



One-pot construction of mediatorless bi-enzymatic glucose biosensor based on organic–inorganic hybrid

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ABSTRACT

A new methodology for the fabrication of bienzymatic amperometric glucose biosensor based on the use of an organic–inorganic hybrid is presented. The fabrication involves a self-assembly directed one-pot electrochemical process. Bi-enzymes, horseradish peroxidase (HRP) and glucose oxidase (GOx) are immobilized into the porous and electroactive silica–polyaniline hybrid composite through electrochemical polymerization of N[3-(trimethoxysilyl)propyl]aniline in the presence of enzymes. The modified electrode is designated as PTMSPA/HRP–GOx. The direct electron transfer of HRP is achieved at the modified electrode. Also, the electrode exhibits excellent bio-electro-catalytic activity for the reduction of hydrogen peroxide. The response current at PTMSPA/HRP–GOx modified electrode revealed a good linear relationship with concentration of glucose range between 1 and 20 mM with a response time of 7 s. Thus, the modified electrode shows the combined advantages of polyaniline and silica networks through synergistic influence from the individual components. The PTMSPA assembly has shown the potential for a third generation amperometric biosensor.

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1. Introduction

The quantification of glucose is one of the most popular and well-known biosensor applications (Wang, 2008; Santhosh et al., 2009a,b; Manesh et al., 2008a). Electrochemical biosensors have gained increasing interest due to their intrinsic sensitivity. Research activities on glucose oxidase (GOx) based amperometric sensors attract continuous interest owing to their importance in the field of glucose monitoring and quantification (Wang, 2008). The detection of glucose in such sensors is often based on electrochemical oxidation of hydrogen peroxide (H_2O_2), product of the enzymatic reaction. However, the main drawback in this approach is the high over-potential required for the oxidation of H_2O_2 . At this high potential, endogenous or exogenous compound such as uric acid, ascorbic acid and acetaminophen commonly present in biological samples can be electrochemically oxidized leading to a high level of interference for the quantification of the analyte (Santhosh et al., 2006a). To overcome these problems, several approaches have been proposed. One promising method to suppress the interference and improve the sensitivity of the sensors

involves the use bi-enzymes—oxidase and peroxidase. In these bi-enzymes based biosensors, GOx acts as the source of substrate for the second enzyme horseradish peroxidase (HRP), producing H_2O_2 from oxidation of glucose in presence of oxygen. In these systems, the H_2O_2 produced by GOx is reduced by HRP. The enzyme and the analyte are electrically connected to the electrode by a freely diffusing mediator (Matsumoto et al., 2002) or directly attached at the electrode surface (Dai et al., 2008). Cascade schemes, where an enzyme is catalytically linked to another enzyme can produce signal amplification and therefore increase biosensor efficiency.

Generally, the bi-enzymes based electrodes have been fabricated by entrapping the enzymes within a polymer layer (De Benedetto et al., 1996). Besides the polymer layer, several other matrices, such as hydrogels, resins, layered double hydroxides, and so on have been used for the immobilization of enzymes. The various factors such as biocompatibility, electro-catalytic performance and loading capability of the matrix are to be considered and optimized (McMahon et al., 2006).

Mesoporous silica-based materials are well suitable for enzyme immobilization due to their large surface area, biocompatibility and adaptable surface immobilization mechanism (Wang, 1999; Wu et al., 2007). Silica matrices are highly porous with additional features such as physical rigidity, chemical and biological inertness. Moreover, their particle size and morphology could be controlled by the method of preparation. Sol–gel methods have been proved

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to be an effective way to immobilize biomolecules (Coradin and Livage, 2007; Takahashi et al., 2001). Also, sol–gel network induces stabilizing properties to the enzymes from the high viscosity and restricted mobility inside the matrix. The stabilization process is mediated through conformational flexibility limitation for the protein within the rigid framework as well the generation of a protective microenvironment (Zheng and Brennan, 1998). Enzymes have been successfully encapsulated into sol–gel matrices by physical adsorption (Lei et al., 2002), covalent binding (DeLouise and Miller, 2004) and reverse micelle techniques (Schuleit and Luisi, 2001).

In recent years, silica-based organic hybrid materials gain interest as a matrix for enzyme immobilization owing to the synergy resulting from the combination in one material of the intrinsic properties of the two components (Walcarius, 2001). These materials were prepared by co-condensation reactions of organic silanes (MacLachlan et al., 2000) or post-grafting of functional molecules onto the surface of silica (Moller and Bein, 1998) or non-covalent interactions (Lim and Stein, 1999). Generally, preparation of silica-conducting polymer hybrids involves initial modification of silica walls with organic functional molecules to obtain the precursor. In the subsequent step, the functional groups in the modified silica are used to nucleate the conducting polymer chains within the channels (pore) of mesoporous silica (Lee et al., 2005).

In most of these reports on sol–gel electrodes, the activity/sensitivity was tapered, due to phase separation, cracking and low adhesion to the electrode surface (Narang et al., 1994). Hence, it becomes highly demanding to develop new fabrication protocol for imparting the over all performance of bi-enzyme electrodes. We present here a one-pot electrochemical methodology for the fabrication of bi-enzymes based biosensor and minimizes the tediousness in the multi-step procedures (Dong et al., 2009; Alvaro et al., 2002). The work involves the fabrication of a new amperometric enzyme electrode for the detection of glucose through immobilization of bi-enzymes (HRP and GOx) in a poly(N[3-(trimethoxysilyl)propyl]aniline) (PTMSPA) matrix. Mesoporous silica-organic hybrids are well suitable to entrap enzymes. However, there is mismatching between the pore diameters in the silica pores and size of enzyme molecules. Generally, the pore diameter of silica is comparable or far smaller than the size of the enzyme molecules (Hecht et al., 1993; Ispas et al., 2009; Kim et al., 2006). Enzyme entrapment was limited in mesoporous silica due to the small pore size and/or repulsive interaction between the negative charges on the surfaces of silica and the enzyme molecules. Herein, we have chosen ‘ship-in-bottle’ approach to load the enzyme molecules (HRP and GOx) within PTMSPA matrix.

