Horseradish Peroxidase and Horseradish peroxidase-SWNT Composites for Hydrogen Peroxide Detection

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Abstract **The Horse radish peroxidase enzyme (HRP) displays good biosensing properties by catalyzing reduction of hydrogen peroxide. Here, we present the immobilization and biosensor properties of HRP enzyme on self-assembled monolayers (SAM) by covalent modification, non-covalent modification and electrochemical modification. The properties of the HRP was enhanced by forming the single walled carbon nanotubes composites. The immobilized surface was characterized by atomic force microscopy (AFM), cyclic voltammetry (CV) and chronoamperometry (CA). The developed sensor shows the excellent electrocatalytic activities towards the reduction of hydrogen peroxide. The detection limits were found to be 10, 1, and 0.8 µM for covalent, non-covalent and electrochemically immobilized HRP, respectively. Linear calibration plots were obtained in the concentration range of 50 µM to 275 µM.**

Keywords —Biosensors, Cyclic Voltammerty, Horse radish peroxidase, Hydrogen peroxidase, Self-assembled monolayers, Single walled carbon nanotubes.

I. INTRODUCTION

Development of a biosensor with high reproducibility and reliability is a challenging. The stability of the biosensor depends on the orientation of the bio-elements on the surface. The favorable orientations on the surfaces depend on the method of immobilization of bio-elements on the surface. The enzymes like cytochrome C, Myoglobin, Horse radish peroxidase, glucose oxidase etc. have been used as the bio-element for the sensing application [1].

Horseradish peroxidase (HRP) is a 44,173.9 dalton protein. HRP is a single chain polypeptide containing four disulfide bridges. The 18% carbohydrate is constituted of galactose, arabinose, xylose, fructose, mannose, mannosamine, and galactosamine depending upon the specific isozyme. The isoelectric point of HRP is around 6.8 [2].

HRP readily combines with hydrogen peroxide (H2O2) and the resultant [HRP-H2O2] complex can oxidize a wide variety of hydrogen donors. HRP is ideal in many applications because it is more stable and less expensive than other popular alternatives such as alkaline phosphatase. It also has a high turnover rate that allows generation of strong signals in a relatively short time span.

Horseradish peroxidase (HRP), functioning physiologically as in the biocatalysis of H2O2, has been extensively investigated. The direct electron transfer catalysis of immobilized HRP has been achieved on different modified surfaces [3]-[6]. There are some reports in literature on HRP-SWNT modified electrodes [7]-[9].

Here, we have studied the HRP immobilization by various techniques and studied their electrocatalytic activity towards hydrogen peroxide. The prepared sensors are characterized by AFM, CV and CA.

II.EXPERIMENTAL

Chemicals

We have used the following analytical grade reagents: Horseradish peroxidase (Sigma), 4-aminothiophenol (Aldrich), sodium phosphate dibasic Na2HPO4 (Rankem), sodium dihydrogen phosphate (Merck), hydrogen

peroxide (H2O2) solution 30% (Merck). All the solutions were prepared using Milli-Q water of resistance 18 MΩ cm. protein solution was made by dissolving 2 mg of protein in 0.3 ml of phosphate buffer solution of pH 6.5.

Purification of Single Walled Carbon Nanotubes (SWNTs)

The single walled carbon nanotube obtained from Carbolex Co. USA has been purified as reported earlier [36]. In brief, the raw soot SWNT was subjected to air oxidation by heating the sample in air at 350 0C for 4 hours to oxidize the non-tubular form of the carbon. The SWNT material was then refluxed with 6N nitric acid for 30 min in order to dissolve the metal particles. After cooling, the sample was filtered using a

cellulose nitrate filter paper with 0.2 μm pore size by applying vacuum suction. A clear green colored supernatant acidic solution was collected at the bottom of filtering unit. Successive washing with Milli-Q water removes the substantial amount of trapped acid form the sediment. The SWNTs collected in the filter paper was dried in an air oven at 50 0C for 30 min.

