An HRP based biosensor using sulphonated polyaniline

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Abstract

The properties of poly (2-methoxyaniline-5-sulfonic acid) (PMAS), a water-soluble polymer, has been investigated as a potential mediator for biosensors. Addition of poly (L-Lysine) (PLL) to the PMAS renders this material water-insoluble; an important feature for aqueous based biosensors. Characterisation of various PMAS/PLL mixtures showed that a uniform and stable film could reproducibly be fabricated on ITO coated Mylar using 0.1% (w/v) PMAS and 0.05% (w/v) PLL. Immobilisation of enzyme horseradish peroxidase (HRP) onto the PMAS/PLL film was also investigated. Amperometric detection (-0.1 V vs. Ag/AgCl reference electrode) of hydrogen peroxide was investigated using the PMAS/PLL/HRP electrode as the working electrode in a three electrode cell containing phosphate buffer saline (pH 6.4) solution. Amperometric responses were observed upon the addition of hydrogen peroxide. HRP immobilised concentration was optimised to be 0.35% (w/v) at 35 μL. Loading volume was also studied and optimised to be 35 μL. The optimal condition for amperometric test was using constant applied potential at -100 mV. Detection limit of the sensor in PBS solution was 0.01 mM H_2O_2. Calibration curve in PBS pH 6.4 shows a linear response range between 0.01 and 0.1 mM H_2O_2 with a sensitivity of 24.91 $\mu A/cm^2\ mM^{-1}$ and correlation coefficient of 0.9966.

Keywords: polyaniline and derivatives, conducting polymers, HRP biosensors

1. Introduction

Horseradish peroxidase (HRP) based biosensors are important in many fields of analyses such as pharmaceutical and dairy industries [1]. Generally, biosensors consist of a base electrode substrate coated with a mediator and immobilised biomolecules. In this work, indium tin oxide coated polyester (ITO Mylar) was used as the electrode substrate due to its low cost. Polythiophene [2], polypyrrole [3] and polyaniline [4-8] have been used as mediator in HRP based biosensors. Following from the work of Tatsuma et.al [4], we have prepared water insoluble PMAS by complexing with poly (L-Lysine) PLL. In the present work, we used PMAS-PLL as the mediator for fabrication of an HRP-H_2O_2 biosensor. HRP was premixed with PMAS-PLL before coating onto ITO Mylar substrates. Amperometric detection was employed to detect the response upon the addition of H_2O_2. The amount of immobilised HRP was optimised. Sensor stability and the effect of interferent were also investigated.

2. Experimental

2.1 Reagents and materials

PMAS was synthesised using the method developed in the IPRI laboratories. Isopropyl alcohol from Univar, di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) from Univar, potassium di-hydrogen orthophosphate (KH_2PO_4) from BDH, sodium chloride (NaCl) from Sigma Aldrich, potassium chloride (KCl) from Univar, hydrochloric acid (HCl) from Univar, poly-L-lysine hydrochloride (PLL) P9404-Sigma, hydrogen peroxide (H_2O_2) from Univar, horseradish peroxidase (HRP) P6782-Sigma, albumin, bovine serum (BSA) A3350-Sigma and L-ascorbic acid A7506-Sigma were used as supplied. Phosphate buffered saline solution (PBS) was prepared as described elsewhere [9]. PBS solution of different pH was

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prepared by mixing 0.1M KH_2PO_4 buffer and 0.1M Na_2HPO_4 until the desired pH was obtained. All solutions were prepared using Milli-Q water.

2.2 Equipment

pH/Conductivity Meter, Denver Model 20 was used to measured pH of PBS solutions. ITO coated polyester Mylar from CPFilms Inc. was pretreated before use. UV cleaner, Jelight Company, Inc. Model No.42-220 was used for pretreatment of the Mylar. Cyclic voltammograms were recorded in a three electrode cell using a polymer modified working electrode with a platinum mesh and Ag/AgCl (3 M NaCl) auxiliary and reference electrodes, respectively. A Bioanalytical Systems (BAS) CV-27 workstation, interfaced with ADInstruments/4e (ADI/4e) MacLab analogue/digital converter to a computer, was used to record the cyclic voltammograms and amperometric response. The cell was purged with nitrogen gas prior to amperometric experiments. All potentials stated are vs. an Ag/AgCl (3 M NaCl) reference electrode.

