Strategies for an enzyme immobilization on electrodes: Structural and electrochemical characterizations

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Abstract. In this paper, we propose various strategies for an enzyme immobilization on electrodes (both metal and semiconductor electrodes). In general, the proposed methodology involves two critical steps viz., (1) chemical modification of substrates using functional monolayers [Langmuir – Blodgett (LB) films and/or self-assembled monolayers (SAMs)] and (2) anchoring of a target enzyme using specific chemical and physical interactions by attacking the terminal functionality of the modified films. Basically there are three ways to immobilize an enzyme on chemically modified electrodes. First method consists of an electrostatic interaction between the enzyme and terminal functional groups present within the chemically modified films. Second and third methods involve the introduction of nanomaterials followed by an enzyme immobilization using both the physical and chemical adsorption processes. As a proof of principle, in this work we demonstrate the sensing and catalytic activity of horseradish peroxidase (HRP) anchored onto SAM modified indium tin oxide (ITO) electrodes towards hydrogen peroxide (H_2O_2). Structural characterization of such modified electrodes is performed using X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) and contact angle measurements. The binding events and the enzymatic reactions are monitored using electrochemical techniques mainly cyclic voltammetry (CV).

1. Introduction

Self-assembled monolayer (SAM) forms the basis to understand the structural arrangement, electron transfer across the interface and the flexibility of macro-molecules to the surrounding environment, which are the key factors for the realization of biomimics [1]. As a first step towards the development of synthetic biomimetic systems, it is very much important to have a thorough knowledge on enzymes and their activity in terms of their structural arrangement and enzymatic reactions. SAM provides a very good platform for immobilizing enzymes and investigating their redox properties that help in understanding their sensing ability and catalytic reactions. Keeping this in mind, in this work we propose a simple methodology to anchor horseradish peroxidase (HRP) on 3-mercaptopropyltrimethoxysilane (MPTMS) monolayer coated ITO electrodes. Gold nanoparticles (GNPs) are introduced into this sensing system in order to enhance the loading of enzymes and to tune their sensitivity and detection limit. Several reviews are available on enzyme immobilization and structural evaluation [2,3] using a variety of spectroscopic, microscopic and optical techniques.

Determination of hydrogen peroxide (H_2O_2) is of considerable interest in recent times. It is not only a by-product of several highly selective oxidases enzymes but also an essential mediator in food, pharmaceutical, clinical, industrial and environmental analysis [4]. H_2O_2 can be detected at a low applied

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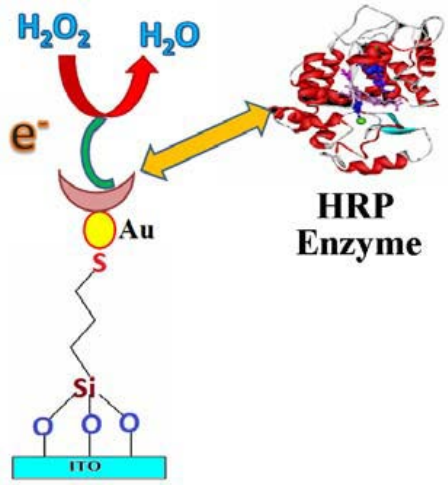
potential by using peroxidase enzyme as an electro-catalyst for monitoring the electrochemical reduction process. The peroxidase enzyme contains heme as a prosthetic group, which is also the protein active site with the resting state of heme iron as Fe(III), and it can catalyze the H_2O_2 reduction. Some of the substrates were reported for this process [5-9]. However, when HRP is immobilized on an electrode, direct electron transfer between enzyme and the electrode becomes difficult because the electro active sites present within the enzyme are deeply buried by the peptide chains. The immobilized enzyme can also partially lose its bioactivity due to an unfavorable orientation at the electrode surface [10]. Therefore, suitable electrode materials and effective enzyme immobilization methods that are capable of facilitating direct electron transfer while retaining the bioactivity of enzyme at the same time are most crucial factors for the success of a biosensor. Several methods including adsorption [11], cross-linking [12], entrapment in sol-gel [13], layer-by-layer assembly [14] have been used to immobilize HRP enzyme on electrodes. Even then the application and development of biosensors have been limited due to the lack of a simple and stable enzyme immobilization approach to monitor the enzymatic reactions. On the other hand, ITO offers an attractive substrate due to their prominent characteristics such as excellent optical transparency, high electrical conductivity, wide electrochemical working window, excellent substrate adhesion and stable electrochemicalproperties [15-18], which could be exploited for immobilizing enzymes and monitoring their catalytic processes.

