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Direct spectroelectrochemistry of peroxidases immobilised on mesoporous metal oxide electrodes: Towards reagentless hydrogen peroxide sensing

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ABSTRACT

In this paper, we employ two peroxidases (horseradish peroxidase, HRP and cytochrome *c* peroxidase, CcP) to demonstrate their ability to retain their redox and biological functions after their immobilisation on mesoporous TiO_2 and SnO_2 electrodes. We will also demonstrate the use of HRP immobilised on the metal oxide electrodes for the development of reagentless optical and electrochemical biosensors for the detection of hydrogen peroxide (H_2O_2) with low detection limit of 0.04 and 1 μM, respectively.

1. Introduction

Enzymes play a highly significant part in biotechnological development, creating a billion dollar businesses including a wide diversity of industrial processes, consumer products, and the field of biosensors. Optimising their potency by immobilising them in a solid support has been one of the top lists in research agenda in many laboratories, mainly due to the simplification of the overall process design and the cost effective reusability. Understanding their properties when immobilised is therefore important for the development of biotechnological devices.

In this paper, attention is directed towards hemeperoxidases, a class of hemoenzymes that employ H_2O_2 in their catalytic reactions. Their chemistry can be considered as sitting between the simple electron-transfer reaction of cytochrome *c* and the oxygen activation characteristics of the cytochrome P450, making them suitable candidates for our model of enzyme functionalised mesoporous metal oxide electrodes.

The peroxidases have been widely used in many field of applications, i.e. medicine, industry, and environment [1]. The three step reaction cycle proposed for the hemeperoxidases in the early 1950s remains unchanged today.

$$\text{Peroxidase(Fe(III))} + H_2O_2 \rightarrow \text{CompoundI}(Fe^{IV}O, P^{+}) + H_2O$$

$$\text{CompoundI}(Fe^{IV}O, P^{+}) + AH_2 \rightarrow \text{CompoundII}(Fe^{IV}O) + AH^{\bullet}$$

$$\text{CompoundII}(Fe^{IV}O) + AH_2 \rightarrow \text{Peroxidase(Fe(III))} + AH^{\bullet} + H_2O$$

However despite the fact that all peroxidases have enzymatic activity, it is the commercially available horseradish peroxidase (HRP) that is mostly used for the development of biosensors [2–6]. The use of HRP is not without obstacles; major drawbacks include relatively slow electron-transfer processes due to the partial shielding of electron pathways by its carbohydrate contents [7] and its relatively low activity in organic solvents due to protein instability [2,8]. Several attempts have been made to replace HRP, including the use of cytochrome *c* peroxidase (CcP), a carbohydrate free peroxidase with higher peroxidase activity [9,10].

Peroxidase modified electrodes have potential use in the rapid monitoring of peroxides, of compounds that yield H_2O_2 in the presence of appropriate oxidases and of hazardous compounds like CN^- that inhibit the enzyme activity. Early attempts utilised the commercially available HRP C, whose performance was however found to be limited by the poor electronic coupling with the electrode. Considerable efforts have been done to develop alternatives to HRP modified electrodes in recent years [8,11–13]. Encouraging results include those of Armstrong showing that the electron transfer can

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be improved by two order magnitudes upon the use of the carbohydrate free CcP [10]. Further improvements were made by Cass, by the use of ferrocene as a mediator [8].

We have previously demonstrated that protein adsorption can be readily achieved on mesoporous TiO_2 and SnO_2 electrodes from aqueous solutions at 4 °C with high binding stability and undetectable protein denaturation [14–16]. We have characterized the properties of such protein/TiO_2 electrodes by cyclic voltammetry and UV–visible spectroscopy and demonstrated that the immobilised proteins can be reduced by the application of an electrical potential to the film without the addition of any electron-transfer mediators [14–16]. Moreover, we and other groups have also shown the adsorption of a range of biomolecules on mesoporous metal oxide electrodes as working electrodes for sensing devices [17–23].

In this paper, we extend these studies to the immobilisation of peroxidases. Both peroxidases employed in this study, HRP and CcP, have well understood structures, providing a basic knowledge for understanding enzyme/electrode interactions. Direct and extensive investigations of a layer of immobilised enzyme on mesoporous metal oxide electrodes can provide a wealth of information on the basic enzymatic reactions and furthermore leads towards biotechnological device development. We will demonstrate the ability of the peroxidases immobilised on mesoporous metal oxide electrodes to retain their redox and biological functions and the viability of using this electrode system as an optical or electrochemical biosensor for H_2O_2.