Electrochemical polymerization of (N[3-(trimethoxysilyl)propyl]aniline) (TMSPA) was performed in the presence of the enzymes (HRP and GOx) to result PTMSPA/HRP-GOx composite. In this one-pot fabrication, the enzymes are in situ entrapped into the sol–gel PTMSPA matrix. The advantage of having PANI for the development of biosensor lies in its usefulness as an enzyme entrapment matrix, coupled with its physicochemical transducer characteristics to convert a biochemical signal into an electrical signal resulting into signal amplification and elimination of electrode fouling (Lee et al., 2009; Santhosh et al., 2006b). Moreover, due to the ability of PANI to bind biomolecules, the two enzymes became electrostatically or hydrophobically adsorbed on the electrode surface and because of the nature of this immobilization; it was assumed that the distribution of the enzyme molecules over the surface was equal. In the present investigation, the use of PTMSPA/HRP-GOx modified electrode as glucose biosensor has been demonstrated.

2. Experimental

2.1. Reagents used

N[3-(Trimethoxysilyl)propyl]aniline (TMSPA), β -naphthalene sulfonic acid (β -NSA), hydrogen peroxide, D-(+)-glucose, horseradish peroxidase (HRP) (E.C 1.11.1.7, ≥ 250 U mg⁻¹, from Horseradish), glucose oxidase (GOx) (EC 1.1.3.4, 256 U mg⁻¹, from *Aspergillus niger*), dopamine, uric acid and ascorbic acid were of analytical grades from Sigma and used as received. Double-distilled water was used throughout the experiments. Aqueous solutions of the analytes were prepared in 0.1 M phosphate buffer saline (PBS; pH 7) afresh at the time of experiments.

2.2. Electrochemical measurements

Electrochemical measurements were performed by using EG&G PAR 283 Potentiostat/Galvanostat with Frequency Response Analyzer 1025. Ag/AgCl (diameter: 4 mm) and platinum wire (diameter: 0.25 mm) were used as reference and counter electrodes, respectively. Indium-doped tin oxide (ITO)-coated glass plate of 1 cm² (1 cm $\times$ 1 cm) area and platinum disc (2 mm diameter) were used for making the sensor electrodes. Before performing each of the experiment, the ITO plate was rinsed with acetone and washed with distilled water.

2.3. Fabrication of PTMSPA/HRP-GOx modified electrode

Electrochemical polymerization of TMSPA and simultaneous enzymes immobilization were performed (in a 2 mL cell) by the application of a fixed potential (potentiostatic) +1.0 V for 10 min. Typically, a solution of TMSPA (50 mM) in β -NSA (1 M) containing HRP and GOx was electropolymerized by applying a constant potential of +1.0 V to fabricate the PTMSPA/HRP-GOx modified electrode. Electrodes were fabricated with solutions containing the two enzymes of different mass ratios (1:9 to 9:1) with a total concentration of 10 mg mL⁻¹. Cyclic voltammetry and hydrodynamic voltammetry experiments were performed with 1.5 mL PBS (in a 2 mL cell).

In a similar condition, electropolymerization of TMSPA was performed in the presence of HRP (without GOx) and the PTMSPA/HRP modified electrode was fabricated.

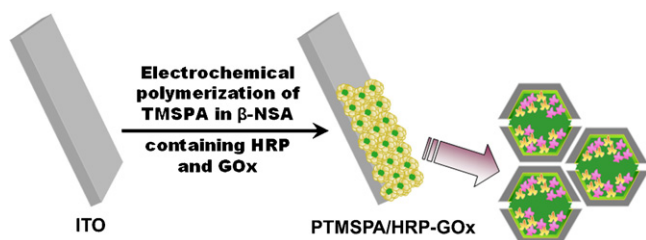
2.4. Characterization

The morphology of the modified electrode was examined by field emission scanning electron microscopy; (FESEM, Hitach-530) and high resolution transmission electron microscopy (HRTEM, Jeol JEM-ARM1300S, Japan). Nitrogen adsorption–desorption isotherms (Brunauer–Emmett–Teller) measurements were carried out using a Quantachrome Autosorb-1 with nitrogen as adsorbate at 77 K. The samples were degassed for 2 h in a vacuum before measurement.

3. Results and discussion

3.1. Fabrication of modified electrode

The fabrication of the biosensor involves electrodeposition of sol–gel TMSPA with simultaneous co-immobilization of HRP and GOx (Scheme 1). In the electrodeposition process, the application of +1.0 V (vs. Ag/AgCl) in the 1 M β -NSA solution containing 50 mM TMSPA results in the oxidation of $-NH_2$ group in TMSPA and initiates the polymerization of aniline units. Meanwhile, the sequential processes, such as hydrolysis of $-OCH_3$ group, condensation of $-OH$ and polycondensation occur to result sol–gel PTMSPA. The sol–gel



Scheme 1. One-pot electrochemical fabrication of PTMSPA/HRP-GOx modified electrode.

process is accompanied by the encapsulation of the two enzymes, HRP and GOx.

