UDK 678.7
Original scientific article/Izvirni znanstveni članek

ISSN 1580-2949
MTAEC9, 48(2)209(2014)

DESIGN OF A MICROBIAL SENSOR USING A CONDUCTING POLYMER OF POLYANILINE/POLY 4,4'-DIAMINODIPHENYL SULPHONE-SILVER NANOCOMPOSITE FILMS ON A CARBON PASTE ELECTRODE

OBLIKOVANJE MIKROBNEGA SENZORJA Z UPORABO PREVODNE POLIMERNE POLIANILINSKE/POLI 4,4'-DIAMINODIFENIL SULFONSKE SREBRNE NANOKOMPOZITNE TANKE PLASTI NA ELEKTRODI Z OGLJIKOVO PASTO

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Prejem rokopisa – received: 2012-11-03; sprejem za objavo – accepted for publication: 2013-06-07

A microbial biosensor based on Gluconobacter oxydans cells immobilized on a conducting polymer of polyaniline/Poly 4,4'-diaminodiphenyl sulphone-silver (PANI/PDDS/Ag) coated onto the surface of a carbon-paste electrode was constructed. The proposed biosensor was characterized using glucose as the substrate. Conducting polymers are electrochemically polymerized at the carbon-paste electrode substrates. The polymer films are modified by electrochemically depositing PANI/PDDS/Ag particles. The effect of changing the size of the Poly 4,4'-diaminodiphenyl sulphone-silver particles and the polymer film thickness on the voltammetric behaviour of the resulting hybrid material showed noticeable changes in the electrocatalytic current in an acid medium. The morphology of the polymer films and the distribution of the PDDS/Ag particles in the film were studied with scanning electron microscopy.

Keywords: polyaniline/Poly 4,4'-diaminodiphenyl sulphone-silver, conducting polymers, gluconobacter oxydans, voltammetric properties, nanoparticles

Konstruiran je bil mikrobni senzor na osnovi glukonobakterijskih oksidacijskih celic, pritrjenih na prevoden polimer iz polianilina/poli 4,4'-diaminodifenil sulfon–srebra (PANI/PDDS/Ag), nanesenega na površino elektrode z ogljikovo pasto. Predlagani senzor je bil karakteriziran z uporabo podlage iz glukoze. Prevodni polimeri so bili elektrokemijsko polimerizirani na elektrodo s podlago iz ogljikove paste. Polimerne tanke plasti so bile modificirane z elektrokemijskim nanosom PANI/PDDS/Ag-delcev. Spreminjanje velikosti poli 4,4'-diaminodifenil sulfonskih srebrnih delcev in debeline polimerne plasti je pokazalo občutne spremembe pri voltametričnem vedenju, rezultirajoči hibridni material pa je pokazal občutne spremembe elektrokatalitičnega toka v kislem mediju. Morfologija polimernih plasti in razporeditev PDDS/Ag-delcev v plasti sta bila pregledana na vrstičnem elektronskem mikroskopu.

Ključne besede: polianilin/poli 4,4'-diaminodifenil sulfon–srebro, prevodni polimeri, glukonobakterijski oksidant, voltametrične lastnosti, nanodelci

1 INTRODUCTION

Many types of microbial sensors have been developed as analytical tools. Such a microbial sensor consists of a transducer and a microbe as the sensing element. The characteristics of microbial sensors are in complete contrast to those of enzyme sensors or immunosensors, which are highly specific for the substrates of interest, although the specificity of the microbial sensor has been improved by genetic modifications of the microbe used as the sensing element. Microbial sensors have the advantages of a tolerance to the measuring conditions, a long lifetime, and cost performance, but they also have the disadvantage of a long response time.

