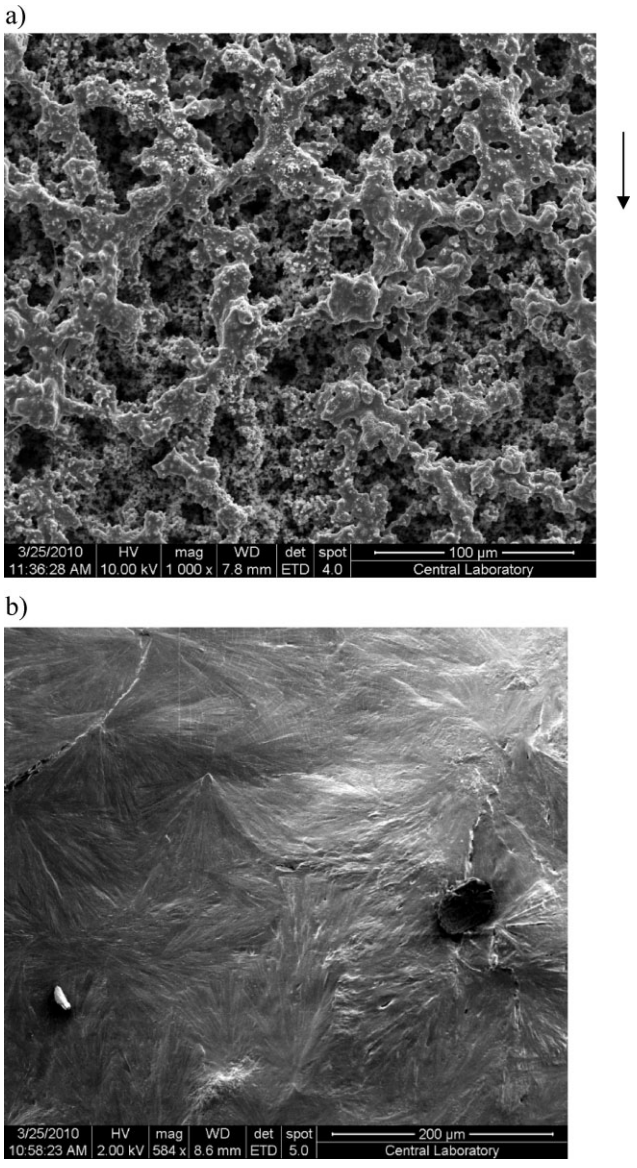


Figure 7. Scanning electron microscopy (SEM) images of TBT polymer before (A) and after biomolecule immobilization (B) at the optimized conditions.

to differentiate it on the polymer surface under fluorescence microscopy as indicated in Figure 8(B).

It is a common fact that as well as the number and the nature of bonds between the enzyme and the carrier, the form of the enzyme affects the stability of immobilized enzyme at unfavorable temperatures and also in the presence of other inactivating agents such as organic solvents.[30] It has been proved that the stability is increased with crystal formation in enzyme immobilization and the activity is prolonged.[31] CLECs can stay active and stable against harsh environments, hence it is highly desired to form CLECs for biosensor preparation. Crystalline structure is supported with cross-linkage by multi-attachment of protein to the support by preventing the unfolding of proteins.[32] By this way, 1–100 μm uniform-sized crystals can be achieved. Moreover, in the same manner, chemical cross-linkers are used to produce cross-linked enzyme aggregates (CLEA).[30] This extreme stability is an outcome of both high hydrophobic and electrostatic (polar) interactions.[32,33] The raise in these interactions among the protein molecules enhances the formation of crystalline structure. In our case, CLEC-like assembles with the regular shapes were observed in the presence of Ile after cross-linking via glutaraldehyde in 250×10^{-3} M buffer solution. Fluorescence microscopy image of GOx prepared in the mentioned conditions was the evident of CLEC-like structure (Figure 9).

The top layer of the electrode was examined with FTIR-ATR; following peaks are the proof of the presence of the enzyme in the biosensor: 1 599 cm^{-1} (C=O); 1 224 cm^{-1} (C–O); 1 108 cm^{-1} (C–N); 965 cm^{-1} (C–H).

As to the analytical characterization, the dependence of current signals on the glucose concentration was shown in Figure 10. A perfect linearity was obtained for 0.1–2.5×10^{-3} M glucose in 50×10^{-3} M sodium acetate buffer pH 5.5 given with an equation of $y = 1.937x + 0.416$ 0.416 and $R^2 = 0.9972$ (given as inset in Figure 10).

On the other hand the biosensor signals corresponding to 1×10^{-3} M glucose standard solutions were measured for ten times in order to achieve repeatability of the biosensor response. The standard deviation (SD) and the relative standard deviation (RSD) were calculated as ±0.064 and 4.2%, respectively. Limit of detection (LOD) was also calculated as 29.3 μM according to $S/N = 3$.

