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Design of a microbial sensor using conducting polymer of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine

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ABSTRACT

A microbial biosensor based on *Gluconobacter oxydans* cells immobilized on the conducting polymer of 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine (SNS-NH_2) coated onto the surface of graphite electrode was constructed. The proposed biosensor was characterized using glucose as the substrate. The linear relation was observed in the range of 0.1–2.5 mM and defined by the equation $y = 0.415x + 0.377$ ($R^2 = 0.986$). Analytical characterization, effects of electropolymerization time, pH, cell amount and the presence of gold nanoparticles (GNP) on the polymer surface together with the biological material were examined as well. Finally, the system was used for ethanol and glucose detection in real samples.

1. Introduction

Since their discovery in the mid-1970s, research on conducting polymers (CP) became an ever growing research area in polymer chemistry [1]. The redox behavior and unusual combination properties of metals and plastics make the conducting polymers a new class of materials [2]. Interest in conducting polymers is largely due to the wide range of possible applications due to their facile synthesis, good environmental stability and long term stability of electrical conductivity. The advantage of using conducting polymers compared to more traditional sputtered metal coatings is that the polymer is soluble enabling the nondestructive analysis of the specimens [3]. CPs were extensively studied in the last decade and used for technological applications in electrochromic devices [4,5], gas separation membranes [6], enzyme immobilization [7] and have been featured in biotechnical applications since the very early days following their discovery. Biosensing approach using CPs have been also widely investigated in previous works [8].

Microbial cells are very promising for biosensor construction due to several advantages: the enzyme does not need to be isolated, enzymes are usually more stable in their natural environment in the cell, coenzymes and activators are already present in the system [9]. Cell-based biosensors are frequently used for determination of biological oxygen demand (BOD), toxic agents and assimilable sugars as well as the selective detection of a single analyte [10].

Gluconobacter oxydans is a gram-negative bacterium belonging to the family of Acetobacteraceae and an obligate aerobe, having a respiratory type of metabolism using oxygen as the terminal electron acceptor [11]. These cells contain several quinoprotein membrane-bound dehydrogenases specific for D-glucose, D-fructose, D-sorbitol and D-mannitol [12]. In addition, alcohol dehydrogenase acts on linear and branched monoalcohols up to C_4 to give the corresponding acids and ketones [13]. Various *Gluconobacter* based biosensors for xylose, glucose, total sugars in lignocellulose hydrolysate, 1,3-propanediol, ethanol, glycerol and BOD have previously been reported [14–19].

The synthesis of the monomer, 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine (SNS-NH_2) and its electrochemical polymerization and characterization were described in a previous work [20]. Here we report the use of SNS-NH_2 matrix as a bacterial biosensing platform. *G. oxydans* cells were entrapped on this novel conducting polymer behind a dialysis membrane onto the surface of graphite electrode. The measurement was based on the respiratory activity of the cells. As well as the optimization and characterization, application of the proposed system on real samples was carried out.

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2. Experimental

2.1. Reagents

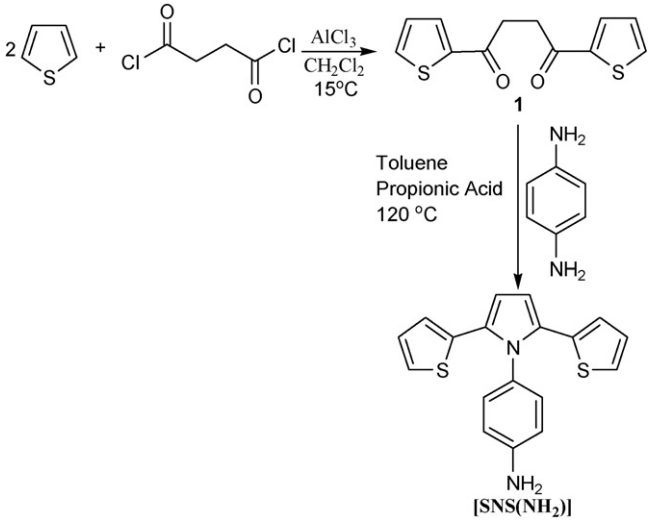
$LiClO_4$, $NaClO_4$, dialysis membrane (cut off 12 000), $AlCl_3$, succinyl chloride, benzene-1,4-diamine propionic acid, nitromethane, iron(III) chloride, propylene carbonate, poly(methylmethacrylate), dichloromethane, toluene, D-glucose, ethanol and gold colloidal (~0.75 A_{520} units/mL, 10 nm) were purchased from Sigma (St. Louis, USA, www.sigmaaldrich.com). Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany, www.merck.com). All other chemicals were of analytical grade and purchased either from Merck (Darmstadt, Germany) or from Sigma (St. Louis, USA).

