An amperometric H_2O_2 biosensor based on hemoglobin nanoparticles immobilized onto a gold electrode

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Keywords: H_2O_2; Hemoglobin nanoparticles; Gold electrode; Serum; H_2O_2 biosensor.

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Abstract

The nanoparticles (NPs) of hemoglobin (Hb) were prepared by desolvation method and characterized by transmission electron microscopy (TEM), ultraviolet (UV) spectroscopy and Fourier-transform infrared spectroscopy (FTIR). An amperometric H_2O_2 biosensor was constructed by immobilizing HbNPs covalently onto a polycrystalline Au electrode (AuE). HbNPs/AuE was characterized by scanning electron microscopy (SEM), cyclic voltammetry (CV) and electrochemical impedance spectra (EIS) before and after immobilization of HbNPs. The HbNPs/Au electrode showed optimum response within 2.5s at pH 6.5 in 0.1M sodium phosphate buffer containing 100μM H_2O_2 and 30^0C, when operated at -0.2V against Ag/AgCl. The HbNPs/AuE exhibited Vmax of 5.161±0.1 μA cm^{-2} with Michaelis-Menten constant (Km) of 0.1±0.01mM. The biosensor showed lower detection limit (1.0μM), high sensitivity (129±0.25μA cm^{-2} mM^{-1}) and wider linear range (1.0-1200μM) for H_2O_2 as compared to earlier biosensors. The analytical recoveries of added H_2O_2 in serum (0.5μM and1.0μM) were 97.77% and 98.01% respectively and within and between batch coefficients of variation (CV) were 3.16% and 3.36% respectively. There was a good correlation between sera H_2O_2 values obtained by standard enzymic colourimetric method and the present biosensor (R^2=0.99). The biosensor measured H_2O_2 level in sera of apparently healthy subjects and persons suffering from diabetes type II. The HbNPs/Au electrode lost 10% of its initial activity after 90 days of its regular uses, when stored dry at 4°C.

Keywords: H_2O_2, Hemoglobin nanoparticles, Gold electrode, Serum, H_2O_2 biosensor.

1. Introduction

Hydrogen peroxide (H_2O_2) is incompletely reduced metabolite of oxygen that has a diverse array of physiological and pathological effects within living cells, depending on the extent, timing, and location of its production. Characterization of the cellular functions of H_2O_2 requires measurement of its concentration selectively in the presence of other oxygen metabolites and with spatial and temporal fidelity in live cells.[1] H_2O_2 is one of the reactive oxygen species (ROS) among others like superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydroxyl ion (OH^-). The highest concentration of H_2O_2 is associated with diabetes, atherosclerosis, and aging, as it generates free hydroxyl radicals, which cause oxidative damage to the tissue components such as lipids and proteins besides DNA.[2-3] For the measurement of H_2O_2 in biological fluids, several sensitive methods based on horseradish peroxidase and artificial substrates (such as Amplex Red and 3,5,3'5'-tetramethylbenzidine) or on ferrous oxidation in the presence of xylenol orange (FOX) have been developed.[4] The rapid and accurate determination of hydrogen peroxide is very important, as it is not only the product of the reactions catalyzed by many highly selective oxidases, but also employed in various fields such as food, pharmaceutical, biology, medicine, industry and environmental analysis.[5] Hyperglycemia is one of the major ways of inducing reactive oxygen species (ROS) and endothelial dysfunction in patients with diabetes.[6] The imbalance between the production of reactive oxygen species (ROS) and the endogenous antioxidant mechanism to counteract the effect of ROS or repair the resulting damages generate oxidative stress.[7] The determination of hydrogen peroxide in diabetics helps in evaluating their oxidative stress status. By maintaining a good diabetic control and implementing strategies including supplementation of antioxidant micronutrients (vitamin E and C, beta-carotene) will reduce the oxidative stress and thus help in reducing the risk of chronic complications.[8] Several methods, such as titrimetry[5], spectrometry[9], chemiluminescence[10], fluorimetry[11], chromatography[12], and electrochemical techniques[13,14] have been reported for this purpose. Among these techniques, the electrochemical techniques are preferable because of their simplicity, low cost, high sensitivity, rapidity, and selectivity. The proteins containing heme groups, such as hemoglobin (Hb), myoglobin and cytochrome C possess peroxidase-like for reduction of H_2O_2, due to the electroactive center of heme[17] and therefore have been employed for the construction of H_2O_2 sensors.[18-19] Among these, hemoglobin (Hb) is more suitable for H_2O_2 biosensor, because of its known structure, commercially availability at low cost and

relatively more stability. Thus the biosensors using native Hb was more specific with reduced fabrication cost.[20] The native Hb has been immobilized onto various types of nanocomposites for construction of H_2O_2 biosensor such as Hb/Pluronic P123-nanographene platelet (NGP)[21], Hb/Collagen micro belt[22], Hb/Ag sol films[23], Hb/AuNPs/L-Cysteine(L-Cys)/paminobenzene sulfonic acid(p-ABSA)/Pt disk[24], Hb/Collagen-mWCNT[25], DNA–Hb/Au[26]. The various support used for the H_2O_2biosensors are Platinum nanoparticles PtNPs/ Reduced graphene oxide RGO/ Chitosan CS/ Ferrocene Fc[28], Self-assembled dipeptide DP- Gold nanoparticles AuNP/ Horseradish peroxidase HRP/ Glassy carbon electrode GCE[29], Graphene capsules GRCAPS/HRP/ Indium tin oxide ITO[30], HRP/ Poly (aniline-co-N-methyllanthionine)PAN-PNMThH[31], Electrochemically reduced graphene oxide ERGO/GCE[32], TMB-3,3′,5,5′-teramethylbencidine /HRP/PDMS Polydimethylsiloxane /TEOS Tetraethylorthosilicate /SiO_2NPs Silicon oxide nanoparticles[33], TTP Turnip tissue paper /SPCE[34], GPtNPs[35], PtRu/3DGF[36], Cytochrome c Cytc/ Nickel oxide nanaoparticles NiONPs/ Multiwall carbon nanotubes c-MWCNT/ Polyaniline PANI/Au[37]. However, these methods involve the complex strategies for construction of working electrode and have poor detection limit and narrow linear range. Hence, there is a need for development of an efficient and reliable analytical device that can provide high sensitivity, facile operation and quick response to the H_2O_2 molecule. In recent years, protein nanoparticles have been employed in the construction of improved biosensors due to their exceptional electronic, optical, mechanical, thermal properties.[15] Au electrode offers many attractive features such as good conductor of electricity, low cost, low background current and easy availability.[16] The present work describes the construction of an H_2O_2 biosensor based on covalent immobilization of HbNPs onto Au electrode, its characterization, evaluation and application for amperometric determination of H_2O_2 in sera of diabetic patients.

2. Experimental Methods

2.1 Materials

Hemoglobin, TRIS-buffer, NAD+, D-Glucose, uric acid, urea, glucose, ascorbic acid, potassium chloride, silica gel from SRL, Mumbai, sulphuric acid from Qualigens Fine Chemicals, Mumbai were used. Au wire (diameter-2mm) for the preparation of Au electrode was purchased from local Jewellery shop. Sera samples of apparently healthy subjects and persons suffering from

diabetes type II were collected from local PGIMS hospital. All other chemicals were analytic reagent (AR) grade. Double distilled water (DW) was used throughout the experimental studies.

2.2 Instruments used

Potentiostat/Galvanostat (Make: Autolab, model: AUT83785, manufactured by Eco Chemie. The Netherland) with a three electrode system consisting of a Pt wire as an auxillary electrode, an Ag/AgCl electrode as reference electrode and LDH immobilized onto GrONPs) modified PGE as a working/enzyme electrode. Scanning electron microscope (SEM) (Zeiss EV040, USA, UV Spectrophotometer (Make: Shimadzu,Japan, Model 1700), X-ray diffractometer (XRD), (Make: 122 Rigaku, D/Max2550, Tokyo, Japan), Fourier transform Infra-red spectrometer (FTIR) (Thermo Scientific, USA), were used.

