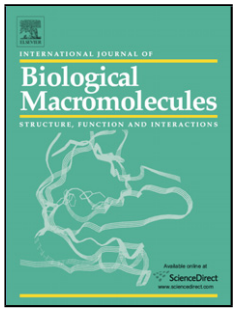

Title: Preparation, characterization and application of haemoglobin nanoparticles for detection of acrylamide in processed foods

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DEPARTMENT OF BIOCHEMISTRY

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To

September 17, 2017

Dr. A. Dong, Editor-in-chief

International Journal of Biological Macromolecules

Sub: Submission of revised MS entitled **"Preparation, characterization and application of haemoglobin nanoparticles for detection of acrylamide in processed foods"** by Neelam Yadav[1], Anil Kumar Chhillar[1], Chandra S Pundir[2] *

Dear Dr. A. Dong,

Thank you for your email dated 12 Sept.2017 regarding the revision of our MS entitled, **"Preparation, characterization and application of haemoglobin nanoparticles for detection of acrylamide in processed foods"** by Neelam Yadav[1], Anil Kumar Chhillar[1], Chandra S Pundir[2] *. We have revised the MS in view of reviewers' comments, which led to the definite improvement of the MS. I am uploading herewith the revised MS of above article along with Author's response to Reviewer's comments for your kind consideration of its publication in your esteemed journal International Journal of Biological Macromolecules. The paper has not been submitted to any other journal for consideration of its publication. There is no conflict of interest among the authors of this research paper. Kindly acknowledge the receipt of the above research article.

With regards!

Yours sincerely,

Emeritus Professor **CS Pundir,** Ph.D., MACS (USA), FRSC (UK)

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Sept.17, 2017

Preparation, characterization and application of haemoglobin nanoparticles for detection of acrylamide in processed foods

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Highlights

- Prepared and characterized Haemoglobin nanoparticles (HbNPs)
- Constructed an improved amperometric acrylamide biosensor employing HbNPs onto Au electrode
- Biosensor was evaluated and applied for determination of acrylamide
- Limit of detection (LOD) of proposed acrylamide biosensor was 0.1 nM, within and between batch co-efficient variation 3.85% and 4.67% respectively
- Analytical recovery of HbNPs /AuE at 5mM and 10 mM was 99% and 98.2% respectively
- Storage stability of working enzyme electrode was four months when stored at 4°C.
- HbNPs /AuE is very simple, sensitive, selective for the detection of acrylamide with better Limit of detection (LOD), co-efficient of variation (CV), storage time and stability than earlier reported biosensor.

Abstract

The nanoparticles of hemoglobin (HbNPs) were prepared by desolvation method and characterized by transmission electron microscopy (TEM),UV-visible spectroscopy, Fourier transformation infra red (FTIR) spectroscopy and X-ray diffraction (XRD) and atomic force microscopy (AFM). Protein profile of HbNPs was also studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). An amperometric acrylamide biosensor was constructed by immobilizing covalently HbNPs onto polycrystalline Au electrode. The Au electrode was characterized by scanning electron microscopy (SEM) and electrochemical impedance spectra (EIS) before and after immobilization of HbNPs. The biosensor showed optimum current response within 2 s at 0.26V, pH 5.0 at room temperature (20° C). The biosensor measured the acrylamide concentration in processed foods. The working range of biosensor was 0.05 nm-100mM with a limit of detection (LOD) as low as 0.1nM. The biosensor measured acrylamide concentration in various processed foods such as biscuits, bread, potato crisps, "kurkure", nuts and fried cereals. The analytical recovery of added acrylamide in aqueous extract of food was 99% and 98%. Within-and between-batch, co-efficient of variations were 3.85% and 4.67% respectively. The structural analogs of acrylamide such as acrylic acid and propionic acid had practically no interference on the biosensor.

Keywords: Haemoglobin nanoparticles; Desolvation method; Acrylamide biosensor; Carcinogen; Neurotoxin

Introduction

Acrylamide, an unsaturated amide, is found in various thermally processed foods. It is generated in food products containing high content of reducing sugars such as glucose and proteins specially rich in asparagines, amino acid, when heated at high temperature (>170 °C) (Mottram et al, 2002, Stadler et al, 2003). The formation of acrylamide occurs through Maillard reaction, when processed foods containing asparagines and reducing sugars are heated at high temperature. The reaction involves the formation of Schiff base by N-glycosidic pathway

(Claeys et al, 2005), followed by the decarboxylation of organic acid by acrolein pathway (Medeiros et al, 2012 and Yaylayan and Stadler, 2005).

Acrylamide is a potent carcinogen, neurotoxin and toxic compound for reproductive system (Sufian 2009). The toxicity of acrylamide is because of formation of glycidamide, an intermediate, which is highly mutagenic and responsible for inducing point mutations in various systems (Fazendeiro, 2013). Secondly, acrylamide acting as a Michael acceptor, forms adduct with the functional groups such as thiol, hydroxyl and amino groups. This adduct damages DNA (Watzek et al, 2012 and Erkekoglu and Baydar, 2012).

Various analytical methods such as gas chromatography (GC), mass spectroscopy (MS) (Motaghi et al, 2012), GC tandem MS (MS/MS) (Castle et al, 2003), high performance liquid chromatography (HPLC) (Ono et al, 2003) are available for detection of acrylamide. But these methods are cumbersome and require time consuming sample preparation, expensive instruments and trained persons to operate. Biosensing methods/biosensors overcome these problems, as these are simple, rapid, sensitive, specific and available on line. A comparison of various analytical parameters of different methods for the detection of acrylamide in processed foods has been illustrated in Table 1.

The proteins such as Hb, myoglobin and cytochrome C possess four heme groups in their redox center (Ye, 1988, Wu et al, 2015, Liu et al, 2012 and Sayyad et al, 2012) and thus employed in construction of various biosensors (Reed, 1987 and Sezgintürk and Dinçkaya, 2009). However, Hb is more appropriate in construction of acrylamide biosensor, due to its commercial accessibility at low cost and relatively more stability and configuration [N-(2-carbamoyl-ethyl)-L-valine] similar to one of the glycidamide [N-(2-carbamoyl-2-hydroxyethyl)-RS-valine), which facilitate the formation of Hb-acrylamide adduct (Friedman, 2003). Hence, the biosensors constructed with native Hb are more specific and cost effective (Xu et al, 2011).