The mechanism for the formation of PTMSPA is detailed. The electrolyte, β -NSA with its amphiphilic character forms spherical micelles in aqueous solution. The electrostatic interactions between the $-\text{NH}_2$ group in TMSPA and $-\text{SO}_3^-$ group in β -NSA induces self-assembly of TMSPA molecule in the spherical micelles environment. The self-assembled TMSPA/ β -NSA structure was subjected to electropolymerization by the application of +1.0 V. As a result, spherical particles of PTMSPA are formed, in which silica network generates pore structure for PTMSPA. The porous nature of PTMSPA was further evident by N_2 adsorption and desorption measurement (discussed later). It should be noted that the bi-enzymes, HRP and GOx were concurrently immobilized into the silica–polyaniline hybrid composite during the electrochemical process. PTMSPA has the silicate network as well as the covalently linked PANI chains within silica framework. This one-pot methodology used for the fabrication of PTMSPA/HRP-GOx modified electrode avoids the tediousness in the multi-step procedures that are generally reported (Dong et al., 2009; Luo et al., 2007).

3.2. Characterization

3.2.1. Morphology

The morphology of the PTMSPA was studied using field emission scanning electron microscopy, FESEM (Fig. 1). The particles are spherical and stacked on top of each other. The particle diameters are in the range from 0.5 to 3.0 μm . The average diameter of the particle is estimated to be $\sim 1 \mu\text{m}$. An amorphous region is observed (through HRTEM-figure not shown) at PTMSPA, which is due to the existence of amorphous PANI within the pores of silica. Also, HRTEM image shows the presence of well-defined, uniform and ordered nanometer scale pores similar to MCM-41 (Mobile Crystalline Material-41) type porous channel structure of nanospheres (Zhou, 2000). Thus, the FESEM images reveal the presence of both silica network and PANI chains in PTMSPA matrix.

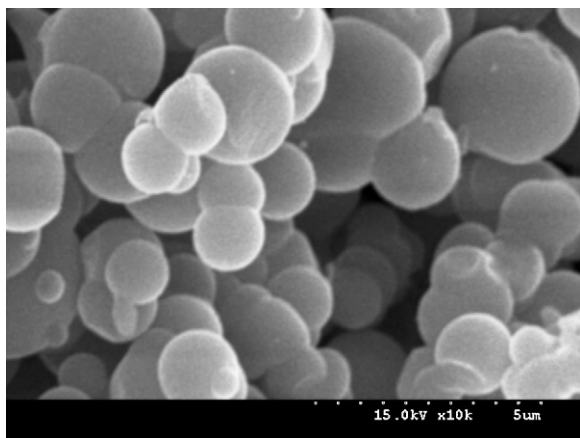


Fig. 1. FESEM image of PTMSPA/HRP-GOx modified electrode.

3.2.2. Electrochemical impedance spectroscopy (EIS)

EIS was performed to characterize the interfacial properties of the modified surfaces (Santhosh et al., 2006b). Fig. S1 (see Supplemental information) shows the impedance spectra recorded at various electrodes (ITO, ITO/PTMSPA, ITO/PTMSPA/HRP and ITO/PTMSPA/HRP-GOx) in the solution containing $\text{K}_3\text{Fe}(\text{CN})_6$ and $\text{K}_4\text{Fe}(\text{CN})_6$ (1 mM each) and 0.1 M KCl. One could observe distinct differences in the impedance spectra. ITO exhibits virtually straight line, a typical characteristic of a diffusion limiting step of the electrochemical process, however, an increase in the R_{ct} (charge transfer resistance) was observed when ITO was modified with PTMSPA matrix. Both interfaces, PTMSPA/HRP and PTMSPA/HRP-GOx show impedance characteristics with a semicircle at higher frequency corresponding to the charge transfer and a linear part due to diffusion limiting step. R_{ct} was found to be higher for PTMSPA/HRP-GOx as compared to PTMSPA. The enzymes are expected to block the electron transfer of the redox probe at the PTMSPA modified electrode. Such an increase in the R_{ct} signifies the incorporation of the biomolecules into PTMSPA matrix.

3.2.3. N_2 adsorption/desorption isotherms

The N_2 adsorption–desorption isotherms were measured at PTMSPA and PTMSPA/HRP-GOx in order to corroborate the successful immobilization of enzymes during the electrochemical process. The N_2 adsorption–desorption isotherm of PTMSPA (prepared without enzymes) shows a typical reversible type IV adsorption (as defined by IUPAC). PTMSPA exhibits H1 type narrow hysteresis loop with a change in adsorption around $P/P_0 = 0.20$ that are characteristics of materials having uniform pores with open cylindrical geometry (Kruk et al., 2000). PTMSPA has a pore diameter of 2.4 nm, a pore volume of $16.1 \times 10^{-3} \text{ cm}^3 \text{ g}^{-1}$ and a surface area of $40.0 \text{ m}^2 \text{ g}^{-1}$ (determined from BET isotherm and BJH method). The pore size (2.4 nm) and wider wall thickness (6.11 nm) indicate that the PANI chains are formed within the silica pores. On the other hand, after the immobilization of enzymes, PTMSPA/HRP-GOx exhibited H1 type narrow hysteresis loop and have lesser surface area ($21.8 \text{ m}^2 \text{ g}^{-1}$). The decrease in the surface area of PTMSPA/HRP-GOx signifies that enzymes (HRP and GOx) were in situ entrapped/immobilized into PTMSPA matrix during the electrochemical polymerization (Dai et al., 2008).