In this work, we propose a simple methodology to immobilize HRP on SAM modified ITO electrode to monitor its sensing and catalytic behavior towards H_2O_2 as shown in scheme 1. MPTMS is used for monolayer formation and GNPs are used as a matrix to bind the enzyme effectively and to facilitate direct electron transfer between the enzyme and the electrode surface.



Scheme 1: Schematic representation of the proposed methodology for HRP immobilization on SAM modified ITO electrode.

2. Experimental section

2.1. Chemicals and materials

Horseradish peroxidase (HRP, RZ>3.0, A>250U/mg) enzyme was purchased from Sigma Aldrich. 3-mercaptopropyltrimethoxysilane (MPTMS), 80% (Across Organics) was used for monolayerformation on ITO electrodes. H_2O_2 (bio-chemica), KH_2PO_4 (HiMedia), NaOH (Merck) and other chemicals procured were of analytical grade. In addition potassium ferrocyanide, potassium ferricyanide, sodium chloride (all GR grade) were purchased from Merck. All these chemicals were used as received without any further purification. Millipore water having a resistivity of 18 MΩcm was used to prepare all the aqueous solutions used in this work. ITO sheets having a sheet resistance of R_s = 4 – 8□ were obtained from Delta Technologies Limited, Stillwater, MN, USA. This plate (25mm x 75mm x 0.7mm) is a single-side polished,

SiO_2 passivated float glass coated with ITO film of 200 – 500nm thickness. For other characterizations including electrochemical studies, this fairly big plate was cut into small pieces of pre-defined geometric area and was used as strips.

2.2. Preparation of characterization of MPTMS monolayer films on ITO electrodes

Before SAM formation, ITO strips were cleaned ultrasonically using acetone and water for 15min, respectively. After that it was immersed in an aqueous solution consisting of hydrogen peroxide, liquid ammonia, and water in the ratio of 1:3:5 for about 1h. Then these electrodes were rinsed several times with millipore water and immediately used for monolayer formation and subsequent analyses. We have used MPTMS (a short aliphatic chain with thiol terminal functionality) for SAM formation and it was prepared by immersing the pre-cleaned ITO electrodes into 1mM solution in toluene for about 14-15 hrs. After that the SAM of MPTMS coated ITO electrode was taken out; rinsed with excess of toluene and washed thoroughly with millipore water. Further, these electrodes were dried under a stream of nitrogen gas and immediately used for structural and electrochemical characterizations. XPS, AFM and contact angle measurements were used for the structural characterization of SAM modified electrodes.

Electron transfer (ET) behaviour and barrier property of these SAM modified ITO electrodes were evaluated by investigating the ET process of a redox probe using CV and EIS. A three-electrode electrochemical cell was used for electrochemical characterization. A platinum wire and saturated calomel electrode (SCE) were used as counter and reference electrodes respectively. Bare ITO, SAM modified ITO and HRP enzyme immobilized ITO electrodes were employed as working electrodes. Prior to use, the counter electrode was cleaned by dipping into 1:1 mixture of water and conc. HNO_3 for about 5min followed by a thorough cleaning with millipore water; and the reference electrode, SCE was rinsed thoroughly with millipore water. CV experiments were carried out at a potential scan rate of 50mV/s in an aqueous solution of 0.1M NaCl having 1mM potassium ferrocyanide as a redox probe within a potential range from -0.1V to 0.6V vs. SCE. Impedance measurements were carried out using an ac signal of 10mV amplitude at a formal potential of the redox probe (0.17V vs. SCE) using a wide frequency ranging from 100kHz to 100mHz. The electrolyte solution containing equal concentrations of both the oxidized and reduced forms namely, 1mM potassium ferrocyanide and 1mM potassium ferricyanide in 0.1M NaCl aqueous medium was employed for the study. In addition, the impedance data were useful in determining the kinetics parameters such as charge transfer resistance (R_{ct}), surface coverage (θ), pinholes – defects analysis and heterogeneous electron transfer rate constant (k_{ET}) associated with the monolayer film formation on ITO electrodes. All these experiments were performed at room temperature.

