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Short communication

Capability of parasulfonato calix[6]arene, as an anion dopant, and organic solvents in enhancing the sensitivity and loading of glucose oxidase (GOx) on polypyrrole film in a biosensor: A comparative study

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ABSTRACT

In this study, the effects of two solvents (acetonitrile and water) and an anion dopant (para sulfonato calix[6]arene ($(C[6]S)^{-6}$)), on the manufacturing and properties of a polypyrrole (Ppy)-based, glucose oxidase amperometric biosensor were studied. Pyrrole was polymerized using galvanostatic mode in two different solvents, and the effect of $(C[6]S)^{-6}$ was studied in aqueous solution. The morphology of the obtained polypyrrole films was studied by scanning electron microscopy (SEM). Glucose oxidase (GOx) was adsorbed on the Ppy films via cross-linking method. Then the amperometric responses of the Pt/Ppy/GOx electrodes were measured using the amperometric method at the potential of 0.7 V in steps of adding a glucose solution to a potassium phosphate buffer. We found that acetonitrile and $(C[6]S)^{-6}$ increase the sensitivity of the enzyme electrode up to 79.30 $\mu A\, M^{-1}\, cm^{-2}$ in comparison with 31.60 $\mu A\, M^{-1} cm^{-2}$ for the electrode synthesized in calixarene free aqueous solvent. Also $(C[6]S)^{-6}$ has the main role in preventing leaching the enzyme from the electrode. This fact increases loading of the enzyme and stability of the biosensor. So that the steady state current density of the aforementioned electrode increases linearly with increasing glucose concentration up to 190 mM. Whereas the linearity was observed up to 61 mM and 80 mM for the electrodes made using calixarene free acetonitrile and aqueous solutions, respectively.

1. Introduction

Using enzyme electrodes such as glucose oxidase (GOx) electrode is very interesting in vast studies especially biomedical analysis (Sung and Bae, 2003). Development of these electrodes for responsible, explicit, and low cost biosensors is one of the important zones of science. These electrodes are constructed by combining an electrochemical sensor with a thin layer of immobilized enzyme (Bélanger et al., 1989). Conducting polymers offer a suitable matrix for the enzyme immobilization (Borole et al., 2005). The most reported methods for the enzyme immobilization include adsorption (Genies and Marchesiello, 1993; Hämmerle et al., 1992), covalent attachment (Schuhmann et al., 1990; Şenel, 2011; Şenel et al., 2011), cross-linking (Mala Ekanayake et al., 2008; Gaikwad et al., 2006), and entrapment of an enzyme in polymer matrix during electropolymerization (Nien et al., 2006).

Various polymers have been utilized for immobilization of glucose oxidase (GOx) such as polypyrrole (Ppy) (Shin and Kim, 1996a, 1996b), polyaniline (Gaikwad et al., 2006), polythiophene (Abasıyanık and Şenel, 2010), polyvinylalcohol (Guascito et al., 2011), etc. Since Ppy films have several advantages such as stability at ambient conditions, ease of preparing them in aqueous and organic solvents, good redox properties, etc., they have been extensively used for development of glucose biosensors (Singh et al., 2009). There are different processing parameters effective on the physical and electrochemical properties of conducting polymers. The nature of the dopant anion is one of the most considerable ones. Depending on the largeness of the dopant anion, the Ppy films can behave as an anion exchange (Yuan et al., 1999) or cation exchange polymer (Akieh et al., 2010). Interestingly, the films formed by the use of bulky anions particularly large aromatic sulfates, have shown more stability and posed better electrochemical and mechanical properties than the films formed using smaller anions (Ingram et al., 2004; Kupila and Kankare, 1995; Suematsu et al., 2000; Yuan et al., 1999). Para sulfonato calix[*n*]arenes are bulky cavitary anions potentially applicable in building up ionic sensors. They also act as actuators for trapping and releasing of ions or molecules (Duncan and Cockayne, 2001; Evtugyn et al., 2007; Gendi et al., 2009; Mousavi et al., 2005; Kaneto and Bidan, 1998). It has been confirmed that even if para sulfonato calix[*n*]arenes be scattered in the polymeric matrix, keep their recognition properties (Bidan and Niel, 1997).