2.2. Apparatus

Chronoamperometry measurements were carried out with a Radiometer electrochemical measurement unit (Lyon, France, www.radiometer.com). Graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, type RW001, 3.05 mm diameter and 13% porosity) were used as the working electrode. Ag/AgCl (3 M KCl saturated with AgCl as an internal solution, Radiometer Analytical, REF321) and a Pt electrode (Metrohm, Switzerland, www.metrohm.com) were used as the reference and counter electrodes, respectively. Palm Instruments (PalmSens, Houten, The Netherlands, www.palmsens.com) with three electrode configurations was used for cyclic voltammetry experiments. Scanning electron microscope (JEOL JSM-6400) was used for surface imaging of the microbial electrodes.

2.3. Synthesis of SNS-NH_2

The monomer SNS-NH_2 was synthesized from 1,4-di(2-thienyl)-1,4-butanedione and benzene-1,4-diamine in the presence of catalytic amount of propionic acid. 1,4-di(2-thienyl)-1,4-butanedione was synthesized with the double Friedel–Crafts reaction. $AlCl_3$ was used as the Lewis acid catalyst (Scheme 1). The reaction mixture was refluxed for 4 h (yield 78%). A round-bottomed flask equipped with an argon inlet and magnetic stirrer was charged with 1,4-di(2-thienyl)-1,4-butanedione, benzene-1,4-diamine, propionic acid and toluene. The resultant mixture was stirred and refluxed for 24 h under argon. Evaporation of the toluene, followed by flash column chromatography (SiO_2 column, elution with dichloromethane), afforded the desired compound as pale yellow powder [20].



Scheme 1. Synthesis of 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine (SNS-NH_2).

2.4. Cell cultivation of G. oxydans

The strain of *G. oxydans* was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, www.dsmz.de) and maintained on the agar containing ($g\,L^{-1}$): D-glucose, 100; yeast extract, 10; calcium carbonate, 20; agar, 20 [10]. The cell biomass was prepared by aerobic cultivation at 28 °C on a rotary shaker in 250 mL flasks filled with 50 mL of media. The growth medium contained 5 $g\,L^{-1}$ glucose and 5 $g\,L^{-1}$ yeast extract. The culture, inoculated from the slant agar, was incubated for 12 h to reach the late exponential phase. The cell growth was followed spectrophotometrically via measuring optical density at 600 nm [21]. The cells from one of the cultivation flask were collected by centrifugation for 10 min at 3500 × *g*, resuspended in sterile 0.9% NaCl solution and centrifuged again. Biomass was used for the biosensor preparation [16].

2.5. Preparation of microbial electrode

Prior to the electropolymerization, graphite electrodes were polished on wet emery paper (Tufback Durite, P1200) and washed thoroughly with distilled water, sonicated for 2–3 min, rinsed with bi-distilled water and dried at 105 °C [22]. Electrochemical polymerization of SNS-NH_2 (5 mg/mL) was carried out scanning the potential between −0.5 V and +1.2 V via cyclic voltammetry with the scan rate of 500 mV/s in $NaClO_4$ (0.1 M) and $LiClO_4$ (0.1 M)/acetonitrile medium. The concentration of the monomer was and for the polymerization of $SNSNH_2$.