2.3 Preparation of hemoglobin nanoparticles (HbNPs)

The HbNPs were prepared by desolvation method. To Hb solution (1mg/ml), 5ml of absolute ethanol was added dropwise at a rate of 0.1-0.2ml/min under continuous stirring at a speed of 500rpm. The desolvating agent encouraged the aggregation of protein molecules into small nanoparticles (NPs) by eliminating water molecules in between them. In the same way, process was followed by addition of 1ml, 1% glutaraldehyde solution to reaction mixture. The mixture was kept under the similar stirring condition at 4°C for 24h to ensure complete crosslinking of HbNPs. Such a high concentration of glutaraldehyde is likely to provide intermolecular crosslinking of HbNPs molecules through Schiff base. Besides above all, the enzyme NPs thus formed were thiol functionalized by adding 0.02g/ml of cysteamine solution (0.02g/ml) to HbNPs suspension under constant stirring for 5-6h. Lastly, HbNPs were separated from free enzyme by centrifuging NPs suspension at 1200×g for 10min at 4°C, followed by redispersion of HbNPs in 0.1M phosphate buffer pH (7.3) and sonication for 5min. Moreover, it is assumed that alpha-NH_2 group of cysteamine reacts with excess unreacted –CHO groups of glutaraldehyde cross-linked Hb molecules to form Schiff base. Thus, the glutaraldehyde cross-linked HbNPs get functionalized with –SH groups. These –SH functionalized HbNPs were stored at 4°C, until use.[38]

2.4 Characterization of HbNPs

HbNPs were characterized by recording their transmission electron microscopic (TEM) images, UV and visible spectra and Fourier transform Infra-red (FTIR) spectra. The size and shape of HbNPs were measured by a high resolution transmission electron microscope, Zeiss EM 912, at an accelerating voltage of 120kV. TEM samples of the HbNPs were prepared by placing of the product solution onto the carbon-coated copper grids, allowing the solvent to evaporate in air. UV absorbance of HbNPs dispersed in 0.1M sodium phosphate buffer pH (7.0) was recorded in the range from 200 to 600nm at an interval of 50nm, using UV Spectrophotometer (Make: Shimadzu, Japan, Model 1700). FT-IR spectra of the HbNPs were measured by Fourier transform Infra-red spectrometer (FTIR) (Thermo Scientific, USA) using the standard KBr method over a range of 500 to 4000cm^{-1}.

2.5 Preparation of HbNPs modified Au electrode

Thiol groups on the surface of HbNPs provide a facile way for attachment of HbNPs on the surface of Au electrode. Before the immobilization, the cleaned bare Au electrode was scanned over the 0 to 0.5V range in freshly prepared 0.2M H_2SO_4 until the voltammogram characteristic of the clean polycrystalline Au was established. The polycrystalline Au electrode was placed in the HbNPs suspension under mild stirring at 4°C for 12h to get covalent coupling between thiol-functionalized cross-linked HbNPs and polycrystalline Au electrode via Au-thiolated bond through dehydration process (Fig.1). The HbNPs/Au electrode was rinsed with 0.1M of phosphate buffer (PB, pH 7.0) carefully and stored in a PB buffer at 4°C, when not in use. The characterization of HbNPs/Au electrode was done by SEM and EIS. Below is a representation of the chemical reaction that describes the attachment of the HbNPs to the Au electrode

$$\mathbf{Au_n\text{-}AuO + 2(HbNPs)\text{-}SH \xrightarrow{\text{Dehydration}} Au_n(Au\text{-}S\text{-}HbNPs) + H_2O}$$

Polycrystalline -AuE **Functionalized HbNPs** **Hb-E (Hb-electrode)**

2.6 Construction and response measurement of H_2O_2 biosensor

To construct the H_2O_2 biosensor, HbNPs/Au, as working electrode, Ag/AgCl as reference electrode and Pt wire as counter electrode were connected through potentiostat. Cyclic voltametry (CV) of working electrode was recorded in Potentiostat–Galvanostat at the potential range, -0.75 to -0.25V in 25 ml of 0.1M sodium phosphate buffer (pH 6.5) containing 0.1ml

100μM H_2O_2. The current (in mA) was measured at different time (in seconds).

2.7 Optimization of H_2O_2 biosensor

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time of incubation and substrate (H_2O_2) concentration on biosensor response were studied. To determine optimum pH, the pH was varied between pH 4.0 to 8.0 at an interval of pH 0.5 using the following buffer, each at a final concentration of 0.1M: pH 4.0 to 5.0 sodium acetate buffers and pH 5.5 to 8 sodium phosphate buffer. Similarly to determine optimum temperature and incubation time the reaction mixture was incubated at different temperature (5–70ºC) and time duration (1-10s). The effect of H_2O_2 concentration on biosensor response was determined by varying the concentration of H_2O_2in the range 1-1200μM. The standard error was calculated using the formula: Standard Error (SE) =SD/√n, where SD= standard deviation, n=number of samples. Basically, it just tells the general variability of the points around their group means. The limit of detection (LOD) was calculated using the following formula: LOD = 3σ/slope, where σ is standard deviation. The correlation coefficient (R^2) was calculated by regression equation.

2.8 Applications of H_2O_2biosensor

Blood samples (1ml each) were drawn from apparently healthy male and female (20 each) and persons with a diagnosis of diabetes type II at Pt. BDS PGIMS, Rohtak hospital and centrifuged at 2000rpm for 10–15min and their supernatant (serum) was collected. H_2O_2 content in sera was determined by the present biosensor in the similar manner as described above for its response measurement, under its optimal working conditions except that H_2O_2 was replaced by serum sample. The H_2O_2 content in serum was interpolated from standard curve between H_2O_2 concentrations versus current in mA prepared under optimal assay conditions of biosensor (Fig. 2).

3. Results and discussion

3.1 Characterization of HbNPs

The average size of HbNPs as measured by TEM was between 5nm to 100nm (Fig. 3a), and showed an average size of 20nm, which enhanced the surface area of the electrode as compared to the native protein. However, this TEM image is also showing diameter of HbNPs in the higher range, i.e. 103.47 to 630.24nm (average 236.95), which might be due to aggregation of HbNPs. UV and visible spectra exhibited strong absorbance peak at 280nm, revealing), revealing a high degree of absorption of aromatic amino acids of peptide chain confirming their NPs formation. The FTIR spectrum of native Hb showed distinct peaks at 1062, 1317, 1553, 1679 and 3497cm $-^{1}$. The strong absorption band at 1317cm $-^{1}$ may arise due to the stretching vibrations of C-N aromatic functional groups of protein. The medium absorption bands located at 1679 and 1553cm $-^{1}$ corresponds to carbonyl stretch and NH- stretch vibrations in the amide II linkage of the protein respectively. The band at 1062cm $^{-1}$ indicates presence of amine group. In addition, there was a located at 3497cm $^{-1}$, which could be assigned to the O H stretching vibrations, indicating the presence of hydroxyl groups. The FTIR spectrum of HbNPs showed distinct peaks at 1090, 1305, 1561, 1701 and 3488cm $-^{1}$(Fig. 3b). In the comparison with the FTIR spectrum of the Hb and HbNPs, the major shift was observed in the carbonyl, hydroxyl and amino group of protein which is due to ethanol, glutaraldehyde and cysteamine treatment of HbNPs during their preparation.