Earlier, a number of Hb based biosensors involving Hb/sol-gel film modified carbon paste electrode (Wang et al, 2004), Hb/gold nanoparticles(AuNPs)/carboxylated multiwalled carbon nanotubes (cMWCNT)/glassy carbon(GC) electrode (Chen et al, 2007), Poly(maleic anhydride-alt-butyl vinyl ether (AM41)-polyethylene glycol(PEG)/Hb/citric acid NPs (Dessy et al, 2011), Hb/ silver nanoparticles)(AgNPs) modified borron dopped diamond electrode (BDDE) (Jiang et

al, 2015) and dimethyldioctadecyl-ammonium bromide(DDAB)/Hb/glassy carbon(GC) electrode) (Wu et al, 2015) have been fabricated for diverse applications.

A number of voltametric and amperometric biosensors have been constructed for the detection of acrylamide. The voltametric acrylamide biosensor employed Hb/DDAB/CP electrode (Stobiecka et al, 2007), Hb/SWCNT/GCelectrode/(Krajewska et al, 2008), while amperometric acrylamide biosensors were based on Hb/AuNPs (Garabagju et al, 2011), Hb/cMWCNT/CuNPs/polyaniline(PANI)/pencil graphite(PG) electrode (Batra et al, 2013), Hb/cMWCNT-Fe_3O_4NP/ chitosan(CHIT)/Au electrode (Batra et al, 2012). However, these methods have some drawbacks such as complicated preparation of working electrode and its poor analytical performance. Moreover, direct immobilization of native Hb onto Au surface leads to slow rate of electron transfer from the surface of Hb to Au electrode, due to presence of heme groups (redox center) inside the intensified globular structure of Hb (not exposed) (Stobiecka et al, 2007). These problems can be overcome by use of HbNPs in place of native Hb molecules, as protein nanoparticles(100-200nm in size) have unique advantages such as high optical, electrical, electronic, thermal properties, chemical and catalytic (ability to facilitate electron transfer) properties, increased surface area, rapid detection of traces amount of acrylamide with high sensitivity (Pundir, 2015).

During past decade, various improved biosensors were developed employing enzymes NPs such as horse reddish peroxidase (HRP) (Liu et al, 2005), glucose oxidase (GOD) (Sharma et al, 2010 and Kundu et al, 2010), uricase (Chauhan et al, 2014), cholesterol esterase (Aggarwal et al, 2016), cholesterol oxidase (ChOx) (Chawla et al, 2013), lipase, glycerokinase and glycerol-3-phosphate oxidase immobilized onto metal electrode for the determination of H_2O_2, glucose, uric acid, cholesterol and triglycerides respectively (Pundir and Aggarwal, 2017).

We describe herein the preparation of Hb nanoparticles, their physic-chemical properties and use in construction of an improved amperometric biosensor for detection of acrylamide in processed foods. The use of Hb nanoparticles in amperometric acrylamide biosensor is expected not only to simplify & economize its construction but also improve its analytical performance as well as its storage stability.

Materials and methods

Reagents

Human Hb from Sigma-Aldrich, acrylamide, potassium hexacyanoferrate (II) trihydrate (Carrrez I), acetic acid, zinc sulfate heptahydrate (Carrez II) and gluteraldehyde from Merk, cysteamine dihydrochloride, potassium ferrocyanide, potassium ferricyanide and copper sulfate from SISCO Reseach Lab (Mumbai, India), ethanol from Changshu Yangyuan Chemical China have been used. All other chemicals were of AR grade. Au wire purchased from local jeweler from market, different brands of biscuits, kurkure, crisps, fried cereals and diverse bread items were used, purchased from local market.

Instruments used

A potentiostat/galvanostat (Autolab, AUT83785, Eco Chemie) with a three electrode system consisting of a Au wire as a working electrode, Pt wire as a counter electrode, Ag/AgCl as a reference electrode. Scanning electron microscope (SEM) (Zeiss EV040), ultraviolet (UV) spectrophotometer (Dynamica HALO DB-20, UK), transmission electron microscope (TEM), X-ray diffractometer (XRD) (Rigaku, Smartlab guidance, 2011), Fourier transform infrared (FTIR) spectrophotometer (Thermo Scientific, USA), refrigerated centrifuge (Sigma 3-30K, Germany) Spectronic-20, electronic balance, temperature controlled water bath and atomic force microscope (AFM)(NT-MDT, 2011), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Construction of HbNPs/Au electrode

Preparation of HbNPs

HbNPs were prepared by desolvation method (Batra et al, 2013). Hb solution was prepared by dissolving 4 mg Hb powder in 2ml 0.02 M sodium phosphate buffer (pH 5.0). Four ml of ethanol was added to Hb solution at dropping rate of 0.5 ml/min. under constant stirring (500rpm), followed by addition of 1.8 ml of 2.5% glutaraldehyde and stirring for 24 hours. Gluteraldehyde helps in the intermolecular crosslinking with the functional groups present on the exposed surface of the Hb through Schiff base and thus mediates the formation of aggregate of HbNPs by removing water. Now 0.12g cysteamine hydrochloride was added to this solution and kept this mixture for 5-6 hours under constant stirring. Cysteamine hydrochloride provides the thiol (-SH) groups onto the HbNPs for their immobilization onto Au. The resulted solution was centrifuged

at1200 rpm, at 4°C for 10 min. The supernatant was separated from the pellet and the pellet was dispersed in 0.1M sodium phosphate buffer (pH 7.0) and stored it at 4°C until it used. The yield of HbNPs was calculated by using following formula:

$$\% \text{ Yield} = \frac{\text{Weight of HbNPs (mg)} \times 100}{\text{Total amount of Haemoglobin powder used (mg)}}$$

Characterization of HbNPs

The shape and size of HbNPs were studied by taking their image in a transmission electron microscope at Indian Agriculture Research Institutes (IARI) Pussa, New Delhi. HbNPs were also characterized by recording their UV-visible spectra in a UV-visible spectrophotometer and Fourier transform infrared (FTIR) spectra and electrochemical impedance (EIS) spectra. HbNPs were also examined by taking their AFM at Jamia Islamia University, New Delhi.

X-ray diffraction (XRD)

Proteomics of HbNPs was investigated by recording their XRD pattern in reflection mode at Jamia Islamia University, New Delhi. The sample was scanned from 5° to 90° (2 Â) in steps of 0.05°. The crystallite domain sizes (D) of HbNPs was scrutinized from XRD peaks based on the Scherrer's equation: $D = 0.9 / (W \cos Â)$, where the wavelength of X-ray was (= 0.15418 nm), Â was the Bragg's diffraction angle and W was the true half-peak width of the X-ray diffraction lines.