Prior to the assessment of influence of spatial arrangement of the two enzymes (HRP and GOx) on the performances of the modified electrode for glucose detection, we performed several experiments to characterize the electrochemical response of mono-enzyme modified electrode (containing only HRP) towards the detection of H_2O_2 . The experimental conditions for the fabrication and performance of the modified electrode were thoroughly investigated. Elaborate optimization experiments were done with PTMSPA/HRP modified electrode. These studies have been performed keeping into consideration that the ultimate performances of the PTMSPA/HRP-GOx modified electrode towards electrochemical detection of glucose would depend on the sensitivity of electrochemical detection of H_2O_2 at PTMSPA/HRP assembly.

3.3. Electrochemical characteristics of PTMSPA/HRP modified electrode

3.3.1. Cyclic voltammetric studies

The electrochemical characteristics of the PTMSPA/HRP modified electrode were investigated by cyclic voltammetry. Fig. 2(ii) shows the cyclic voltammogram recorded at PTMSPA/HRP modified electrode in PBS (1.5 mL) at a scan rate of 10 mV s^{-1} . In this potential region, the PTMSPA/HRP modified electrode displays a pair of well-defined peaks at -152 mV (E_p^c) and -107 mV (E_p^a) that correspond to the redox peaks of the redox reaction of the active site ($\text{HRP-Fe}^{3+/2+}$) (Tong et al., 2007). The CV of PTMSPA elec-

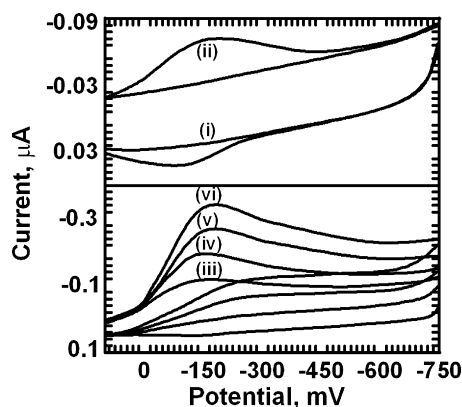


Fig. 2. Cyclic voltammograms of (i) PTMSPA and (ii) PTMSPA/HRP modified electrodes in PBS (pH 7); CVs of different concentration of H_2O_2 at PTMSPA/HRP modified electrode in PBS; (iii)–(vi) 0.2–0.8 mM H_2O_2 ; scan rate: 10 mV s^{-1} .

trode (without HRP) in PBS did not show any redox peak in this potential region (Fig. 2(i)). Thus, it is apparent that PTMSPA/HRP could facilitate direct electron transfer (DET) between HRP and the electrode (Wang et al., 2009). The silica environment surface provides biocompatibility and favorable microenvironment for HRP to preserve the native structure and exchange electrons directly with the electrode. The anodic and cathodic peak current values were found to show linearity with the scan rates between 10 and 150 mV s^{-1} . Also, E_p^c and E_p^a values were shifted to the negative and positive directions, respectively, while increasing the scan rates (Fig. S2 (see Supplemental information)). These results demonstrate that DET of HRP at PTMSPA/HRP modified electrode is a surface-confined and quasi-reversible process. The DET at PTMSPA/HRP modified electrode is prominent as compared to HRP-chitosan (Huang et al., 2002) and HRP-gluten (Liu and Hu, 2003) modified electrodes. The lower peak separation signifies that HRP molecules have performed orientation in PTMSPA environment to make effective DET from the active site to the electrode.

Voltammograms were then recorded to realize the bio-electrochemical catalytic reduction of H_2O_2 at PTMSPA/HRP modified electrode. The electrochemical behavior of various electrodes (Pt, ITO, PTMSPA and PTMSPA/HRP) for the reduction of 0.5 mM H_2O_2 in PBS was compared and presented (Fig. S3 (see Supplemental information)). PTMSPA/HRP modified electrode shows an enhanced current response as compared to the aforementioned electrodes. The current response of PTMSPA/HRP modified electrode is approximately 10 times higher than PTMSPA electrode. CV recorded at PTMSPA/HRP modified electrode in presence of H_2O_2 (0.2 mM) in PBS is shown in Fig. 2(iii). A significant increase in the cathodic peak current (at -150 mV) with almost complete disappearance of oxidation peak current was observed. The result is in accordance to a typical bio-electrochemical catalytic reduction process of H_2O_2 . The peak current at -150 mV was found to increase linearly with increase in H_2O_2 concentration between 0.2 and 0.8 mM (Fig. 2B(iii–vi)).

3.3.2. Hydrodynamic amperogram

The most appropriate working potential suited for the bio-electrochemical catalytic reduction of H_2O_2 at the PTMSPA/HRP modified electrode was further ascertained by hydrodynamic measurements. Dynamic current responses for the successive addition of $100 \mu\text{M}$ H_2O_2 at PTMSPA/HRP modified electrode in PBS (1.5 mL) were recorded under various potentials between -200 and $+50 \text{ mV}$ (by 50 mV increments) (Fig. 3). The response current was negligible at $+50$ and 0 mV . However, an increase in cathodic current was noticed from -50 to -200 mV . A highest current response was observed at -150 mV . It can be seen that, the