3.2 Characterization of working electrode (HbNPs/Au) at different stages of its construction

3.2.1 By scanning electron microscopy (SEM)

The SEM images of the surface of bare Au and HbNPs/Au are shown in Fig. 3c respectively. The stepwise modification of electrode could be seen clearly from these SEM images. The SEM image of the bare Au showed a smooth and featureless morphology. On immobilization of HbNPs, a globular structural morphology was appeared due to the interaction between HbNPs and Au, which revealed larger and effective surface area.

3.2.2 By electrochemical impedance spectroscopy (EIS)

EIS provides the useful information on impedance changes of the electrode surface during the fabrication process. It was carried out to investigate immobilization of HbNPs onto Au electrode. The diameter of the semicircle at higher frequencies corresponds to the electron transfer

resistance (R_{CT}) which controls the electron transfer kinetics of the redox probe at the electrode interface, while the linear part at lower frequencies corresponds to Warburg diffusion process. The Nyquist plot (Fig. 3d) displays EIS of HbNPs/Au in 5mM $K_3Fe(CN)_6/K_4Fe(CN)_6$. The R_{CT} value for the native Hb was 200Ω which is lower than HbNPs/Au i.e. 300Ω. These results showed that the increased R_{CT} value of HbNPs/Au electrode was due to the immobilization of Hb nanoparticles onto Au surface. This increase in R_{CT} is attributed to the fact that most biological molecules, including Hb nanoparticles, are poor electrical conductors at low frequencies (at least <10 kHz; applied voltage: 0.2V) and cause hindrance to the electron transfer.

3.3 Construction &optimization of H_2O_2biosensor

A novel amperometric H_2O_2 biosensor was developed based on covalent immobilization of HbNPs onto Au electrode. The bare AuE electrode had no oxidation and reduction peaks. The HbNPs/ AuE electrode i.e. working electrode showed optimum oxidation/reduction peak i.e. current at a potential of -0.2 V (Fig. 4), which is similar to HRP/PAN-PNMThH (-0.2V)[31], ERGO/GCE (-0.2V)[32] Hb/NGP (0.2V)[20], but higher to those involving Hb/Collagen-MWCNT (-0.365V)[24] Hb/Collagen microbelt (-0.38V)[21], Hb/Ag sol films/GCE (-0.40V)[22], GRCAPS/HRP/ITO(-0.45V)[30] , DNA–Hb/Au (-0.75V)[25], and lower to TTP/SPCE (-0.1V)[34],DP-AuNP/HRP/GCE (-0.05V)[29], PtNPs/RGO/CS/Fc (-0.05V)[28] Hb/AuNPs/ L-cys/ p-ABSA/Pt disk (0.1V)[23], PtRu/3DGF (0.2V)[36] , Cytc/NiONPs/c-MWCNT/polyaniline/Au (0.28V)[37], GPtNPs (0.45V)[35] .The biosensor showed optimum current at pH 6.5 in 0.1 M sodium phosphate buffer (Fig. 5a), which is similar to that of biosensor based on Cytc/NiONPs/c MWCNT/polyaniline/Au[37] but lower to those involving Hb/NGP (pH 7.0)[20], Hb/Collagen microbelt (pH 7.0)[21], Hb/Ag sol films/GCE (7.0)[22], Hb/AuNPs/ L-cys/ p-ABSA/Pt disk (pH 7.0)[23], Hb/Collagen-mWCNT (7.0)[24], , DP-AuNP/HRP/GCE (7.0)[29], GRCAPS/HRP/ITO(7.0)[30], , ERGO/GCE (7.0)[32], ,TTP/SPCE (7.0)[34], PtRu/3DGF (7.4)[36] and higher to HRP/PAN-PNMThH (6.0)[31],TMB/HRP/PDMS/TEOS/SiO_2NPs(5.0)[33] and DNA–Hb/Au (5.0)[25]. The optimum incubation temperature was observed at 30ºC (Fig. 5b),which is similar to the Cytc/NiONPs/c-MWCNT/polyaniline/Au (30ºC) but higher to than those of Hb/NGP (25 ºC)[20], Hb/AuNPs/ L-cys/ p-ABSA/Pt disk (25 ºC)[23] , Hb/Collagen microbelt (20 ºC)[21], and lower to those of

PtNPs/RGO/CS/Fc (37 ºC)[28], GRCAPS/HRP/ITO (37 ºC)[30]. Fig. 5c showed that there was a sharp increase in biosensor response with the increase in incubation time upto 2.5 s, after that it became constant. Hence the optimum response time of biosensor was considered as 2.5 s. The faster response time of biosensor provides the real time analysis of the H_2O_2 in the serum sample. Hence, all the subsequent electrochemical experiments were carried out in 0.1 M sodium phosphate buffer, pH 6.5at 30°C. There was a linear relationship between biosensor response and H_2O_2 concentration in the range 1-1200μM, the response was constant after 1200μM (Fig.5d). The apparent Km is an indication of protein nanoparticles affinity. It can be calculated for immobilized protein nanoparticles by the amperometric method, because the biosensor response is kinetic. The Km and V*max* values for the HbNPs/AuE electrode were found to be 0.1±0.01μM and 5.161±0.1μA cm^{-2}, respectively. The Km value of the biosensor was lower than those of the earlier reported biosensors (45.35μM [20]and 77.7μM [21]).The smaller the Km value, the stronger is the affinity between HbNPs and substrate, implying that the present electrode exhibits a higher affinity for H_2O_2.

3.4 Evaluation of H_2O_2 biosensor

There was a linear relationship between the biosensor response i.e. current (in mA) and the H_2O_2 concentration in the range 1.0-1200μM, which is better than earlier biosensors, 10-150μM[20], 10-120μM[25], 5 -30μM[21], 0.21 - 31μM[23], 1 -25μM[22] (Supplementary Table 4).The detection limit of the present biosensor was 0.0001μM, which is also better/lower than those of earlier biosensors, 8.24μM[20], 0.91μM[24], 0.4μM[25], 0.37μM[21], 0.1μM[22], 0.07μM[23]. This could be attributed to the use of nanoparticles of Hb instead of native Hb. As the detection limit of biosensor is 0.0001μM, it can even detect a single molecule of H_2O_2, showing the high sensitivity of biosensor. The average recoveries of H_2O_2 added in sera at levels of 0.5μM and 1.0μM were 97.77 and 98.01% respectively (Supplementary Table 1), demonstrating the reliability of the present biosensor. The within and between-batch coefficients of variation for determination of H_2O_2 in five sera samples on the same day and after one week of storage were 3.16% and 3.36%, respectively (Supplementary Table 2). These high precisions reveal the good reproducibility and consistency of the present method. The H_2O_2 level in 20 sera of apparently healthy subjects and persons suffering from diabetes type II, as measured by present biosensor were compared with those

obtained by the standard Enzo kit method. There was a good correlation (R^2=0.99) between these two methods (Fig.6a) showing the high accuracy of biosensor.

3.5 Interference study

The interference study was performed to assess the selectivity of the present biosensor. The HbNPs biosensor exhibited excellent anti-interferences ability with various major blood components such as citric acid, glutamic acid, uric acid, urea and ascorbic acid at their physiological concentrations. The change in relative response in presence of citric acid, glutamic acid, uric acid, urea and ascorbic acid was 4.20%, 2.70%, 1.50%, 3.80% and 2.80% respectively under the standard assay conditions. These changes (1.50-4.20%) had practically no effect on biosensor response. This practically no interference could be attributed to functioning of biosensor at lower applied potential (–0.2V). Hence, the present biosensor could be highly selective/specific for H_2O_2.

3.6 Applications of H_2O_2biosensor

The H_2O_2 content as measured by the present biosensor was in the range 23.9±0.04-45.6±0.05 (n=20)μM for sera of apparently healthy persons, which is in normal established range and 68.2±0.05-143±0.05μM (n=20) for persons suffering from diabetes type II (Supplementary Table 3).

