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Use of a thiophene-based conducting polymer in microbial biosensing

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Abstract

Immobilization of whole viable *Pseudomonas fluorescens* cells was achieved on a graphite electrode modified with a thiophene-based conducting polymer. Microbial electrodes were constructed by the entrapment of bacterial cells on conducting copolymer matrix using a dialysis membrane. The biosensor was characterized using glucose as the substrate. As well as analytical characterization, effects of electropolymerization time, pH and temperature on the sensor response were examined. Finally, operational stability was also tested.

Keywords: Conducting polymer; Electrochemical polymerization; Microbial electrode; *Pseudomonas fluorescens*; Electrochemical biosensor

1. Introduction

Conducting polymers (CP) have extended π-conjugation along the polymer backbone. This structure provides them with unusual electrochemical properties, like low energy optical transitions, high electrical conductivity, low ionization potential, high electronic affinities [1,2]. CPs have widespread applications such as electrochemical batteries, gas separation membranes, EMI shielding, electrochromic display devices, metal protection against corrosion, solar cells, ion exchange membrane in fuel cells and drug release systems [3–8]. CPs have numerous features, which allow them to act as an excellent material for immobilization of biomolecules and rapid electron transfer for the fabrication of efficient biosensors or as sensitive components (e.g. gas sensors) [9,10]. The entrapment of enzymes in conducting copolymer matrices during electrochemical polymerization is an alternative procedure for biosensor construction [11].

Polyheterocycles, such as polypyrrole (PPy), polythiophene (PT), polyaniline (PANI), and poly (3,4-ethylenedioxythiophene) (PEDOT), were characterized in the 1980s [12]. Among these materials, polythiophenes are one of the more extensively studied classes of π-conjugated systems. Both the conducting and semiconducting forms are very stable and readily characterized. Applications of these materials in light emitting devices, field effect transistors, as well as other molecular electronic devices have been stimulated by the improved solubility and process ability of mono-, di-, and ring-substituted polythiophenes [13–18]. The possibility of modifying the structure to alter some physical properties is the main advantage of polythiophenes [19–22]. Furthermore, poly (3,4-ethylendioxythiophene) (PEDOT) exhibits relatively high electrical conductivity and also is very stable even during the electrochemical charging and discharging [23].

Biosensor technology based on enzyme or microorganisms is well suited for rapid, cost effective, sensitive, selective and on-line/field monitoring [24]. Microbes have a number of advantages as biological sensing materials in the fabrication of biosensors [25]. Although purified enzymes have very high specificity for their substrates or inhibitors, their application in biosensors construction may be limited by the tedious, time-consuming and costly enzyme purification, requirement of multiple enzymes to generate the measurable product or need of cofactor/coenzyme. Microorganisms provide an ideal alternative to these bottlenecks [26]. Enzymes are usually more stable in their natural environment in the cell, coenzymes and activators are already present in the system [25,27]. On the other side, the major restriction of microbial biosensors compared to enzyme sensors is the slow response, which has been attributed to diffusional problems associated with the

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cell membranes. Cell-based biosensors are frequently used for determination of BOD, toxic agents and assimilable sugars [28].

In this study, bacterial cells were entrapped on a thiophene-based conducting polymer behind a dialysis membrane onto the surface of graphite electrode to form microbial biosensor. *Pseudomonas fluorescens* was used as a biological component and the measurement was based on the respiratory activity of the cells. The optimization and characterization of the sensor were performed.

2. Experimental

2.1. Apparatus

Chronoamperometric measurements were carried out with a Radiometer electrochemical measurement unit (Lyon, France, www.radiometer.com). Ag|AgCl (3 M KCl saturated with AgCl as an internal solution, Radiometer Analytical, REF321) and a Pt electrode (Radiometer Analytical, M241PT) were used as the reference and counter electrodes, respectively.

2.2. Reagents

Glucose, EDOT (2,3-dihydrothieno [3,4-*b*]-1,4-dioxin), SDS (sodium dodecylsulfate) were purchased from Sigma Chem. Co. (St. Louis, MO, USA, www.sigmaaldrich.com). All other chemicals were of analytical grade. Dialysis membranes with a cut-off of 6000–8000 Da were used. Mineral salt medium (MSM) with the following composition was used as the growth medium for *P. fluorescens*; 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4{\cdot}7H_2O$, 0.005% $CaCl_2{\cdot}2H_2O$. The trace element solution (10 mL/L) was prepared from reagent grade chemicals. The pH of the growth media was adjusted to 6.9 [29].