Since their discovery in the mid-1970s, research on conducting polymers (CPs) has become an ever-growing research area in polymer chemistry.[1] The redox behaviour and an unusual combination of the properties of metals and plastics make conducting polymers a new class of materials.[2] The interest in conducting polymers is largely due to the wide range of possible applications due to their facile synthesis, good environmental stability and the long-term stability of the electrical conductivity. The advantage of using conducting polymers compared to more traditional sputtered metal coatings is that the polymer is soluble, enabling a non-destructive analysis of the specimens.[3] CPs were extensively studied in the past 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. The biosensing approach using CPs has also been widely investigated in previous studies.[8]

There have been several attempts to produce nanoparticle polymer composites. Overall, we note four different approaches used to date. The first technique consists of the in-situ preparation of the nanoparticles in the polymer matrix. This is affected by a reduction of the metal salts dissolved in the polymer matrix.[9] The second technique involves polymerizing the matrix around the

nanoparticles.[10] The blending of pre-formed nanoparticles into pre-synthesized polymer can be considered as the third technique for the preparation of a nanoparticle polymer composite material.[11] The fourth process involves the in-situ synthesis of the composite material, with the metal nanoparticles being formed from an ionic precursor and the polymer being produced from the monomer. A major advantage of the latter method lies in the provision of a better particle-polymer interaction.[12]

Microbial cells are very promising for biosensor constructions due to them having several advantages: the enzyme does not need to be isolated, the enzymes are usually more stable in their natural environment in the cell, the co-enzymes and activators are already present in the system.[13] Cell-based biosensors are frequently used for a determination of the biological oxygen demand (BOD), the toxic agents and the assimilatable sugars as well as the selective detection of a single analyte.[14]

In this study different amounts of polymer nanocomposites aniline were examined for the detection of glucose and ethanol. The measurement was based on the respiratory activity of the cells. As well as the optimization and characterization, an application of the proposed system was carried out on real samples.

2 EXPERIMENTAL

2.1 Reagents

$LiClO_4$, $NaClO_4$, dialysis membrane, $AlCl_3$, succinyl chloride, benzene-1,4-diamine propionic acid, nitromethane, iron(III) chloride, propylene carbonate, poly(methylmethacrylate), dichloromethane, toluene, d-glucose, ethanol and gold colloidal were purchased from Sigma. Methanol and acetonitrile were purchased from Merck. All other chemicals were of analytical grade and purchased either from Merck or Sigma. The DDS were purchased from Merck, and silver nitrate from Alderich.

2.2 Apparatus

Scanning electron microscopy (SEM) images were taken using a VEGA HV (high potential) 1500 V at various magnifications. All the experiments were carried out in air atmosphere at room temperature (25 °C). A conventional three-electrode set up was employed for the CV studies. The counter electrode was a platinum sheet with a surface area of 2 cm^2. A SCE electrode was employed as the reference electrode. Electropolymerization and all other electrochemical studies were carried out using a Potentiostat/Galvanostat EG&G Model 263 A; USA well equipped with M 270 software.

2.3 Synthesis of the composite material

In a typical reaction, 0.8 g DDS was dissolved in a magnetically stirred 25 mL of methanol in a 50 mL conical flask. After complete dissolution, 100 mL dilute silver nitrate (10^{-1} mol dm^{-3}) was added drop wise. After the addition of the entire silver nitrate, the precipitated material collected at the bottom of the flask. This work accomplished for the solution of silver nitrate that the colloidal solutions were observed as milky coloured.

2.4 Cultivation of G. oxydans

The strain of *G. oxydans* was obtained from DSMZ and maintained on agar containing (g L^{-1}): d-glucose, 90; yeast extract, 15; calcium carbonate, 18; agar, 25.[15] The cell biomass was prepared by aerobic cultivation at 28 °C. Then, the cells were collected by centrifugation after reaching the late exponential phase and washed twice with 0.9 % sodium chloride solution containing $CaCl_2$ 2 mM. The biomass concentration was expressed as the weight matter determined by drying to a constant weight at 105 °C.

2.5 Microbial electrode method of making

Prior to the electropolymerization, graphite electrodes were polished on wet emery paper and washed thoroughly with distilled water, sonicated for 2–3 min, rinsed with bi-distilled water and dried at 105 °C. The electrochemical polymerization of DDS/Ag (1 mg/mL) was carried out by scanning the potential between -0.5 V and 0.8 V via cyclic voltammetry with the scan rate of 300 mV/s in $NaClO_4$ (0.2 M) and $LiClO_4$ (0.2 M)/acetonitrile medium. The concentration of the monomer was as for the polymerization of the DDS/Ag.

For the cells of bacteria to prove G. oxydans on electrode coated with polymer, first we spray it and leave the electrode surface to dry for 1 h. After the 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. Daily-prepared electrodes with fresh cells were used in all the experimental steps, unless otherwise stated. Control experiments using carbon paste electrode were covered with bacterial cells and a polymer coating that was not.