2.3 Preparation of ITO Mylar

The ITO coated Mylar (1 x 3 cm^2) was sonicated in detergent solution for 5 minutes, washed by tap-water twice, washed by Isopropyl alcohol twice and left to dry in open air before cleaning in a UV ozone cleaner for 15 minutes. The pretreated Mylar was masked by adhesive tape to give a constant exposure area (1 x 1 cm^2).

2.4 Optimisation of polymer modified electrode

While keeping the mass ratio of PMAS: PLL = 1:1, mixtures of PMAS-PLL at various PMAS concentration were prepared. The mixtures were drop-coated onto the pretreated ITO Mylar. The films were allowed to dry overnight in a desiccator at 2 °C. CVs were obtained in 0.1M HCl (-0.2 V to + 1.1 V) for 10 cycles at 100 mV/S. The magnitude of the oxidation peak at E= 0.65 V (third scan) was plotted against PMAS concentration. The optimal PMAS concentration was chosen and the PLL concentration was varied. In the same manner, the solution containing various ratios of PMAS: PLL was drop-coated and CVs recorded as above. The optimal PMAS: PLL ratio was used to immobilise HRP by premixing. All error bars in all graphs represent the standard deviation calculated from the data set (n).

2.5 Immobilisation of HRP into the polymer solution before drop-coating onto electrodes

HRP or BSA was dissolved at various concentrations in the PMAS solution. This solution was further mixed with PLL. The mixture was drop-coated onto UV treated ITO Mylar. The films were dried in a desiccator in the refrigerator. The amperometric test was conducted the following day. Using the optimal HRP concentration the effect of the volume deposited on the electrode was investigated (10 to 70 µL). The optimal volume was used for all further experiments.

2.6 Amperometric detection using the PMAS-HRP-PLL modified electrodes

The modified ITO Mylar was placed in a 10 ml cell. Reference and auxiliary electrodes used were Ag/AgCl (3 M NaCl) and platinum mesh, respectively. 10 ml of PBS solution was added into the cell. The cell was connected to amperometric analyser. The solution was stirred throughout the experiment. A constant potential was applied to the cell. After the constant current was obtained, desired amount of hydrogen peroxide was added into the cell. The catalytic signal was obtained by subtract signal response from background response.

2.7 Stability Test

Two series of PMAS-HRP-PLL sensors were kept in a closed container or in PBS solution pH 6.4 at 2° C. Amperometric test were carried out for six months.

2.8 Interferent Test

The effect of 200 µM ascorbic acid was investigated.

3. Results and Discussion

A cyclic voltammogram was obtained in 0.1M HCl after coating the ITO Mylar with PMAS-PLL. Two redox couples that are tentatively assigned to conversion from the leucoemaraldine to emeraldine salt form (A/A') and emeraldine salt to pernigraniline (B/B') were observed (Fig. 1a).

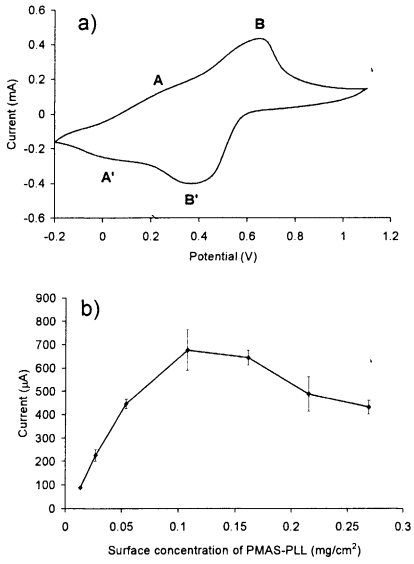

Fig. 1 a) CV of film from PMAS-PLL (0.11 mg/cm^2), PMAS:PLL ratio = 1:1, on UV treated ITO Mylar in 0.1M HCl at scan rate 100 mV/s and b) Dependence of anodic peak current ($i_{p,B}$) on the surface concentration of PMAS-PLL (n=3).

The PMAS/PLL surface concentration used to drop-coat on the Mylar surface was varied from 0.01 to 0.25 mg/cm^2 using a ratio of PMAS:PLL = 1:1 (Fig. 1b). The maximum oxidation current (peak B) was observed at 0.11mg/cm^2. This implies that the concentration of PMAS was high enough to allow conductivity between PMAS and the ITO. At PMAS/PLL surface concentrations greater than 0.11

mg/cm^2 the current decreased. This may be due to a thicker film forming, reducing the charge transfer between the PMAS and the ITO. At 0.11 mg/cm^2 PMAS, the PLL concentration was varied to obtain the highest charge transfer while maintaining the best film stability. The higher the PLL content, the lower the charging current observed (Fig. 2).

