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A conducting polymer with benzothiadiazole unit: Cell based biosensing applications and adhesion properties

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ABSTRACT

Poly(4,7-di(2,3)-dihydrothienol[3,4-b][1,4]dioxin-5-yl-benzo[1,2,5]thiadiazole) (PBDT) was electrochemically deposited on graphite electrodes and used as a matrix for microbial biosensing studies. Moreover, protein adsorption property of the surface was investigated using bovine serum albumin (BSA). For the biosensor preparation, after electrochemical deposition of the polymeric matrix, *Gluconobacter oxydans* cells were immobilized on the modified electrode. Glucose was used as the substrate and biosensor response was followed successfully at −0.7 V vs Ag/AgCl due to the respiratory activity of the cells which is directly proportional with the substrate concentration. Characterizations were carried out in terms of several parameters such as operational and storage stabilities and surface morphologies. Finally, the effect of antimicrobial agent on the cell based response was tested. As a matrix, conducting polymers enable the preparation of sensitive and stable electrochemical microbial biosensors.

1. Introduction

Conducting polymers serve as an appropriate matrix supplying prevalent properties for biomolecule immobilization [1]. Their compatibility with biological molecules, easy preparation, high reproducibility and electrochemical properties make them fascinating in biosensor design [2–4]. Conducting polymers were widely used in many applications such as electrochromic devices [5–7], light emitting diodes [8], solar cells [9,10], enzyme immobilization matrices [11–13], drug release systems [14] and rechargeable batteries [15]. They exhibit a well-organized molecular structure so that they have the ability to function as a three dimensional matrix for immobilization of biomolecules [16–18]. While transferring the electrical charge, they serve as a suitable microenvironment for the immobilization [19]. The resulting polymers were used as matrices to immobilize microorganisms [20–22].

A microbial biosensor is constructed via immobilizing desired cells which are in contact with a transducer to monitor a specific change in its microenvironment [23–25]. In many biosensor applications, microbial cells were used [26–28]. To choose the appropriate matrix for immobilization is a crucial step for the biosensor. With the help of their redox behavior, conducting polymers have been developed as a suitable immobilization matrix while serving as a transducer [1,29]. Microbial biosensors present some advantages. The desired enzymes are not necessarily to be isolated hence the enzymes can stay in their natural surroundings in the cell. Moreover, the necessary co-enzymes and activators are already present in the system [23]. Bound-enzymes in the cell have tolerance to environmental changes like pH or temperature.

Gluconobacter oxydans is a gram-negative bacterium having a respiratory type of metabolism using oxygen as the terminal electron acceptor [30]. Oxidative enzymes of *G. oxydans* locate in the membrane of the cells; therefore, *G. oxydans* can be used as a biocatalyst for saccharide and alcohol [31]. Since the reactive cites of the related enzymes are located in the periplasmic space, there is no need to transport the substrates into the cells [32]. Table 1 contains a number of results of the studies on *G. oxydans* biosensors as a comparison.

Protein adsorption on the surface of materials is a considerable factor that regulates subsequent biological responses. Surface properties and the protein adsorption capacity of the surfaces are important and may influence the cell attachment [40]. Since the adsorbed protein layer drives the cell attachment, it is important to investigate the protein adsorption ability of the surfaces [41].

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Table 1
Comparison of various *G. oxydans* biosensors reported in the literature.

Electrode	Target	Detection mode	Limit of detection	Range of detection	Operational stability	Reference
Clark type	Xylose	Batch	NR	0.5–20 mM	40% decrease after 35 days of dry storage at 4 °C	[33]
Clark type	Glucose	Batch	NR	1.0–10.0 mM	NR	[34]
Clark type	Ethanol	Batch	0.05 mM	0.05–10 mM	50% decrease after 8 days	[35]
Gold disk	Ethanol	Batch	NR	0.25–2.5 mM	50% decrease after 51 h	[36]
Gold disk	Gylserol	Batch	NR	2.5–45.0 mM	50% decrease after 68 h	[36]
Glassy carbon	Ethanol	Batch	0.85 μM	2–270 μM	75% decrease within 4.5 h	[37]
Graphite	Glucose	Batch	NR	0.25–4.0 mM	6% decrease after 5 h	[38]
Graphite	Glucose	Batch	NR	0.1–2.5 mM	11% decrease after 5 h	[22]
Graphite	Ethanol	Batch	NR	0.1–5.0 mM	11% decrease after 5 h	[22]
Graphite	Glucose	Batch	NR	0.05–1.0 mM	24% decrease after 5 h	[39]
Graphite	Glucose	Batch	0.13 mM	0.5–2.0 mM	16.7% decrease after 4 h	This work