2.6 Measurements

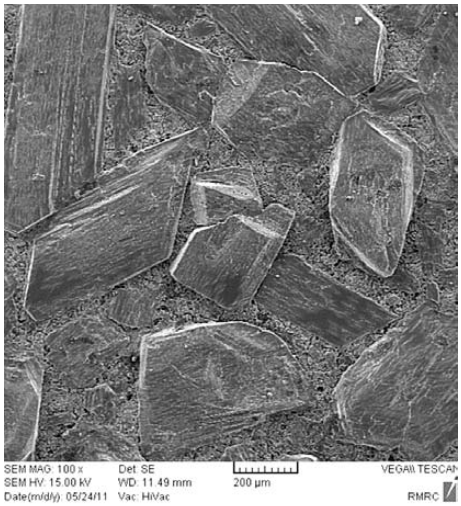
All the measurements were carried out at 30 °C under constant stirring. After each run, the electrode was washed with distilled water and kept in a phosphate buffer (pH 7) solution 50 mM at 30 °C for 7 min. The working buffer solution was renewed after each measurement. The microbial sensor was initially equilibrated in the buffer and then the substrate was added to the reaction cell. After 30 min the substrate was added to the reaction cell. The biosensor responses were registered as current densities by following the oxygen consumption at –0.7 V due to the metabolic activity of the bacterial cells in the presence of glucose.[16]

3 RESULTS AND DISCUSSION

Enzymes and cells have been used in biosensor construction for many decades. Both concepts have some advantages and challenges.[17] There have been various strategies to modify the microbial cells for applications in microbial biosensors. The principle of the bacterial biosensor is rather simple, and sensor productions only require the growth of the microorganisms. There are multiple strategies for 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; an additional benefit for the biosensor performance is that the enzymes are still linked to the respiratory chain.

Since conducting polymers can act as transducers in biosensors and coating electrodes with CPs under mild conditions this opens up various possibilities for the immobilization of biomolecules and biosensing materials, the enhancement of their electrocatalytic properties, rapid electron transfer and direct communication. The co-immobilization of redox mediators or cofactors by entrapment within electropolymerized films or by covalent binding on the surface allows the simple fabrication of reagent-less biosensors.[18] The CPs have an organized molecular structure on different transducers, 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. This process is reproducible with a high operational stability.[19] In this work the use of an electrochemically polymerized aniline/4,4'-diaminodiphenyl sulphone-silver as a microbial biosensing platform was examined for G. oxydans cells.

a)



b)

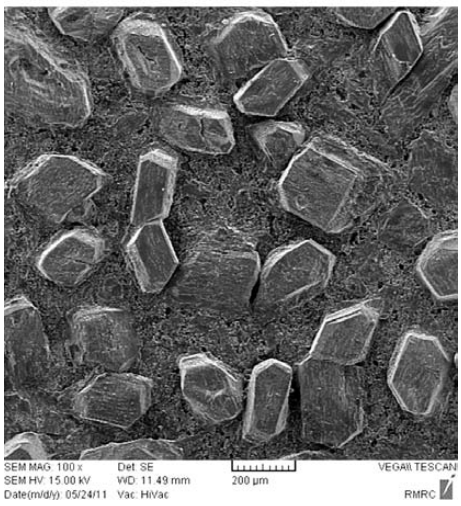


Figure 1: SEM images of bare graphite surface: a) PANI/PDDS/Ag polymer, b) bacteria-PANI/PDDS/Ag

Slika 1: SEM-posnetka gole površine grafita: a) polimer PANI/PDDS/Ag, b) bakterija PANI/PDDS/Ag

The morphologies of the bacterial sensing surfaces provide the most precise information about the cells and matrices used in the system. The scanning electron microscopy (SEM) technique is utilized to monitor the surface characteristics and shows the interaction between the biological materials and the immobilization matrices. The morphologies of bare graphite, and a polymeric matrix with and without cells were shown in **Figure 1**. Unbound cells were removed by washing the electrode surfaces several times before analysis. As seen from the micrographs, PANI/DDS/Ag provided an efficient immobilization platform with a compact structure for the cell immobilization. Hence, cells could be kept on the surface where higher sensor responses with high operational stabilities are obtained. The presence of amino groups in the structure may also contribute to attaching the micro-organisms on the matrix due to the ionic interactions between the cell surface and this functional group.