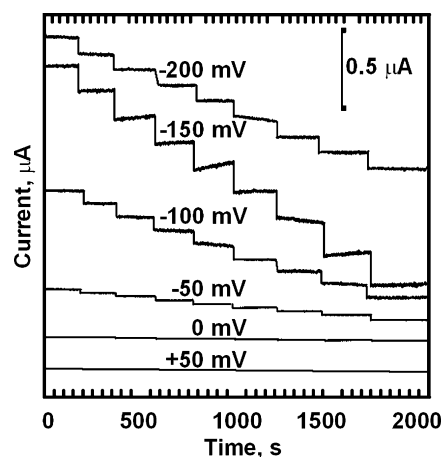


Fig. 3. Hydrodynamic amperometric responses of PTMSPA/HRP modified electrode to the successive addition of $100 \mu\text{M}$ H_2O_2 at various applied potentials (-200 to $+50 \text{ mV}$ vs. Ag/AgCl) in PBS at a rotation rate of 1700 rpm.

amperometric response currents decreased at the working potential beyond -150 mV . Interestingly, no apparent reduction current was observed between -200 and $+50 \text{ mV}$ for PTMSPA electrode (in the absence of HRP). Hence, a working potential of -150 mV vs. Ag/AgCl was chosen for subsequent investigations to achieve high sensitivity and also to minimize possible interferences in the biosensing of H_2O_2 (Lei and Deng, 1996).

3.3.3. Optimization of experimental variables

3.3.3.1. Influence of pH. The influence of pH on the current response for the detection of 0.5 mM H_2O_2 at the PTMSPA/HRP modified electrode was studied in the pH between 5 and 9, Fig. S4 (see Supplemental information). The current value reaches its maximum at pH 7. This indicates that the PTMSPA matrix did not alter the optimum pH for redox activity of the immobilized HRP.

3.3.3.2. Effect of film thickness on enzyme loading. The thickness of PTMSPA film was varied and their influence on the loading of HRP was followed. The film thickness was determined from the anodic charge, which was involved in the first anodic scan of the cyclic voltammogram run in 0.5 M H_2SO_4 solution (Santhosh et al., 2007). The amount of immobilized HRP was estimated through UV–vis spectroscopy (Santhosh et al., 2009b). Fig. S4 (see Supplemental information) shows the influence of film thickness on the loading of HRP into the PTMSPA matrix. A gradual increase in HRP loading with increase in thickness (up to $4 \mu\text{m}$) of PTMSPA film was witnessed. Thereafter, increase in the thickness of PTMSPA does not have any significant influence on the loading of HRP. Hence, film thickness of about $4 \mu\text{m}$ was selected for further studies.

3.3.3.3. Effect of HRP loading. The PTMSPA/HRP modified electrode loaded with different amount of HRP was tested for the electrochemical detection of 0.5 mM H_2O_2 (Fig. S4 (see Supplemental information)). A steady increase in the current response was noticed for an increase of HRP loading (up to 65%; w/v, based on the wt% of HRP loaded to the volume of electrolyte used in the preparation of PTMSPA/HRP modified electrode). Beyond this, the current did not change much. Hence, a loading of 65% HRP was chosen for the fabrication of the modified electrodes.

3.3.3.4. Influence of temperature. The effect of temperature on the current response for 0.5 mM H_2O_2 at PTMSPA/HRP modified electrode was investigated in the temperature range of 280 – 350 K . The current response showed an increasing trend up to 320 K and decreased thereafter (Fig. S4 (see Supplemental information)). The

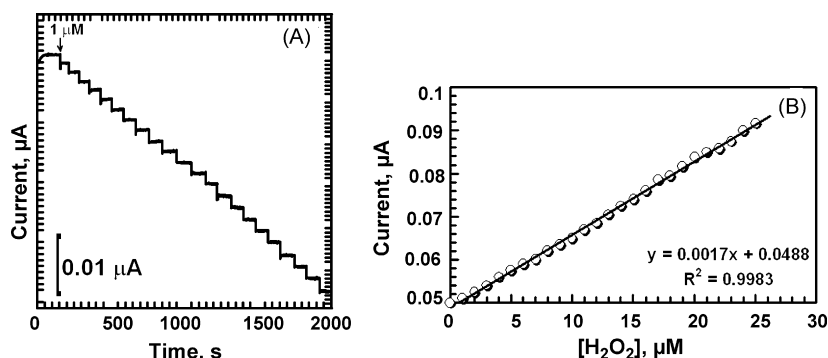


Fig. 4. (A) Amperometric *i*-*t* responses of PTMSPA/HRP modified electrode to the successive addition of 1 μM H₂O₂ at the potential of -150 mV in PBS; (B) calibration curve.

decrease in current response at the elevated temperatures may be due to the denaturation of HRP. The dependence of current response on temperature (280–325 K) is evaluated through the Arrhenius relationship,

$$I(T) = I_0 \exp \left(\frac{-E_a}{RT} \right)$$

where $I(T)$ represents the current value at a specified temperature T , I_0 is proportional factor, R is gas constant, T is temperature in Kelvin, and E_a is the energy of activation. E_a for the overall electrochemical process of enzymatic detection of H₂O₂ at PTMSPA/HRP modified electrode was determined from the slope of the plot, $\ln I$ vs. $1/T$ as 2.52 kJ mol⁻¹. Previously, for the enzymatic detection of H₂O₂, much higher E_a values (14.84 and 21.9 kJ mol⁻¹) has been reported for HRP immobilized in a hydrogel and chitosan/silica hybrid film electrodes, respectively (Sun et al., 2007a; Tan et al., 2005). The lower value of E_a , as noticed in the present study, signifies an efficient enzyme activity of the modified surface leading to fast enzymatic reaction. Though the current response at PTMSPA/HRP modified electrode is maximum at 320 K, by considering the convenience of the practical application, measurements were performed at 298 K.