2.3. Immobilization of HRP enzyme on SAM modified ITO electrodes

SAM modified ITO electrodes were used as substrates for the immobilization of HRP enzyme. Basically we have adapted three methods. First method involves an electrostatic anchoring of HRP enzyme on SAM coated electrodes and in the other two methods gold nanoparticles (GNPs) were introduced to mediate the enzyme binding events. Second method consists of pre-concentration with copper followed by a galvanic replacement with gold and the other method involves the chemical interaction between GNPs and the thiolate (-SH) terminal functionality from the monolayer. Further these GNPs coated ITO electrodes were employed for HRP enzyme immobilization. During preconcentration step, initially SAM modified ITO electrode was dipped into 1mM aqueous solution of copper sulphate for 30mins. After that it was taken out; washed well with millipore water followed by potential cycling in a phosphate buffer solution with a pH of 7.4 within a potential ranges from -0.6V to 0.6V vs. SCE using CV. Finally, the Cu pre-concentrated ITO electrode was dipped into 1mM gold chloride ($AuCl_4$) solution for the galvanic replacement which is further confirmed by performing CV in aqueous phosphate buffer solution (pH = 7.4) between the potential range from -0.4V to 1.2V vs. SCE at a fixed scan rate of 50mV/s. In the case of third method, the monolayer coated ITO electrode was directly dipped into an aqueous solution of citrate stabilized GNPs for 30mins. CV and XPS were used to confirm the presence of GNPs. Finally these electrodes were dipped into 0.1M phosphate buffer solution containing HRP (0.1mg/2.5ml) for a specified time, which results in the anchoring of this enzyme on these chemically modified electrodes. After the immobilization, enzyme modified electrodes were washed well with phosphate buffer; thoroughly washed with millipore water and used for further characterizations and analysis.

2.4. Instrumentation

CV and EIS measurements were carried out using an electrochemical impedance analyzer, Model 6310, EG&G instruments obtained from Princeton Applied Research, USA and an AUTOLAB instrument, received from The Netherlands. CV experiments and their analysis of data were performed using *echem* software provided by EG&G. The potential ranges and scan rates used for the study were shown in the respective diagrams. The impedance data were fitted to an appropriate equivalent circuit model using the Autolab equipment and *Zsimpwin* software (from EG&G) developed on the basis of Boukamp's model. Based on this procedure several kinetics parameters mentioned above associated with the formation of monolayer were also determined. XPS measurements were carried out using Multilab 2000 model, Thermo Scientific, UK. A source of Al K_α having a binding energy of 1486.4eV was used for this study. Initially a full scan from -10 to 1100eV was performed and later on individual scans of 30-50eV having a step energy of 0.5eV were carried out in order to analyze the presence of specific elements and their corresponding oxidation states. AFM studies were carried out using a Pico Plus instrument made by Molecular Imaging (MI). The images were recorded in a tapping mode (ac mode) at a frequency of 175kHz with a cantilever of n_+-silicon type (PPP-NCL-50) obtained from Nanosensors, USA and these images were corrected for plane tilt using a software. The contact angle equipment was purchased from AST Products Inc., USA, having an automated model of VCA Optima XE. The measurement was carried out at room temperature on SAM modified substrates using water based on static sessile drop method.

3. Results and discussion

3.1. Characterization of SAM using XPS

ITO electrodes were chemically modified with a monolayer of MPTMS and the structural characterization of such a modified electrode was carried out using XPS, AFM and contact angle measurements. XPS is a surface chemical analysis technique in which the nature of adsorbing materials even up to the presence of a monolayer could easily be analyzed. We have investigated the formation of SAM of MPTMS on ITO using XPS and for comparison the bare ITO was also studied. Figure 1 shows the XPS spectra of S (2p), Si (2p), C (1s) and O (1s) regions of MPTMS monolayer on ITO surface. It can be seen that the formation of a peak at 163eV (2p) and at 154eV (2p) showed the presence of sulphur and silicon respectively. Further, the formation of peaks corresponding to carbon (1s) and oxygen (1s) could also be noted. Bare ITO showed a peak formation at 444.2eV ($3d_{5/2}$) and at 451.8eV ($3d_{3/2}$) corresponding to indium and at 486.3eV ($3d_{5/2}$) and 494.7eV ($3d_{3/2}$) corresponding to tin respectively, in addition to the usual carbon and oxygen peaks [19,20]. From XPS studies and analysis, it is clear that MPTMS molecules form a monolayer film on ITO electrodes.

3.2. AFM and contact angle measurements

Figure 2 displays a representative AFM image of SAM of MPTMS on ITO electrode. Formation of various domains (topography, 2A) of size ~15-20nm (surface profile, 2C) is clearly evident from these reproducible AFM images. We have measured a surface height of ~43-45nm in Z range from Fig. 2B (3D representation) and these domains extend vertically with the formation of pores in between them, suggesting the immobilization of MPTMS molecules onto ITO surface to form a monolayer film. Surface wettability in terms of hydrophilic and hydrophobic nature of the SAM modified ITO electrode was evaluated using contact angle measurements. We have used a static sessile drop method and the values reported were the average of contact angles measured at five different locations of the sample. Figure 2D shows the water static contact angle of monolayer film MPTMS on ITO electrodes. The equilibrium water contact angle of bare ITO (before modification) exhibits a strong dependence on the pre-treatment method adapted for cleaning the ITO surface. As received ITO surface provides a contact angle of 60±1°, which reflects a higher degree of hydrophobic nature of the substrate and upon pre-treatment by the method explained in experimental section 2.2, contact angle was found to decrease drastically to 18±2° implying a more hydrophilic nature. This change indicates a significant increase in the composition of hydroxyl groups on ITO surface as a result of this cleaning method. Similar observation was reported earlier in literature [21]. After the formation of MPTMS monolayer, contact angle value was found to increase significantly and was measured to be 76±1°.