3.1 Effect of electropolymerization time

The most convenient electrochemical method employed for characterization is cyclic voltammetry. Cyclic voltammograms of bare graphite electrode carbon paste electrode (A), PDDS/Ag polymer (B), bacteria – PDDS/Ag (C), bacteria – PANI/PDDS/Ag (D) onto the graphite electrode are shown in **Figure 2**. 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 an 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 degradation and an incompact microstructure of the films.[20] In order to observe the effect of electropolymerization time, aniline/4,4'-diaminodiphenyl sulphone-silver was polymerized onto the carbon paste surface for different periods ((5, 10 and 20) min, **Figure 3**), and then modified electrodes were used to form microbial biosen-

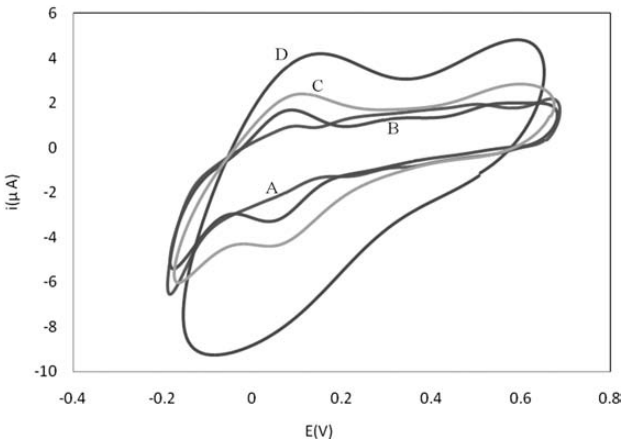


Figure 2: Cyclic voltammograms of bare carbon paste electrode (A), PDDS/Ag polymer (B), bacteria – PDDS/Ag (C), bacteria – PANI/PDDS/Ag (D) onto the graphite electrode (number of scans: 100, in potassium phosphate buffer (50 mM, pH 7))

Slika 2: Krožni voltamogrami elektrode z ogljikovo pasto (A), polimera PDDS/Ag (B), bakterije – PDDS/Ag (C), bakterije – PANI/PDDS/Ag (D) na grafitni elektrodi (število pregledovanj: 100, v kalij fosfatnem pufru (50 mM, pH 7))

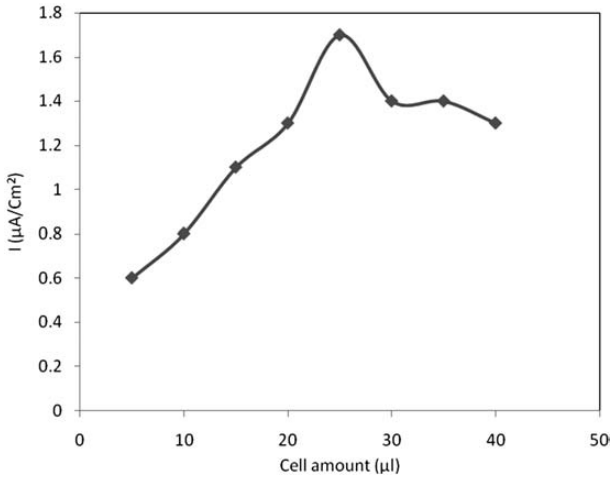


Figure 4: Effect of cell loading on the biosensor response (in potassium phosphate buffer (50 mM, pH 7), –0.7 V, 10 mM glucose)

Slika 4: Vpliv obremenitve celice na odziv biosenzorja (v kalijevem fosfatnem pufru (50 mM, pH 7), –0,7 V, glukoza 10 mM)

sors, as described in the experimental section. The best current values were obtained for 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 to the thickness after 10 min of the electropolymerization time for the cell immobilization.

3.2 Effect of cell amount

In order to determine the appropriate amount of cells, different biosensors containing (5, 10, 15, 20, 25, 30, 35 and 40) μL of bacterial cells were prepared. The highest current responses were obtained with the 25 μL of cells. When the 5 μL of cells was used the lowest current response was obtained. On the other hand, when the amount of cells was increased to 30 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 the 25 μL cell (**Figure 4**).