For the immobilization of bacterial cells, *G. oxydans* suspension were spread over the polymer coated electrode and allowed to dry at ambient conditions for 1 h. After removal of unbound cells by washing with distilled water, the layer was covered with a dialysis membrane, pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring [22]. Daily prepared electrodes with fresh cells (25 μL) (35×10^9 cell titer) were used in all experimental steps unless otherwise stated.

For the preparation of gold nanoparticle (GNP) modified microbial sensors, cellular paste containing 2.5 μL of gold colloidal solution (with the average size of 10 nm) was used as the biological sensing material.

Control experiments were done using entrapped cells behind dialysis membrane on the polished graphite electrode without the polymer.

2.6. Measurements

All experiments were done at ambient conditions in a vessel containing 10 mL buffer. After each run, the electrode was washed with distilled water and kept in phosphate buffer (50 mM, pH 6.5) for 5 min. The microbial sensor was initially equilibrated in buffer and then the substrate was added to the reaction cell. Current response due to glucose addition was recorded at 120–130th s. The biosensor responses were registered as current densities ($\mu A/cm^2$) at −0.7 V by following the oxygen consumption resulting from metabolic activity of microbial cells in the presence of glucose substrate. Buffer was refreshed after each measurement [22].

2.7. Sample application

The microbial sensors were tested to analyze glucose in fruit juice (Cappy, Mersin/TURKIYE) and ethanol content in vodka (Mey,

Bilecik/TURKIYE) and whisky (Jack Danniel's, Tennessee/USA) samples. No sample pretreatment was required for the analysis.

Additionally, a commercial enzyme assay kit based on spectrophotometric Trinder reaction (Cromatest, Glucose MR, Cat. No. 1129010) was used as the reference method for independent analysis of the glucose content. In this reaction, the glucose is oxidized to D-gluconate by the glucose oxidase (GOD) with the formation of hydrogen peroxide. In the presence of peroxidase (POD), a mixture of phenol and 4-aminoantipyrine (4-AAP) is oxidized by hydrogen peroxide to form a red quinine imine dye proportional to the glucose concentration in the sample [23]. As far as alcohol analysis was concerned, amount of ethanol detected in the sample by the proposed sensor was compared with the label value of the product.

3. Results and discussion

The use of conducting polymers in the area of bioanalytical sciences is of great interest since their compatibility opens up the possibility of using them in different biosensing applications [24–27]. CPs have an organized molecular structure on different tranducers, which allows them to function as a three dimensional matrix for the biomolecule immobilization and preserve the activity for a long period. This property of the matrix with their functionality as a membrane has provided opportunities for sensor development [8,28]. Immobilization of whole viable *Pseudomonas fluorescens* cells were previously trapped on a thiophene-based conducting polymer. This process is reproducible with a high operational stability [22]. In this work, use of an electrochemically polymerized 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine (SNS-NH_2) as a microbial biosensing platform was examined for *G. oxydans* cells.

Morphologies of bacterial sensing surfaces provide the most precise information on the cells and matrices used in the system. Scanning electron microscopy (SEM) technique is utilized to monitor the surface characteristics and shows the interaction between biological materials and immobilization matrices. Morphologies of bare graphite, polymeric matrix without and with cells were shown in Fig. 1(A)–(C), respectively. Unbound cells were removed by washing the electrode surfaces several times before analysis. As seen from the micrographs, poly(SNS-NH_2) provided an efficient immobilization platform with the compact structure for the cell immobilization. Hence, cells could be kept on to the surface where higher sensors responses with high operational stabilities are obtained. The presence of amino groups in the structure may also contribute to attach the microorganisms on the matrix due to the ionic interactions between the cell surface and this functional group.

3.1. Effect of electropolymerization time

The advantages of electropolymerization can be summarized as the achievement of new properties using various supporting electrolytes or monomers and the control of the film thickness by regulating the amount of charge passed [29]. The most convenient electrochemical method employed for characterization is cyclic voltammetry. Cyclic voltammograms of bare graphite electrode (A), $SNSNH_2$ polymer (B), bacteria adsorbed $SNSNH_2$ matrix (C) and bacteria adsorbed polymeric matrix containing gold nanoparticles (D) onto the graphite electrode were shown in Fig. 2. It is clear that GNP modification caused higher current values in compared with the biosensing system without nanoparticles due to its physical properties.

