A simple means to immobilize enzyme in conducting polymers via entrapment

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Abstract

Poly(3,4-ethylenedioxy thiophene) (PEDT), obtained by chemical vapour phase polymerisation can shrink to 5% of its original thickness during a washing step. This phenomenon was exploited in order to immobilize the active enzyme, horse-radish peroxidase (HRP), into PEDT films by rinsing with an enthanol solution containing HRP. The use of the PEDT-HRP as an amperometric biosensor was demonstrated by sensing 5mM H_2O_2 in phosphate buffered saline (PBS) solution with a sensitivity of 190 ?A cm^{-2}. The stability of the immobilization of HRP into the 'stuffed' PEDT matrix was also characterised by repeated amperometric detection and scanning electron microscopy (SEM) images obtained before and after exposure to H_2O_2.

Keywords: amperometric biosensor; vapour phase polymerisation; PEDT; horse-radish peroxidase; hydrogen peroxide, conducting polymer.

Introduction

One of the simplest methods of immobilising biomolecules on polymer surfaces is adsorption [1]. This includes both physical adsorption and electrochemical adsorption techniques. The latter is achieved by applying a potential to the polymer electrode surface, which enhances electrostatic interactions and hence immobilisation [2]. The main disadvantage of adsorption however, is that the biomolecule may easily desorb from the surface during use.

Covalent attachment of biomolecules to the surface of polymers ensures immobilisation without leaching of the biomolecule from the substrate surface [3]. Covalent attachment of biomolecules to the monomer prior to polymerisation has also been reported [4,5]. This strategy however requires lengthy syntheses to produce the monomer or to modify a preformed surface. Since covalent attachment is usually achieved using a linker, the separation of the biomolecule from the polymer backbone can also have disadvantages in terms of signal transduction [6].

Self assembly techniques have been investigated for biomolecule immobilisation onto various support materials. Layers formed by self-assembly require a poly-ion to effect biomolecule attachment through strong electrostatic interaction [7] and can result in tightly packed structures which limit diffusion [8]. Furthermore, careful control of pH is required in order to achieve these assemblies and for them to retain stability.

Biomolecules have been trapped within conducting polymers as dopant molecules when the appropriate conditions are met [9,10]. To function as a dopant for oxidised conducting polymers, anions are required. This restricts the number of biomolecules that can be incorporated using this approach.

We have recently reported the successful vapour phase polymerisation of polypyrrole (PPy), poly-terthiophene (PTTh) and poly(3,4-ethylenedioxy thiophene) (PEDT), which allows the incorporation of guest molecules within the polymer matrix via a simple washing step [11,12]. This procedure obviates the need for covalent attachment of biomolecules and specific charges on either the biomolecule or polymer surface. The maximum possible size of molecules that can be loaded into a conjugated host polymer by this method is yet to be determined, but PEG (M.W. 600) has been incorporated without difficulty [12]. In this publication we describe the successful incorporation of the enzyme horse-radish peroxidase (HRP, M.W. 44,000), a molecule of far greater size than previously incorporated, into a PEDT film [11,12]. This biomolecule-conducting polymer composite film displayed an amperometric response to hydrogen peroxide demonstrating promise as a biosensor material.

Experimental

Phosphate buffered saline solution (PBS) was prepared as described elsewhere [2]. All other chemicals, 3,4-ethylenedioxythiophene (EDT, Bayer AG), ferric toluenesulfonate (Fe(III) tosylate) (Bayer AG), ethanol (Univar), butanol (Univar), 30% (v/v) hydrogen peroxide solution (Merck), horse-radish peroxidase (HRP) (Sigma), disodium hydrogen orthophosphate anhydrous (Univar), potassium di-hydrogen orthophosphate (BDH), sodium chloride (Sigma-Aldrich), potassium chloride (Univar), hydrochloride acid (Univar) and poly-L-lysine hydrochloride (Sigma), were used as received.