3.3 Effect of pH

The effect of pH on the microbial sensor based on PANI/PDDS/Ag was achieved by adjusting the pH between 6.0 and 8 when using phosphate buffer (50 mM). The response of the microbial sensor towards glucose (10 mM) at different pH values was shown in **Figure 5**. Since pH 7 has the maximum current response, it was chosen as the optimum pH and all the other experiments were conducted with this pH value.

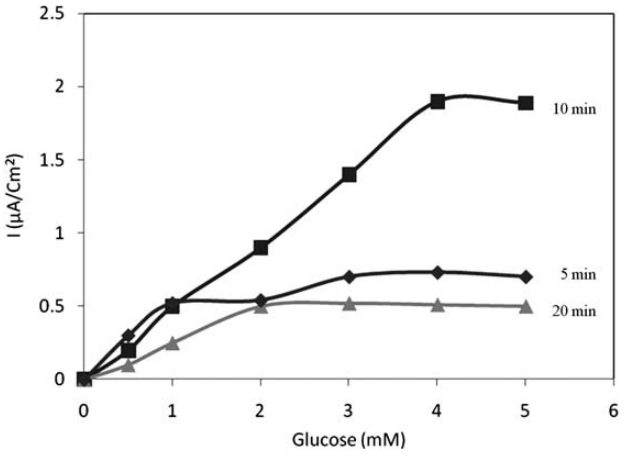


Figure 3: Effect of electropolymerization time on the biosensor response (in phosphate buffer, 50 mM, pH 7, 30 °C, –0.7 V)

Slika 3: Vpliv časa elektropolimerizacije na odzivnost biosenzorja (v fosfatnem pufru, 50 mM, pH 7, 30 °C, –0,7 V)

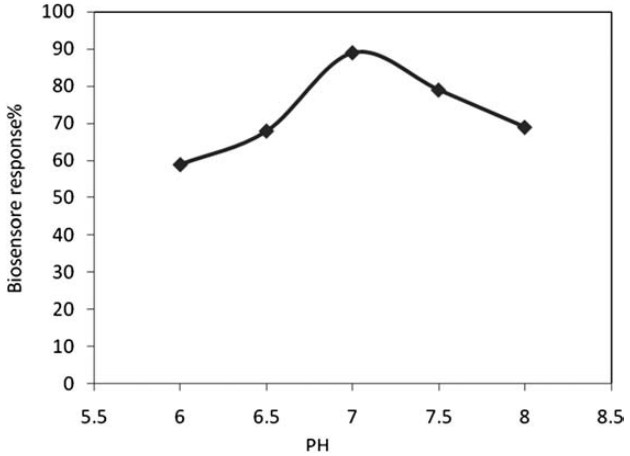


Figure 5: Effect of pH on the biosensor response (pH 6.0–8.0 phosphate buffer, 30 °C, –0.7 V)

Slika 5: Vpliv pH na odzivnost biosenzorja (pH 6,0–8,0 fosfatni pufer, 30 °C, –0,7 V)

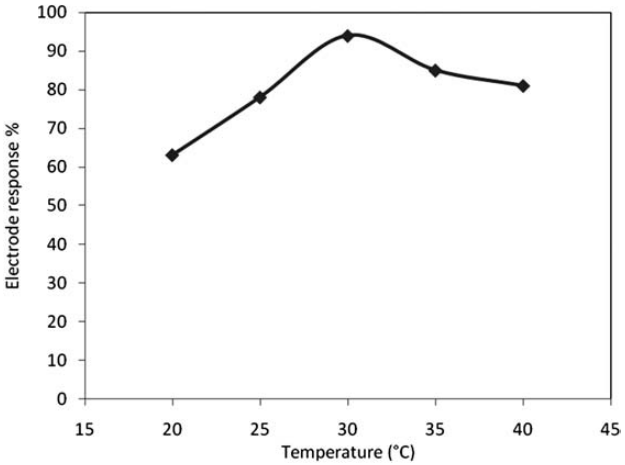


Figure 6: Effect of temperature on the electrode response for microbial biosensors (in potassium phosphate buffer, 50 mM, pH 7, –0.7 V)
Slika 6: Vpliv temperature na odzivnost elektrode pri osnovnem mikrobnem biosenzorju (v kalijevem fosfatnem pufru, 50 mM, pH 7, –0,7 V)

3.4 Effect of temperature

The amperometric response of the microbial sensors to glucose (50 mM) was followed at different temperatures, varying from 20 °C to 40 °C. From 20 °C to 25 °C an increase was observed up to 30 °C and then the signal started to decrease at 35 °C (**Figure 6**). As a result, the optimum temperature was found to be approximately 30 °C. And further experiments were conducted at this temperature.