Electrode Pretreatment

All electrochemical experiments were carried out using gold disc of geometric area 0.002 cm2 as a working electrode. Immediately before use, the poly crystalline gold disc electrode was polished with emery paper of grade 800 and 1500, followed by polishing in aqueous slurries of 1 μm, 0.3 μm and finally with 0.05 μm alumina slurries, ultrasonicated in water to remove alumina particles for 1 minute and then cleaned with dil. aquaregia (3:1:4 mixture of conc. HCl, conc. HNO3 and water) for one minute before each experiment. Finally, it was rinsed in distilled water thoroughly, followed by rinsing in Milli-Q water and ethanol before SAM formation.

Self-Assembled Monolayer Formation

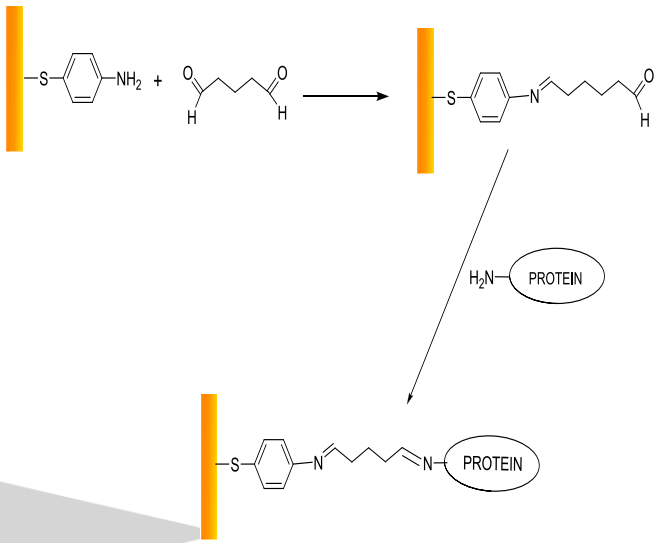
The self-assembled monolayer of 4-Aminothiophenol (4-ATP) was formed by placing the cleaned gold disc electrode in 1 mM ethanol solution of 4-ATP for 12 hours. The electrode was taken out from the thiol solution, rinsed with ethanol and finally with milli-Q water.

Proteins Immobilization on 4-ATP

Covalent immobilization of proteins were carried out by immersing 4-ATP modified Au electrode in a 30% glutaraldehyde for 24 hours. The electrode was taken out from gluaraldehyde, rinsed with water and placed in protein solution at 4 0C and pH 6.5 for 24 hours. The electrode was taken out from the protein solution, washed with phosphate buffer solution for several times, followed by Milli-Q water to remove any non-specific bound protein on the surface. The schematic representation of the covalent modification of proteins to the amine terminated surfaces (protein = HRP) is as shown scheme 1.

Non-covalent adsorption was carried out at 4 0C onto the 4-ATP self-assembled monolayer modified gold surface by immersing in a 2 mg of protein in a 0.3 ml of 0.1M sodium phosphate buffer solution of pH 6.5. The electrode was taken out from the protein solution, washed with phosphate buffer followed by rinsing in Milli-Q water.

Proteins were immobilized at room temperature onto the 4-ATP self-assembled monolayer modified gold surface by potential cycling in a microcell containing protein solution. The electrode was taken from protein solution, washed with phosphate buffer solution followed by rinsing in Milli-Q water. Different methods of immobilization are shown in the Table 1.



Scheme 1. Schematic representation of the covalent modification of proteins to the amine terminated surfaces (protein = HRP).

Immobilization of Protein-SWNT on Monolayer Modified Surface

Dispersion of SWNT in Protein Solution

Homogeneous and well-dispersed CNTs in aqueous and non-aqueous media were reported earlier [10]. Here, we have used analogous method to disperse the SWNTs in phosphate buffer solution. Firstly, 2 mg of protein was dissolved in 0.3 ml of 0.1M phosphate buffer solution of pH 6.5, then purified SWNTs were added into the protein solution and sonicated. The protein helps in better solubilization of SWNTs in phosphate buffer.