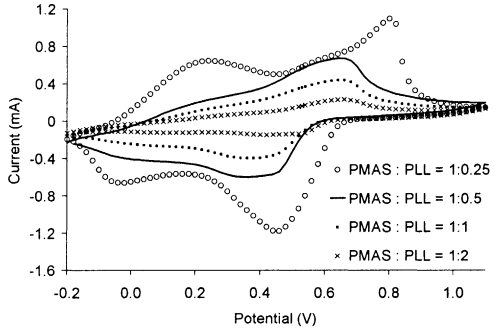

Fig. 2 CVs in 0.1 M HCl for various ratios of PMAS:PLL (50 µl volume) on ITO Mylar. 0.1% PMAS was used for all drop-coatings.

The best film stability was obtained when a ratio of PMAS: PLL = 1:0.5 was used. This ratio was used to optimise the immobilised HRP or BSA on the electrode. Amperometric test has been conducted at applied potential -100 mV and 10 mM H_2O_2 was added. The typical response from this format showed steady state as in Fig. 3.

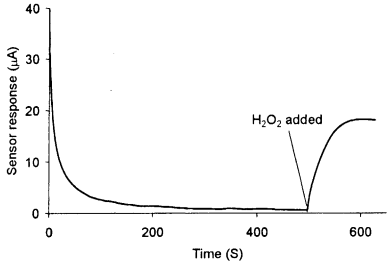

Fig. 3 Typical sensor response from modified PMAS-HRP-PLL biosensors upon addition of 10 mM H_2O_2. The constant potential -100 mV was applied at t = 0.

Various concentrations of HRP (%w/v) were premixed with PMAS/PLL before coating onto the electrode. The catalytic response to H_2O_2 using various concentrations of immobilised HRP was determined (Fig. 4).

The response obtained from premixed PMAS-HRP-PLL compared to PMAS-BSA-PLL confirms the activity of HRP. The highest response was obtained at 0.35% HRP. At concentrations greater than 0.35%, the signal decreased. This response may be attributed to the formation of HRP multilayers which may reduce the electron transfer kinetics due to large insulating layer formation.

The effect of the volume of PLL-HRP-PMAS solution loaded on to the electrode was investigated (Fig. 5).

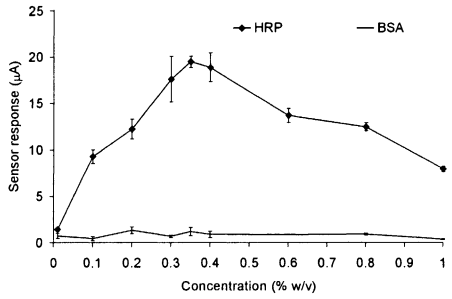

Fig. 4 Dependence of catalytic response on [HRP] or [BSA] premixed with PMAS-PLL mixture (n=3). Concentration of H_2O_2 used was 10 mM.

The higher the loading volume the higher the sensor response obtained. However, consideration of the sensor cost, two different slopes was drawn to find the most optimal point between sensor response and the loading volume. At the interception, the loading volume was optimised to be 35 µL. Moreover, the response time was longer at higher volume (Fig. 6). Therefore, the optimal condition for PMAS/HRP/PLL sensor was 0.1% PMAS-0.35% HRP-0.05% PLL (w/v) at 35 µL loading volume.

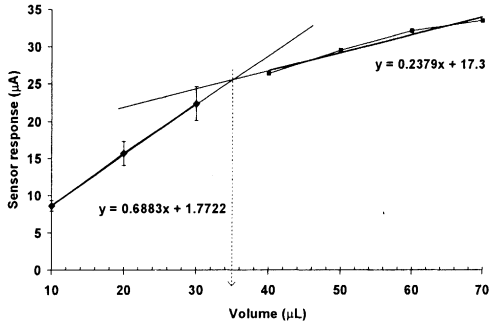

Fig. 5 Dependence of catalytic response on loading volume (µL) of 0.1% PMAS-0.35% HRP-0.05 % PLL (n=3).