2.3. Biological material

P. fluorescens (*Pseudomonas putida* DSM6521) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, www.dsmz.de) and sub-cultured on nutrient agar. Cells were inoculated into 50 mL of MSM containing 250 mg/L glucose and incubated at 28 °C on an orbital shaker at 175 rpm. After 16 h, the biomass was harvested by centrifugation at 10,000 rpm and suspended in MSM and then re-centrifuged. The supernatant was removed and the cellular paste was used for the construction of biosensor. Bacterial cells in logarithmic phase were used during the experiments and the cell growth was followed spectrophotometrically via measuring optical density at 560 nm [30,31]. Daily-prepared electrodes with fresh cells, which have 1.34×10^9 cell titer were used in all experimental steps. Since respiratory activity of intact cells was followed during the experiments, microbial electrodes were immersed into MSM containing glucose when not in use [29,31].

2.4. Electrode preparation

Prior to the electropolymerization, spectrographic graphite rods, (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were polished on wet emery paper (Tufback Durite, P1200) and washed thoroughly with distilled water, sonicated for 2 min, rinsed with bi-distilled water and dried at 105 °C.

Electrochemical polymerization of EDOT (0.1 M) on the graphite electrode via constant potential electrolysis was performed at +1.0 V in the presence of SDS (1 mg/mL) as the supporting electrolyte. The polymer film was washed with bi-distilled water to remove the supporting electrolyte and unreacted monomer [32].

For immobilization of bacterial cells, 25 μL of bacterial cell were spread over the modified electrode and allowed to dry at ambient conditions for 30 min. Then, the layer was washed with working buffer solution and MSM, respectively, to remove unbound cells from the surface and covered with a dialysis membrane, pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring.

2.5. Measurements

All measurements were carried out at 30 °C under constant stirring. After each run, the electrode was washed with distilled water and kept in 50 mM phosphate buffer (pH 7.5) solution at 30 °C for 10 min. Working buffer solution has been renewed after each measurement. The microbial sensor was initially equilibrated in MSM solution and phosphate buffer. After 30 min, substrate was added to the reaction cell. The biosensor responses were registered as current densities ($\mu A/cm^2$) by following the oxygen consumption at −0.7 V due to the metabolic activity of bacterial cells in the presence of glucose [33].

Control experiments were done using entrapped cells behind a dialysis membrane on the electrode, however, without polymer no significant response signal was observed at the working potential in the presence of substrate.

3. Results and discussion

Enzymes and cells have been used in biosensor construction for many decades. Both concepts have some advantages and challenges [34]. There have been various strategies to modify the microbial cells for application to microbial biosensors. The principle of the bacterial biosensor is rather simple, and sensor productions only require growth of the microorganisms. There are multiple strategies how to use catalytic activities present in microbial cells ranging from using viable cells, non-viable cells, permeable cells, or membrane fractions. These cell-derived biocatalysts serve as an economical substitute for enzymes; the additional benefit for the biosensor performance is that the enzymes are still linked to the respiratory chain [35].

Conducting polymers can act as transducers in biosensors and coating electrodes with CPs under mild conditions opens up various possibilities for the immobilization of biomolecules and biosensing materials, the enhancement of their electrocatalytic

properties, rapid electron transfer and direct communication. Co-immobilization of redox mediators or cofactors by entrapment within electropolymerized films or by covalent binding on the surface allows simple fabrication of reagentless biosensors [36].

Different electrochemical detection methods have been used to integrate microbes with various immobilization matrices. *Bacillus subtilis* were immobilized in a sol–gel composite material of silica and poly(vinyl alcohol)-grafted-poly(vinylpyridine) copolymer to form optical BOD sensor [37]. Previously, intact *P. fluorescens* cells were entrapped together with osmium redox polymers onto the surface of cysteamine modified gold electrode and the sensor response were investigated for catechol, phenol and glucose in both batch and flow mode [29]. In this study, bacterial cells were fixed on the outer layer of conducting polymer and then covered with a dialysis membrane to avoid leakage of biological material during the measurements. Response characteristics of the resulted system were examined for glucose. In addition, developed system could be adapted for many purposes such as estimation of biochemical oxygen demand (BOD), which is the most important and widely used environmental index especially for monitoring organic pollutants in wastewater. It can be also used as a toxicity sensor and a bioanalyzer for certain substances. In case of BOD detection, usage of microorganisms with broad-range substrate specificity like *P. fluorescens* could be more advantageous due to the need for the rapid oxidation of diverse range of compounds [38].