The polymerization of PEDT by vapor phase polymerization (VPP) was carried out as reported previously [11,12]. Ferric *p*-toluenesulfonate (Fe(III) tosylate) (20 wt %) was coated onto poly(ethylene terephthalate) (PET) foils from a butanol solution. When the coating was almost dry, but before the Fe(III) sulfonates formed crystals, the samples were put into an oven between 50-90 °C. After heating for between 1/2 and 3 min, the colour of the coating changed to darker yellow, indicating that the solvent had evaporated. The samples were then exposed to 3,4-ethylenedioxythiophene (EDT) monomer vapor in the VPP chamber at room temperature. After one hour, the samples were removed from the chamber with an observed colour change from yellow to blue. Following air drying for half an hour, these PEDT modified PET foils were washed twice in an ethanol solution containing 0.5% (w/w) HRP (20 minutes each). The significant shrinkage of these films during the washing step allows the successful entrapment of molecules within the PEDT films. Rinsing with an ethanol solution containing HRP effects immobilisation of the enzyme into the PEDT films. Subsequent to air drying for half an hour, these PEDT/HRP samples were washed in pure ethanol solution for 30 minutes to remove the excess HRP from the surface of the PEDT films.

UV-visible spectra of PEDT/HRP films were obtained using a Shimadzu UV1601 spectrophotometer, over the range of 310-1100nm.

Amperometric sensing tests were performed by using an electrochemical hardware system comprising of an EG&G PAR 363 Potentiostat/Galvanostat, a MacLab 400 with Chart v3.5.7/EChem v 1.3.2 software (ADInstruments), and a PC computer. A three-electrode electrochemical cell was used which comprised a working electrode (PEDT/HRP modified Pt-coated PET), a platinum mesh auxiliary electrode and an Ag/AgCl reference electrode with salt bridge.

Scanning electron microscopy (SEM) mapping was performed with a Hitachi S 3000N scanning electron microscope.

Results and Discussion

The steps used to form the bioactive conducting polymer surface are summarized in Schematic 1. After immobilization of HRP into the PEDT, the PEDT/HRP film obtained shows typical redox activity (high capacitance square-like shape) during cyclic voltammetry due to oxidation/reduction of the PEDT in aqueous solution scanned between -0.6 V to +0.6 V.

[pic]
Schematic 1. Loading HRP molecule into the PEDT films.

UV-visible spectroscopy was used to verify incorporation of HRP into the PEDT polymer matrix. Figure 1 presents the UV-vis spectra of the PEDT/HRP film (Fig.1a), pure PEDT (Fig.1b) and pure HRP (Fig.1c). The peak at 317 nm (peak X) in Fig.1a can be assigned as a combined contribution of HRP and PEDT, while the peak at 198 nm (peak Y) is attributed solely to the presence of HRP in the PEDT film. This confirms that the enzyme, HRP, has been successfully immobilised into the PEDT matrix.

Following the successful immobilisation of horse-radish peroxidase (HRP) into the PEDT film, the PEDT/HRP film modified PET electrode was placed in phosphate buffered saline (PBS) solution (10 mL, pH6.8) as the working electrode in a stirred batch cell. Amperometric experiments were performed potentiostatically at -100 mV (vs. Ag/AgCl). The potential applied during the amperometric test ensures that PEDT will be in the reduced form. The reduced PEDT was necessary as an electron transfer mediator to complete the electron transfer cycle as described in [13]. Hydrogen peroxide (5 mM) was added once the current had reached steady state. Figure 2a shows a typical amperometric response obtained from the PEDT/HRP film upon the addition of H_2O_2 (5 mM). It shows that the cathodic current increased sharply after the addition of H_2O_2 with a response time of less than 2 s. The response obtained from the PEDT/HRP film confirms the electrochemical activity of HRP which was immobilised within the PEDT matrix. Various concentrations of H_2O_2 in PBS were investigated to test the sensitivity of the PEDT/HRP films to H_2O_2. The catalytic response of PEDT/HRP to various concentrations of H_2O_2 is shown in Fig.2b and shows that the catalytic current increased with the increased amounts of H_2O_2 added.

The distribution of HRP immobilized within the PEDT film was studied by carrying out SEM mapping of the PEDT/HRP films before (Fig.3a) and after (Fig.3b) the amperometric biosensing test. The bright spots correspond to Fe sites within the HRP**.** It was found that the HRP enzyme was uniformly distributed throughout the PEDT film without significant clustering. The SEM images of PEDT/HRP obtained before (Fig.3a) and after (Fig.3b) exposure to H_2O_2 confirm that this structure was retained. Further evidence to confirm this was obtained from elemental analysis which showed a change in the molar ratio of HRP(Fe):EDT(S) from 1:9.1 to 1:9.5 (less than 5% change) after exposure to H_2O_2. This drop is attributed to the desorption of surface bound HRP.