3.7 Storage stability of HbNPs/Au electrode

The HbNPs/Au electrode was tested every fifth day over a period of 90days, while being stored dry at 4^0C. Fig. 6b shows the % of initial response of HbNPs/AuE electrode vs storage time. The electrode lost 10% of its initial activity after 90 days of its regular uses when stored dry at 4°C. The storage stability of the present biosensor is better than earlier reported biosensors as 25days[34], 30days[37] which could be credited to covalent immobilization of HbNPs onto Au electrode.

The present biosensor could be advanced with respect to use in biomedicine or clinical settings by miniaturizing it into commercial model/portable model, which could be used at the bedside of the patient. The miniaturization means designing of an electronic chip for current measuring

device (potentiostat) and transformation of HbNPs/Au electrode into a microelectrode (like screen-printed electrode), which could be dipped into one drop of biological sample and an adopter to connect the miniaturized current device with the microelectrode. The current device needs to be battery-operated and finally calibrated according to the concentration of H_2O_2. However, biosensor has some drawbacks such as lack of substrate specificity, requirement of expensive Au wire for construction of working electrode and cold temperature for its storage.

Conclusion

The covalent immobilization of HbNPs onto Au electrode in the construction of H_2O_2 biosensor has resulted into its better analytical performance in terms of lower working potential (-0.2V), which is similar to HRP/PAN-PNMThH (-0.2V)[31], ERGO/GCE (-0.2V)[32] Hb/NGP (0.2V)[20], but higher to those involving Hb/Collagen-MWCNT (-0.365V)[24] Hb/Collagen microbelt (-0.38V)[21], Hb/Ag sol films/GCE (-0.40V)[22], GRCAPS/HRP/ITO(-0.45V)[30] , DNA–Hb/Au (-0.75V)[25], and lower to TTP/SPCE (-0.1V)[34],DP-AuNP/HRP/GCE (-0.05V)[29], PtNPs/RGO/CS/Fc (-0.05V)[28] Hb/AuNPs/ L-cys/ p-ABSA/Pt disk (0.1V)[23], PtRu/3DGF (0.2V)[36] , Cytc/NiONPs/c-MWCNT/polyaniline/Au (0.28V)[37], GPtNPs (0.45V)[35] , LOD (0.0001μM) which is also better/lower than those of earlier biosensors, 8.24μM[20], 0.91μM[24], 0.4μM[25], 0.37μM[21], 0.1μM[22], 0.07μM[23], wider linear range (1 to 1200μM) which is better than earlier biosensors, 10-150μM[20], 10-120μM[25], 5 -30μM[21], 0.21 - 31μM[23], 1 -25μM[22] , rapid response (2.5s), and higher storage stability (90days) compared to earlier biosensors (Supplementary Table 4). Thus covalently bound protein nanoparticles onto Au electrode could be used for the construction of other improved biosensors also.

Conflict of interest

The authors have no conflict of interest.

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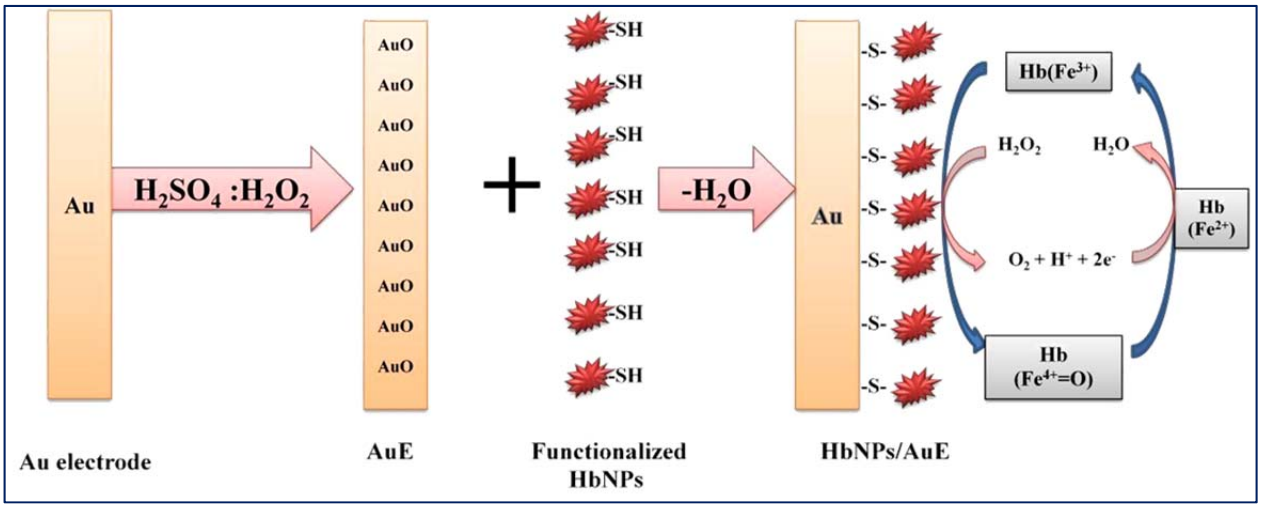
Au
$H_2SO_4 : H_2O_2$
AuO
AuO
AuO
AuO
AuO
AuO
AuO
AuO
AuO
SH
SH
SH
SH
SH
SH
$-H_2O$
Au
-S-
-S-
-S-
-S-
-S-
-S-
-S-
$Hb(Fe^{3+})$
H_2O_2
H_2O
$O_2 + H^+ + 2e^-$
Hb
(Fe^{2+})
Hb
$(Fe^{4+}=O)$
Au electrode
AuE
Functionalized
HbNPs
HbNPs/AuE

Fig.1

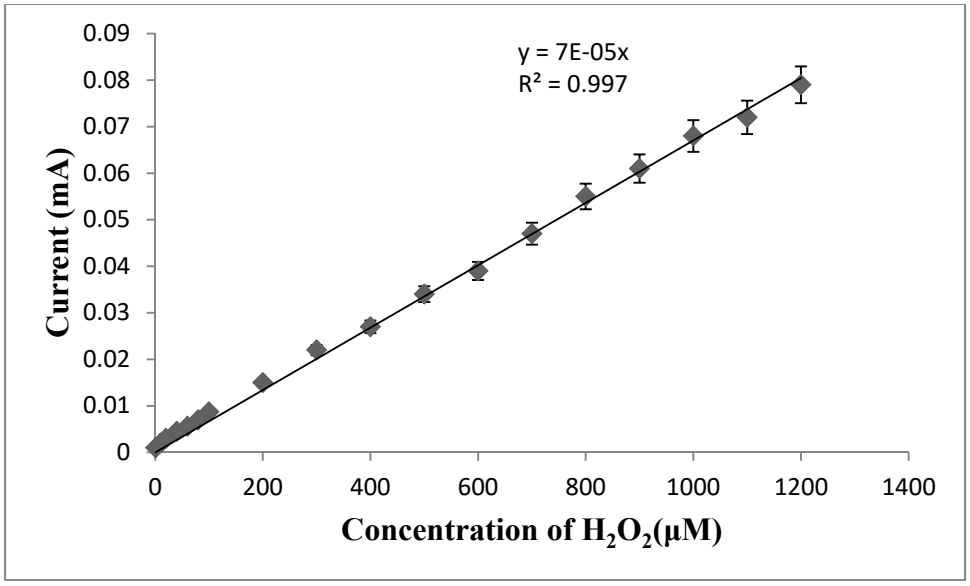
y = 7E-05x
R² = 0.997
Current (mA)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
200
400
600
800
1000
1200
1400
Concentration of H2O2(µM)