Proteomics of HbNPs

Proteomics of HbNPs were carried out by SDS-PAGE.

Immobilization of HbNPs onto Au electrode

To immobilize HbNPs onto Au electrode, firstly Au wire was washed with pirrahna solution (Concentrated H_2SO_4 and H_2O_2, in 3:1 ratio), followed by cleaning the exposed surface of Au electrode from unwanted materials by silica slurry. This silica slurry was a mixture of silica gel and distilled water. This silica gel exhibits high adsorption capacity, non-soluble in many solvents, better thermal stability, high surface area that makes the electrode suitable for various

oxidation reactions (Salama et al, 2016). After washing the Au electrode with silica gel, finally washed with ethanol.

This Au wire was sonicated and washed with distilled water. After this Au wire was dipped into 2 ml suspension of HbNPs (1 mg/ml, 0.2 M sodium phosphate buffer, pH 7.0, temperature (4°C) for 12 hours. The HbNPs modified Au electrode was removed and rinsed with 0.1 M sodium phosphate buffer (pH 7.0) and was stored at 4°C in 0.1 M sodium phosphate buffer (pH 7.0) until its use.

Characterization of HbNPs/Au electrode

SFM: The images of bare Au electrode and HbNPs/Au electrode were taken in a scanning electrode microscope.

Construction of HbNPs based acrylamide biosensor

To construct the acrylamide biosensor, HbNPs/Au, as a working electrode, Ag/AgCl as reference electrode and Pt wire as auxiliary electrode were connected through Potentiostat. The current (in mA) was measured at different time (in seconds).

Response measurement of HbNPs/Au electrode

The cyclic voltametry (CV) of working electrode was recorded in Potentiostat Galvanostat in the potential range of -0.750 to 0.500 V 500V in 25 ml of 0.2 M sodium acetate buffer (pH 6.0) containing 0.1ml 3.5µM acrylamide with scan rate of $20mVs^{-1}$. The maximum response (current in mA) was observed at 0.26 V (Figure 1), hence, succeeding reading was recorded at this voltage and the current was recorded at different time (in seconds). This response of biosensor (in current) was recorded toward the acrylamide in the range of 0.1-100 nM. With the simultaneous increase in Hb–acrylamide adduct concentration on the surface of electrode; consequently, parallel decrease in current was recorded**.** The electrochemical reaction between haemoglobin and acrylamide is shown in Figure 2a. Figure 2b illustrates the mechanism of Hb-acrylamide adduct formation in thermally processed foods. This decrease in current treated was taken as analytical signal and was the base for highly selective and sensitive acrylamide detection.

Optimization of acrylamide biosensor

The acrylamide biosensor was optimized by studying the effect of pH, time and substrate (acrylamide) concentration on biosensor response. Optimum pH of the biosensor was determined by varying the pH of reaction buffer between pH 4.0 to 8.0 at an interval of 0.5 pH using the following buffer, each at a final concentration of 0.1 M, pH 4.0 to 6.0, sodium acetate buffers; pH 6.5 to 8.0, sodium phosphate buffers. The effect of acrylamide concentration on biosensor response was measured by changing the acrylamide concentration (in reaction mixture) in the range of 0.05-100nM.

Application of HbNPs based acrylamide biosensor

Different brands of diverse processed food items such as biscuits (A) (Brand: A_1, A_2, A_3, A_4, A_5, A_6. A_7, A_8, A_9, A_{10} and A_{11}), cakes (B) (Brand: B_1, B_2. B_3, B_4, B_5, B_6, B_7, B_8 and B_9), chips (C) (C_1, C_2, C_3 and C_4), fried cereals (D) (D_1, D_2, D_3 and D_4), nuts (E) (E_1, E_2 and E_3) and kurkure (Corn puffs) (F) (F_1, F_2, F_3 and F_4) (4 mg) were purchased from the local market and then crushed to form the fine powder in the mortar and pestle separately. The weighed quantity of food powder was dissolved in 100 ml DW and kept at room temperature for 20 min to swell. Now, this suspension was stirred for 1 hour at 60°C and centrifuged at 4,500 rpm, at 4°C for 20 min. The supernatant was collected and purified by adding 2.5 ml Carrez I (0.78 M) and Carrez II (0.5 M). The upper aqueous layer of the supernatant was removed, filtered and tested for acrylamide. The acrylamide content was measured in this extract of processed foods in the similar standard conditions i.e. 25 ml sodium acetate buffer having pH 5.0 at 0.26 V with scan rate $20mVs^{-1}$ at room temperature (20°C) as for standard curve for acrylamide. The sample (100µl) was added into 25ml sodium acetate buffer (pH 5.0) and the current was measured at 0.26 V with scan rate $20mVs^{-1}$ vs Ag/AgCl. The acrylamide content in sample was extrapolated from standard curve for acrylamide.

As the concentration of acrylamide in food sample increases the cyclic volatametry of acrylamide biosensor shows the decrease in current, which is due to formation $HbFe2^{+}$-acrylamide adduct onto Au electrode that interferes the redox reactions of core haem part of haemoglobin (removal of electrons) or vice-versa. This decreased current acted as an analytical signal for the determination of acrylamide in various thermally processed food samples.

Evaluation of acrylamide biosensor

The acrylamide biosensor was evaluated by investigating its various parameters such as linearity, limit of detection (LOD), % analytical recovery, precision, and correlation with the standard HPLC method. LOD was calculated using the formula: LOD=3.3 × SD/S where SD is standard deviation of the response, S is the slope of calibration curve (Valipour, 2015). To determine the analytical recovery of the HbNPs based acrylamide biosensor, known amount of acrylamide (5mM and 10mM) was added into food sample. The acrylamide content of this extract was determined before and after addition of acrylamide. The precision of the proposed acrylamide biosensor was determined by studying the within- and between-sample coefficients of variation (CVs) for the determination of acrylamide in potato crisps on the same day and after1 week of storage at -20 °C. A comparison of various analytical parameters of various electrochemical biosensors for detection of acrylamide in processed foods has been depicted in Table 5

Storage stability of HbNPs/Au electrode

The storage stability of the HbNPs/AuE working electrode was tested weekly for 4 months during its storage at 4°C in dry condition.

