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Horseradish peroxidase and toluidine blue covalently immobilized leak-free sol-gel composite biosensor for hydrogen peroxide

K. Thenmozhi [a,*], S. Sriman Narayanan [b]

[a] Department of Chemistry, School of Advanced Sciences, VIT University, Vellore 632 014, India
[b] Department of Analytical Chemistry, University of Madras, Guindy Campus, Chennai 600 025, India

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ABSTRACT

The enzyme horseradish peroxidase and the water-soluble mediator toluidine blue were covalently immobilized to 3-aminopropyl trimethoxy silane precursor through glutaraldehyde crosslinker. A rigid ceramic composite electrode was fabricated from this modified silane along with graphite powder, which resulted in an amperometric biosensor for H_2O_2. The electrochemical behaviour of the modified biosensor was monitored using cyclic voltammetry in the potential range of 0.2 V to −0.4 V vs SCE. The biosensor exhibited a stable voltammogram with cathodic peak at −0.234 V and anodic peak at −0.172 V, with a formal potential of −0.203 V. Various factors influencing the performance of the biosensor such as buffer solution, pH, temperature and potential were examined for optimizing the working conditions. The modified biosensor exhibited a good catalytic behaviour for the reduction of H_2O_2 at a lower potential of −0.25 V without any barrier from possible interferents. The analytical working range was found to be 0.429 μM to 0.455 mM of H_2O_2 with a detection limit of 0.171 μM. The fabricated biosensor is robust for long-term usage in addition to the high sensitivity, rapid response and having an advantage of surface renewability by simple mechanical polishing.

1. Introduction

Development of simple and reliable methods for the immobilization of enzymes is a crucial step in the fabrication of biosensors. The stability, reproducibility, sensitivity as well as lifetime of the biosensor depend on the strategy of immobilization of the enzymes. Immobilized enzymes are more robust and have the advantages of convenient handling, easy separation from the product, operational stability, more resistant to environmental changes and feasibility of reuse compared to free enzymes in solution. Many methods such as physical or chemical adsorption at a solid surface [1,2], incorporation into conducting polymers [3], bulk modified composites [4], and covalent immobilization [5], anchoring on arrays [6,7] or crosslinking to a matrix [8] have been used as valuable techniques for immobilization. But, the development of new strategies of immobilization is still an active field of research for enhancing the performance of the existing biosensors. The simplest method of biosensor fabrication is entrapment or adsorption of the biocatalyst (e.g. proteins, enzymes), which encounters several problems. In addition to the leaching of the biocatalysts, various factors hinders the direct electron transfer between the electrode and the enzymes or proteins, including slow electron transfer due to the deep burial of the electroactive cofactors in the proteins shell, denaturation of the protein at the electrode surface, and unfavorable orientation of the protein. To overcome these shortcomings, much effort has been focused on facilitating the electron transfer between proteins and electrodes, including the use of electron transfer mediators [9,10]. In the construction of mediated biosensors, immobilization of the mediator is also an important task as that of the enzyme [11]. The immobilization procedure should maintain the integrity and activity of the enzyme while utilizing mediators for enhancing the electron transfer rates.

Appropriate choice of mediator is crucial for added efficiency in electron transfer between the active site of the enzyme and the electrode surface. A variety of organic dyes have been used as electron shuttling agents, such as toluidine blue [12], thionine [13], brilliant green [14], methylene blue [15], methylene green [16], prussian blue [17], azure A [18] and phenazines [19]. All these dyes have been reported to have an excellent mediating ability in the bioelectrocatalytic reduction of H_2O_2. The problem associated with these low molecular weight dyes is their leachability from the electrode surface into the solution, which may lead to a significant signal loss and affects the performance of the biosensor. Hence efficient immobilization methods should be adopted while employing these mediators to enhance the performance of the biosensor [20].