The 4-ATP modified gold disc electrode was placed in the freshly prepared dispersion of protein-SWNT for 24 hours at 4 0C. The electrode was taken out and washed with phosphate buffer solution followed by Milli-Q water. During the formation of the dispersion by sonication under ice cold water, the protein in the solution functionalizes the SWNTs by hydrophobic interaction.

Protein-SWNT was immobilized at room temperature onto the 4-ATP self-assembled monolayer modified gold surface by potential cycling in a microcell containing protein-SWNT dispersion solution. The electrode was taken from protein-SWNT solution, washed with phosphate buffer solution followed by rinsing in Milli-Q water. Table 1 gives the different proteins and protein-SWNT composites immobilized on 4-ATP surface.

Method of Immobilization	
Covalent	HRP/GA/4-ATP/Au
Non-covalent	HRP/4-ATP/Au

Electrochemical	EHRP/4-ATP/Au
Non-Covalent (protein-SWNT)	HRP-SWNT/4-ATP/Au
Electrochemical (protein-SWNT)	EHRP-SWNT/4-ATP/Au

Table 1. Different methods used to immobilize proteins and HRP-SWNT composites on 4-ATP (Horseradish peroxidase = HRP, 4-ATP = 4-Aminothiophenol, GA = glutaraldehyde.)

III. RESULTS AND DISCUSSIONS

Atomic Force Microscopy (AFM) Studies

Figure 1 shows the AFM image of the HRP-SWNT on 4-ATP modified gold surface. There are rod like features, with heights which are in the range 10-30 nm. These features clearly show that the HRP wrapped SWNTs has been immobilized on the surface. The heights of more than 10 nm implies that multilayer of protein molecules was formed on the surface.

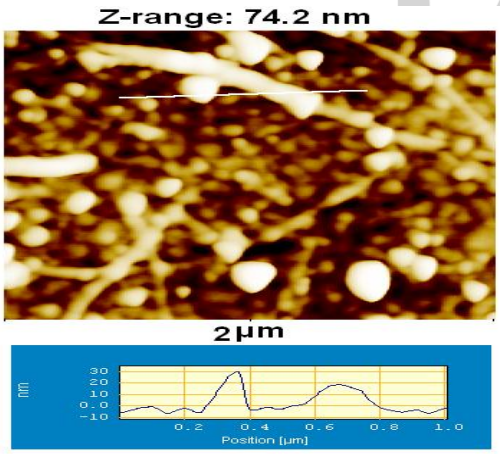


Figure 1. AFM image of the HRP-SWNT immobilized on 4-ATP modified Au surface.

Redox Properties

Figure 2 shows the HRP-SWNT cyclic voltammograms taken in a phosphate buffer solution at different scan rates immobilized on 4-ATP modified gold surface. The cyclic voltammograms shows redox peaks corresponds to Fe2+/ Fe3+ reaction of immobilized HRP. The peak current varies linearly with scan rate following the expression ip= n2F2νAΓ/4RT, where n=number of electrons, F=Faraday constant, Γ=the number of redox active sites on the surface, A=area, ν=scan rate as shown in the Figure 37(II), indicating a diffusionless surface bound species. The half peak potential for the electron transfer process is -110 mV verses SCE.

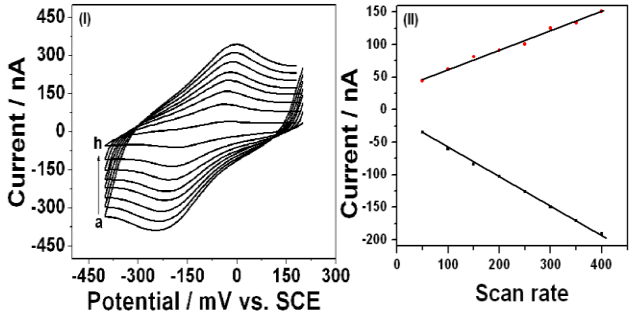


Figure 2. (I) Cyclic voltammograms of HRP/SWNT/4-ATP/Au electrode in 0.1 M phosphate buffer solution of pH 6.5 under N_2 atmosphere at scan rates (mVs^{-1}) of (a) 50, (b) 100, (c) 150, (d) 200, (e) 250, (f) 300, (g) 350, and (h) 400 (II) The plot of Peak current verses scan rate.