Results and discussion

Preparation of HbNPs

The nanoparticles of Hb from Hb suspension were prepared by desolvation method with 95% yield. The formation of brown reddish colour indicated the formation of HbNPs.

Characterization of HbNPs

TEM

The typical TEM image of HbNPs (Figure 3) showed their spherical shape. The diameter of these HbNPs lie in the range 84.07 to 103.47nm with average diameter is 187.54nm with an average size of 93.77 nm, which is in the range of protein nanoparticlesi.e.100-200nm (Pundir, 2015).

UV-Spectra

UV-visible spectra of HbNPs and Hb molecules exhibited a strong absorbance at 280 nm **(Figure 4a)** and 410nm respectively, which could be due to small sized HbNPs (Figure 4b).

FTIR spectra

FTIR spectrum of native Hb showed distinct peaks at 1062, 1317, 1553, 1679 and 3497 cm $^{-1}$. The strong absorption band at 1317 cm-^{1} which may arise due to the stretching vibrations of C-N aromatic functional groups of protein. The medium absorption bands located at 1679 and 1553 cm $^{-1}$ corresponds to carbonyl stretch and N-H stretch vibrations in the amide II linkage of the protein respectively. The band at 1062 cm^{-1} indicates presence of amine group. In addition, there was a located at 3497 cm $^{-1}$, 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 3488 cm^{-1}(Figure 5a). 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 may be due to ethanol, glutaraldehyde and cysteamine treatment of HbNPs during their preparation. The FTIR spectra of HbNPs in the presence of acrylamide showed an additional peak (Figure 5b) at 1635cm-1 which was due to the formation of –NH- bond between Hb-acrylamide adduct.

XRD

The XRD patterns of the HbNPs, showed clearly the characteristic diffraction peaks of HbNPs at 37°, 43°, 64°, 77° and 82° which revealed the polycrystalline nature of HbNPs that increase the surface area of the working electrode. Diffraction peaks indexed the pure face –centered cubic (f.c.c) lattice of HbNPs without any adulteration of impurities (Figure 6).

AFM

The size of HbNPs was also calculated from by taking averages (viz. 2.5 nm, 5 nm, 7.1 nm, 50 nm, 7.5 nm, 33.3nm, 78.4 nm) of all the particles that are visible in AFM images and the average size of HbNPs was 25nm (Figure 7a). Figure 7b shows the 3-D image of these HbNPs.

Proteomics of HbNPs

Proteomics of HbNPs was carried out by SDS-PAGE that separates the charged molecules according to their charge, size and shape. But there was no appearance of any band, which was due to neutralization of HbNPs aggregates during their preparation by desolvating agent (ethanol) which removed the water in between the molecules and leads to the formation of aggregates which has no charge.

Characterization of HbNPs/Au electrode by SEM

The SEM images of the surface of bare Au electrode, HbNPs/Au electrode are shown in (Figure 8a) and (Figure 8b) respectively. The stepwise modification of electrode can be seen clearly from these SEM images. The SEM images of the bare Au electrode showed a smooth and featureless morphology. The HbNPs/Au electrode exhibited a net structure. The film was more uniform and porous; hence, the effective surface area was larger. On immobilization of Hb, the globular structural morphology appeared due to attachment of Hb onto Au electrode. HbNPs was immobilized onto the Au electrode covalently via thiolate bond.

Characterization of HbNPs/Au electrode by EIS

The charge transfer process in HbNPs/Au electrode was investigated by monitoring charge transfer resistance (R_{CT}) at the electrode and electrolytic interface. The diameter of the semicircle at higher frequencies corresponds to the electron transfer resistance (R_{CT}) that controls the electron transfer kinetics of the redox probe at the electrode interface, while the linear part at lower frequencies corresponds to Warberg diffusion process. The Nyquist plot (Figure 9) displays EIS of HbNPs/Au in 5mM $K_3Fe(CN)_6/K_4Fe(CN)_6$. The R_{CT} value for the native Hb was 200 Ω, which is lower than that of HbNPs/Au i.e. 300Ω. The decreased (R_{CT}) value in the presence of Hb revealed that the electron transfer through redox couple was enhanced by the presence of Hb on the electrode surface.

Response measurement of HbNPs/Au electrode

The response of present acrylamide biosensor was measured by obtaining the decline in current peaks by cyclic voltagram. This decrease in current peaks was due to the formation of Hb-acrylamide adduct during the electrochemical reaction on the surface of the working electrode. Therefore, it reduces the flow of electrons from the working Au electrode immobilized

with HbNPs, as a result the electroactivity of Hb changed. The scheme for electroactivity of immobilized Hb on the surface of Au electrode has been described in Figure (10). The working range of potential of biosensor was observed at -0.750 to +0.500V (current in mA) and maximum current was recorded at 0.26V (Figure 1) which is slightly higher than Hb-DDAB carbon-paste modified electrode ($0.2Vs^{-1}$) (Stobiecka et al, 2007), Hb/cMWCNT-Fe3O4NP/CHIT/Au (0.182 V) (Batra et al, 2012), Hb/cMWCNT/CuNP/PANI/PG (0.194 V) (Batra et al, 2013). Hence, all the parameters were studied subsequently at this voltage (0.26V). The proposed biosensor showed electrochemical response toward the acrylamide with an inverse linear relationship in the range of 0.1-100nM. This decreased current peak could be a base for very specific as well as sensitive biosensors for acrylamide determination.

Optimization of biosensor

The optimal pH of the biosensor was pH 5.**0** (Figure 11) which is higher from Hb- DDAB modified carbon paste electrode (pH 4.8) (Krajewska et al, 2008), similar to Hb/cMWCNT-Fe3O4NP/CHIT/Au (pH 5.0) (Batra et al, 2012) and lower than Hb/cMWCNT/CuNP/PANI/PG (pH 5.5) (Batra et al, 2013), biosensor based on glassy carbon electrode with single walled carbon nanotubes/Hb (pH 5.0) (Stobiecka et al, 2007) . There was an inverse relationship between sensor response and acrylamide concentration in the range of 0.1-100nM (Figure 12).

Evaluation of biosensor

Linearity

The working range of HbNPs/AuE biosensor was 0.05-100nM. There was an inverse linear relationship between the current (nA) and the acrylamide concentration in the range of 0.1- 100 nM (Figure 13), which is better than those of earlier reported biosensors (Table 5) (Batra et al, 2013), (Batra et al, 2012), (Krajewska et al, 2008) and (Stobiecka et al, 2007).