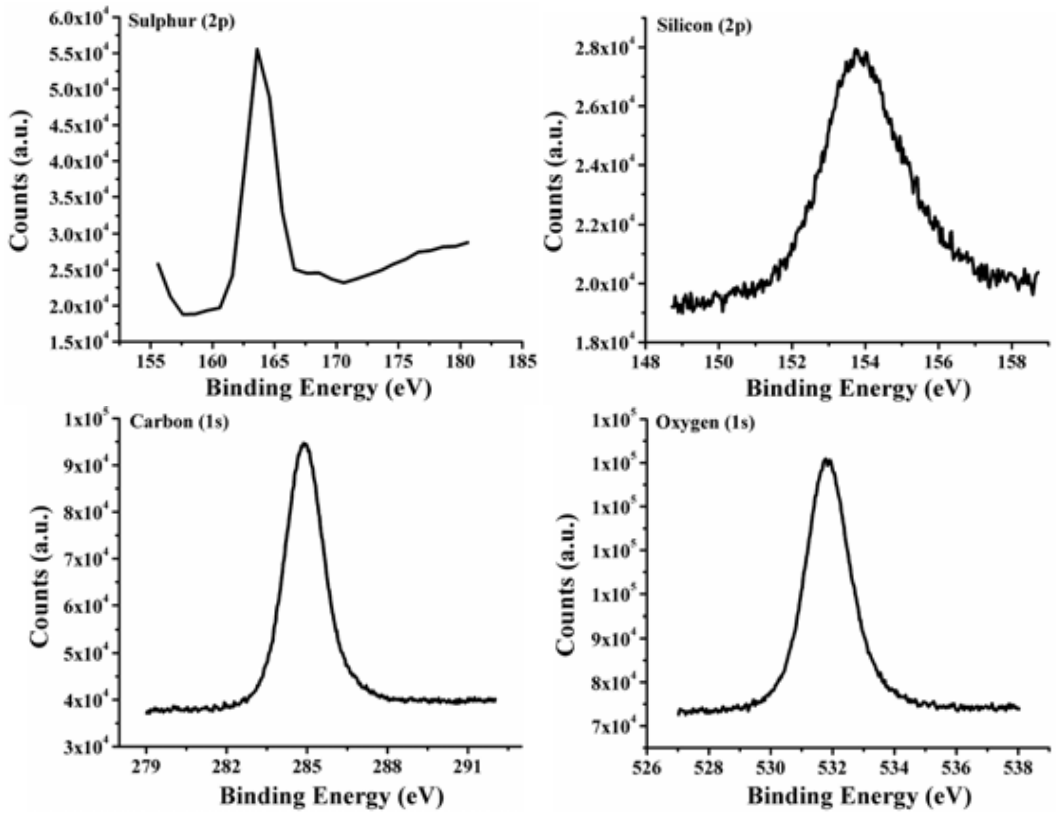


Figure 1: XPS spectra of MPTMS monolayer on ITO electrodes displaying S (2p), Si (2p), C (1s) and O (1s) regions.