In blank experiments, bioactive layer at the optimized conditions as described before was adsorbed on directly

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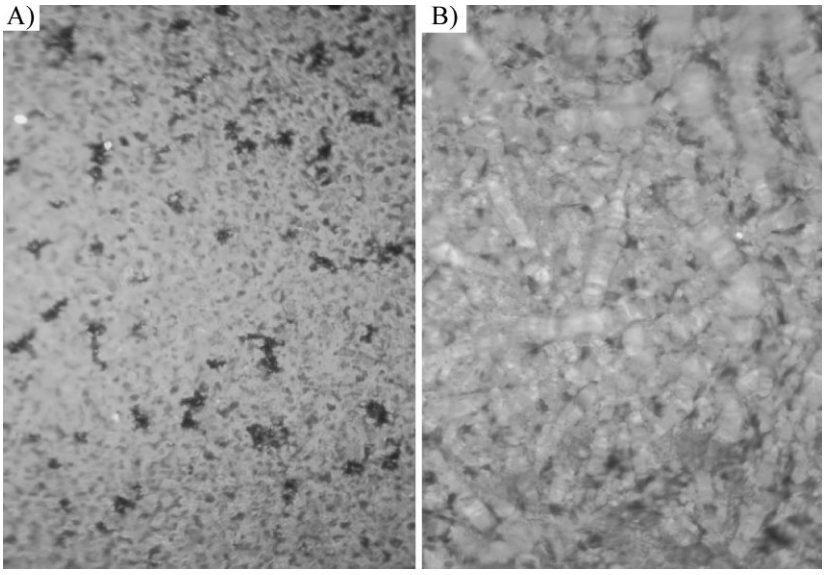

Figure 8. Fluorescence images of TBT polymer (A) and GOx (B) on polymer surface coated on ITO glass (with 200 × magnification).

graphite surface with no the polymer behind the dialysis membrane. However, lower response signals with worse repeatability were registered. In this case, linearity was observed between 0.25 and 1×10^{-3} M glucose with an equation of $y = 0.816x + 0.159$ and $R^2 = 0.980$ and also SD and the RSD were calculated as ±0.279 and 27%, respectively.

Stability of the biosensor was also tested. Operational stability of biosensor was investigated for 1×10^{-3} M glucose under optimized conditions. In this part, the biosensor was kept in buffer by stirring at the optimized working conditions until the next measurement. Signal responses were measured every hour for 8 h and no activity loss was observed. Apart from operational stability, the shelf life of the biosensor was also examined. The measurements were performed every other day during two weeks using 1×10^{-3} M glucose. Biosensor system was stored at +4 °C in contact with sodium acetate buffer (pH 5.5) in between consecutive measurements. For a period of 2 weeks no activity loss was observed.

Moreover, interference studies were also carried out. For this purpose, interference effect of ascorbic acid, cholesterol, and urea as the possible interfering compounds (between 0.01 and 10×10^{-3} M) and Schatzman medium (used in the fermentation experiments as the growth medium) were examined in different amounts (between 10 and 1 000 μL). However, no interference was observed at the potential of –0.7 V under optimized working conditions. It can be claimed that interference free measurements especially during the sample application could be done via using the proposed biosensor.

Some characteristic properties including kinetic parameters (K_m, I_{max}), sensitivity as well as stabilities were shown in Table 2. It was found that proposed biosensor obeyed the Michaelis–Menten kinetics and K_m and I_{max} values were calculated using Lineweaver–Burk diagram. The K_m value of free GOx (from *A. niger*) was previously reported between 33 and 248×10^{-3} M depending on the conditions used in the activity measurement.[34] On the other hand, when GOx was immobilized in poly(*N*-methylpyrrole)/polyvinyl sulfonic acid/sodium nitrate,

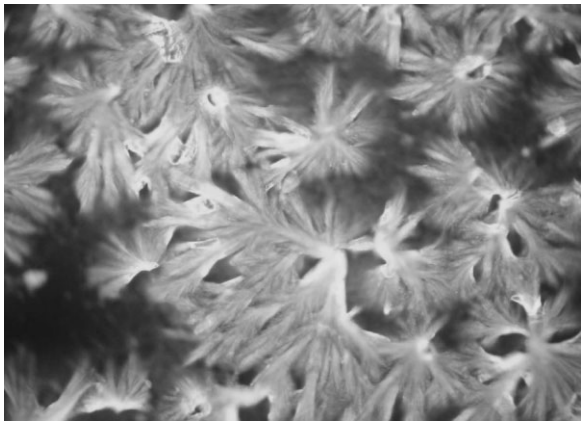

Figure 9. Fluorescence microscopy image of CLEC-like GOx structure obtained after crosslinked via glutaraldehyde in the presence of Ile in sodium phosphate buffer, 250×10^{-3} M, pH 7.0, with 200 × magnification.