Electrochemical methods surpass the defects of the conventional methods with attractive features of quick response, high sensitivity and abilities to be miniaturized [21–24]. Electrochemical enzyme based biosensors have gained marked attention as sensitive and

* Corresponding author.
E-mail address: kt.thenmozhi@gmail.com (K. Thenmozhi).

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selective tool due to amplification of substrate turnover and the specificity of biological recognition unit [25–27]. Quantitation of H_2O_2 with reliable and accurate sensors is of great interest in many disciplines such as food, industrial and environmental monitoring, and particularly in biosensing since H_2O_2 is a by-product of several reactions catalysed by various oxidase enzymes. [28–34].

Sol-gel-derived materials have emerged with attractive features to incorporate all kinds of species [35–38]. These new kinds of inorganic materials are particularly attractive for electrochemical biosensor development, since they possess the advantages of both carbon based electrodes and sol-gel electrochemistry [39,40]. The smart features of sol-gel materials would further be enhanced by the use of bifunctional sol-gel precursors such as (3-aminopropyl) trimethoxy silane and (3-mercaptopropyl) trimethoxy silane which provide free amino groups and thiol groups that could be used as the binding site for covalent attachment of mediators and biomolecules [41,42]. These functional sol-gel precursors facilitate the construction of sensing materials with possible control over the selective modification, orientation and distribution of catalytic sites [43,44].

We report in this work for the first time, an efficient route for the covalent immobilization of both the enzyme and the mediator to the sol-gel matrix derived from 3-aminopropyl trimethoxy silane (APTMOS) and methyl trimethoxy silane (MTMOS) and develop a renewable amperometric biosensor for H_2O_2. In the ceramic composite composition, APTMOS provides the immobilization site for both the enzyme, Horseradish peroxidase (HRP) and the mediator, Toluidine blue (TB). The additional monomer, MTMOS serves to maintain the hydrophobicity/hydrophilicity of the composite. Here, graphite powder provides conductivity, APTMOS and MTMOS serves as binder and attribute to the rigidity and porosity, HRP acts as biocatalyst and TB for shutting electrons between the enzyme and the electrode surface. The enzyme composite electrode has been further applied for the determination of H_2O_2 in both static and dynamic conditions, after thorough optimization of the electrocatalytic performance.

2. Experimental

2.1. Reagents and solutions

HRP (RZ 3.0, 250 U mg^{-1}) was purchased from Sisco Research Laboratories and used as received. APTMOS, MTMOS and Graphite powder were obtained from Aldrich. Hydrogen peroxide (50%) was purchased from Merck. Toluidine blue was received from s.d. fine chemicals. All other chemicals were of reagent grade and used without further purification. Double distilled water was used for the preparation of solution and further dilutions. Electrochemical experiments were carried out in the background electrolyte of 0.05 M phosphate buffer. The solutions were purged with high purity N_2 for 15 min before commencing the experiments. The accurate concentration of H_2O_2 was determined by titration with $KMnO_4$.

2.2. Apparatus

The electrochemical experiments were performed in a conventional one-compartment cell with a three electrode configuration using a CHI 400 A Electrochemical Analyzer. The working electrode was HRP and TB modified ceramic composite biosensor (HRP/TB/CCB), the auxiliary electrode was a platinum wire and the saturated calomel electrode (SCE) served as the reference electrode. All the potentials measured and quoted in this paper are against SCE. Impedance measurements were performed in 2.0 mM $[Fe(CN)_6/]^{3/4-}$ with 0.1 M KCl as supporting electrolyte, within the frequency range of 100 kHz–1 Hz, using an alternating current voltage of 5 mV. A magnetic stirrer and a stirring bar provided the convective environment for the amperometric studies. An Elico pH meter (Model LI 120) was employed for pH measurements. FT-IR spectra were recorded on a PerkinElmer FTIR Spectrometer (Spectrum RX1) in KBr pellets.