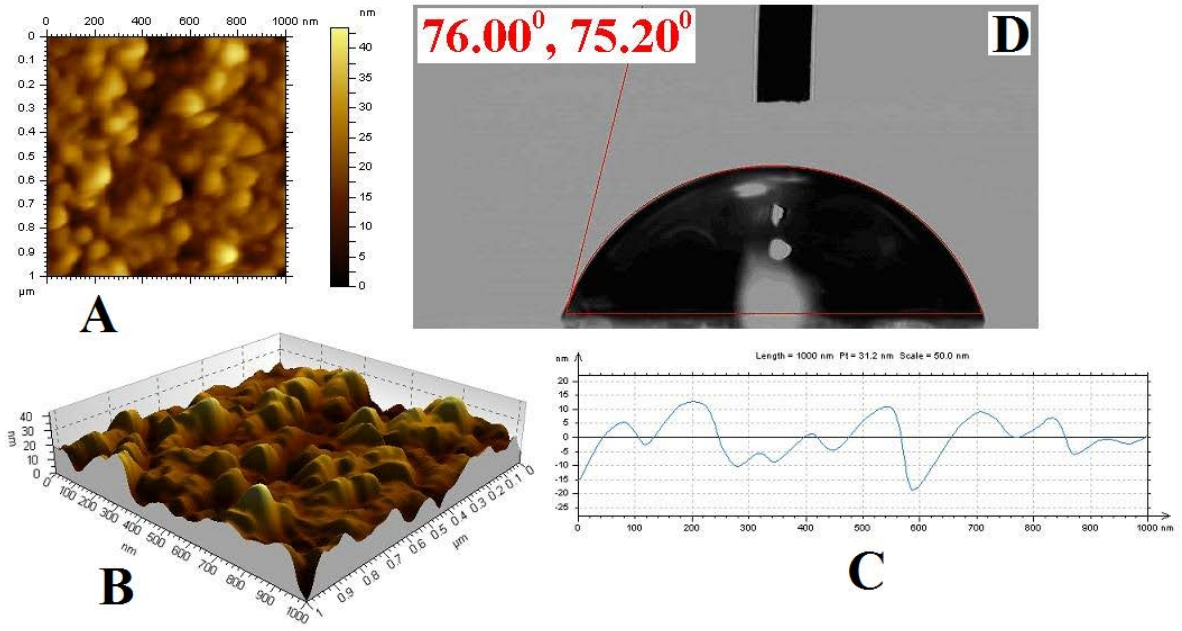


Figure 2: AFM [Topography (A), 3D representation (B) and surface profile (C)] and contact angle images of SAM of MPTMS on ITO electrode.

3.3. Electrochemical characterization of SAM

CV and EIS are the most popular techniques in electrochemistry and these methods are demonstrated to be the most effective techniques to assess the quality of a monolayer film. Generally, the blocking behaviour of such monolayer films towards ET reaction of a redox probe is investigated using these techniques by employing potassium ferro/ferricyanide redox couple as a probe. Figure 3A shows the CVs of bare ITO and SAM of MPTMS coated ITO electrodes in 1mM potassium ferrocyanide with 0.1M NaCl as a supporting electrolyte at a potential scan rate of 50mV/s. It can be seen that bare ITO electrode (Fig. 3A[a]) exhibits a peak shaped CV for the redox reaction indicating that the ET reaction is under diffusion controlled and reversible. On the other hand, monolayer of MPTMS (Fig. 3A[b]) on ITO electrode does not show any distinct peak formation indicating that the ET reaction of a redox probe is completely suppressed and shows a perfect blocking behaviour with the formation of very less pinholes and defects. Interestingly, beyond a positive potential range of > 0.05V vs. SCE, the magnitude of redox current is found to be two orders of magnitude lower in the case of SAM of MPTMS when compared to bare ITO electrode. This clearly shows that the redox reaction is blocked on SAM modified electrodes due to the formation of highly ordered, well-packed, compact monolayer films with ultra low defect density.

Structural integrity and barrier property of this monolayer film were further evaluated using electrochemical impedance spectroscopic studies. Using this technique, a parameter, namely charge transfer resistance (R_{ct}) is determined, which provides the resistance offered by these monolayer films towards the ET process across the SAM modified electrode – electrolyte interface. In addition, the impedance data were also used to determine the surface coverage (θ) and other kinetic parameters of the monolayer coated electrodes. Figure 3B shows the impedance plots (Nyquist plots) of bare ITO and monolayer of MPTMS coated ITO electrodes in equal concentrations (1mM) of potassium ferrocyanide and potassium ferricyanide having 0.1M NaCl as a supporting electrolyte. The impedance measurements were carried out at a formal potential of $[Fe(CN)_6]^{3-/4-}$ redox couple, viz., 0.17V vs. SCE. It can be noted from these figures that bare ITO electrode (inset) shows a low frequency straight line with a very small semicircle at high frequency region indicating that the ET process of a redox couple is reversible and essentially diffusion controlled. On contrary, monolayer coated electrode exhibits a semicircle formation over the entire range of frequency used for the study indicating that the redox reaction is inhibited by displaying a perfect blocking behaviour. In such case the process of ET is considered to be under charge transfer control. Formation of a very large semicircle in the case of SAM of MPTMS on ITO electrodes confirms an excellent electrochemical blocking ability of these monolayer films against the diffusion of a redox probe molecule. Higher the charge transfer resistance, higher is the blocking behaviour. These results are in good agreement with our CV results discussed earlier.