3.3.4. Amperometric studies

Under the chosen (optimum) conditions, the amperometric responses of PTMSPA/HRP modified electrode (at -150 mV vs. Ag/AgCl) for successive addition of 1.0 μM H₂O₂ were recorded in a stirred PBS (1.5 mL) (Fig. 4A). The PTMSPA/HRP modified electrode responds effectively to the addition of H₂O₂ through current signals within 2 s. The calibration curve showing the current response for various concentration of H₂O₂ is presented in Fig. 4B. A linear increase in current signals between 1.0 and 25 μM H₂O₂ can be

seen (Fig. 4B), which has a sensitivity of 1.7 mA M⁻¹ (R^2 : 0.9983). Successive addition of 10 μM H₂O₂ ($n=5$) showed a relative standard deviation (R.S.D.) of 2.16% signifying the good reproducibility at the modified electrode under this hydrodynamic condition.

Fixed potential amperometry was also performed at PTMSPA/HRP modified electrode for a series of H₂O₂ solutions over the range 0.05–0.5 mM in the quiescent PBS (Fig. 5A). Current measurements were made after 30 s and the corresponding calibration plot is shown in Fig. 5B. The response current shows a good linear relationship with the concentration of H₂O₂ over 0.05–0.5 mM with the sensitivity of 0.84 mA M⁻¹ (R^2 : 0.9875) (Fig. 5B). A plateau in current response was observed for H₂O₂ concentration beyond 0.5 mM. This signifies the operation of the Michaelis–Menten kinetic mechanism for the enzyme-catalyzed process.

3.4. PTMSPA/HRP-GOx as a glucose sensor

Results from the electrochemical studies at PTMSPA/HRP modified electrode clearly demonstrated that the electrode can be used as an electron transducer for the quantitative determination of H₂O₂. Hence, we have fabricated a glucose biosensor with co-immobilization of GOx (and HRP) in the PTMSPA matrix. The working principle of PTMSPA/HRP-GOx modified electrode towards detection of glucose is depicted in Scheme 2. Glucose is transferred from bulk solution to the PTMSPA/HRP-GOx modified electrode through diffusion and GOx in the presence of the natural co-substrate O₂, converts glucose to gluconic acid and O₂ to H₂O₂. The H₂O₂ thus produced from the enzymatic reaction serves as substrate for HRP and the oxidized state of HRP is in turn recycled at the electrode surface. PTMSPA serves as an electron transducer. Thus, the amount of glucose is quantitatively estimated at PTMSPA/HRP-GOx modified electrode.

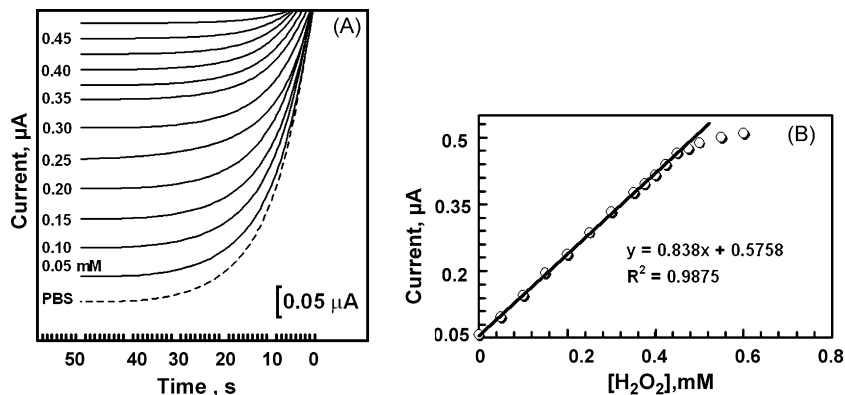
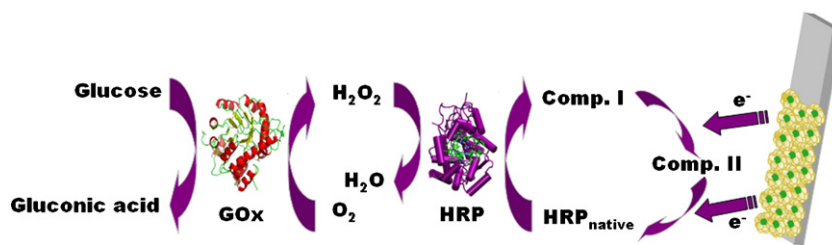


Fig. 5. (A) Amperograms obtained at PTMSPA/HRP modified electrode in the absence and presence of various concentration of H₂O₂ between 0.05 and 0.55 mM in quiescent PBS, $E = -150$ mV vs. Ag/AgCl; (B) calibration curve.



Scheme 2. Bio-electro-catalytic reaction catalyzed by GOx and HRP for the detection of glucose at PTMSPA/HRP-GOx modified electrode.

3.4.1. Electrochemical characteristics of PTMSPA/HRP-GOx modified electrode

PTMSPA/HRP-GOx modified electrodes were fabricated with different ratios of loading of HRP and GOx and tested for their activity towards 7.5 mM glucose in PBS. The experimental results show that the modified electrode with equal molar ratios of the two enzymes yields the optimal sensor response. Further, the electrochemical response of PTMSPA/HRP-GOx modified electrode towards 7.5 mM glucose was evaluated and compared with ITO/TMSPA/HRP-GOx, ITO/silica/HRP-GOx and ITO/silica-graphite/HRP-GOx in PBS. The details regarding the fabrication process and voltammetric response of the aforementioned electrodes are presented in Fig. S5 (see Supplemental information).