Electropolymerization time is directly related with the thickness of the polymer on the graphite electrode. The thickness can

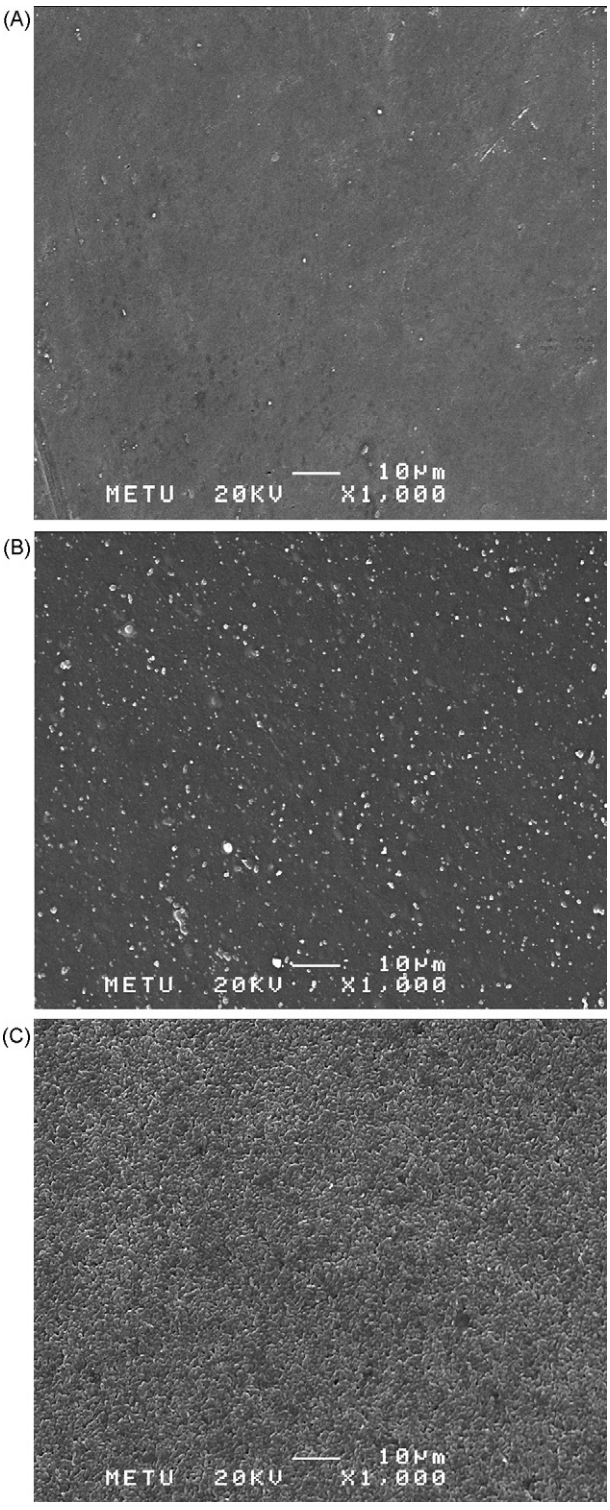


Fig. 1. SEM images of bare graphite surface, bare graphite (A), 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine (B) and 4-(2,5-di(thiophen-2-yl)-1H-pyrrole-1-l) benzenamine based *G. oxydans* biosensors (C).

be measured in terms of charge. After 5, 10, 15, and 20 min of electropolymerization (referring to 50, 100, 150 and 200 scan numbers), the charges required to prepare the polymer coated electrodes were measured as 9.50×10^{-4}, 1.05×10^{-3}, 7.50×10^{-4} and 4.10×10^{-4} C, respectively. These electrodes were used to form microbial sensor as mentioned in the experimental part. As shown in Fig. 3, maxi-