Detection limit

The detection limit of the current sensor was 0.1nM (S/N=3), which is lower than that of earlier biosensor (Table 5) based on HbNPs, (Batra et al, 2013), (Krajewska et al, 2008) and (Stobiecka et al, 2007).

Analytical recovery

The mean analytical recoveries of acrylamide added to few of food samples at a concentration of 5 mM and 10 mM were 99% and 98.2%, respectively revealing the high reliability of the present biosensor (Table2). This analytic recovery was better than earlier reported biosensor (Table 5) (Stobiecka et al, 2007), (Krajewska et al, 2008), (Batra et al, 2013) and (Garabaigu et al, 2011).

Precision

Within-and between-sample coefficients of variation (CVs) for the determination of acrylamide in food samples on the same day and after 1 week of storage were 3.85% and 4.67% respectively (Table 3). This high precision reveals the unique reproducibility as well as consistency of the present biosensor, which could be due to use of HbNPs and their covalent immobilization onto Au electrode.

Storage stability of HbNPs/Au electrode

The storage stability of the HbNPs/AuE working electrode was tested every tenth day for 4 months, while being stored at 4°C in dry condition. Figure12 shows the initial response of HbNPs/AuE electrode vs storage time. The % activity of HbNPs/AuE has been depicted in Figure 14. The electrode lost 10% of its activity after 120 days of its regular use. This storage stability of present biosensor is better than earlier reported biosensor (Table 5) (Batra et al, 2013) (Batra et al 2012), (Krajewska et al, 2008) (Stobiecka et al, 2007) which could be due to covalent immobilization of HbNPs onto Au electrode.

Interference study and selectivity

Two structural analogs of acrylamide namely, acrylic acid and propionic acid were tasted at 1.42µM concentration in 0.1 M sodium acetate buffer pH 5.0. None had shown any significant effect, as there was only 7.6% inhibition by propionic acid in D_3 food sample, but no effect of acrylic acid and propionic acid in other food samples.

Detection of acrylamide in food samples

Table 4 shows the acrylamide level in six different types of food samples viz. biscuits (A), cakes (B), chips (C), fried cereals (D), nuts (E) and kurkure (Corn puffs) (F). The mean ±SD of acrylamide concentrations in these foods were 44.69±35.68mM (A), 20.11±10.44mM(B), 59.8±0.021mM(C), 23.5±10.82mM(D), 12,33±7.1mM(E) and 47.25±26.60mM(F), as measured

by the present biosensor. These values are almost comparable to earlier reported results (Friedman, 2003).

Conclusion

An improved amperometric acrylamide biosensor was developed by immobilizing HbNPs covalently onto Au electrode. HbNPs based acrylamide biosensor showed better analytic performance in terms of lower detection limit (0.10nM), wider working range (0.05nM to 100mM) and longer storage stability (120 days) compared to earlier Hb based biosensors. The use of HbNPs not only improved the analytic performance of the biosensor but also simplified its construction process. HbNPs could also be employed for the fabrication of other amperometric biosensors.

Conflict of interest

All the authors of this paper have no conflict of interest.

Acknowledgement

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Figure Captions for HbNPs based Acrylamide Biosensor

Figure1:- Cyclic voltammogram for formal potential testing of HbNPs /AuE in 25ml 0.1 M sodium acetate buffer (pH 5.0) containing 100µM acrylamide (0.1ml) at a scan rate of 20mVs^{-1}.

Figure2a:- Electrochemical reactions involved in functioning of acrylamide biosensor based on HbNPs

Figure2b:- Reaction for adduct formation between HbNPs and acrylamide

Figure3:- Transmission electron microscopic (TEM) images of HbNPs.

Figure4a:- UV-visible spectra of haemoglobin (Hb)

Figure4b:- UV-visible spectra of haemoglobin nanoparticles (HbNPs)

Figure5a:- FTIR spectra of HbNPs dispersed in 0.1 M sodium phosphate buffer (pH-7.0).

Figure5b:- FTIR spectra of HbNPs and acrylamide dispersed in 0.1 M sodium phosphate buffer (pH-7.0).

Figure6:- X-ray diffraction pattern of HbNPs

Figure7a:- AFM images of HbNPs

Figure 7b:- 3-D Structure of HbNPs

Figure8a:- Bare SEM image of Au electrode

Figure 8b:- SEM image HbNPs immobilized Au electrode

Figure9:- Electrochemical Impedance Spectra (EIS) of bare AuE and HbNPs/AuE.

Figure 10:-Schematic representation of chemical reaction involved in the fabrication of HbNPs onto Au electrode.

Figure11:- Influence of applied pH on the current response of HbNPs/AuE in 25ml 0.1 M sodium acetate buffer (pH 5.0) containing 100µM acrylamide (0.1ml) at an applied potential of 0.26V (vs Ag/AgCl) with scan rate of 20mVs^{-1}. The error bars show standard deviation for n=3.

Figure 12:-Influence of time on the current response of HbNPs/AuE in 25ml 0.1 M sodium acetate buffer (pH 5.0) containing 100μM acrylamide (0.1ml) at an applied potential of 0.26V (vs Ag/AgCl) with scan rate of $20mVs^{-1}$.

Figure 13:- Standard inverse relationship of acrylamide concentration of HbNPs/AuE in 25ml 0.1 M sodium acetate buffer (pH 5.0) containing 100μM acrylamide at an applied formal potential of 0.26V (vs Ag/AgCl) with scan rate of $20mVs^{-1}$. The error bars show standard deviation for n=3.