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Solvent effect is another important parameter to be considered. Using aqueous solutions in the polymerization stage could result in forming fragile and powdery films with poor physical strength, shorter conjugation length, and lower conductivity values (Otero and Arévalo, 1994). However, applying non aqueous solutions for this purpose has been less studied because doing immobilization at the same time of polymerization requires the use of aqueous solutions (Deshpande and Amalnerkar, 1993).

Considering the importance of the dopant anion and solvent effect, we investigate the role of these two parameters on the characteristics and morphology of the Ppy films as a matrix for enzyme immobilization. In this study we use galvanostatic electropolymerization to manufacture the Pt/Ppy/GOx biosensor in: (a) acetonitrile containing lithium perchlorate as a supporting electrolyte; (b) an aqueous solution containing para sulfonato calix[6]arene ($(C[6]S)^{-6}$) as a dopant anion, and lithium perchlorate as a supporting electrolyte; and (c) an aqueous solution containing lithium perchlorate. Eventually we compare the amperometric responses of these electrodes to the addition of glucose.

2. Experimental

2.1. Reagents

Glucose oxidase (GOx) (EC 1.1.3.4) type X-S (Aspergillus niger, 181, 800 U/g), β-D(+)-glucose, and phosphate buffer saline (PBS, pH 7) were obtained from Sigma. Pyrrole was purchased from Merck and was purified by distillation and kept cool in dark prior to use. $(C[6]S)^{-6}$ was obtained from Acros Organics. All other chemical substances were of analytical reagent grade. The glucose solution was prepared in distilled water and it was kept at room temperature for 24 h before using in order to make sure of the existence of the β-D-glucose formation.

2.2. Instrumentation

The electrochemical polymerizations were carried out in a one compartment three electrodes electrochemical cell connected to a μ Autolab typeIII electrochemical system. The working electrode was a platinum disk electrode (2 mm diameter). A platinum plate electrode was used as the auxiliary electrode. All potentials were referred to a KCl (3 M) Ag/AgCl electrode. During amperometric measurements, homogenization of the solutions after the addition of glucose was done by a stirring bead and magnetic stirrer. The scanning electron microscopy and energy dispersive X-ray spectroscopy were carried out using an electron microscope (VEGA//TESCAN).

2.3. Preparation of the enzyme electrode

Pyrrole was electropolymerized by galvanostatic method because this technique results in a better and more porous polymer and consequently more sensitive enzyme electrode (Hämmerle et al., 1992). Also, by the use of this method, the rate of polymerization is controlled (Wallace et al., 1999).

Before polymerization, the working electrode was polished with 0.3 μm alumina powder, rinsed with distilled water, and cleaned electrochemically by cycling the potential within the range of −0.2 to 1.4 V for 25 cycles in H_2SO_4 (1 M) until the stable state was attained. Then it was washed with ethanol and distilled water again and dried at room temperature. After this stage, the polymerization was done at a constant current density of 0.3 mA/cm^2 for 90 s using a solution of pyrrole (0.05 M) in: (a) an acetonitrile solution containing $LiClO_4$ (0.1 M); (b) an aqueous solution containing $(C[6]S)^{-6}$ (1×10^{-3} M) as dopant anion, and $LiClO_4$ (0.05 M) as supporting electrolyte; and (c) an aqueous solution containing $LiClO_4$ (0.1 M). Right after the film was deposited; each electrode was washed with PBS and dried at room temperature.

The GOx was immobilized by cross-linking via glutaraldehyde solution 0.1% on the Ppy films (Shirale et al., 2006; Gaikwad et al., 2006). In order to immobilize the GOx by cross-linking, 5 μL of glutaraldehyde was carefully placed on the Pt/Ppy electrode. Then 5 μL of the GOx (from a 6-mg/ml solution) was adsorbed. After immobilization of the enzyme, each enzyme electrode (Pt/Ppy/GOx) was carefully washed with distilled water and kept at 4 °C in PBS (pH 7.0) until applying in the amperometric measurements.

2.4. Amperometric measurements of the glucose

The Pt/Ppy/GOx electrode was held at 0.7 V versus Ag/AgCl/KCl (3 M) in PBS (pH 7.0) and over oxidized in order to stabilize the background current. The volume of the sensing electrolyte was 5 ml, and the temperature was kept at room temperature (25 ± 1 °C). The amperometric measurements of the glucose sensing were done at the same potential in O_2-saturated phosphate buffer in which aliquots of the glucose solution (1 M) were added.