This technique offers a novel, simple and effective immobilisation process to load enzyme molecules into conducting polymer films if these enzymes are present in the rinsing fluid used in the post-polymerisation washing step. The immobilisation process described has great potential to achieve high density enzyme containing polymers for the development of stable biosensors and catalysts.

Conclusion

This study illustrates the successful immobilization of HRP into a PEDT matrix via the shrinkage phenomenon encountered during the post-polymerisation washing step. The PEDT polymeric matrix exhibited a uniform distribution of HRP throughout. This type of biomolecule-conducting polymer film displayed a stable amperometric response to hydrogen peroxide; demonstrating its

promise as a biosensor material. This kind of sensor also has a high sensitivity to the concentration of hydrogen peroxide and very good stability when stored under 0°C. It is hoped that this novel and simple method to load enzyme into conducting polymers will lead to the further development of conducting polymer-based biosensors with improved stability.

Acknowledgement

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Figure Captions

Fig. 1. UV-vis spectra of (a) PEDT/HRP, (b) PEDT and (c) HRP.

Fig. 2. (a) Typical amperometric response of PEDT/HRP biosensor to H_2O_2. The applied constant potential: -0.1 V (vs. Ag/AgCl). *Indicates addition of H_2O_2 (5 mM); (b) Catalytic current responses of PEDT/HRP biosensors to various concentrations of H_2O_2 in PBS (pH 6.4) buffer solution. The applied constant potential: -0.1 V (vs. Ag/AgCl).

Fig. 3. Scanning electron microscopy (SEM) mapping images of PEDT/HRP films before (a) and after (b) amperometric biosensing test of H_2O_2.