Figure 14:-Effect of storage stability onto the activity of acrylamide biosensor

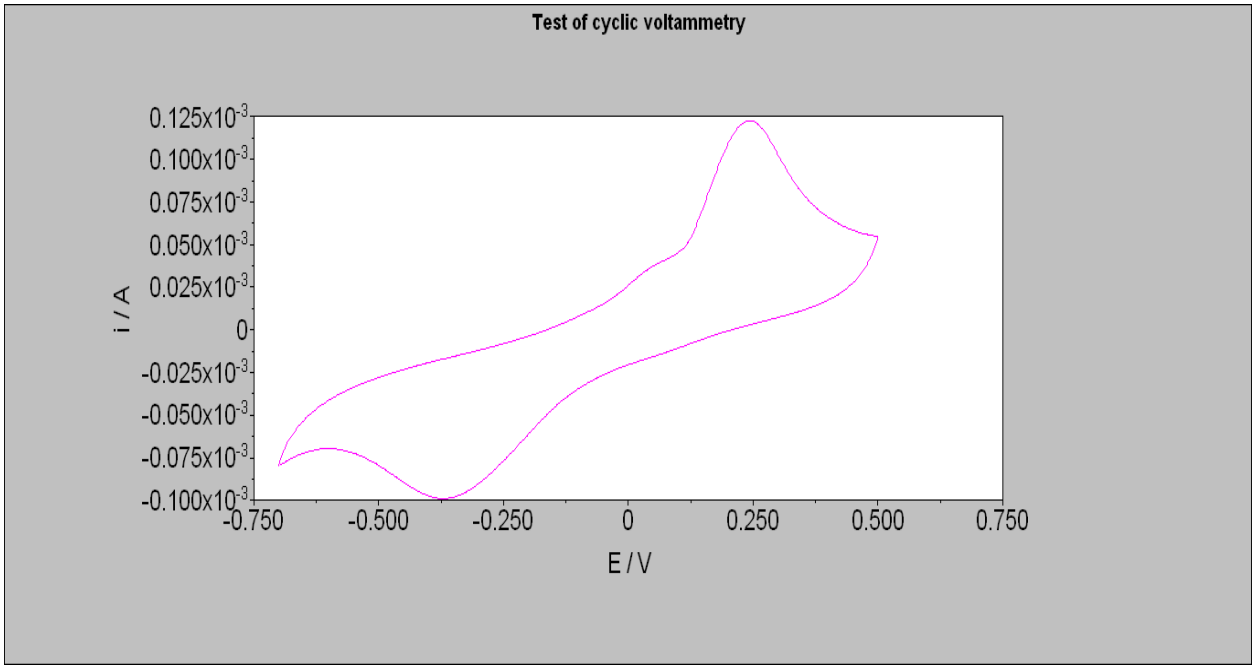

Figure 1

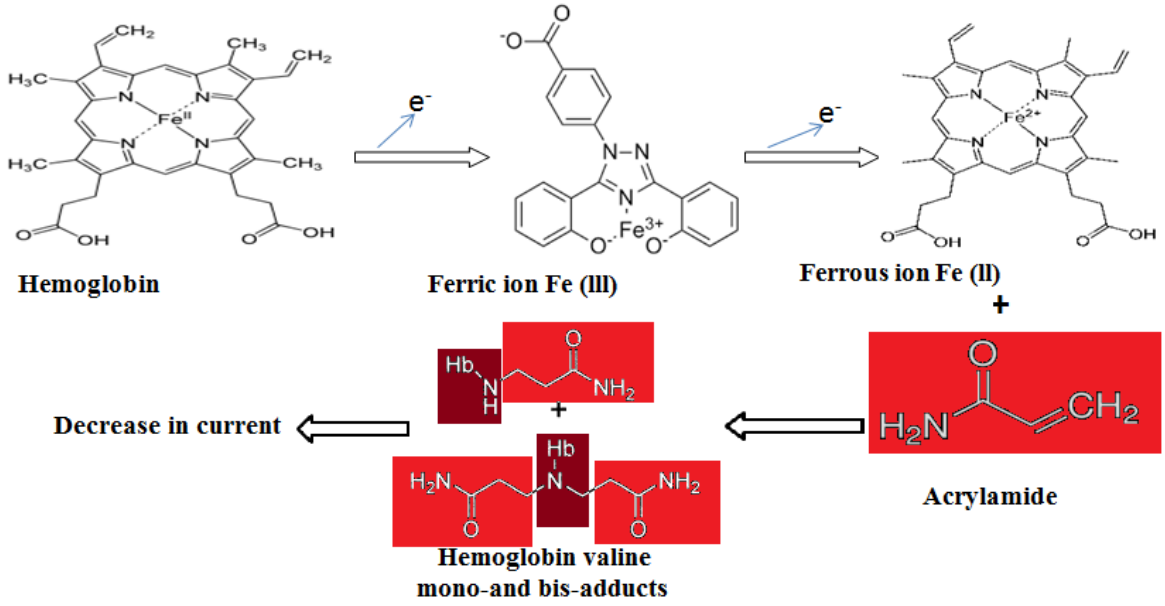