Fig. 2

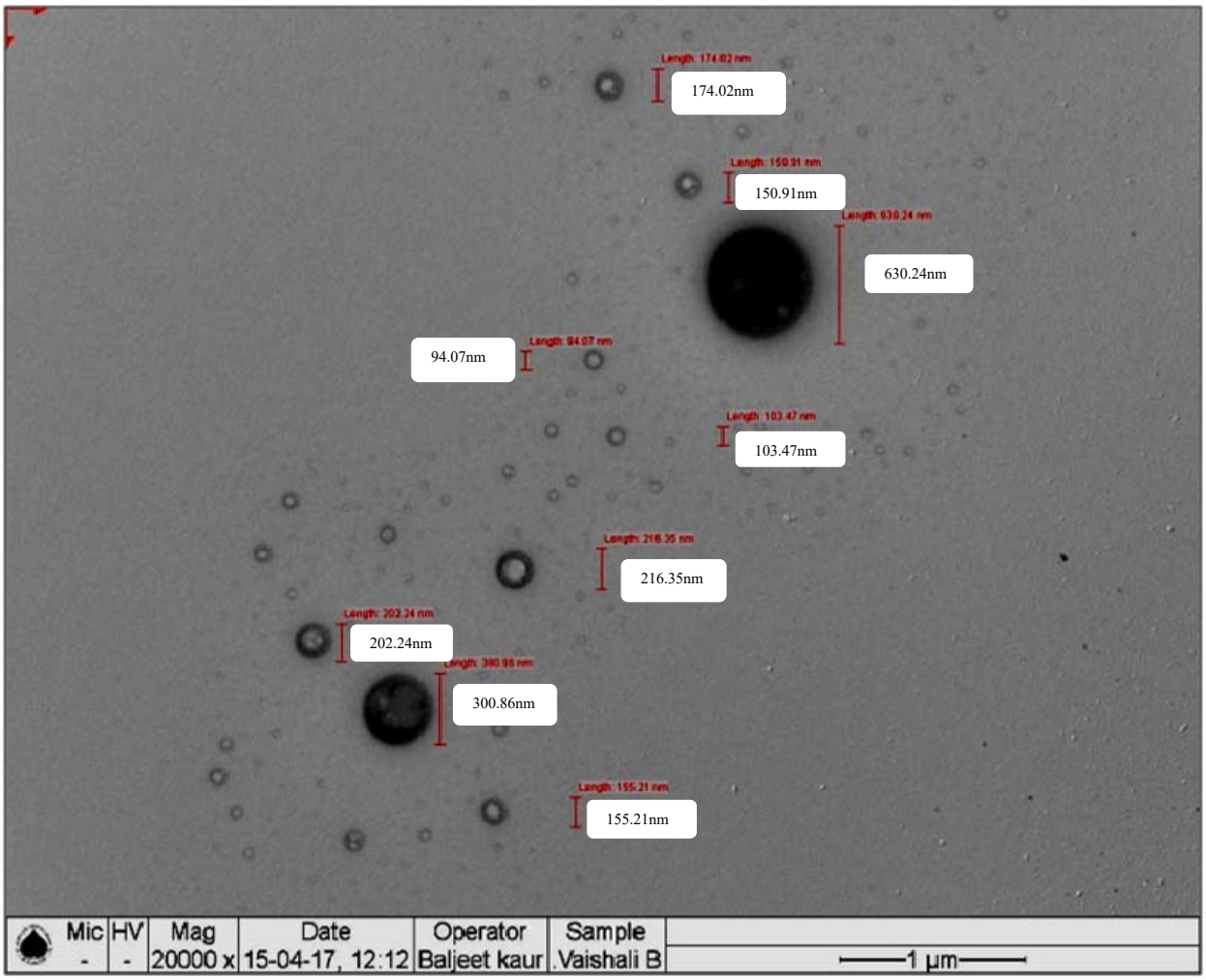
174.02nm
150.91nm
630.24nm
94.07nm
103.47nm
216.35nm
202.24nm
300.86nm
155.21nm
Mic
-
HV
-
Mag
20000 x
Date
15-04-17, 12:12
Operator
Baljeet kaur
Sample
Vaishali B
1 µm

Fig.3a

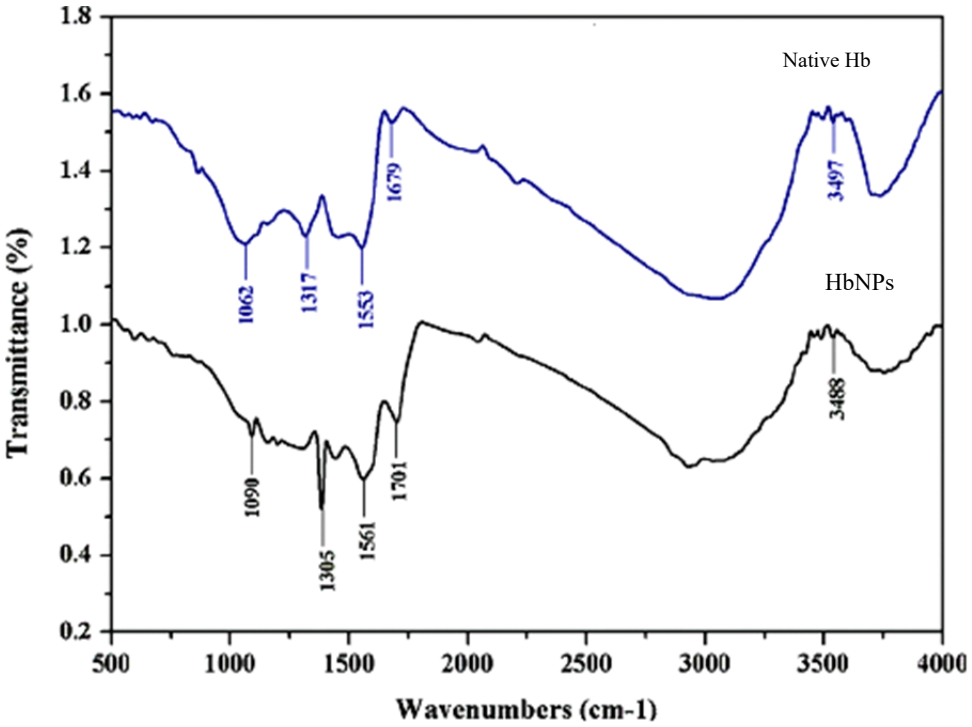
Native Hb
HbNPs
1062
1317
1553
1679
3497
1090
1305
1561
1701
3488
Transmittance (%)
Wavenumbers (cm-1)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
500
1000
1500
2000
2500
3000
3500
4000

Fig.3b

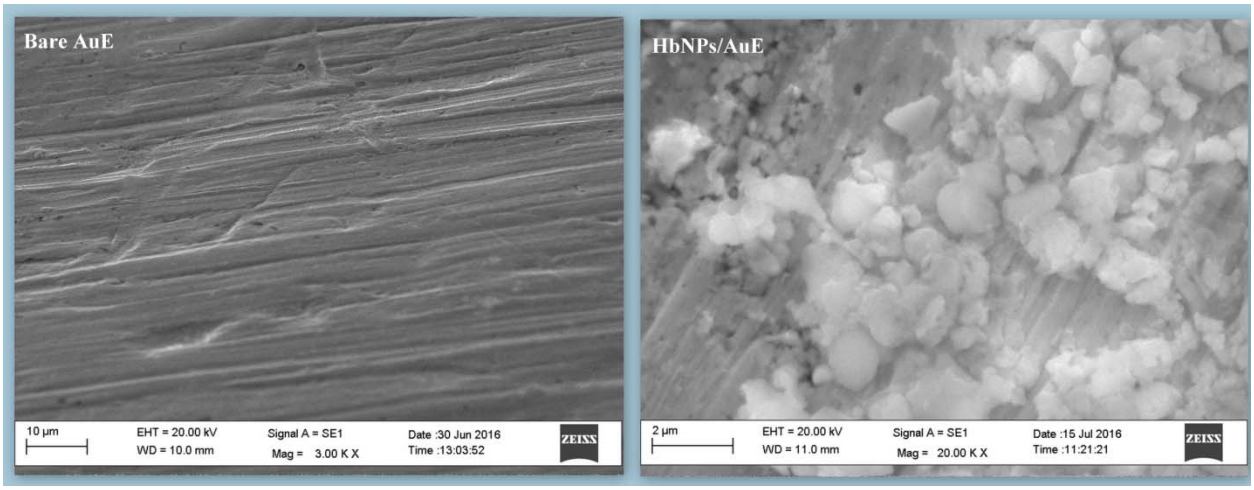
Bare AuE
10 µm
EHT = 20.00 kV
WD = 10.0 mm
Signal A = SE1
Mag = 3.00 K X
Date :30 Jun 2016
Time :13:03:52
ZEISS
HbNPs/AuE
2 µm
EHT = 20.00 kV
WD = 11.0 mm
Signal A = SE1
Mag = 20.00 K X
Date :15 Jul 2016
Time :11:21:21
ZEISS