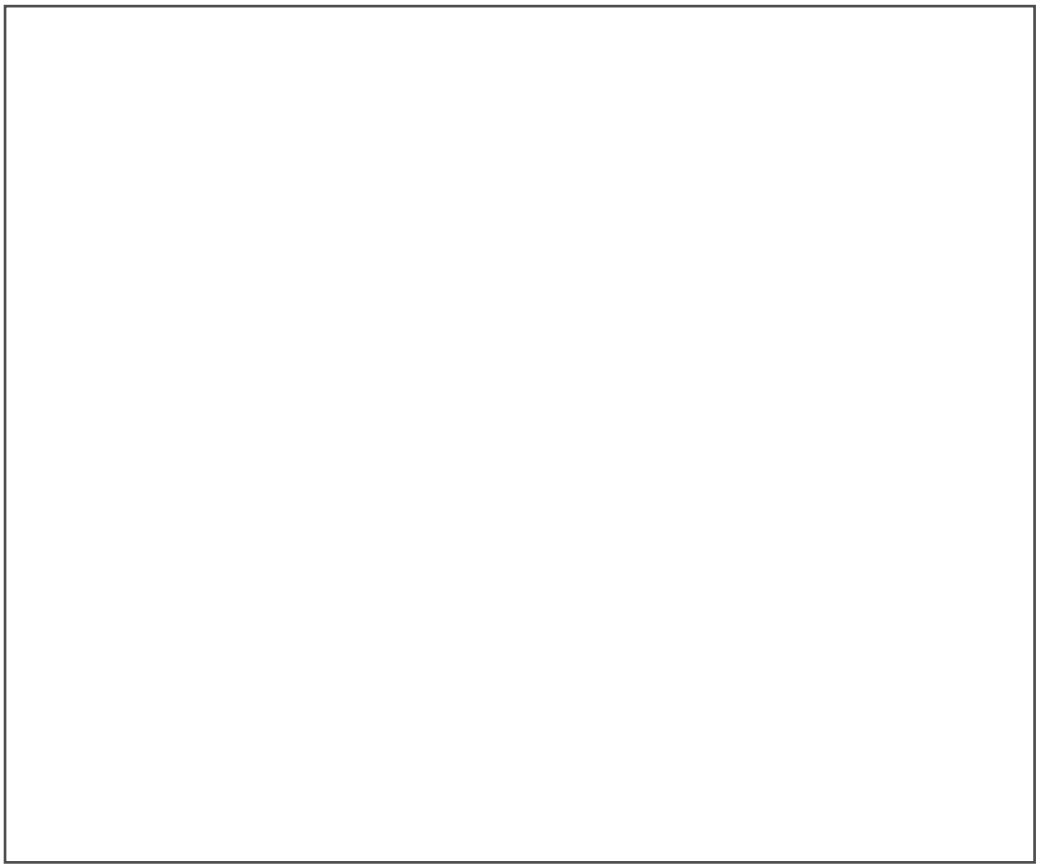
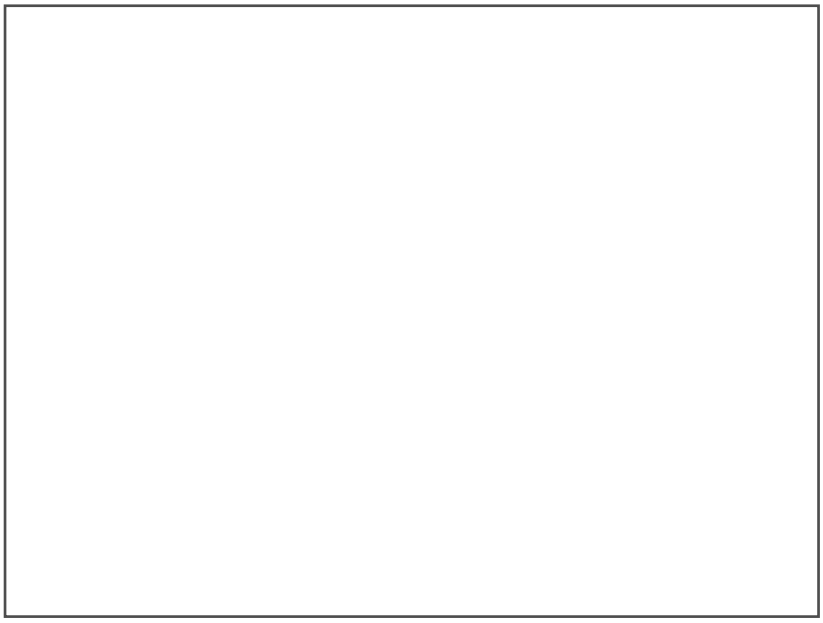


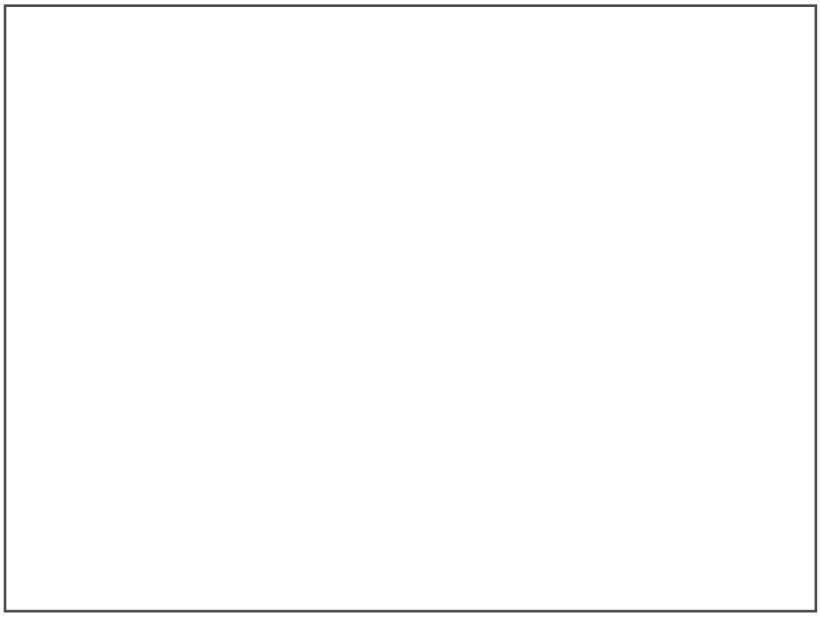
Figure 1

[pic]

Figure 2



(a)



(b)

Figure 3

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Cathodic current
after adding 5 mM H2O2