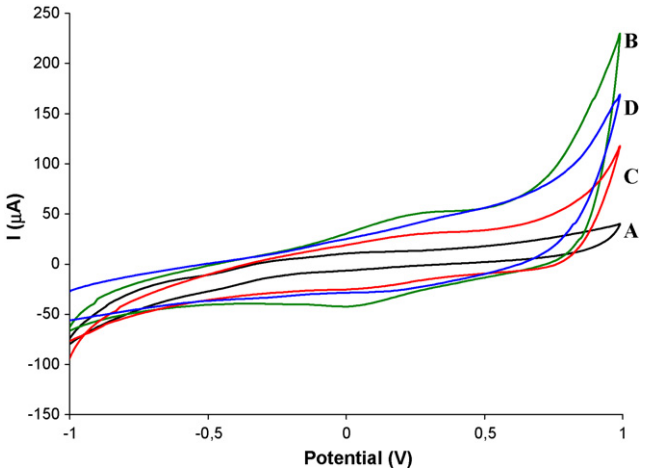


Fig. 2. Cyclic voltammograms of bare graphite electrode (A), $SNSNH_2$ polymer (B) bacteria-$SNSNH_2$ (C), bacteria-GNP-$SNSNH_2$ (D) onto the graphite electrode [number of scans: 100, in potassium phosphate buffer (50 mM, pH 6.5)].

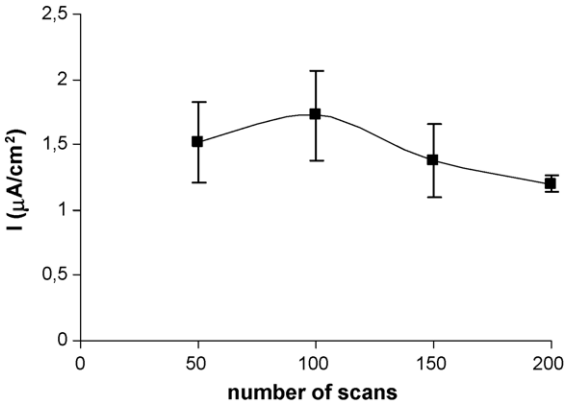


Fig. 3. Effect of number of scan on the biosensor response. Error bars have been deduced from measurements in triplicate [in potassium phosphate buffer (50 mM, pH 6.5), −0.7 V, 10 mM glucose].

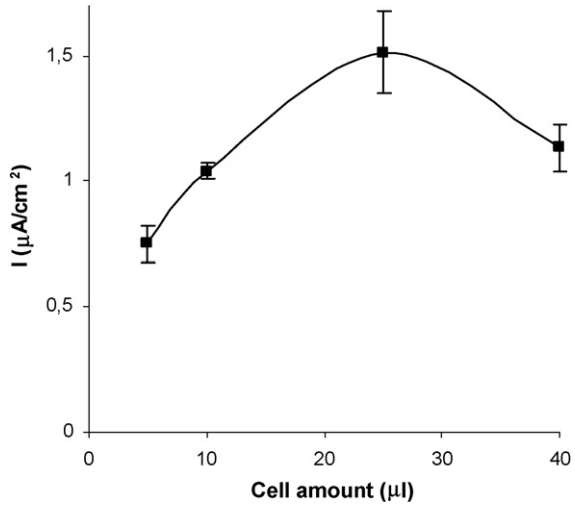


Fig. 4. Effect of cell loading on the biosensor response [in potassium phosphate buffer (50 mM, pH 6.5), −0.7 V, 10 mM glucose]. Error bars have been deduced from measurements in triplicate.

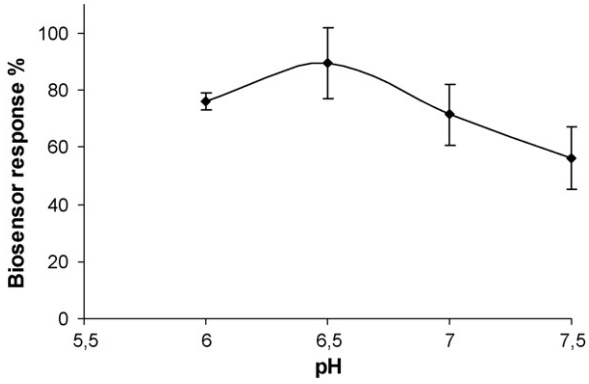


Fig. 5. Effect of pH [potassium phosphate buffer (pH 6.0–7.5, 50 mM), −0.7 V, 10 mM glucose]. Error bars have been deduced from measurements in triplicate.