Fig. 3c

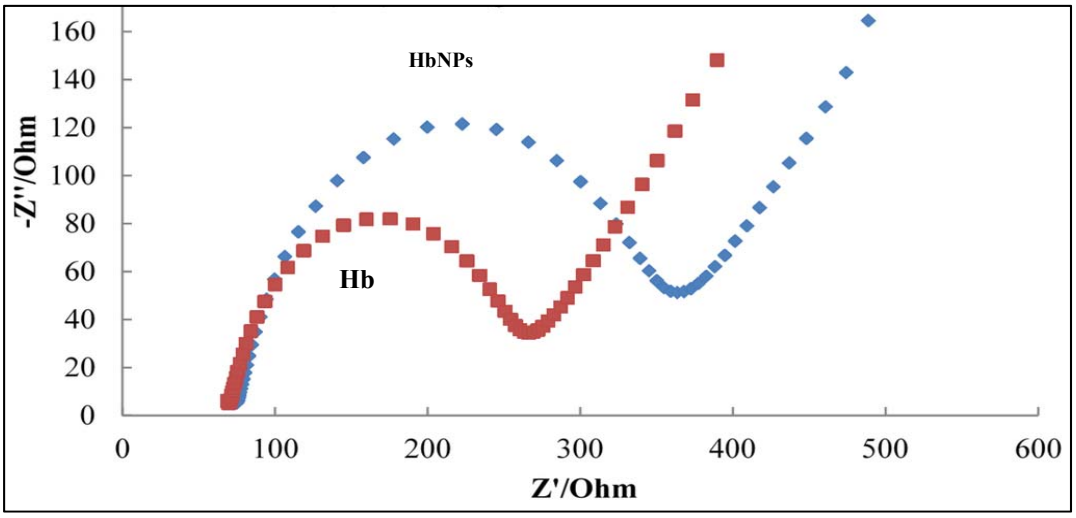
HbNPs
Hb
-Z''/Ohm
Z'/Ohm
0
20
40
60
80
100
120
140
160
0
100
200
300
400
500
600

Fig. 3d

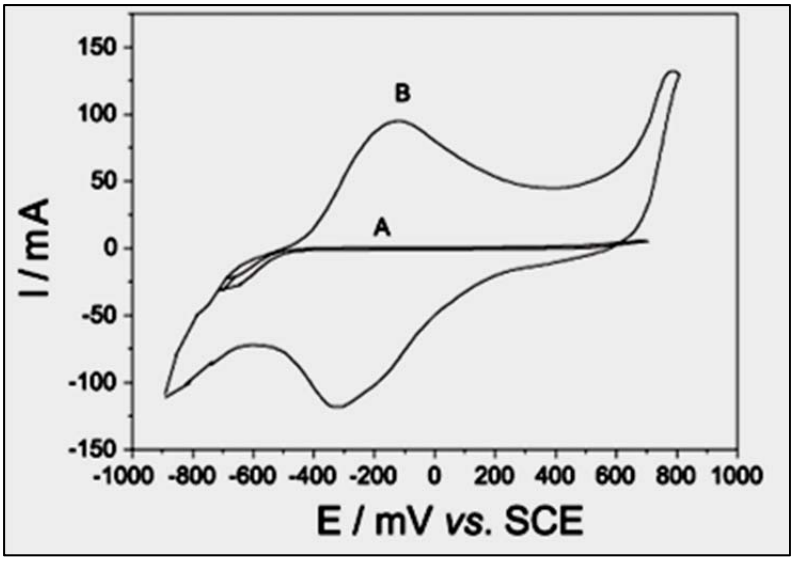
150
100
50
0
-50
-100
-150
I / mA
B
A
-1000 -800 -600 -400 -200 0 200 400 600 800 1000
E / mV vs. SCE

Fig. 4

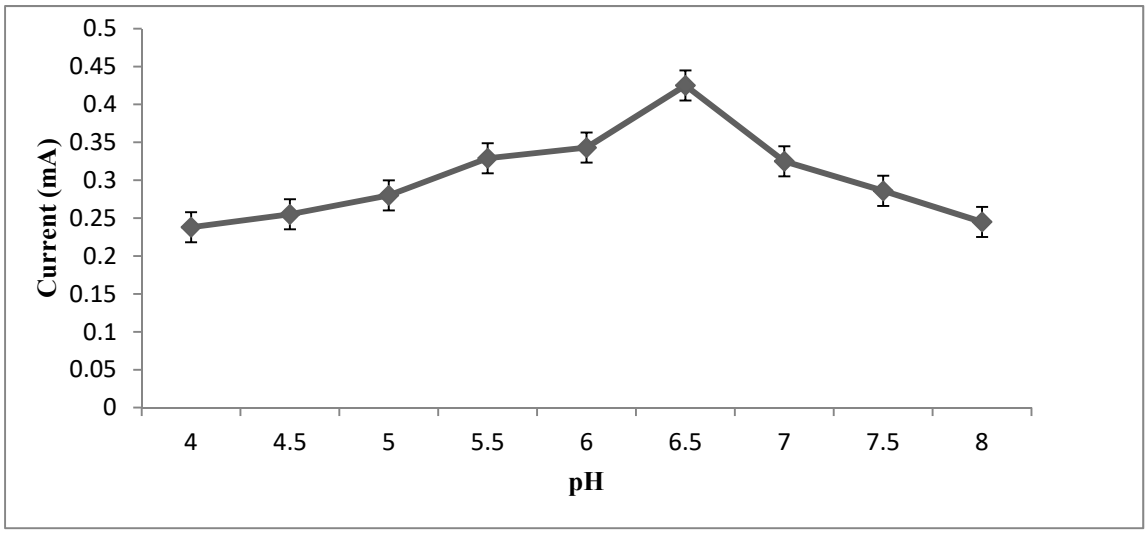
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Current (mA)
4
4.5
5
5.5
6
6.5
7
7.5
8
pH