In this work, the use of a previously described conducting polymer of 4,7-di(2,3)-dihydrothienol[3,4-b][1,4]dioxin-5-yl-benzo[1,2,5]thiadiazole (BDT) [42] as a *Gluconobacter oxydans* based biosensing platform was reported. Since the matrix property of PBDT for enzymatic biosensor application was achieved successfully [11], its microbial biosensor efficiency as well as protein adsorption properties were tested. Microbial cells were entrapped behind a dialysis membrane onto the surface of the conducting polymer modified graphite electrode. Dialysis membrane was used to prevent the leakage of the cells during the measurements. Scheme 1 describes the general constructions of the biosensors. Other than the optimization and characterization studies, analysis of antimicrobial agent with designed system was carried out.

2. Material and methods

2.1. Materials

D(+)-glucose, D(+)-galactose, D(+)-xylose, D(+)-mannose and dialysis tubing cellulose membrane (25 mm × 16 mm) were purchased from Sigma (St. Louis, USA; www.sigmaaldrich.com). Dicloromethane (DCM), acetonitrile (ACN) and ethanol (>99.2%) were purchased from Merck (Darmstadt, Germany; www.merck.com). Tetrabutylammonium hexafluorophosphate ($TBAPF_6$) were purchased from Aldrich. All chemicals needed for the synthesis of monomer were purchased from Aldrich except tetrahydrofuran (THF) which was purchased from Acros (Geel, Belgium, www.acros.com). Zefiran® Forte Solution (100cc) was purchased from Sandoz (Kocaeli, Turkey). All other chemicals were analytical grade.

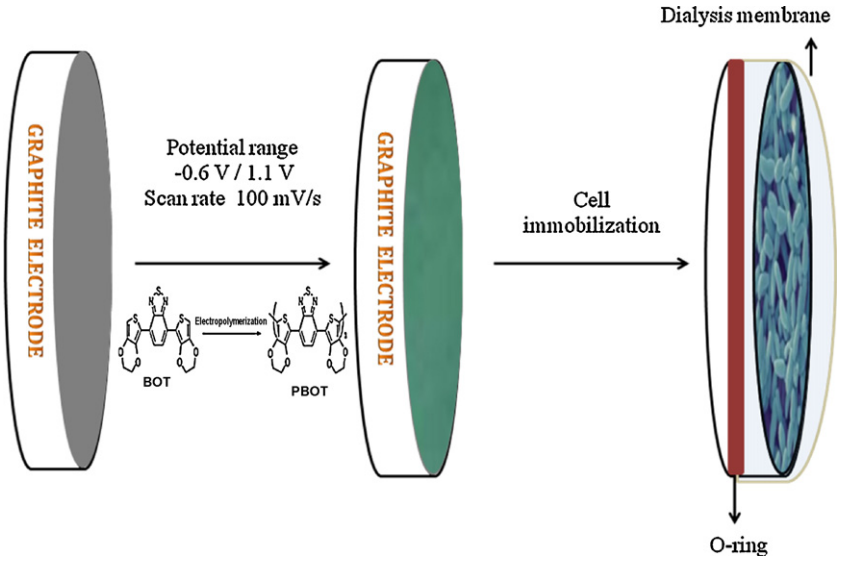
2.2. Biological material

The strain of *G. oxydans* DSM 2343 was provided from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, www.dsmz.de). *G. oxydans* was maintained on the agar containing (g/L): D-glucose, 100; yeast extract, 10; calcium carbonate, 20; agar, 20 [37].