mum activity was recorded when the working electrode was coated using 1.05×10^{-3} (0.5 μ) C. This finding well agrees with the previous work which utilized a thiophene-based conducting polymer for a microbial biosensing where the best response was obtained with 10 min of electropolymerization [22]. It is clear that the most proper film structure related with the thickness was obtained by 10 min of electropolymerization time for the cell immobilization. Longer deposition times might cause the degradation and incompact microstructure as reported in a previous work [30].

3.2. Effect of cell amount

In order to determine the appropriate cell amount, different biosensors containing 5 μL, 10 μL, 25 μL and 40 μL of bacterial cells were prepared. The highest current responses were obtained with 25 μL cell amount (35×10^9 cell titer). When 5 μL cell (with 7×10^9 cell titer) was used, lowest current response was obtained. On the other hand, when cell amount was increased to 40 μL, a lower signal than that for 25 μL was obtained. This is an expected result and caused by the diffusion problem due to the high cell density. Since both amounts caused lower current values, further experiments were conducted using 25 μL cell (Fig. 4).

3.3. Effect of pH

The effect of pH on microbial sensor based on poly($SNS\text{-}NH_2$) was achieved by adjusting the pH between 6.0 and 7.5 of using phosphate buffer (50 mM). The response of the microbial sensor towards glucose (10 mM) at different pH was shown in Fig. 5. Since pH 6.5 has the maximum current response, it was chosen as the optimum pH and all the other experiments were conducted with this pH value.

3.4. Analytical approach

Microbial sensor was prepared as mentioned in the experimental part to examine the analytical characteristics. Linear dynamic ranges and the equations were obtained based on optimized conditions. For the proposed system, a linear calibration graph (Fig. 6) was obtained for current density versus substrate concentration between 0.1 and 2.5 mM glucose. A linear relation was defined by the equation of $y = 0.415x + 0.377$ ($R^2 = 0.986$) (y is the sensor response in current density ($\mu A/cm^2$) and x is the substrate concentration in mM). Repeatability of the microbial sensor was tested for 2.3 mM glucose ($n = 4$) and the standard deviation and coefficient of the variation were calculated as ±0.067 mM and 2.9%, respectively.

Another type of biosensing system including GNP as the modifier was also prepared to observe the effect of nanoparticles on the

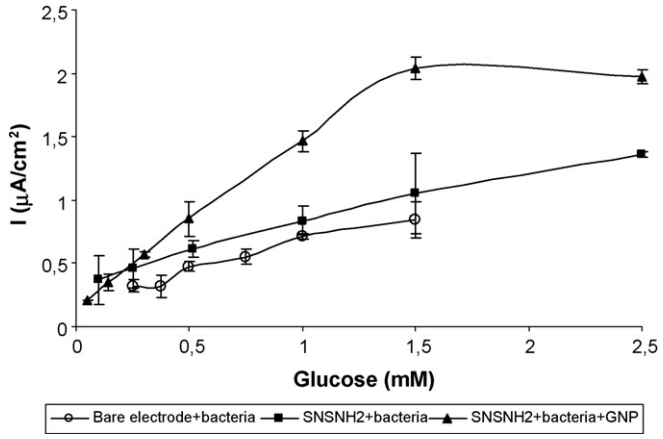


Fig. 6. Calibration curves for three types of microbial biosensor including bacteria immobilized on the graphite electrode with and without $SNSNH_2$ and GNP modified $SNSNH_2$ [in potassium phosphate buffer (50 mM, pH 6.5), −0.7 V].