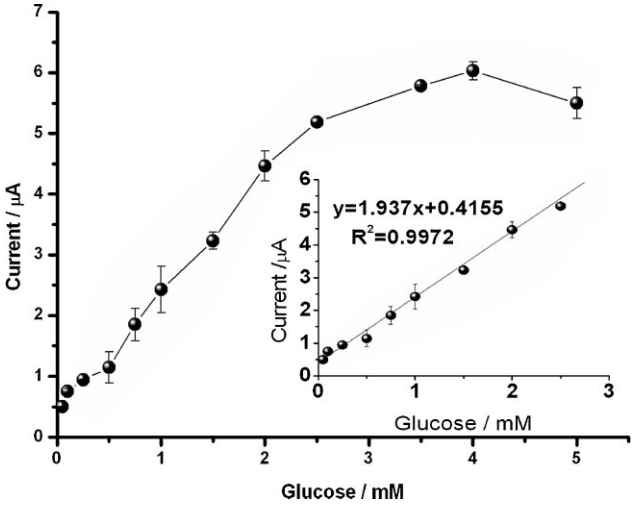

Figure 10. Calibration curve for glucose (in 50×10^{-3} M sodium acetate buffer, pH 5.5; 25 °C; –0.7 V). Error bars show SD.

Table 2. Some characteristics of the proposed biosensor (conditions used: in sodium acetate buffer (50×10^{-3} M, pH 5.5) at 25 °C, applied potential; –0.7 V).

Parameter	
K_m	4.6×10^{-3} M
I_{max}	2.49 μA
Linear range	$0.05–2.5 \times 10^{-3}$ M
Sensitivity	$0.011\ \mu A \times 10^{-3}\ M^{-1}$
LOD[a)]	0.029×10^{-3} M
Response time	52 s
Operational stability	No decrease for 8 h
Shelf life	No decrease during 15 d

[a)]LOD was calculated according to $S/N = 3$.

this value was given as 14.4 and 16.9×10^{-3} M in phosphate and acetate buffers, respectively.[8] Moreover, in one of the previous works, immobilization of GOx was carried out by entrapment within three different polypyrrole/poly(-methyl methacrylate (PMMA)-*co*-thienyl methacrylate (PMTM)) matrices and K_m values were calculated as 40, 74, and 92×10^{-3} M, respectively.[9] For both free and immobilized enzymes compare to previous works where GOx were entrapped on the electrode surface via different CPs, lower K_m value was observed. This may as well be due to the CLEC-like structure on the PTBT matrix that exhibits higher affinity toward the glucose substrate as the other CLEC or CLEA structures.[35]

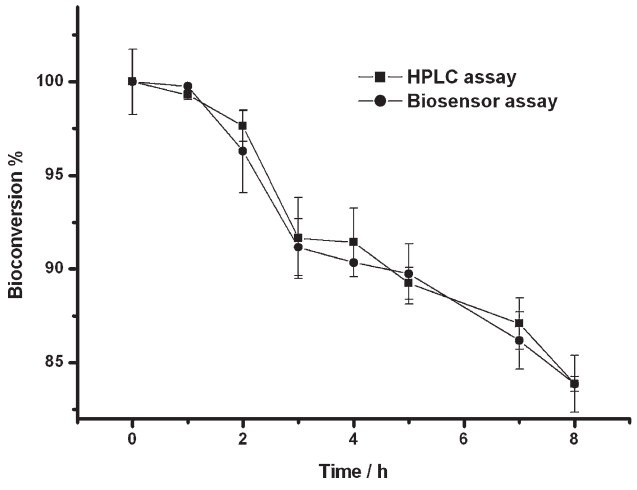

Figure 11. Biomonitoring of time dependent glucose consumption (or bioconversion) in yeast culture in fermentation medium (in sodium acetate buffer, 50×10^{-3} M, pH 5.5; 25 °C; –0.7 V). Error bars show SD of 3–4 measurements.

Sample Application

Finally, the biosensor was used for monitoring the concentration of the glucose in fermentation medium. For this purpose, samples taken from the *S. cerevisiae* cultivation were analyzed. *S. cerevisiae* was cultivated in Schatzman medium according to literature.[36] During the process, 100-fold dilutions of the samples were required for adjusting the glucose concentration in the sample to the linear range of the GOx biosensor. On the other hand, HPLC was performed for glucose assay as a reference method. Both data from biosensor and HPLC responses are shown in Figure 11. As seen results from biosensor applications are in a great conformity with the reference method.

Conclusion

An amperometric biosensor based on a CP has been developed successfully by the formation of cross-linked enzyme molecules. This provides robust, highly active immobilized enzymes with substantial operational and shelf life stability. Remarkable results were obtained by taking in consideration of the effect of hydrophobic interactions between deposited polymer and the enzyme. The results according to this research clearly indicated that even though native GOx has very limited hydrophobic sites; these sites are still available for an interaction with alkyl residues on a hydrophobic carrier. By optimizing ionic strength of enzyme solution for immobilization and using a cross linker, enzyme crystal formation can be enhanced and more stable and active enzyme electrodes were achieved. By compatible results obtained from the biosensor and HPLC, immobilization of GOx enzyme on CP electrodes was proved to be an alternative way for detection of the glucose amount in fermentation medium for yeast cultivation.

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