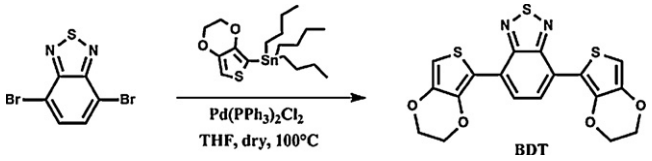
G. oxydans cells were inoculated in the growth medium (50 mL) containing (g/L): glucose, 5; yeast extract, 5 and incubated at 28 °C for 17 h with continuous shaking at 175 rpm. After the cell growth under this condition, 10 mL of growth medium was centrifugated for 10 min at 3500 × *g* and the cellular paste was collected. Then, it was resuspended in 5.0 mL sterile 0.9% NaCl solution and centrifuged again. The cellular paste was used for the biosensor preparation. The cell growth was monitored spectrophotometrically via measuring optical density at 600 nm [38]. The *G. oxydans* cells in late exponential phase were used during the trials. In all experimental steps, daily-prepared electrodes with fresh cells were used.

2.3. Apparatus

In the amperometric and cyclic voltammetry measurements Palm Instrument (PalmSens, Houten, The Netherlands, www.palmsens.com) was used. In all electrochemical



Scheme 1. General construction of designed microbial biosensor.



Scheme 2. Synthesis of the monomer 4,7-di(2,3)-dihydrothienol[3,4-b][1,4]dioxin-5-yl-benzo[1,2,5]thiadiazole (BDT).

measurements, a three electrode cell was used. Graphite electrode (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) as the working electrode, platinum electrode (Metrohm, Switzerland, www.metrohm.com) as the counter electrode, Ag/AgCl (3.0 M KCl) as the reference electrode were used. For surface imaging of the microbial electrode, scanning electron microscope (SEM) (FEI Quanta250 FEG) was used. Contact angle measurements were conducted with CAM 100 KSV (KSV, Finland). With the help of a drop of water (2.0 μL), contact angle measurements were done using the sessile drop method. Recording the drop profile with a CCD camera leads to monitor the changes in contact angle. All reported data were given as the average of five measurements ±standard deviation (SD). The experiments were conducted at ambient temperature (25 °C).

3. Experimental

3.1. Synthesis of the monomer

To synthesize the desired molecule, previously described procedure was carried out [42]. Benzo[c][1,2,5]thiadiazole was brominated using the mixed solution of HBr/Br_2. Then using Stille coupling reaction, the donor acceptor type monomer was synthesized (Scheme 2).

3.2. Protein adhesion studies

Protein and cell adhesion were tested on the surface of the polymer PBDT using bovine serum albumin (BSA). Polymer was generated on the ITO (Indium tin oxide) coated PET (Polyethylene terephthalate) electrochemically with described procedure. After electropolymerization, polymer coated electrodes were washed thoroughly with distilled water. For the protein adsorption experiments, firstly, polymer covered surface was washed with phosphate-buffered saline solution (PBS, 0.1 M, pH 7.4). Then, it was incubated at room temperature in 0.1 M PBS containing 1.0 mg/mL BSA for 120 min. After that, it was washed with PBS and distilled water, respectively. The amount of adsorbed protein on the polymer surface was estimated by following the initial and final concentrations of protein within the adsorption medium and washing solutions using Coomassie Brilliant Blue with crystalline BSA as the standard [43]. Experiments were performed for three times; mean and standard deviation values were determined.

3.3. Preparation of PBDT/G. oxydans biosensors

For the construction of the bioactive layer on the graphite electrode, spectrographic graphite rods were polished on emery paper and washed completely with distilled water.

Electrochemical polymerization of BDT (1.15×10^{-3} M) on the graphite electrode was carried out in 0.1 M tetrabutylammonium hexafluorophosphate ($TBAPF_6$)/dichloromethane medium via cyclic voltammetry. The polymerization was performed between the potentials −0.6 V and +1.1 V with the scan rate of 100 mV/s [42], (Fig. S1). After polymerization, polymer film was washed with acetonitrile and distilled water to remove un-reacted monomer.