Fig. 5a

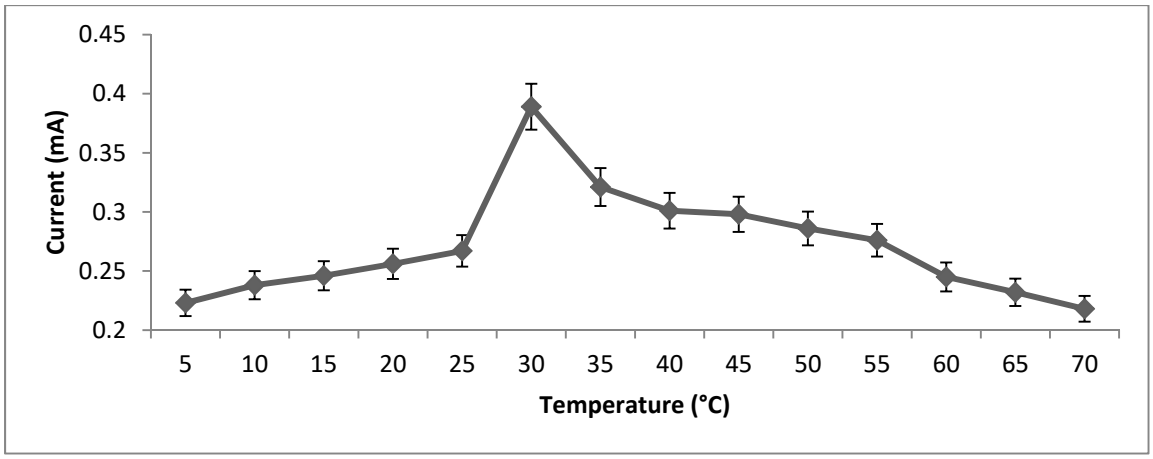
0.45
0.4
0.35
0.3
0.25
0.2
Current (mA)
5
10
15
20
25
30
35
40
45
50
55
60
65
70
Temperature (°C)

Fig. 5b

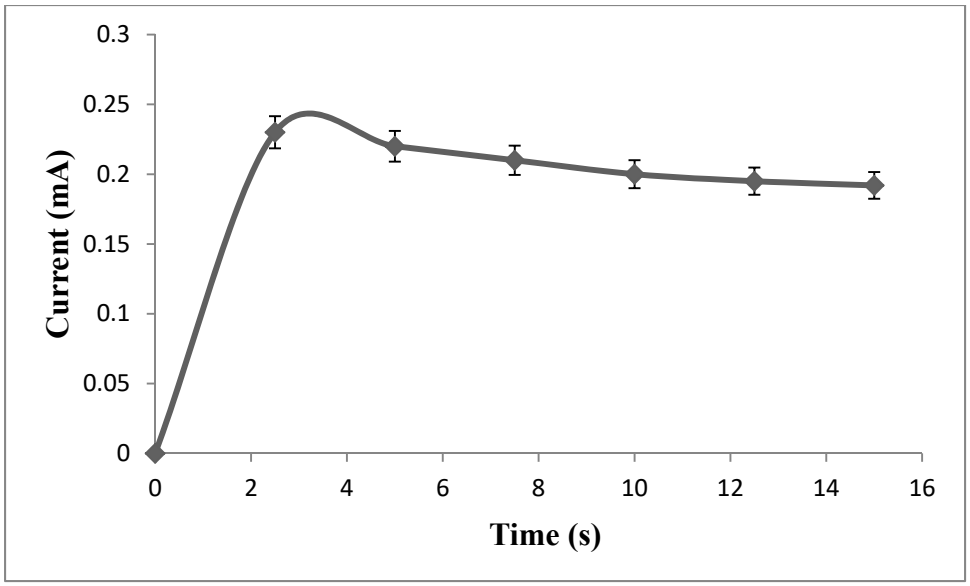
0.3
0.25
0.2
0.15
0.1
0.05
0
Current (mA)
0
2
4
6
8
10
12
14
16
Time (s)

Fig. 5c

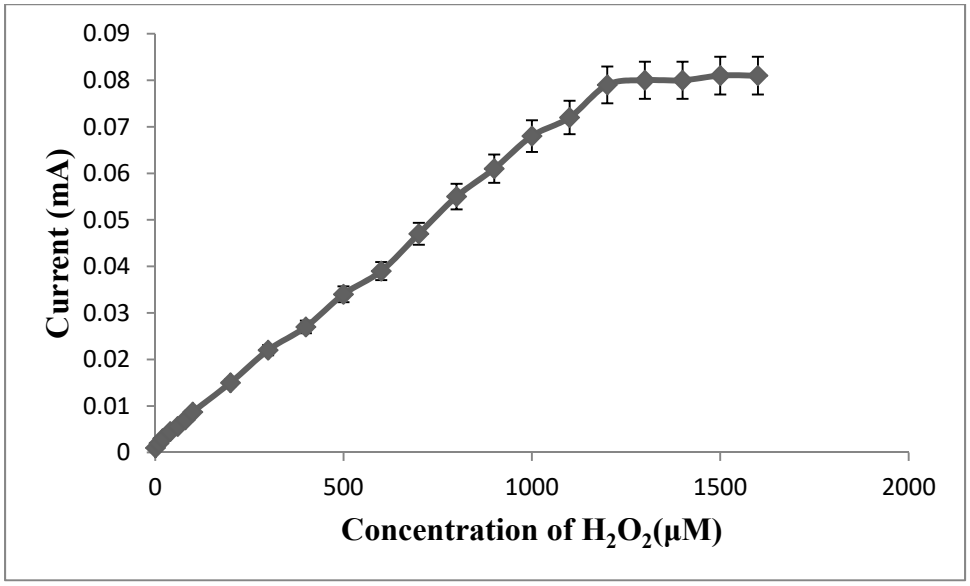
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Current (mA)
0
500
1000
1500
2000
Concentration of H_2O_2(μM)

Fig. 5d

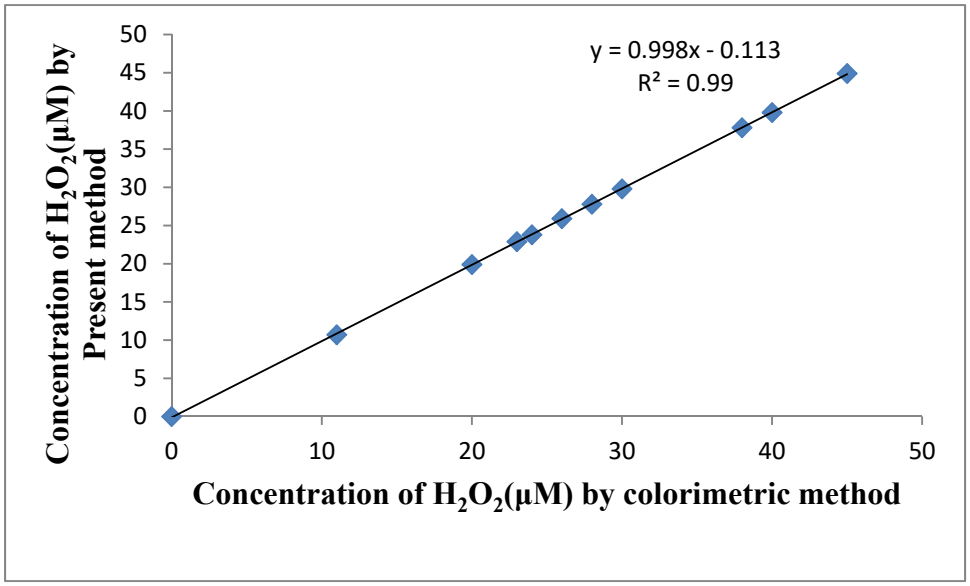
y = 0.998x - 0.113
R² = 0.99
Concentration of H2O2(µM) by Present method
Concentration of H2O2(µM) by colorimetric method
0
5
10
15
20
25
30
35
40
45
50
0
10
20
30
40
50