3.1. Optimization of the microbial biosensor

3.1.1. Effect of electropolymerization time

The amount of conductive polymer on the electrode surface can be controlled by adjusting the polymerization time, which has a direct effect on the resulting current values. It has been previously reported that the microstructure of a conducting polythiophene film changes with the increase in the thickness. As the thickness depends on the deposition time, more and more defects such as voids and large molecule agglomerates could appear, causing the degradation and incompact microstructure of the films [39]. In order to observe the effect of electropolymerization time, EDOT was polymerized onto the graphite surface for different periods (5, 10 and 20 min) (Fig. 1), and then modified electrodes were used to form microbial biosensors as given in experimental section. The best current values were obtained by 10 min of polymerization time. However, a decrease was observed when the polymerization time was higher. This could be due to the improper film structure related with the thickness after 10 min of electropolymerization time for the cell immobilization.

3.1.2. Optimum pH

According to the optimization studies, the effect of pH on the electrode response was investigated in 50 mM phosphate buffer (pH 6.0–8.0). The current response of the microbial electrode to glucose (1 mM) increased significantly from pH 6.0 to 7.5 and then a sharp decrease was observed at higher pH values (Fig. 2).

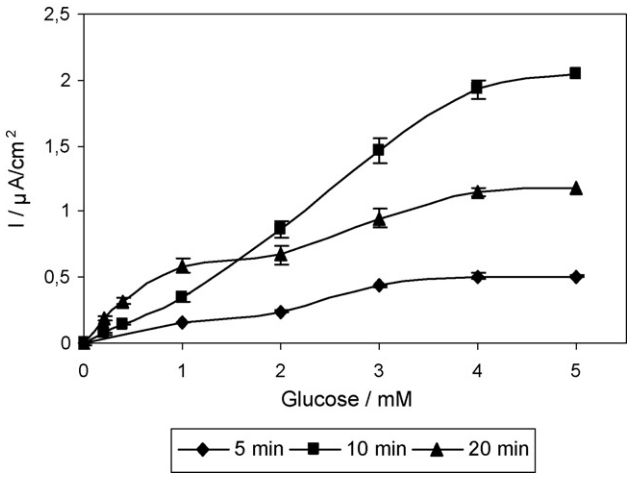

Fig. 1. Effect of electropolymerization time on the biosensor response (in phosphate buffer, 50 mM, pH 7.5, 30 °C, −0.7 V).

As a result, pH 7.5 was chosen as the optimum pH and further studies were conducted with this value.

3.1.3. Analytical characteristics

The analytical characteristics of the developed microbial sensor were examined under optimized conditions. A linear calibration graph between current density and substrate concentration was obtained between 0.25 and 4.0 mM glucose with an equation, $y = 0.472x$ ($R^2 = 0.992$) (where y is the sensor response in terms of current density (μA/cm^2) and x is the substrate concentration in mM). As it is stated before different types of microbial sensors based on *Pseudomonas* sp. were previously developed and characterized by using glucose as the substrate [29–31]. Some characteristics of these systems in terms of linearity for glucose detection, immobilization matrices, and transducers were summarized in Table 1.

The repeatability of the biosensor was tested for 1.0 mM glucose ($n = 5$) and the standard deviation (S.D.) and the variation coefficient (cv) were calculated as ±0.024 mM and 2.4%, respectively.

A calibration curve was also obtained for the microbial electrode containing adsorbed bacterial cells on the modified surface

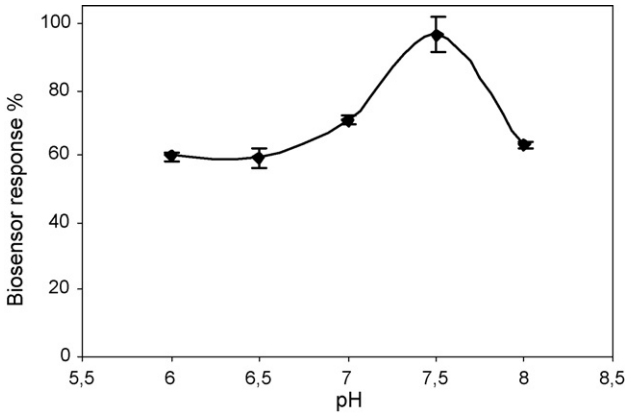

Fig. 2. Effect of pH on the biosensor response (pH 6.0–8.0 phosphate buffer (50 mM), 30 °C, −0.7 V).

Table 1
Some characteristics of microbial sensors based on *Pseudomonas* sp.