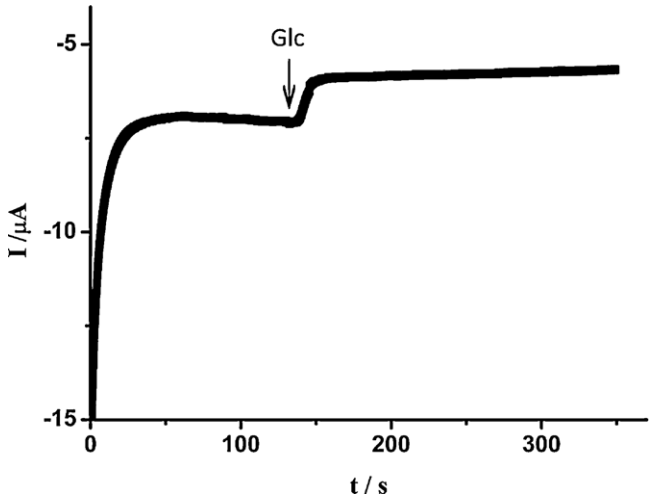


Fig. 1. The general shape of the current response.

For the purpose of immobilization of the bacterial cells, 5.0 μL of *G. oxydans* suspension (7.5×10^9 cell titer) were spread over polymer modified electrode and then, left to dry for 1.5 h at ambient conditions. Then, the layer was covered with a dialysis membrane which was pre-wetted. The membrane was fixed firmly with a silicone rubber O-ring.

3.4. Measurement procedure

All trials were actualized at ambient conditions in a reaction cell containing 10 mL sodium phosphate buffer (50 mM, pH 6.5) under regularly and mildly stirring. After each measurement, working buffer was refreshed and electrodes were washed with distilled water and kept in the buffer under stirring for 3 min.

Monitoring oxygen consumption at −0.7 V due to metabolic activity of microorganism in the presence of substrate (glucose) was the basics of the measurements. After reaching a steady-state current, proper amount of glucose solution was added to the medium; the current changed and reached a new stable value. The difference between first and second steady-state currents was recorded as the microbial sensor response to the substrate [39]. Fig. 1 shows the current responses.

4. Results and discussion

4.1. Optimization studies

There are many parameters that affect the activity of the microbial biosensor. The construction of the microbial biosensor is simple. To keep the cells alive and maintain the immobilization successfully, cells have to be immobilized and fixed strongly to prevent the leakage or death. By the help of conducting polymers, these obstacles can be overcome and immobilization of the biomolecules and microorganisms becomes easier. Combination of microorganisms and conducting polymers results in efficient biosensor designs. Table S1 contains a number of results of the studies on conducting polymer and microbial biosensors as a comparison.

In this part, scan number in electropolymerization, cell loading for immobilization and pH were optimized to achieve the best design.

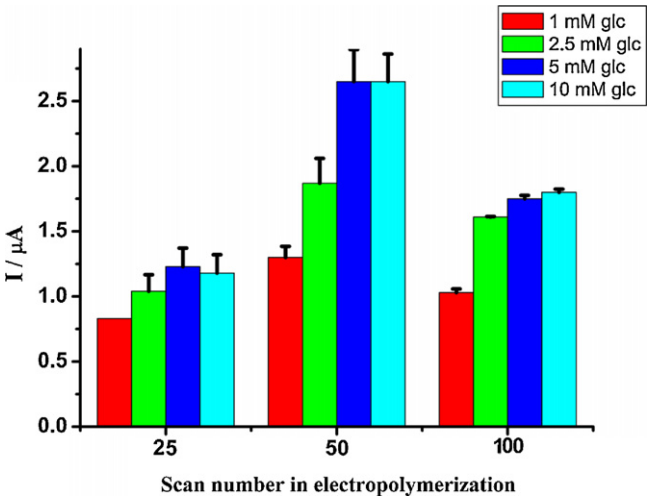


Fig. 2. Effect of scan number during electropolymerization on the biosensor responses (pH 6.5, 50 mM phosphate buffer, ambient conditions, 25 °C, −0.7 V, different glucose concentrations. Error bars show standard deviation (SD) of five measurements).