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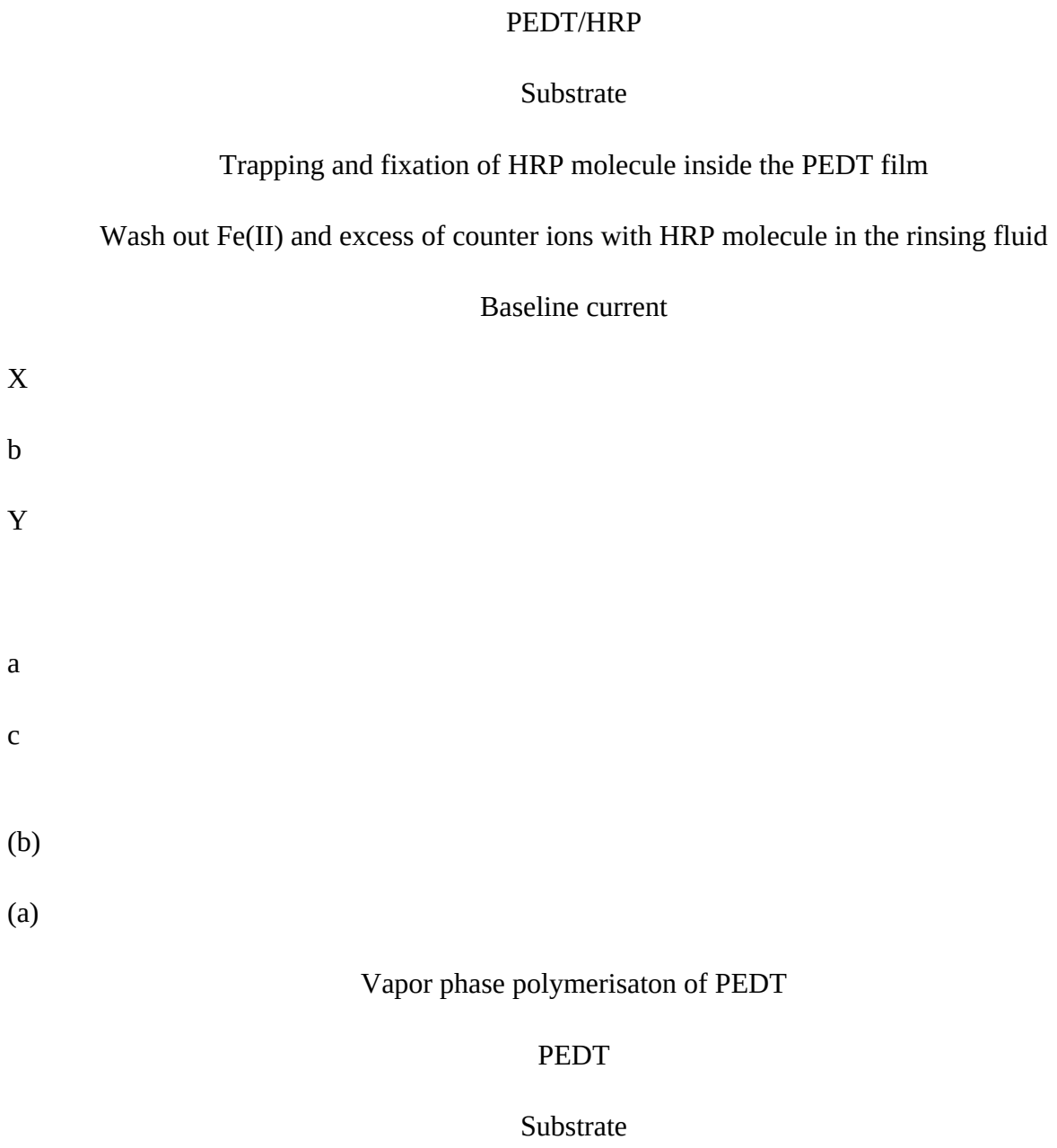
PEDT/HRP
Substrate
Trapping and fixation of HRP molecule inside the PEDT film
Wash out Fe(II) and excess of counter ions with HRP molecule in the rinsing fluid
Baseline current
X
b
Y
a
c
(b)
(a)
Vapor phase polymerisaton of PEDT
PEDT
Substrate