Microorganism	Transducer/immobilization matrix	Linear range for glucose (mM)	Reference
P. fluorescens	CNT-GECE gelatin	0.5–4.0	[31]
P. putida	CNT-CPE Os-redox polymer, (Type I)	0.05–2.0	[30]
P. putida	Gold Os-redox polymer, (Type I)	0.2–1.4	[29]
P. fluorescens	Gold Os-redox polymer, (Type II)	0.05–1.0	[29]
P. putida	Gold Os-redox polymer, (Type I)	0.1–2.2	[29]
P. fluorescens	Gold Os-redox polymer, (Type II)	0.2–2.0	[29]

CNT-GECE: carbon nanotube-graphite epoxy composite electrode; CNT-CPE: carbon nanotube-carbon paste electrode, Os-redox polymer, Type I: poly(1-vinylimidazole)12-[Os-(4,4′-dimethyl-2,2′-dipyridyl)$_2$Cl$_2$]$^{2+/+}$; Os-redox polymer, Type II: poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$.

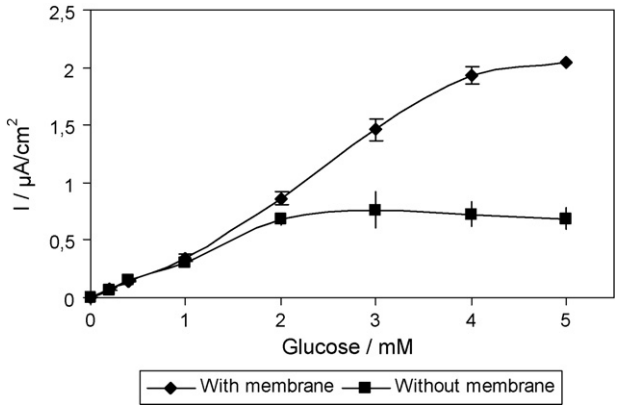

Fig. 3. Effect of the presence of dialysis membrane on the calibration graph (in phosphate buffer, 50 mM, pH 7.5, 30 °C, −0.7 V).

in the absence of dialysis membrane (Fig. 3). Lower and insignificant current responses were observed due to the cell leakage from the surface after 9–10 measurements. It is clear that dialysis membrane keeps the cells on the electrode and provides operational stability at the working conditions.

The substrate specificities of the biosensor to different compounds (galactose, xylose, mannose, sucrose, phenol and ethanol) were tested and results were given in Fig. 4. As seen from the figure, proposed system showed response towards galactose and xylose and lower signals were obtained for these sugars compared to the glucose. No response was observed for

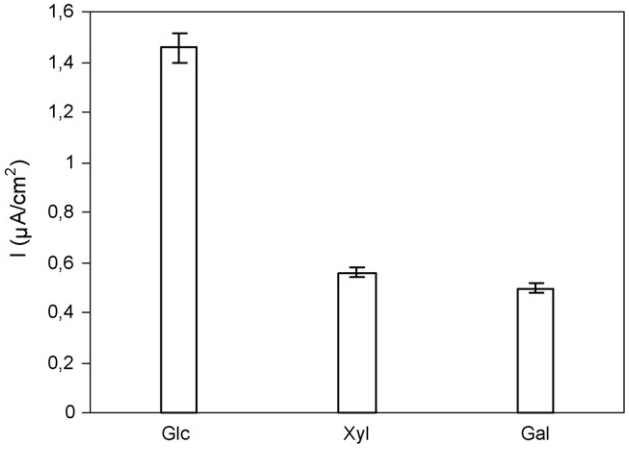

Fig. 4. Substrate specificity of the biosensor (in phosphate buffer (50 mM, pH 7.5), 30 °C, −0.7 V, substrate concentration; 3.0 mM)).

mannose, sucrose, phenol and ethanol in our working conditions. It is known that *P. fluorescens* cells were adapted to grow in the presence of phenol as the sole source of organic carbon. After adaptation process, it could be possible to enhance the substrate specificity and to prepare microbial sensors for the detection of phenolic compounds [29]. Additionally, *Pseudomonas* sp. based microbial sensors were reported to be used for different compounds such as benzene, caffeine as well as phenol [40–42].

To investigate the operational stability of the system, microbial biosensor was immersed in the reaction cell containing the buffer solution for 5.5 h at optimized working conditions where no decrease at the initial activities for the biosensor was observed. During this period, 22 measurements were done and it can be concluded that even more measurements can be carried out for longer times. Operational stability might be also be attributed by the proper microstructures of the polymer film that was optimized by adjusting the electropolymerization time.

4. Conclusion

CPs, which have an organized molecular structure, serves as proper and functional immobilizing platforms for biomolecules. These matrices provide a suitable environment for the immobilization and preserve the activity for long duration [2]. In the present paper, we have described a microbial biosensor in which combination of bacterial cell and a thiophene based conducting polymer was utilized. The proposed system does not require any complicated immobilization procedure for the construction of biosensor. The preparation is simple, cheap and not time consuming. The biosensor showed a good linear range, a good repeatability and a high operational stability. It can be concluded that the proposed system could also be a good alternative for BOD and toxicity estimation.

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