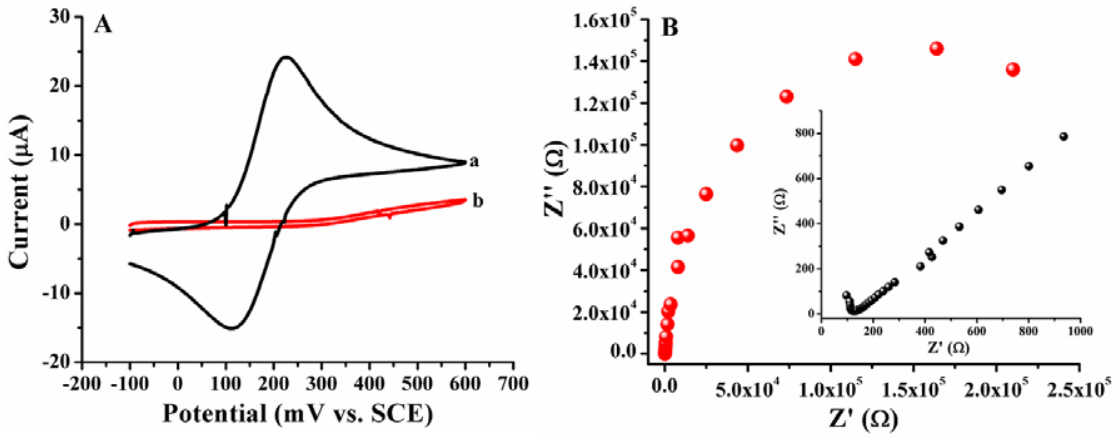


Figure 3: (A) CVs of bare ITO and SAM of MPTMS modified ITO electrodes in 1mM potassium ferrocyanide with 0.1M NaCl as a supporting electrolyte at a fixed scan rate of 50mV/s. (B) Impedance (Nyquist) plots in equal concentrations (1mM) of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ aqueous solution containing 0.1M NaCl for the MPTMS monolayer on ITO. Inset shows the same plot for bare ITO electrode.

By equivalent circuit fitting procedure using the impedance data we have determined R_{ct} values of 17.65Ω cm^2 and 5.44 x 10^4Ω cm^2 for bare ITO and SAM modified ITO electrodes respectively [22]. Basically the monolayer of MPTMS exhibited 4 orders of magnitude higher R_{ct} values implying a better blocking behaviour of SAM towards the redox reaction. Using this R_{ct} value, we have calculated the surface coverage of > 0.999 in the case of SAM of MPTMS suggesting the formation of wellpacked, highly dense monolayer films on ITO electrodes. From the R_{ct} values, one can also determine the heterogeneous electron transfer rate constant (k_{ET}) of $[Fe(CN)_6]^{3-/4-}$ redox couple on the unmodified bare ITO and SAM modified ITO electrodes using the following Eq. (1).

$$k_{ET} = RT / F^2 R_{ct} C \qquad (1)$$

where R is the gas constant, T is the temperature, F is the Faraday's constant, C is the concentration of the redox probe and R_{ct} is the charge transfer resistance respectively. These values were calculated to be 0.1508 cm/s and 4.895 x 10^{-5} cm/s for the cases of bare ITO and MPTMS monolayer coated ITO electrodes [22]. It can be noticed that the monolayer displayed almost 4 orders of magnitude lower rate constant when compared to the rate constant of bare ITO electrode. This suggests that the SAM inhibits ET process by slowing down its kinetics due to the formation of a monolayer film on ITO electrode. The extent of sluggishness depends mainly on the quality and the chemical structure of monolayer films formed on ITO electrodes.

3.4. Immobilization of HRP enzyme

After SAM formation, GNPs were anchored onto the monolayer coated ITO electrodes. As mentioned earlier, two methods namely pre-concentration of Cu followed by a galvanic replacement with Au and functionalization of terminal thiol group with GNPs using chemical interactions. Both these methods result in the formation of ordered assemblies of GNPs on monolayer coated ITO electrodes and the presence of gold is confirmed using CV and XPS. In the case of CV carried out in an aqueous phosphate buffer solution at a constant scan rate of 50mV/s, appearance of redox peaks corresponding to Au reveals the anchoring of GNPs on SAM modified ITO electrodes. Further, the formation of Au 4f (5/2 and 7/2 spins) peaks at 84.4eV and 88eV during XPS studies confirms the presence of GNPs (Fig. 4A inset).