2.3. Construction of HRP/TB/CCB

A mixture of 0.4 ml APTMOS, 0.6 ml MTMOS and 20 μl of 10 mM HCl were thoroughly shaken for 5 min. The composition of APTMOS/MTMOS was optimized to this value for maintaining the hydrophobicity/hydrophilicity of the composite. 30 mg of TB in 1 ml MeOH was allowed to stir with 40 μl glutaraldehyde (25%) for 30 min to permit the crosslinking of mediator with the linker.Separately, 0.5 ml of HRP solution (10 mg/ml in 0.05 M phosphate buffer; pH 7.00) was mixed with 40 μl of glutaraldehyde. Both the mediator and the enzyme linked glutaraldehyde solutions were sequentially added to the above sol-gel mixture and continued shaking for another 30 min. This would permit the unreacted aldehydic group of the glutaraldehyde to couple with the free amino groups of the sol-gel mixture.

Thereafter, 0.8 ml portion of this mediator and enzyme immobilized sol-gel mixture was added to 550 mg of graphite powder and blended totally and packed tightly into a glass vial (3 mm diameter). The tip was smoothened over weighing paper and used as working surface after allowing to dry at 4 °C in a refrigerator for 4 days. A copper wire inserted at the other end provided the electrical contact. Also, an unmodified ceramic composite electrode (CCE) was prepared in the absence of TB and HRP. Further a TB/CCE was prepared in the absence of HRP for comparative purposes.

3. Results and discussion

3.1. Covalent immobilization of TB and HRP

The use of glutaraldehyde as a cross-linker for covalent crosslinking of various reagents and enzymes possessing primary amino group through the formation of Schiff's base has been studied well [45]. In the present work, one of the terminal aldehyde group of the bifunctional cross-linker, glutaraldehyde condensed with amino group of HRP and TB by Schiff's base reaction separately as shown in the first step of the scheme. In the next step, the other free aldehyde group of these compounds was attached to primary amino groups (of APTMOS) in the sol-gel matrix by Schiff's base reaction to result in the covalent immobilization of sol-gel binder with TB and HRP. The steps involved in mediator and enzyme immobilization are illustrated in Scheme 1.

The covalent crosslinking of HRP and TB to APTMOS was confirmed by FTIR. Fig. 1 (curves a, b and c) represents the FTIR spectra of free TB, free HRP and sol-gel immobilized with TB and HRP. Fig. S1 represents the FT-IR spectrum of sol-gel matrix before the immobilization of TB and HRP showing the characteristics silane peaks. The spectrum of free TB shows (Fig. 1 (curve a)) two bands at 3380 cm^{-1} and 3145 cm^{-1} due to the N—H stretching vibration of the primary amino group which were not found in the spectrum of HRP/TB sol-gel matrix (Fig. 1 (curve c)). Also the sharp peak found at 1600 cm^{-1} corresponding to the N—H bending vibration of the free amino group was found to disappear in Fig. 1 (curve c) and a strong and broad band appeared at 1710 cm^{-1}. This might be due to the presence of imine groups due to the covalent immobilization of the TB (Fig. 1 (curve c)). In the spectrum of free HRP (Fig. 1 (curve b)) the band at 1695 cm^{-1} could be assigned to the C═O stretching mode of amide I and the absorption signal at 1595 cm^{-1} indicated a characteristic amide II band. The spectrum of sol-gel immobilized with both the mediator and the enzyme, shows the appearance of amide II band. The amide I band is expected to overlap with the signal of the imine groups. In Fig. 1 (curve c) the absorption at 3471 cm^{-1} may be due to the H-bonded silanol with water and the peaks at 1127 cm^{-1} and 1026 cm^{-1} showed the presence of Si—O—Si asymmetric stretching vibration [46]. The sharp band at 1277 cm^{-1} is assigned to Si-CH_3 symmetric deformation, and the absorption peak at 776 cm^{-1} may be due to the methyl rocking or Si—C stretching