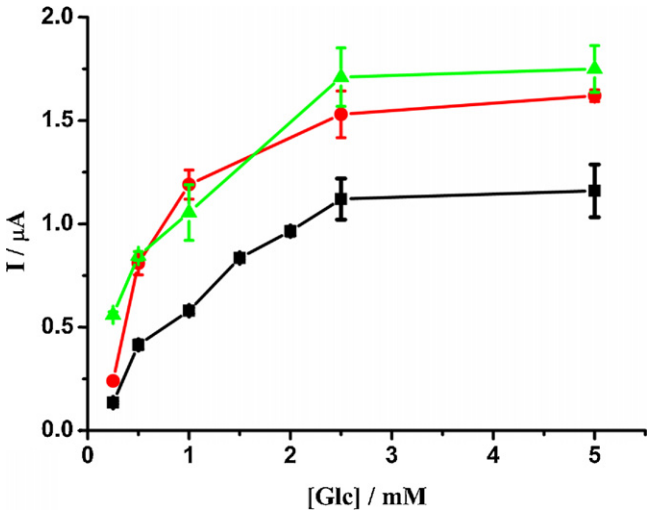


Fig. 3. Effect of cell amount on the microbial biosensor response (pH 6.5; 50 mM phosphate buffer, ambient conditions, 25 °C, −0.7 V. Error bars show SD of five measurements; (■ 3.75×10^9 bacteria; ● 7.5×10^9 bacteria; ▲ 15×10^9 bacteria).

4.2. Effect of scan number in electropolymerization

The conducting polymer thickness can be controlled by adjusting the scan number during electropolymerization. Thus, the stability and microstructure of the polymer film are affected. When the thickness of the conducting polymer film increases, the possibility for defect formation and degradation in polymer increase [44]. Hence the quality of the immobilization matrix decreases. In order to investigate the effect of polymer layer thickness, BDT was polymerized on the graphite electrode in different scan numbers (25, 50 and 100 scans), (Fig. 2). After polymerization, modified graphite electrodes were used for the construction of microbial sensors as given in immobilization procedure. The best response was achieved by 50-scan electropolymerization. The charge and film thickness in the polymer film were calculated as 325 mC and 7.2 μm, respectively.

4.3. Effect of pH

Microbial activity is affected by the pH of the media; hence optimum pH is needed to be determined for efficient measurements. For this purpose, the effect of pH on the microbial electrode response was investigated in 50 mM phosphate buffer (pH 6.0–7.5) in the presence of glucose (5.0 mM). Relative current signals towards pH were offered in Fig. S2. As shown in the figure, pH 6.5 was chosen as the optimum pH for the further measurements. Similar data were also observed in previous work [22]. All the other trials were done with this pH value.

4.4. Effect of biomass (cell) loading

In order to detect the effect of cell amount used in biosensor construction, three different microbial biosensors containing 2.5 μL (3.75×10^9 cell titer), 5.0 μL (7.5×10^9 cell titer), and 10 μL (15×10^9 cell titer) of bacterial cells were prepared. As a result of this study, the responses of the 5.0 and 10 μL cell loaded biosensors were close. Moreover, the 10 μL cell loaded one was not stable as much as the others. 5.0 μL bacterial cell (equivalent to 7.5×10^9 cell titer) was found to be the optimum cell amount. Further measurements were conducted using 5.0 μL bacteria cell (Fig. 3).

4.5. Characterization

To monitor the surface characteristics, SEM and contact angle measurements were performed. Surface morphology of the conducting polymer coated graphite electrode before and after whole microbial cell immobilization were shown in Fig. 4A and B, respectively. In Fig. 4A, the image was taken after 50 cycles of electropolymerization on graphite. SEM micrograph of polymer surface exhibits the characteristic porous and cauliflower structure of the conducting polymer. In Fig. 4B, the presence of microbial cells confirms the successful immobilization of *G. oxydans* onto the conducting polymer matrix.

Protein adsorption experiments were carried out on the polymer PBDT with BSA which is a globular protein found in high concentrations in blood. When 1.0 mg BSA was applied to the polymer surface in 2.0 cm^2 well, the amount of adsorbed protein was determined as 0.12 ± 0.05 mg. To measure the change on the surface hydrophobicity before and after the cell immobilization, contact angle measurements were conducted. For PBDT, before immobilization contact angle was measured as 67.14° (±0.79) and it was found to be 97.47° (±0.79) after immobilization.