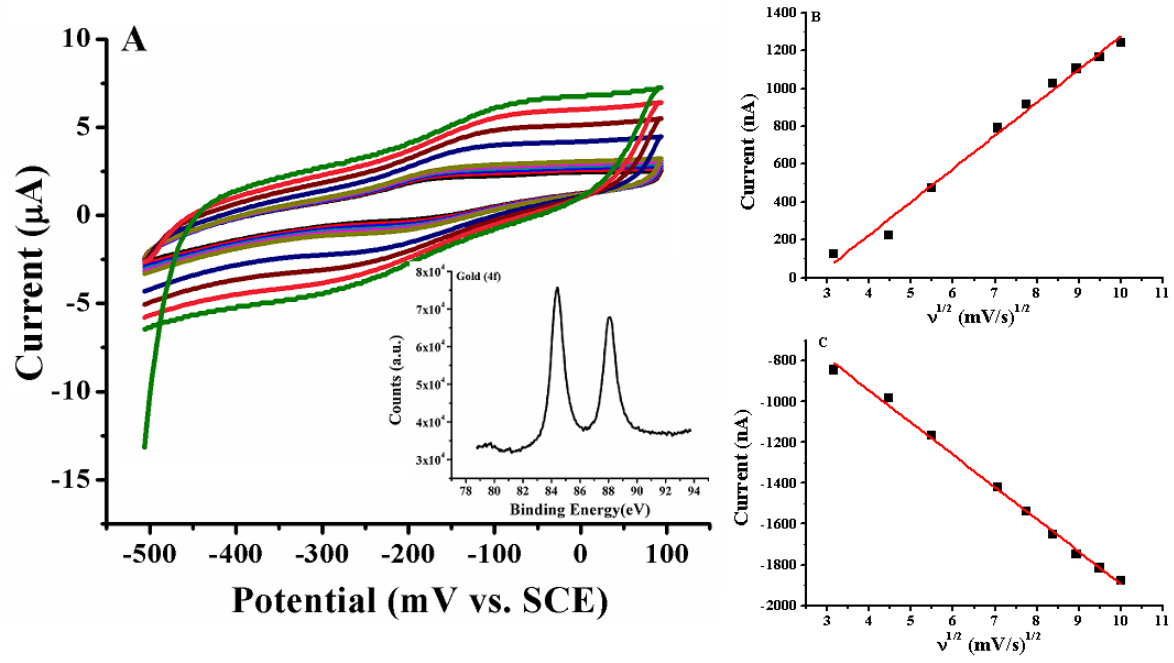


Figure 4: (A) Effect of scan rate on the HRP immobilized ITO electrode in phosphate buffer solution. A wide range of scan rate starting from 10mV/s to 100mV/s was used for the study. Inset shows the XPS spectrum of Au (4f) region. (B) and (C) show the plots of current (oxidation & reduction) vs. square root of scan rate.

HRP enzyme was immobilized on these GNPs coated electrodes by immersing them into a phosphate buffer solution consisting of HRP (0.1mg/2.5ml) for various amounts of time and evaluated their anchoring ability using CV. The redox properties and electron transfer ability of HRP enzyme were investigated with the aid of CV and these studies were carried out in an aqueous phosphate buffer solution possessing a pH of 7.4 at a fixed scan rate of 50mV/s within a potential ranging from -0.5V to 0.1V vs. SCE. The formation of redox peaks at -0.16V and -0.145V vs. SCE reveals the presence of redox proteins (due to Fe from HRP) confirming the immobilization of HRP enzyme on these modified ITO electrodes. Further the effect of scan rate on the redox property of HRP enzyme was studied by varying the scan rate from 10mV/s to 100mV/s and the corresponding CVs were displayed in figure 4A. The formation of redox peaks at all the scan rate suggests that the enzyme was immobilized on these modified electrodes and it is stable over a wide range of scan rate. Figures 4B and 4C show the plots of current corresponding to both the oxidation and reduction processes vs. square root of scan rate. It can be inferred from these plots that there is a linear relationship between the current and square root of scan rate suggesting that HRP enzyme is immobilized on these electrodes and its ET ability is under diffusion controlled. The fact that HRP immobilized ITO electrodes mediate direct ET across the interface implies that the redox sites present within the enzyme is accessible and these molecules structurally anchored on ITO electrodes in such a way that the redox protein is exposed outside. This sort of structural arrangement is very much essential to impart the sensing and catalytic applications so that the enzymatic kinetics can easily be monitored using potentiometry, amperometry and voltammetry. In addition XPS was also used to confirm the HRP immobilization and their results reveal the formation of Fe 2p peak at 719.6eV (Fig. 5A inset) corresponding to redox proteins of HRP enzyme.