Figure 2a

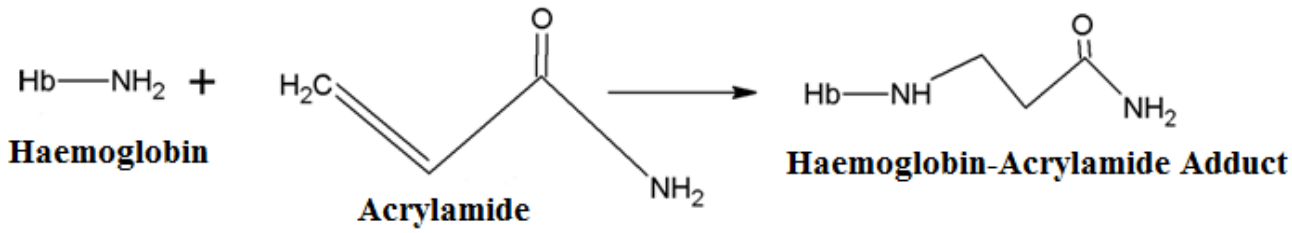

Figure 2b

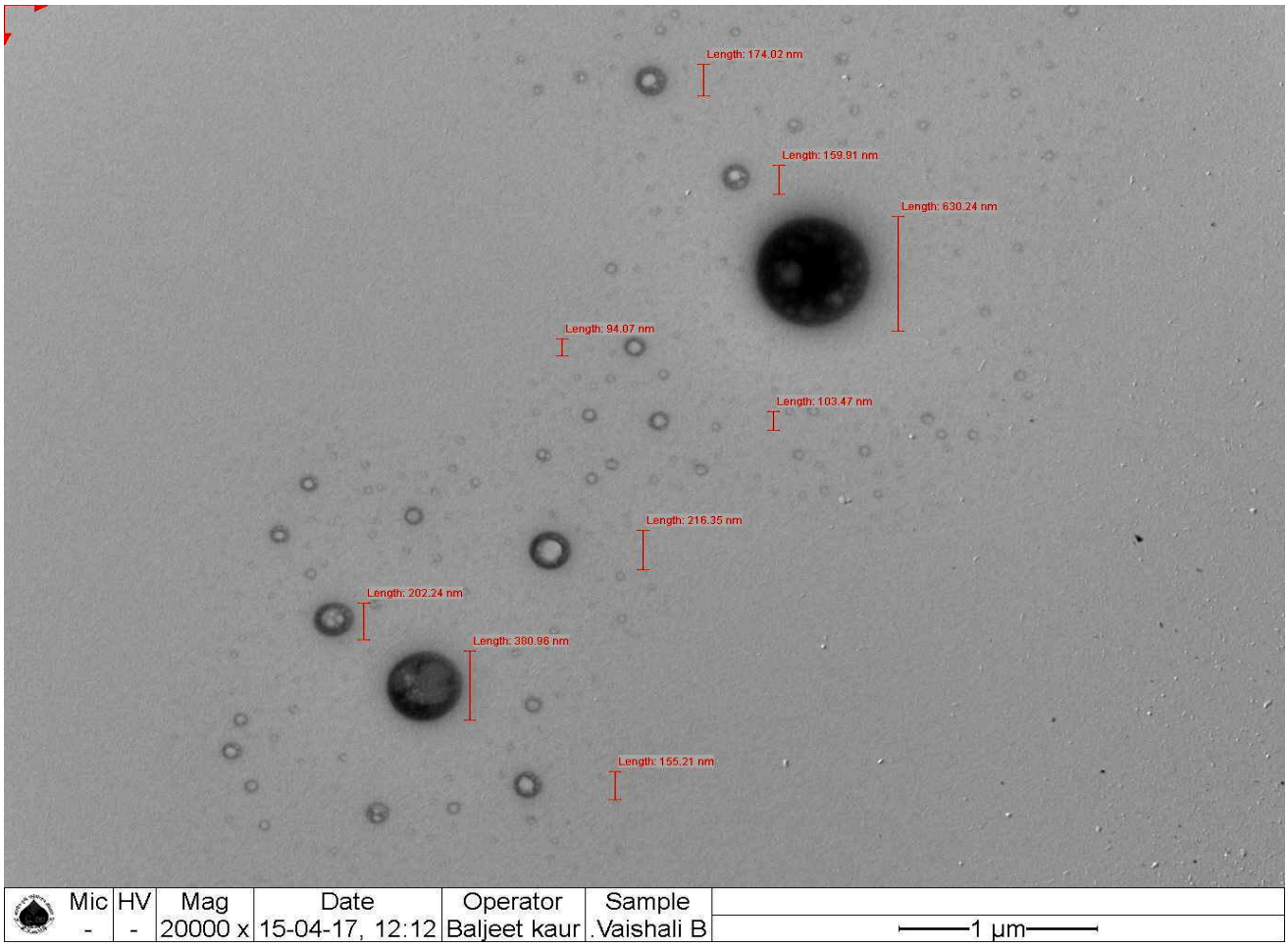

Figure 3

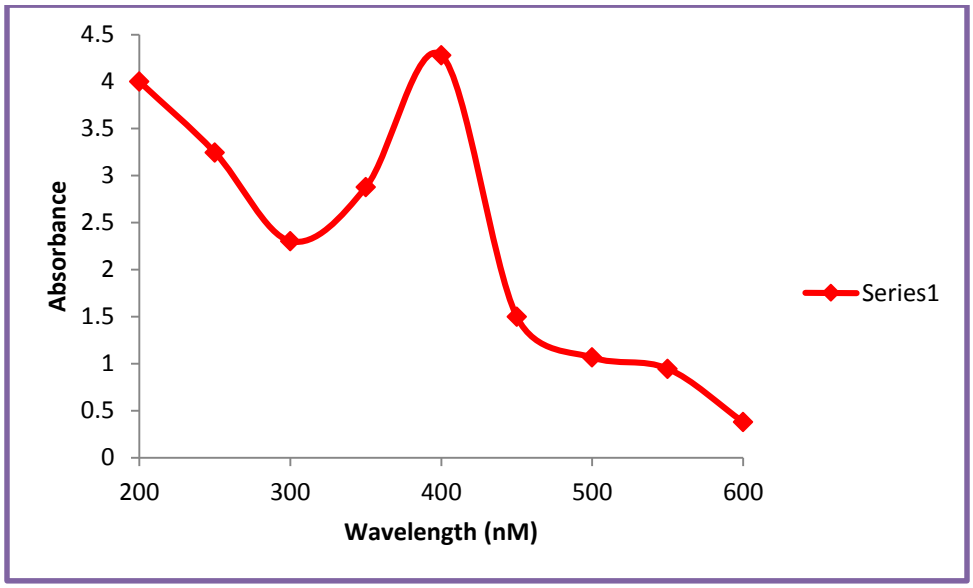