TMSPA/HRP-GOx did not show any significant current response for 7.5 mM glucose in PBS. Also, no obvious electrochemical response was observed for the entire scan range at silica/HRP-GOx modified electrode. Hence graphite particles ($\sim 40 \mu\text{m}$) were dispersed into the silica matrix to achieve an efficient electrical communication between electrode and the enzymes (Guerente et al., 1997) and the electroactivity towards glucose was studied. The incorporation of finite graphite particles into silica matrix provides adequate communication and facilitates DET to the electrode. A broad peak around -100 mV (vs. Ag/AgCl) was observed at silica-graphite/HRP-GOx. However, the peak current is much lower than the current observed at PTMSPA/HRP-GOx modified electrode ($0.48 \mu\text{A}$ vs. $1.0 \mu\text{A}$). The enhanced response of PTMSPA/HRP-GOx

modified electrode compared with silica-graphite/HRP-GOx is due to the presence of covalently linked PANI chains and the close proximity between enzymes and PANI chains which is achieved during their simultaneous immobilization process. These results exemplified the effectiveness of the PTMSPA/HRP-GOx modified electrode towards the detection of glucose and reveal the advantage of utilizing the one-pot electrochemical process for the fabrication of the modified electrode.

3.4.2. Amperometric response

In order to demonstrate the functioning of PTMSPA/HRP-GOx modified electrode as glucose biosensor, amperometric measurements were made by recording the Faradaic current as the glucose concentration was varied (Fig. 6A). The electrode was kept at -150 mV in PBS and the response current was monitored amperometrically during the successive additions of 1 mM glucose in stirred PBS. Upon each addition of glucose into PBS, the response current increased sharply and reaches a stable value. At PTMSPA/HRP-GOx modified electrode, response time required for reaching 90% steady-state current is $\sim 7 \text{ s}$. The response time is much lower than reported for the electrode fabricated with GOx immobilization in silica-based matrixes (Tan et al., 2005; Wang et al., 1997). The enhanced amperometric response time observed here is attributed to the faster diffusion of glucose into the three-dimensional porous inorganic-organic hybrid film.

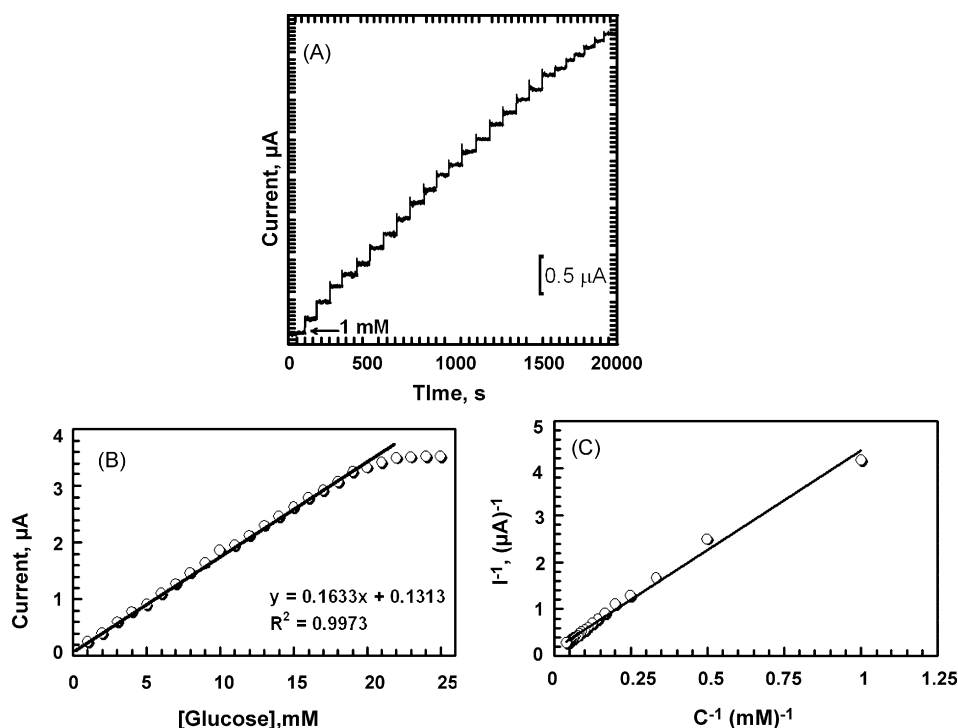


Fig. 6. (A) Amperometric $i-t$ responses of PTMSPA/HRP-GOx modified electrode to the successive addition of 1 mM glucose at the potential of -150 mV in PBS; (B) calibration curve (C) Lineweaver-Burk plot.

Table 1

Comparison of the performance of different bi-enzymatic (HRP-GOx) glucose biosensors.