Electrocatalysis of HRP and HRP-SWNT Modified Electrodes Towards the Reduction of Hydrogen Peroxide (H2O2)

The electrocatalytic reduction of H2O2 at protein modified surface was studied using CA. The CA measurements were conducted at ambient temperature in phosphate buffer solution of pH 7.0 under N2 atmosphere with constant stirring. The amperometric response of protein modified electrodes was recorded as -250 mV. This potential was chosen based on the CVs for the H2O2 reduction at HRP modified surfaces, which exhibits the reduction peak of H2O2 at -250 mV. Figure 2A is the amperometric response of HRP immobilized by covalent, non-covalent and electrochemical methods on 4-ATP. The concentration of H2O2 was increased in steps of 25 µM. The current due to H2O2 reduction is considerably higher in the case of electrochemically immobilized HRP compared to the covalent and non-covalent methods. Detection limits for H2O2 were determined by adding very small amounts of H2O2 until there is a detectable (few nA) change in current. The detection limits were found to be 10, 1, and 0.8 µM for covalent, non-covalent and electrochemically immobilized electrodes, respectively. Linear calibration plots were obtained in the concentration range of 50 µM to 275 µM as shown in the Figure 2B.

Figure 3A is the amperometric response of HRP-SWNT immobilized on 4-ATP. The concentration of H2O2 was increased in steps of 25 µM. Current due to H2O2 reduction is considerably higher in the case of electrochemically immobilized HRP-SWNT compared to the simple adsorption of HRP-SWNT on 4-ATP surface. The detection limits were found to be 0.09 and 0.05 µM for HRP-SWNT and EHRP-SWNT electrodes, respectively. Linear calibration plots are obtained in the concentration range of 50 µM to 225 µM as shown in Figure 3B.

Table 2 shows the detection limits for the HRP immobilized on 4-ATP monolayer. It can be seen from the table that HRP

shows very low detection limits. The results also establishes the catalytic activity of the HRP is significantly enhanced by functionalizing with SWNTs.

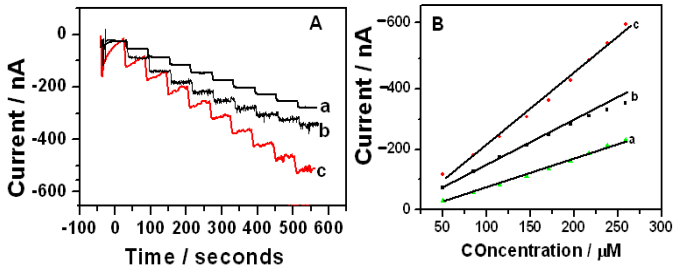


Figure 2. (A) Chronoamperometry measurements of HRP modified electrodes (a) HRP/GA/4-ATP/ Au, (b) HRP/4-ATP/Au, and (c) EHRP/4-ATP/Au electrodes with successive addition of H_2O_2 in phosphate buffer solution (PBS) of pH 7.0 under N_2 atmosphere and stirred conditions (B) corresponding calibration plots.

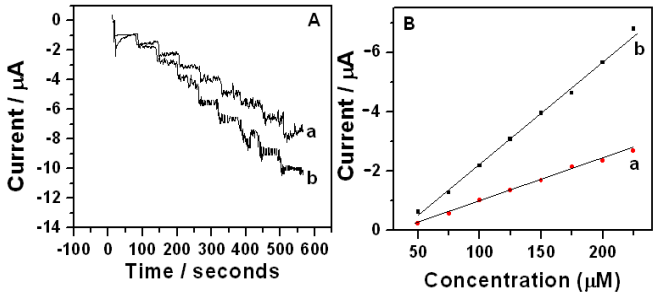


Figure 3. (A) Chronoamperometry measurements of HRP-SWNT modified electrodes (a) HRP-SWNT/4-ATP/ Au and (b) EHRP-SWNT/4-ATP/Au electrodes with successive addition of H_2O_2 in phosphate buffer solution (PBS) of pH 7.0 under N_2 atmosphere and stirred conditions (B) corresponding calibration plots.