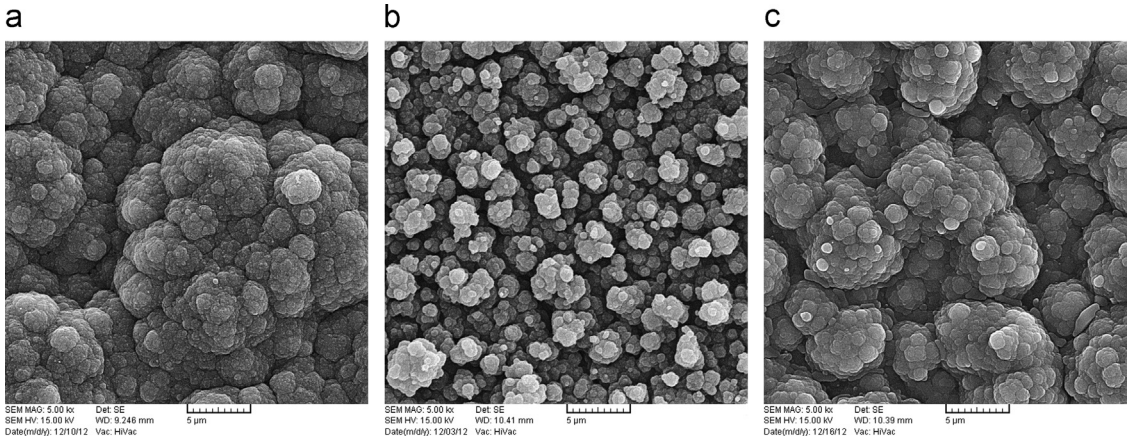

Fig. 1. Scanning electron microscopy images of the polypyrrole grown during 90 s with current density o.3 mA/cm^2 in: (a) acetonitrile containing $LiClO_4$ (0.1 M) ; (b) an aqueous solution containing $LiClO_4$ (0.05 M) and $(C[6]S)^{-6}$ (1×10^{-3} M); (c) an aqueous solution containing $LiClO_4$ (0.1 M).

3. Results and discussion

3.1. Galvanostatic studies of the electropolymerization

The results of the galvanostatic studies are shown in supplementary information.

3.2. The elemental analysis study

The elemental composition of the Pt/Ppy-$(C[6]S)^{-6}$ electrode was checked using energy dispersive X-ray spectroscopy (EDX) facilities (Fig. 2S in supplementary information). The presence of the peaks related to S and Cl confirm that both $(C[6]S)^{-6}$ and $[ClO_4]^-$ have been successfully incorporated into the film.

3.3. Surface morphology of the Pt/Ppy electrodes

Surface morphology of the Ppy films was studied by scanning electron microscopy (SEM) (Fig. 1). The results of the SEM show that the Ppy-$(C[6]S)^{-6}$ (Fig. 1b) has the most porous structure with a quite good uniformity. It seems that the cavitary structure of the calixarene with poly sulfonated anion caused cross-linked and microporous structure of the polymer (Ingram et al., 2004; Suematsu et al., 2000). Other SEM images (Fig. 1a, c) have cauliflower structures, but the one synthesized in acetonitrile shows a more compact structure with less porosity.

3.4. Amperometric responses of the Pt/Ppy/GOx electrodes

The sensitivity of each enzyme electrode and its capability in measuring the glucose concentration was estimated by the electrochemical analysis of H_2O_2 formed from glucose oxidation in the presence of GOx (Shirale et al., 2006).

The amperometric responses of the Pt/Ppy/GOx electrodes to glucose were measured at 0.7 V, and the average steady state current density was recorded at each step of glucose concentrations (Fig. 3S in supplementary information).

3.4.1. Evaluation of the calibration curves

The calibration curves (Fig. 2) display the steady state current density as a function of the glucose concentration. The calibration curve of each electrode (Fig. 2) becomes non-linear at high glucose concentrations and also shows a non linearity at the lowest values of glucose concentrations. The slopes of the linear limits show the lowest sensitivity (31.60 μA M^{-1} cm^{-2}) for the third enzyme electrode (Fig. 2c). The sensitivities of the two other enzyme electrodes (Fig. 2a, b) are approximately the same (79.30 and 79.60 μA $M^{-1}cm^{-2}$ respectively) and are improved more than two times than that of the third enzyme electrode. This could be attributed to the role of calixarene as a large anion (Brânzoi et al., 2007), and acetonitrile as an organic solvent (Deshpande and Amalnerkar, 1993; Otero and Aréνalo, 1994) in enhancing electrical conductivity of the Ppy film. It is expected that increasing the conductivity of the polymer facilitates the electron transfer between the polymer and the enzyme, so the sensitivity of the biosensor would grow higher.