3.5. Hydrogen peroxide (H_2O_2) sensing

Enzymatic reaction corresponding to HRP namely reduction of hydrogen peroxide (H_2O_2) to water was studied using these HRP immobilized ITO electrodes. These electrodes were initially tested towards H_2O_2 sensing in phosphate buffer solution and the corresponding studies were performed using CV. Figure 5A shows the CVs of HRP immobilized ITO electrodes in aqueous buffer solution towards H_2O_2 addition at a fixed scan rate of 50mV/s within the potential range between 0.1V and -0.5V vs. SCE. A wide range of concentration starting from 0.5mM to 5mM was used for the study. For comparison, a CV of HRP immobilized ITO electrode before H_2O_2 addition was also shown. It can be seen from these figures that the HRP anchored electrode displayed the redox peaks corresponding to the enzyme (confirmed by XPS – See inset) before the introduction of H_2O_2. On adding 0.5mM H_2O_2 to the buffer solution, a change in CV was

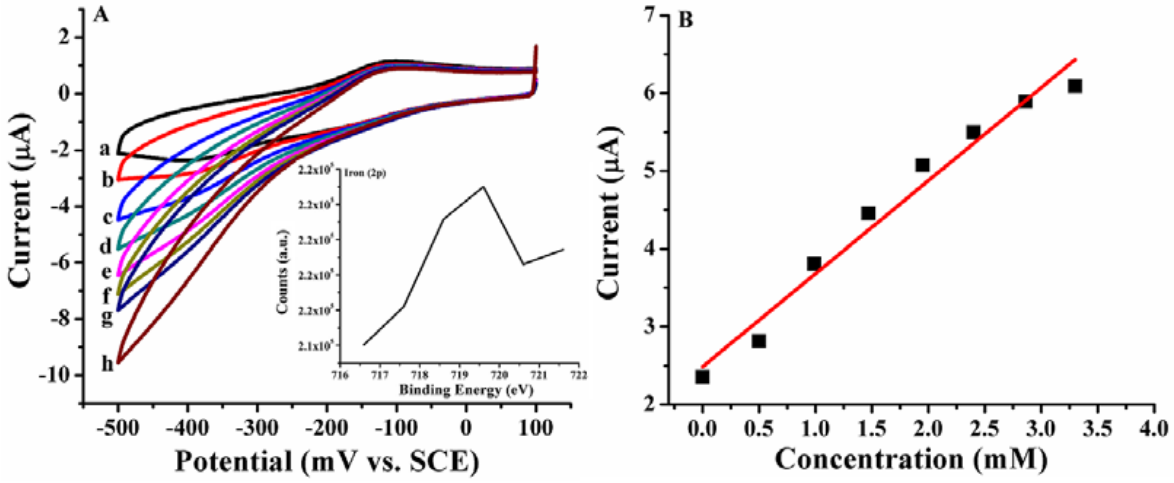


Figure 5: (A) CVs of HRP immobilized ITO electrode in a phosphate buffer aqueous solution towards H_2O_2 addition for various concentration ranging from (b) 0.5mM, (c) 1mM, (d) 1.5mM, (e) 2.0mM, (f) 2.5mM, (g) 3.0mM and (h) 3.5mM respectively. For comparison, the CV of HRP anchored electrode before H_2O_2 addition (a) was also shown. Inset shows the XPS spectra of Fe (2p) region of HRP enzyme immobilized ITO electrode. (B) A plot of current vs. concentration for H_2O_2 addition.

observed indicating a substantial increase in the magnitude of reduction current. Further increase in H_2O_2 concentration results in further increase in the reduction current values suggesting that HRP enzyme retains its activity and it helps in effectively reducing H_2O_2 to water. Figure 5B shows the corresponding plot of current vs. concentration of H_2O_2, from which sensitivity and detection limit values can be determined. In this case, we have determined a sensitivity of 1.2946μA/mM and the detection limit was estimated to be 2.858μM. These values suggest the better performance of this particular sensor when compared to some of the other works reported earlier. The higher sensitivity essentially arises from the introduction of GNPs which in turn helps in higher loading of HRP enzymes on chemically modified ITO electrodes. Further, we observed that either the monolayer of MPTMS coated or GNPs modified ITO electrodes do not contribute to sensing and reduction of H_2O_2 suggesting the effective role of HRP for both the sensing and catalytic applications.

4. Conclusions

Given the importance of bio-mimics, it is absolutely essential to understand the enzymatic reactions, structural arrangement and the associated electron transfer by immobilizing them onto the electrodes. In this work, we have successfully demonstrated a couple of strategies to immobilize and investigate enzymatic reaction of HRP using a simple process of chemical modification on ITO electrodes. Using this process, a sensor for H_2O_2 is fabricated and the performance is evaluated using CV and XPS.

Acknowledgement

Authors would like to acknowledge Department of Science and Technology (DST), India for providing funding towards this research work through Fast Track Scheme for Young Scientists under the project GAP 16/10. Useful discussions and help from Prof. V. Lakshminarayanan of RRI, Bangalore regarding AFM studies are acknowledged. Authors also thank Central Instrumentation Facility (CIF) of CSIR – CECRI for providing XPS facility for surface characterization.

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