current signal. It is previously reported that metal nanoparticles can display unique advantages such as enhancement of mass transport, catalysis, high effective surface area and control over electrode microenvironment over macro electrodes when used for electroanalysis. For instance, Pt and Au nanoparticles are very effective as matrices for enzyme sensors via taking advantage of the biocompatibility and large surface area [31]. In our case, calibration curve for the gold nanoparticle modified system was examined based on the same procedure mentioned in the experimental part. A linear relation for glucose substrate was found between 0.05 and 1.5 mM as represented by the equation $y = 1.258x + 0.181$ ($R^2 = 0.998$) and a response time of 130 s (Fig. 6). When we compare these data with the system without GNP, it is clear that higher current responses and sensitivity were observed. The presence of metal nanoparticles could contribute the facilitated electron transfer between the oxidative enzymes in bacteria and the electrode surface as described previously [31]. It is also possible that high surface area due to the GNP on the polymer matrix provides loading of highest cell amount causing higher biosensor response. The effects of different nanoparticles in terms of sensitivity and stability in microbial biosensing are now under progress.

A calibration curve was also obtained for the microbial electrode containing adsorbed bacterial cells behind the dialysis membrane where there is no coating with poly(SNS-NH_2) (Fig. 6). The currents recorded were low in this case and irreproducible current responses were observed. Linear relation was observed in the range of 0.25–1.0 mM glucose and defined by the equation $y = 0.536x + 0.167$ ($R^2 = 0.954$). This reveals that the polymer provides a good contact for cells on the electrode surface where they can attach and survive during the operational conditions as described in our previous work [22].

Moreover, substrate specificity of the designed microbial biosensor to mannose, galactose, xylose, methanol and ethanol were tested. No response was obtained for mannose, galactose, xylose and methanol. Microbial sensor gives response to ethanol in the range of 0.1–5.0 mM and the relation is described by the following equation: $y = 0.163x + 0.425$ ($R^2 = 0.978$). Results showed that it could be possible to make selective ethanol analysis even in the presence of both methanol and glucose.

The operational stability of microbial sensors was also determined at optimum conditions (in phosphate buffer, pH 6.5) using 1.5 mM glucose. 11% decrease was seen after 5 h. 22 measurements were done in this period and it is clear that it is possible to carry out even more measurements during these conditions.

3.5. Application to real samples

The biosensing system developed was used to for glucose and ethanol detection in fruit juice, vodka and whisky samples. A spectrophotometric method was also used as the reference method to test glucose concentration in the same samples. Samples were used as the substrate solution replacing glucose and ethanol. Signals were recorded and data were calculated from glucose and ethanol calibration curves. For the glucose analysis, values obtained were compared with glucose amounts determined by the spectrophotometric method. Data were given as the average of 3–5 measurements. The glucose amount in fruit juice was determined as 17.64 g/L ± 1.89 by microbial sensor and calculated as 18.19 g/L ± 2.77 by the spectrophotometric method.

As to the ethanol analysis, data were compared with the ethanol label values given by the manufacturer on the product. The alcohol amount in whisky sample (alcohol label 43%) was determined as 46.6 ± 5.71% by the microbial biosensor. Alcohol amount in vodka (40% alcohol on the label) was determined as 35 ± 7.55% by the microbial biosensor. One of the most attractive feature is that the proposed system can be used for both alcohol and glucose detection. It might also be possible to use this system as an electrochemical microbial bioanalyzer (after HPLC separation). Our finding also showed that the proposed system is not affected by the nature of the sample.

4. Conclusion

Conducting polymers have attracted much interest as suitable matrices for biological materials. These are used to increase speed, sensitivity and versatility of biosensors in diagnostics to measure vital analytes [32]. The use of electronically conducting polymers with particular properties together with the immobilized microbial systems enables one to develop novel biomicroelectronic devices. In the present paper, a bacterial biosensor was prepared with *G. oxydans* cells immobilized on a soluble conducting polymer matrix. The biosensor showed a good linear range, and repeatability as well as a high operational stability. Besides, practical use of the present detection system for both ethanol and glucose analyses showed that it can properly be used in a chromatographic set-up as was shown previously with different enzyme electrodes such as pyranose oxidase [33], and sulfhydryl oxidase [34,35].

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