Protein	Detection Limit (µM)
Covalent	10
Non-covalent	1
Electrochemical	0.8
Non-Covalent (HRP-SWNT)	0.09
Electrochemical (HRP-SWNT)	0.05

Table 2. Shows the detection limits for hydrogen peroxide reduction catalyzed by different proteins immobilized on 4-ATP.

IV. CONCLUSIONS

The self-assembled monolayers formed by the chemisorption of thiols on gold offers a platform for immobilization of proteins on surfaces while simultaneously retaining the bioactivity. The electrocatalytic activity of proteins on surfaces depends on the immobilization technique. We have immobilized the proteins by different methods such as covalent coupling, non-covalent adsorption and by electrochemical cycling on self-assembled monolayers. To enhance the electrocatalytic activity of proteins on surfaces, composites of protein-SWNTs have been immobilized non-covalently by simple immersion process and electrochemically by potential cycling the monolayer modified electrode in protein-SWNT composites. The electrochemical studies have been carried out using cyclic voltammetry and chronoamperometry. The electrocatalytic activity of HRP-SWNT is significantly higher than the covalent and non-covalently immobilized HRP on 4-ATP.

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REFERENCES

[1] Nagaraju D. H. "Electrochemical Studies of Some Redox Active Biomolecules and Small Organic Molecules on Nanomaterials Modified surfaces" Ph.D. dissertation, JNU. Delhi, 2011.

[2] C. Phelps, L. Forlani, E. Antonini, "The hydrogen ion equilibria of horseradish peroxidase and apoperoxidase."Biochem. J. vol.124, pp. 605, 1971

[3] Valdemaras J. Razumas, Arvydas V. Gudavičius, Juozas J. Kulys, "Redox conversion of peroxidase on surface-modified gold electrode." J. Electroanal. Chem. vol.151, pp. 311, 1983.

[4] L. Gorton, G. Jönsson-Pettersson, E. Csöregi, K. Johansson, E. Domínguez G. Marko-Varga, **"**Amperometric biosensors based on an apparent direct electron transfer between electrodes and immobilized peroxidases. Plenary lecture," Analyst vol. 117, pp. 1235. 1992.

[5] T. Lötzbeyer, W. Schuhmann, H.-L. Schmidt, "Minizymes. A new strategy for the development of reagentless amperometric biosensors based on direct electron-transfer processes," Bioelectrochem. Bioenerg. vol. 42, pp. 1, 1997.

[6] J. Li, S. Dong, "The electrochemical study of oxidation-reduction properties of horseradish peroxidase," J. Electroanal. Chem. vol. 431, pp. 19, 1997.

[7] Y.-D. Zhao, W.-D. Zhang, H. Chen, Q.-M. Luo, S. F. Y. Li, "Direct electrochemistry of horseradish peroxidase at carbon nanotube powder microelectrode," Sens. Actuat. B vol. 87, pp 168, 2002.

[8] C. Cai, J. Chen, "Direct electron transfer and bioelectrocatalysis of hemoglobin at a carbon nanotube electrode," Anal. Biochem. vol. 325, pp. 285, 2004.

[9] F. Zhao, X. Wu, M. Wang, Y. Liu, L. Gao, S. Dong, "Electrochemcial and bioelectrochemistry properties of room-temperature ionic liquids and carbon composite materials," Anal. Chem. vol. 76, pp. 4960, 2004.

[10] J. Wang, M. Musameh, Y. Lin, "Solubilization of carbon nanotubes by nafion toward the preparation of amerometric biosensors," J. Am. Chem. Soc. vol. 125, pp. 2408, 2003.