Fig. 6a

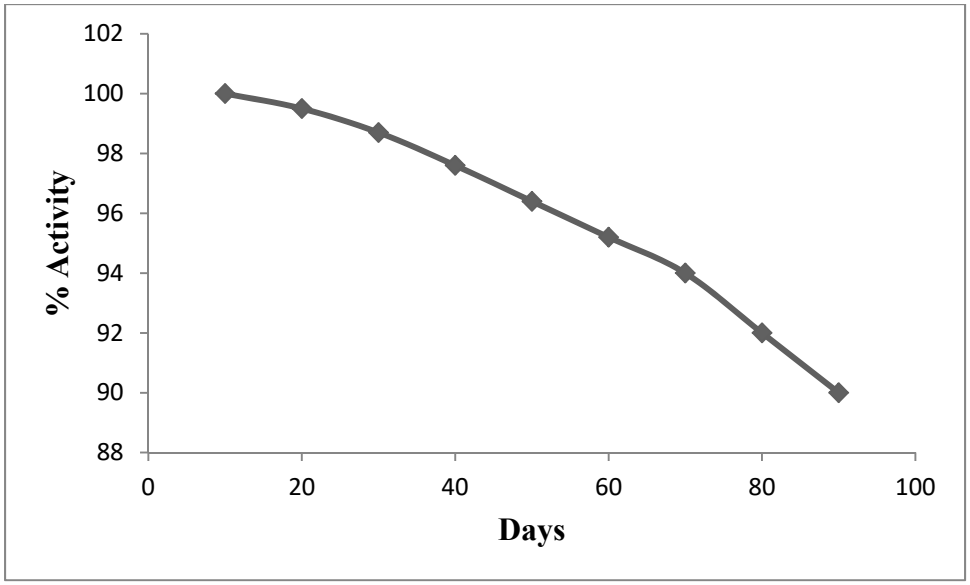
% Activity
Days
102
100
98
96
94
92
90
88
0
20
40
60
80
100

Fig. 6b

List of Supplementary Tables

Supplementary Table: 1 Analytical recovery of added H_2O_2 in the serum samples, as measured by H_2O_2 biosensor based on HbNPs/AuE.

Supplementary Table: 2 Within and between assay coefficients of variation for determination of H_2O_2 in the serum samples, as measured by H_2O_2 biosensor based on HbNPs/AuE.

Supplementary Table: 3 Serum H_2O_2 levels in apparently fasting healthy persons and diabetic patients, as measured by H_2O_2 biosensor based on HbNPs/AuE.

Supplementary Table: 4 Comparison of different analytic parameters of present H_2O_2 biosensor with those of earlier biosensors.

Table 1

H_2O_2 added (µM)	H_2O_2 found (µM)	% Recovery
-	50.8	-
0.5	50.13	97.77±0.06
1.0	50.77	98.01±0.08

Table 2

N	H_2O_2 (µM)	CV (%)
Within assay (5)	50	3.16
50		
51		
52		
48		
49		
Between assay (5)	48.8	3.36
48		
50		
51		
47		
48		

Table 3

Sex	Age	Fasting Healthy Persons (µM)	Sex	Age	Diabetic Patients (µM)
M	32	40.4±.03	M	42	107.2±.04
M	41	41.6±.04	F	35	68.2±.05
F	27	41.3±.02	M	46	88.9±.02
M	29	41.9±.01	M	49	99.0±.01
F	24	44.5±.04	F	60	110.8±.04
F	32	34.0±.05	F	37	118.3±.03
F	24	44.2±.04	M	32	98.7±.02
M	26	24.7±.03	F	54	99.8±.06
M	55	35.9±.03	F	40	95.4±.04
F	25	25.0±.06	F	46	116.2±.01
M	28	24.8±.05	M	46	114.2±.03
M	33	25.6±.03	M	52	102.1±.04
F	38	35.7±.05	F	24	107.5±.02
F	22	45.6±.01	M	49	109.2±.04
M	22	25.2±.03	F	47	124.5±.02
F	39	43.8±.05	F	59	123.1±.06
M	37	33.4±.02	M	39	142.4±.02
M	54	44.5±.05	F	54	151.3±.03
M	35	23.9±.04	M	45	134.2±.02
F	33	25.7±.02	M	52	143.0±.05

Table 4

S. No	Support of immobilization	Method for immobilization	Type of biosensor	Linearity(μM)	Detection limit(LOD) μM	pH	Temp (°C)	Working Potential (V)	Sensitivity (μAmp/ mM/cm^2	Application	Ref.
1.	Hb/NGP	Electrodeposotion	Amperometric	10-150	8.24	7.0	25	0.20	NR	Blood	20
2.	Hb/Collagen microbelt	Electrospinning	Amperometric	5 -30	0.37	7.0	20	-0.38	NR	Blood	21
3.	Hb/Ag sol films/GCE	Covalent binding	Amperometric	1 -25	0.1	7.0	NR	-0.4	NR	Blood	22
4.	Hb/AuNPs/ L-cys/ p-ABSA/Pt disk	Electro-polymerization	Amperometric	0.21 -31	0.07	7.0	25	0.1	NR	Blood	23
5.	Hb/Collagen-mWCNT	Electrospinning	Amperometric	NR	0.91	7.0	NR	-0.365	NR	Blood	24
6.	DNA–Hb/Au	Covalent binding	Amperometric	10-120	0.4	5.0	NR	-0.750	NR	Blood	25
7.	PtNPs/RGO/CS/Fc	Covalent binding	Amperometric	0.002-30	0.02	NR	37	-0.05	NR	Real samples	28
8.	DP-AuNP/HRP/GCE	Covalent binding	Amperometric	0.05-300	0.1	7.0	NR	-0.05	28.3	Plasma	29
9.	GRCAPS/HRP/ITO	Covalent binding	Amperometric	0.01-12	3.3	7.0	37	-0.45	NR	Serum	30
10.	HRP/PAN-PNMThH	Covalent binding	Amperometric	0.005-60	3.2	6.0	NR	-0.25	35	Sera and real samples	31
11.	ERGO/GCE	Covalent binding	Amperometric	1-16	0.7	7.0	NR	-0.25	300	Real samples	32
12.	TMB/HRP/PDMS/TEOS/SiO_2 NPs	Covalent binding	Colorimetric	4-72	1.3	5.0	NR	NR	NR	Real samples	33
13.	TTP/SPCE	Electrodeposition	Amperometric	20-500	4.1	7.0	NR	-0.1	NR	Blood	34
14	GPtNPs	Covalent binding	Amperometric	0-0.32	0.001	NR	NR	0.45	811.26	Blood	35

15	PtRu/3DGF	Covalent binding	Amperometric	NR	0.04	7.4	NR	0.2	1023.1	Biological	36
16	Cytc/NiONPs/c-MWCNT/polyaniline/Au	Covalent binding	Amperometric	3-700	0.2	6.5	30	0.28	3.3	Fruit juices	37
17	HbNPs/Au	Covalent binding	Amperometric	0.001-1.2	0.0001	6.5	30	-0.2	1290.02	Diabetic	Present

Hb-Hemoglobin; NGP-Pluronic P123-nanographene platelet; L-cys-l-Cysteine; p-ABSA-paminobenzene sulfonic acid; PtNPs-Platinum nanoparticles; RGO-Reduced graphene oxide; CS-Chitosan; FC-Ferrocene carboxylic acid nano-hybrids; DP- Self assembled dipeptide; AuNP- Gold nanoparticles; HRP- Horse radish peroxidase; GCE- Glassy carbon electrode; GRCAPS- Graphene capsules; ITO- Indium tin oxide; PAN-PNMThH- Poly (aniline-co-N-methylthionine); ERGO-Electrochemically reduced graphene oxide; TMB- 3,3′,5,5′-teramethylbencidine; PDMS-Polydimethylsiloxane; TEOS-Tetraethylorthosilicate; SiO_2NPs- Silicon oxide nanoparticles; TTP- Turnip tissue paper; Cyt c-Cytochrome c; NiONPs- Nickel oxide nanaoparticles; c-MWCNT-Multiwall carbon nanotubes; PANI- Polyaniline; HbNPs- Hemoglobin nanoparticles; Au- Gold