3.5 Analytical characteristics

A microbial sensor was prepared, as described in the experimental part, to examine the analytical characteristics. The linear dynamic ranges and the equations were obtained based on optimized conditions. For the proposed system, a linear calibration graph **Figure 7** (A) was obtained for the current density versus the substrate concentration between 0.1 mM and 2.5 mM glucose. A linear relation was defined with the equation $y = 0.3116x$ ($R^2 = 0.9376$), where y is the sensor response in the current density (μA/cm^2) and x is the substrate concentration (mM).

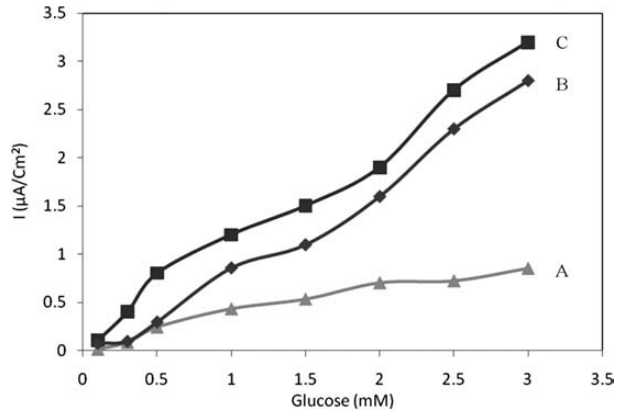


Figure 7: Calibration curves for three types of microbial biosensor including bacteria immobilized on the bare carbon paste electrode (A), PDDS/Ag polymer (B), PANI/PDDS/Ag (C) (in potassium phosphate buffer (50 mM, pH 6.5), –0.7 V)
Slika 7: Umeritvene krivulje za tri vrste mikrobnih biosenzorjev, vključno z bakterijami, pritrjenimi na elektrodi z ogljikovo pasto (A), polimer PDDS/Ag (B), PANI/PDDS/Ag (C) (v kalijevem fosfatnem pufru (50 mM, pH 6,5), –0,7 V)

Another type of electrode was covered using a polymer PDDS/silver and bacteria. It was previously reported that metal nanoparticles can display unique advantages, such as an enhancement of the mass transport, catalysis, a high effective surface area and control over the electrode microenvironment over macro-electrodes when used for electro-analysis. For instance, Pt and Au nanoparticles are very effective as matrices for enzyme sensors by taking advantage of the biocompatibility and large surface area. In our case, the calibration curve for the modified system based on silver nanoparticles was studied using the same method as described in the experimental section. However, these nanoparticles in polymer nanocomposites were a DDS. A linear relation for the glucose substrate was found between 0. 5 mM and 3 mM, as represented by the equation $y = 0.8774x$ ($R^2 = 0.9786$) and a response time of 120 s (**Figure 7** (B)). It is also possible that the high surface area due to the Ag on the polymer matrix provides the loading of the largest amount of cells, causing a larger biosensor response. The effects of different nanoparticles in terms of sensitivity and stability in microbial biosensing are now under investigation.

At the end of the calibration curve the carbon-paste electrode modified with bacteria and polymer PANI/DDS/Ag was observed (**Figure 7** (C)). The currents recorded were low in this case and irreproducible current responses were observed. A linear relation was observed in the range 0.1–3.0 mM glucose and defined by the equation $y = 1.056x$ ($R^2 = 0.9808$). This reveals that the polymer provides a good contact for the cells on the electrode surface where they can attach and survive during the operational conditions, as described in our previous study.

4 CONCLUSIONS

Conducting polymers that have an organized molecular structure can serve as proper and functional immobilizing platforms for biomolecules. These matrices provide a suitable environment for the immobilization and preserve the activity for long durations. In this paper a measurement method and environment for the bacteria and the PANI/DDS/Ag-modified electrode are described. The proposed system does not require any complicated immobilization procedure for the construction of a biosensor. The preparation is simple, cheap and is 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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