2. Experimental

2.1. Chemicals and materials

All chemicals were obtained from Sigma–Aldrich Ltd. unless otherwise stated, distilled water was demineralised to a resistivity of 10 MΩ cm. HRP isozyme C was purchased from Biozyme. It was lyophilised, salt-free preparation, had a purity index (A405/A280) of 3.2 and was used as received. Recombinant CcP was prepared as described previously [9], resulting in CcP solution with a ratio A408/A280 of 1.25 in NaPi buffer of pH 6.5. After adding glycerol as an antifreeze agent, the enzyme was then stored at −20 °C. Prior to immobilisation, the glycerol was removed and the buffer was exchanged to 10 mM NaPi pH 6 using a Centricon or an Amicon centrifugal ultrafiltration system. H_2O_2 stock solution (10 mM or 100 μM) was prepared by diluting H_2O_2 (30%, BDH Aristar) in deionised water. The concentration of H_2O_2 was verified by optical measurements at 240 nm (ε_{240} = 40 M^{-1} cm^{-1}) [24–26].

2.2. Preparation of electrodes

The SnO_2 paste, consisting of 15-nm-sized particles, was prepared following a sol–gel procedure: 345 mL of distilled water was used in a 500-mL glass beaker in a cooling bath (∼−5 °C). A total of 10.8 mL (equivalent to 24 g) of $SnCl_4$ was then slowly added and left stirring until the solution became clear and the amount of SnO_2 in the solution was ∼4%. A dialysis membrane (Membra-Cel TM MD34-14.100 Clear) was used to separate the colloids. The sol–gel was then acidified to pH 1 with HNO_3 prior to autoclaving at 230 °C for 12 h. The solution was then concentrated to 12% before precipitating the SnO_2 with ethanol. After this, the colloidal solution was prepared for spreading on conducting glass slides. We used a 20% SnO_2 paste mixed with 7.3% ethylcellulose 7 mPa, 2.7% ethylcellulose 45 mPa, and 70% terpineol and ethanol as the solvent. The solution was stirred and sonicated until it became homogeneous. The ethanol was removed by the use of a rotor evaporator using a vacuum pump at 40 °C.

The TiO_2 paste, consisting of 15 nm sized particles was prepared from a sol–gel colloidal suspension containing 12.5 wt% TiO_2 particles and 6.2 wt% Carbowax 20,000 as reported previously [27].

The TiO_2 or SnO_2 suspension was then applied to the surface of a conducting glass using the "doctor blade" technique. Masking the glass slide with Scotch tape controlled the thickness and the width of the area spread, with one layer of tape being employed to yield a final film thickness of 4 μm. The spread suspension was then allowed to dry before being sintered for 20 min at 450 °C. The thickness of the mesoporous TiO_2 or SnO_2 films was measured with a DEKTAK profilometer. The TiO_2 or SnO_2 films were cut in 1 cm^2 pieces. Immediately prior to enzyme immobilisation, the films were heated to 450 °C for 15 min and then allowed to cool down to room temperature before being immersed in the protein solution.

Modification of SnO_2 electrodes was achieved by immersing them overnight in 20 μM poly-L-lysine (PLL), a polycationic binding promoter solution.

2.3. Protein immobilisation

Aqueous solutions of CcP and HRP were prepared in 10 mM sodium phosphate (NaPi) buffer of pH 6 and 7, respectively. Prior to immobilisation, the TiO_2 or SnO_2 electrode was heated at 450 °C for 20 min to remove all the dirt and any adsorbed water. After cooling, the electrodes were rinsed with NaPi buffer and then immersed in the protein solutions at 4 °C and the protein adsorption was followed by monitoring the optical absorbance change over one week using a Shimadzu UV-2401 spectrophotometer. Contributions to the spectra from the scatter and absorption by the TiO_2 and SnO_2 electrodes alone were subtracted by the use of protein free reference electrodes. Prior to all spectroscopic measurements, electrodes were removed from the immobilisation solution and rinsed in buffer solution to remove non-immobilised protein.

2.4. Electrochemistry and spectroelectrochemistry

The electrochemistry and spectroelectrochemistry experiments were carried out using and AUTOLAB PGStat12 potentiostat and a home made three electrode cell with quartz windows, a platinum mesh flag as the counter electrode, a Ag/AgCl in 3.5 M KCl reference electrode, and the metal oxide film (with or without enzyme immobilised on it) on conducting glass as the working electrode. The immobilised peroxidases were subject to electrochemical and spectroelectrochemical investigations. The electrolyte solution used was 3 mL (volume of the three electrode cell) of 10 mM NaPi of pH 7 which was deoxygenated with Argon prior to any optical/electrochemical measurements. The H_2O_2 solution was titrated into the electrochemical cell using a GSE microsyringe. Optical biosensing was conducted by monitoring the increase of absorption at 418 nm, a wavelength characteristic of HRP Compound II. Electrochemical biosensing was performed by the chronoamperometric technique, monitoring the increase in catalytic current after titration of hydrogen peroxide into the cell. All experiments were carried out at room temperature.

3. Results

3.1. Immobilisation studies

Immobilisation of peroxidases results in orange-brown coloration of both TiO_2 and SnO_2 electrodes. We found out that CcP and HRP can be immobilised on either bare or polycation modified mesoporous metal oxide electrodes. Typical UV visible absorption spectra are shown in Fig. 1 in comparison with the respective spectra of those proteins in the solution. In this case, CcP was