Another observation from the calibration curves is that the Pt/Ppy-$(C[6]S)^{-6}$/GOx electrode shows a linearity up to 190 mM in comparison with 61 mM and 80 mM for the electrodes made using calixarene free acetonitrile and aqueous solutions respectively. It seems that this observation to be resulted from the cavitary structure with poly sulfonated anions of the $(C[6]S)^{-6}$ and the resulted porous films. These properties could enhance the enzyme loading and also diffusion of the glucose to the active sites of the enzyme. Along with the porosity of the film, the phenol groups in a cyclic array at lower rim of the calixarene molecules can produce an ideal site for selective bindings with a compatible guest molecule (such as an enzyme) via hydrogen bindings (Chan et al., 1997). Therefore, $(C[6]S)^{-6}$ prevents the immobilized enzyme from leaching. So that it leads to an increase in loading the enzyme and also stability of the biosensor.

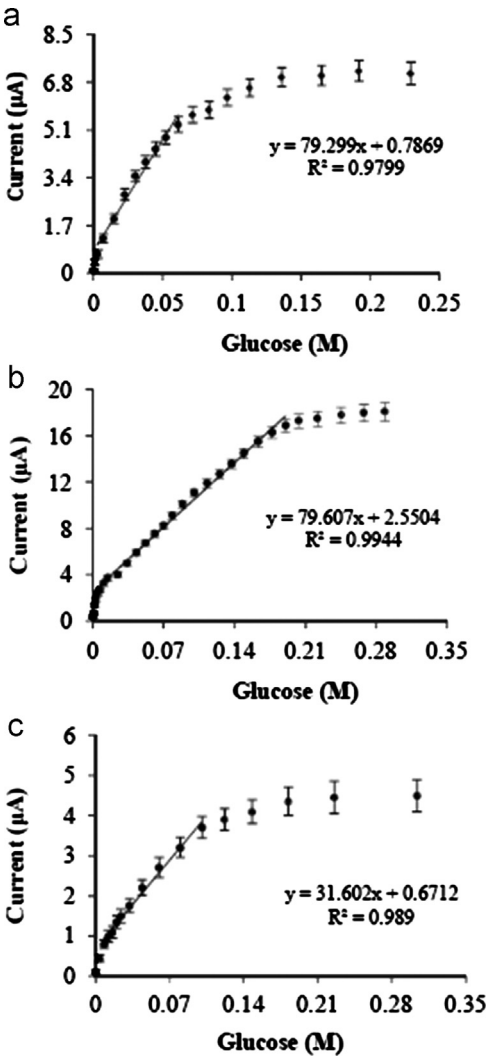

Fig. 2. Calibration curves of the enzyme electrodes mentioned in Fig. 1 ($n=3$).

The comparison of the two other calibration curves with each other (Fig. 2a, c) shows a smaller limit of linearity for the electrode synthesized in acetonitrile than that for the other one. It seems that more compact structure of the films leads to less enzyme loading or less diffusion of the glucose to the active sites of the enzyme.

3.4.2. Michaelis–Menten behavior of the Pt/Ppy/GOx electrodes

In order to give a better explanation on the electrode sensitivity, it is more appropriate to investigate the kinetics parameters in an enzymatic reaction. For this purpose, it is assumed that in the reaction of the immobilized GOx with glucose, the amperometric currents exhibit Michaelis–Menten behavior with respect to glucose concentrations [S] (Eq. (1)).

$$I = \frac{I_{max}[S]}{K'_m + [S]} \quad (1)$$

where I, I_{max}, and K'_m are represented as the steady-state current density, the apparent maximum current density at saturated glucose