4.6. Analytical characteristics of the microbial biosensor

The best working conditions of the microbial sensors were obtained via optimization studies. After these studies, the analytical characteristics of the developed microbial sensor were examined under these conditions. For the proposed system, a linear calibration graph was plotted for current density versus glucose as substrate (Fig. 5). For the microbial sensor, linear range for substrate concentration was obtained between 0.5 and 2.0 mM glucose with an equation, $y = 0.625x + 0.521$ ($R^2 = 0.994$) Limit of detection (LOD) and sensitivity for the biosensor were calculated as 0.13 mM and 0.953 mM, respectively. The response time of the microbial biosensor was 20 s. Despite the use of membrane, there was not any diffusion problem for the proposed system.

4.7. Repeatability and operational stability

For repeatability of the microbial biosensor, six measurements were completed with 1.0 mM glucose solution. With respect to the results, the standard deviation (SD) and the variation coefficient (CV) were calculated as 1.1 ± 0.079 mM and 2.85%, respectively.

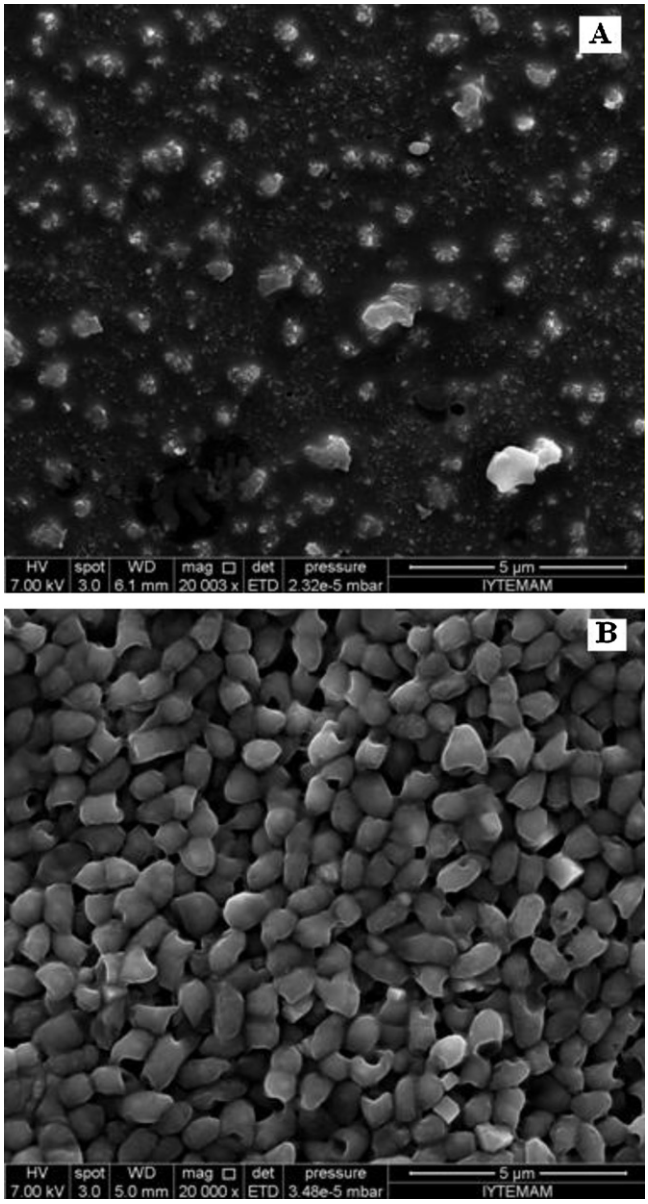


Fig. 4. SEM images of conducting polymer surfaces; before (A) and after immobilization of cells (B).