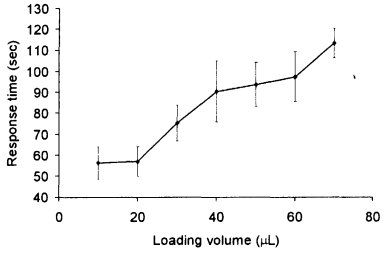

Fig. 6 Dependence of response time on loading volume (µL) of 0.1% PMAS-0.35% HRP-0.05 % PLL (n=3).

The potential applied during the amperometric test means PMAS will be in the reduced form. The reduced PMAS was needed as electron transfer mediator to complete the electron transfer cycle as described in [5]. The applied potential was varied from -500 mV to 0 mV. The amperometric test of the sensor was performed upon addition of 10 mM H_2O_2 in PBS solution pH 6.4 (Fig. 7).

Application of -500 mV provided the highest response but reproducibility was worst. Application of -100 mV presented

relatively high sensor response and the best reproducibility and was used for all further experiments.

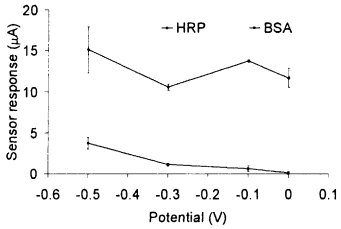

Fig. 7 Responses of biosensors immobilised 35 μL of 0.1% PMAS-0.35% HRP/BSA-0.05% PLL to 10 mM H_2O_2 at various applied potential (n=3).

The effect of pH (4.4, 6.4 and 8.4) of the PBS solution was also studied. Calibration curves in the three different buffers were obtained (Fig. 8). At acidic pH, the sensor response was highest at concentrations of H_2O_2 greater than 1 mM. The sensor response was better at lower concentrations of H_2O_2 in pH 6.4. No detectable sensor response was observed below 0.01 mM H_2O_2 in all buffers, therefore the detection limit was taken as 0.01 mM H_2O_2.

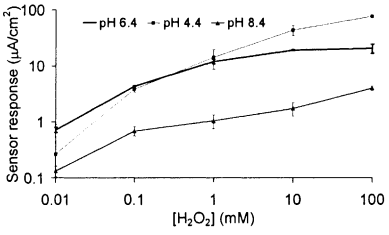

Fig. 8 Cathodic current responses of PMAS/HRP/PLL biosensors to various concentrations of H_2O_2 in PBS pH 4.4, 6.4 and 8.4 (n=3). The constant potential -100 mV was applied.

The linearity of the sensor response in pH 6.4 PBS solution was found in the range of 0.01 to 0.1 mM H_2O_2 with the sensitivity of 24.91 μA/cm^2 mM^{-1} and correlation coefficient 0.9966.

The interference test was performed by using ascorbic acid as the model. The concentration of ascorbic acid to be used was 200 μM which is the normal physiological level [10]. Amperometric test was performed in the absence and presence of ascorbic acid, and the response after addition of 200 μM H_2O_2 was 4.43 and 4.3, respectively. It was found that there was negligible effect from the presence of ascorbic acid.

Stability test of the sensors keeping in closed container and PBS pH 6.4 were shown in Fig. 9.

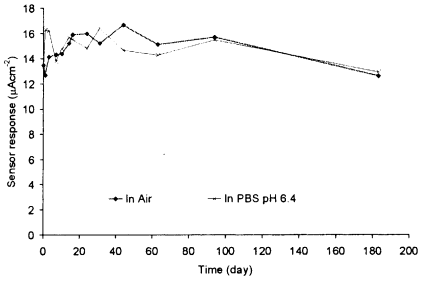

Fig. 9 Stability of the sensor stored at 2 °C in air and PBS solution pH 6.4.

The sensor was very stable. The sensor response was obtained in the same level as initial value in both conditions after being stored for six months.

4. Conclusions

The use of ITO Mylar as the base electrode for the H_2O_2 sensor was demonstrated. PMAS was employed as the insoluble conducting polymer mediator by complexing with PLL. The optimal condition was 0.1% PMAS-0.05% PLL. The maximum sensor response was obtained from 0.35% (w/v) immobilised HRP. Loading volume was optimised to be 35 μL. The sensor has a detection limit of 0.01 mM H_2O_2. A linear range between 0.01 and 0.1 mM H_2O_2 was observed. The sensor has a high selectivity with a constant potential of -100 mV applied. It also has very good stability both being stored in air and in PBS pH 6.4 at 2 °C.

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