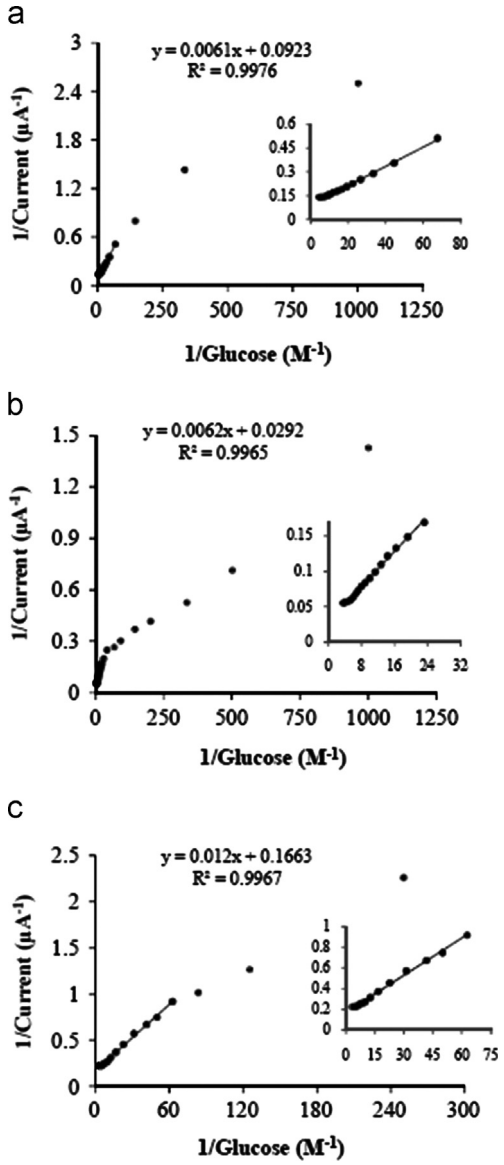

Fig. 3. Line weaver-Burk type plots of the enzyme electrodes mentioned in Fig. 1. Inset: zooms of linear limits.

concentration, and QUOTE the apparent Michaelis–Menten constant, respectively (Wang et al., 2005).

Fig. 3 shows the lineweaver–burk plots which are derived from Eq. (1). Although a fine correlation is expected to be observed, the plots deviate from Michaelis–Menten behavior at high glucose concentrations and also at the lowest values of the glucose concentrations (see inserts in Fig. 3). Values of K'_m and I_{max} for each Pt/Ppy/GOx electrode were estimated by using Eq. (1) (Table 1S in supplementary information). The results from the Table 1S show that the Pt/ Ppy-$(C[6]S)^{-6}$/GOx electrode has the highest K'_m and I_{max}. Whearas, according to Michaelis–Menten behavior for free enzymes in homogenous systems, it is expected that less values of K_m to be observed for an enzyme with higher sensitivity. Therefore the enhanced sensitivity of the enzyme electrode in this study cannot be exactly explained by the values of Michaelis–Menten constant.

Deviation from Michaelis–Menten behavior has been described by Barlett and Pratt. They showed that diffusion coefficient of the substrate should be considered in this issue (Bartlett and Pratt, 1993). However, in most of the times, this effect is ignored and therefore the simple Michaelis–Menten equation is used (Olea et al., 2008). If it is presumed that all H_2O_2 is oxidized at the electrode and GOx activity is not influenced by the immobilization process, the higher current response in amperometric analysis can be attributed to the higher sensitivity of the electrode. Also higher maximum current density, I_{max}, can be resulted from higher loading of the enzyme (Wang et al., 2005).

4. Conclusion

In this study we showed that using parasulfonato calix[6]arene (as a large cavitary dopant anion) in aqueous solution, and acetonitrile (as an organic solvent) during polymerization of pyrrole, enhances the electrode sensitivity at the same level in comparison with a calixarene free aqueous solution. Whereas, increasing the enzyme loading is just observed in the presence of calixarene anion. Therefore, the role of the anion dopant could be more effective than the solvent in improving the capability of the enzyme electrode. In other words, using of an aqueous solvent along with a large anion like calixarene is more effective in improving the enzyme capability than using an organic solvent containing a small anion.

Also, the results of the calibration curves confirmed that loading the enzyme on the electrode, regardless of the enzyme electrode sensitivity, is mainly influenced by its porosity.

Finally, using an organic solution containing a large anion like para sulfonato calix[*n*]arene could be the future study that we propose in the hope of improving the capability of the enzyme electrode even more.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.04.043.

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