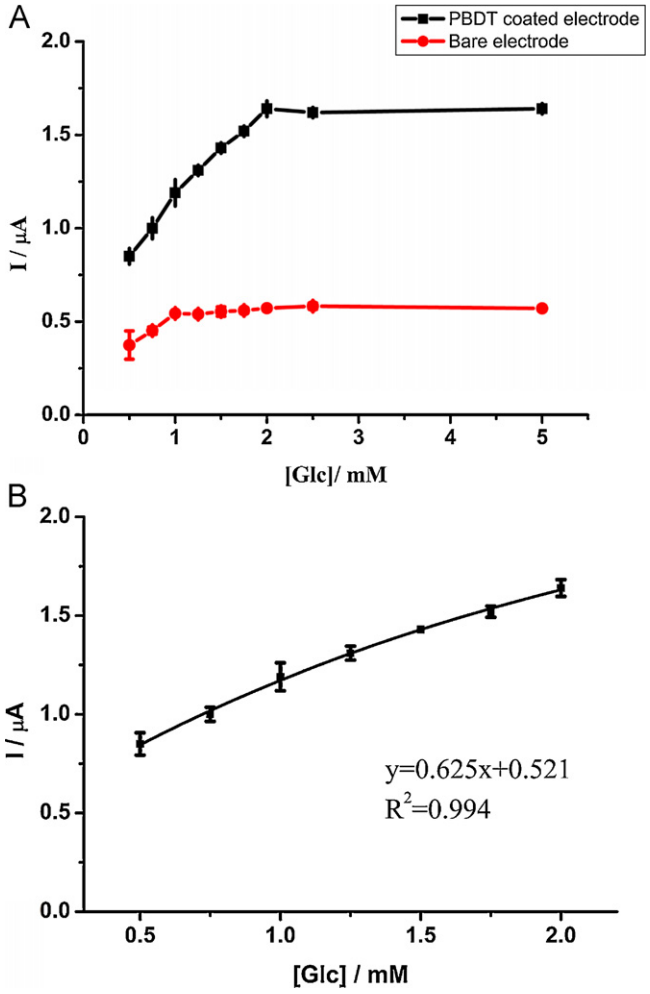


Fig. 5. Effect of glucose concentration on the biosensor response of bare and PBDT/*G. oxydans* modified graphite electrode (A) and linear range for glucose for/*G. oxydans* modified graphite electrode (B) (pH 6.5; 50 mM phosphate buffer, ambient conditions, 25 °C, −0.7 V. Error bars show SD of five measurements).

The operational stability of microbial sensors was determined at optimum conditions (in sodium phosphate buffer, pH 6.5 at 25 °C) using 1.0 mM glucose. 16.7% decrease was observed after 4 h (Fig. S3). 21 repetitive measurements were done in this period.

4.8. Substrate specificity

The substrate specificity of the microbial biosensor was tested with different compounds such as galactose, xylose, mannose and ethanol (Table S2). Microbial sensor gives higher responses to ethanol and lower responses to xylose and galactose. Similar data were also observed in a previous work [33]. No signal was observed for mannose.

5. Antimicrobial agent analysis

The biosensor system was used for antimicrobial agent (Zefiran®) detection. Initially, 1.0 mM glucose was added to the medium in optimum conditions (pH 6.5; 50 mM phosphate buffer, ambient conditions, 25 °C) and current response was registered. In the next step, working buffer was refreshed, proper amount of antimicrobial agent was added to the medium and waited for 15 min for interaction with cells and antimicrobial agent. After that, 1.0 mM glucose was added to the medium and new current response was registered. Four different Zefiran® concentrations were tested (0.05%, 0.1%, 0.25%, and 0.5%) and inhibition response of cells were observed (50%, 56%, 63.6%, 70.6%, respectively), (Table S3). The decrease in biosensor response corresponds to the increase in antimicrobial agent was observed in a linear manner.

6. Conclusion

In this study, we have utilized PBDT conducting polymer and *G. oxydans* bacterial cells to construct a microbial biosensor. The biosensor preparation is simple and not time consuming. The proposed system can be used in antimicrobial agent detection and for different substrates such as glucose, ethanol and galactose analyses. Additionally, noteworthy properties of PBDT like easy preparation, compatibility with biological molecules (enzyme, microorganism and protein) and reproducibility, allow using it in bio-microelectronic devices, immobilization matrix and biofuel cells [45–49].

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2012.04.005.

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