Bi-enzymatic (HRP-GOx) electrodes	Linear range of detection	Detection limit (μM)	Sensitivity (mA M^{-1})	References
Polypyrrole	1000.0 μM to 8.0 mM	–	0.17	De Benedetto et al. (1996)
Sol-gel/graphite	50.0 μM to 5.0 mM	–	2.95	Guerente et al. (1997)
Polypyrrole/sol-gel/graphite/ferrocenecarboxylic acid	80.0 μM to 1.3 mM	10.0	1.11	Tian and Zhu (2002)
SWNT/polypyrrole	30.0 μM to 2.43 mM	30.0	7.0	Zhu et al. (2007)
Au nanoparticles/thionine	1000.0 μM to 3.0 mM	35.0	3.8	Sun et al. (2007b)
SBA-15 (mesoporous silica)	3.0 μM to 34 mM	0.27	90.0	Dai et al. (2008)
Ag nanoparticles/CNT/chitosan	0.5 μM to 0.05 mM	0.1	135.9	Lin et al. (2009)
Concanavalin A/chitosan	1.0 μM to 0.22 mM	0.67	1.18	Li et al. (2009)
MWNT/polyaniline	50.0 μM to 12.0 mM	20.0	0.94	Sheng and Zheng (2009)
PTMSPA	1000.0 μM to 20.0 mM	100.0	0.16	Present work

Table 2

Detection of glucose at PTMSPA/HRP-GOx modified electrode in human serum samples.

Samples	Detected before spike		Spiked (mM)	Detected after spike	
	Reference method ^a (mM)	at PTMSPA/HRP-GOx (mM)		Reference method ^a (mM)	at PTMSPA/HRP-GOx (mM)
Human serum 1	9.22	9.28 (R.S.D. = 1.3)	1	10.21	10.26 (R.S.D. = 0.8)
Human serum 2	7.02	6.98 (R.S.D. = 3.5)	3	10.02	9.95 (R.S.D. = 1.54)
Human serum 3	4.75	4.78 (R.S.D. = 2.7)	5	9.76	9.74 (R.S.D. = 1.1)

Human serum 1: as received. Human serum 2: 25% diluted using PBS. Human serum 3: 50% diluted using PBS.

^a UV-Visible spectroscopy, R.S.D. was calculated for the three replicates.

The PTMSPA/HRP-GOx modified electrode shows linearity for the current response to the glucose concentration in the range between 1 and 20 mM with a sensitivity of 0.16 mA M^{-1} (R^2 : 0.9973) (Fig. 6B). The higher sensitivity is due to the low diffusional resistance of porous silica structure to glucose and high electronic conductivity of PANI within the silica pores. As a result, a very low mass transport barrier has been resulted. K_M at PTMSPA/HRP-GOx modified electrode was deduced as 5.76 mM using the slope and intercept values from Lineweaver–Burk plot (Fig. 6C) (Manesh et al., 2008b). The K_M value is lower than observed at HRP-GOx layered assembly (9 mM) (Ferri et al., 2001). PTMSPA/HRP-GOx modified electrode shows a detection limit of 0.1 mM for glucose (at signal-to-noise ratio of 3). The performance (in terms of linear detection range, LOD and sensitivity) of PTMSPA/HRP-GOx modified electrode as a glucose sensor is compared with earlier reported bi-enzymatic glucose sensors and is presented in Table 1.

3.4.3. Interference study

Discrimination of interfering species having electroactivities similar to the target analyte is one of the most important analytical aspects for an amperometric biosensor. Dopamine (DA), uric acid (UA) and ascorbic acid (AA) are the most common interfering electroactive species during the amperometric detection of glucose. In the present investigation, they were checked individually for their electroactivity at PTMSPA/HRP-GOx modified electrode by varying the potentials between -150 and $+150$ mV in PBS (Fig. S6 (see Supplemental information)). A well-defined current response ($\sim 0.23 \mu\text{A}$) was observed for glucose at -150 mV (vs. Ag/AgCl), whereas, no significant current responses were observed for the other interfering species (DA, UA and AA) at -150 mV. On the other hand, at a higher potential, for instance at $+150$ mV, these electroactive species show augmented current response than glucose (Santhosh et al., 2009a). Thus, at a potential of -150 mV, electrochemical detection of glucose is free from other interference substances, indicating high selectivity towards glucose.

3.4.4. Sensor reproducibility and stability

The reproducibility and stability are the two important parameters for the evaluation of biosensor to be applied for analytical purposes. The detection reproducibility of PTMSPA/HRP-GOx modified electrode in the online assay for 5 mM glucose was measured. High reproducibility of the amperometric response (R.S.D. of

3.8%) was observed for 10 repetitive injections of 5 mM glucose in PBS. The stability of the PTMSPA/HRP-GOx modified electrode was evaluated by recording the response current for 5 mM glucose over 50 days (Fig. S7 (see Supplemental information)). The electrode was stored in PBS at 4°C when it was not in use. A constant current response was noticed for first 10 days. After 15 days, the current response of PTMSPA/HRP-GOx modified electrode decreased to 95.3%. After 50 days, the bio-electrode retained about 55% of its original current response.

3.4.5. Real sample analysis

The applicability of the PTMSPA/HRP-GOx modified electrode in practical purpose was assessed by determination of glucose concentration in human serum sample under the optimized conditions. Serum sample was diluted to known concentrations using PBS and was analyzed without any pretreatment. Table 2 presents the results for the detection of glucose in serum samples. The results are satisfactory and agree with value determined by a spectrophotometric method (Manesh et al., 2007). This shows that PTMSPA/HRP-GOx modified electrode is selective and sensitive towards the quantification of glucose in real samples.

4. Conclusions

We have successfully fabricated a bi-enzymatic (HRP and GOx) biosensor based on porous, periodic and electroactive silica–polyaniline hybrid material through a one-pot electrochemical method. The PTMSPA/HRP-GOx modified electrode exhibited excellent sensitivity and high selectivity for glucose and has adequate practical viability for real sample analysis. The methodology adopted in the present investigation can be conveniently extended to fabricate other bi-enzymatic biosensors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bios.2009.11.015](https://doi.org/10.1016/j.bios.2009.11.015).

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