Figure 4a

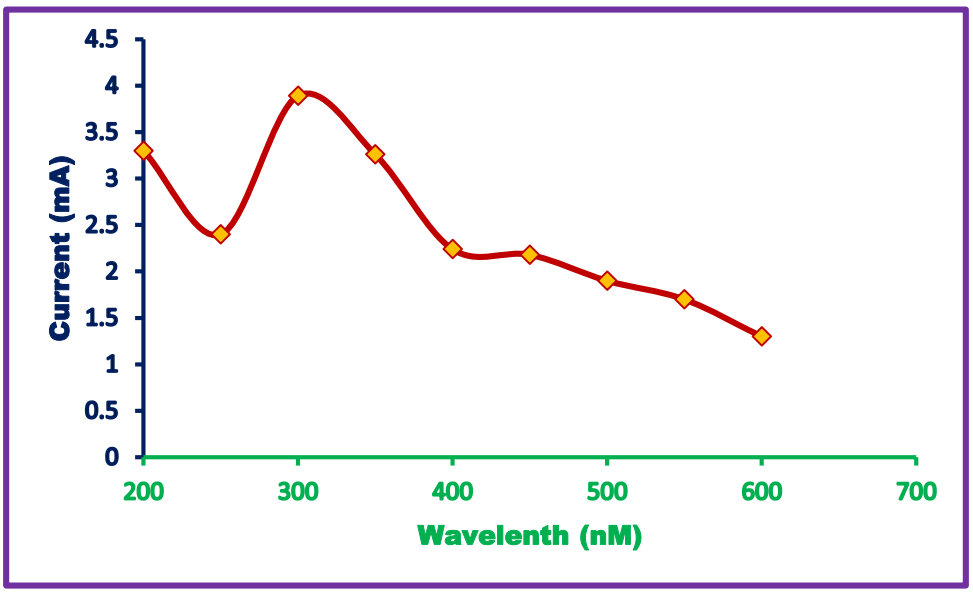

Figure 4b

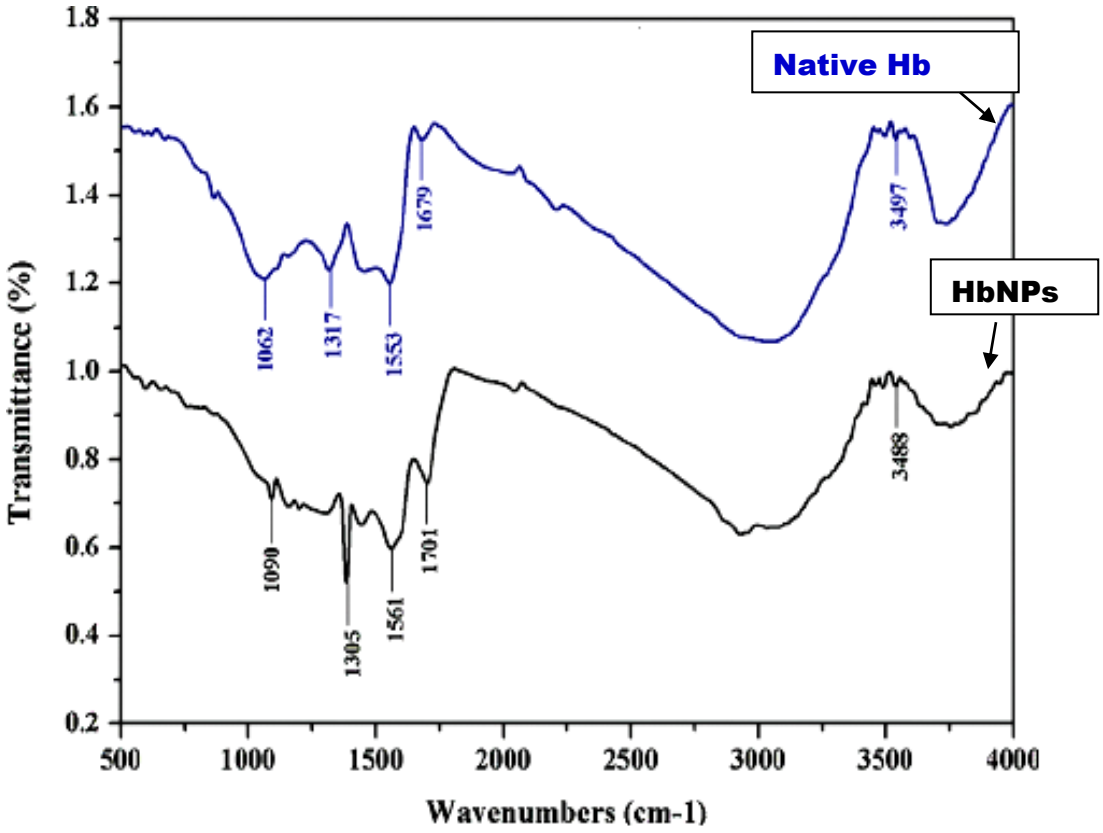

Figure 5a

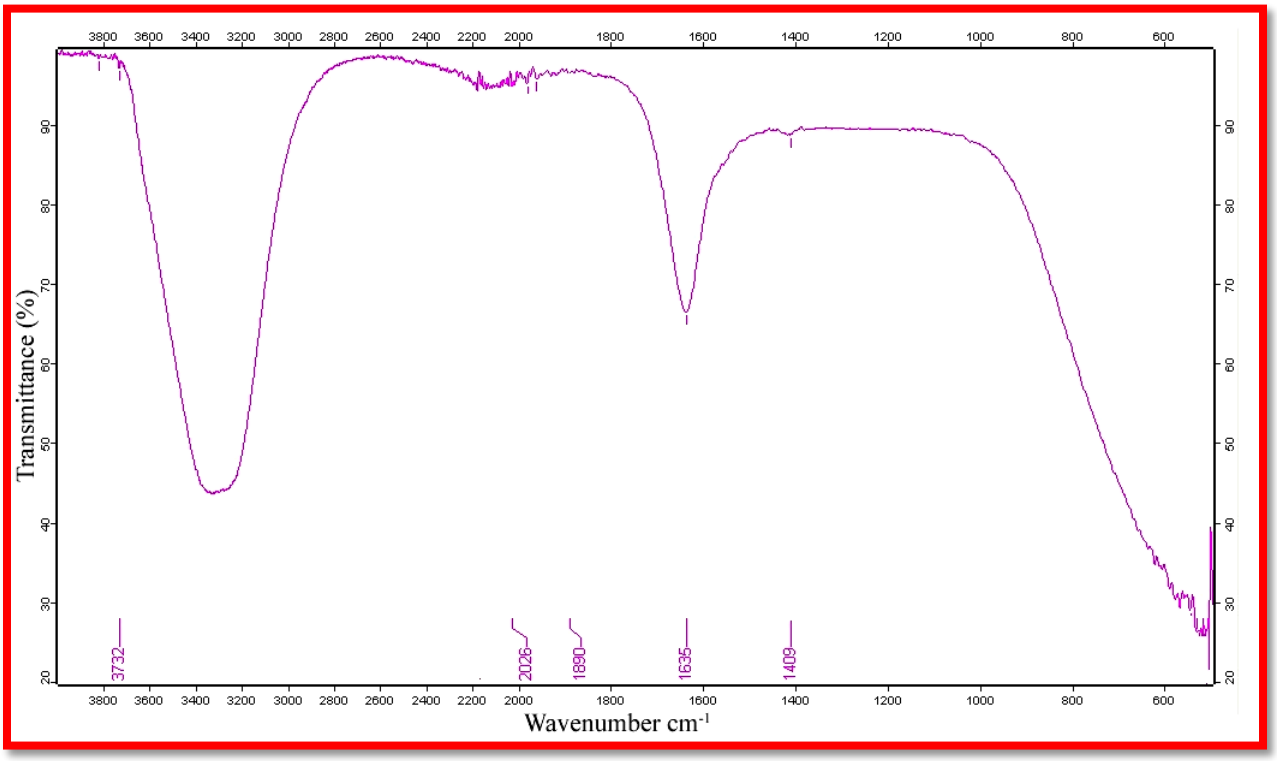

Figure 5b

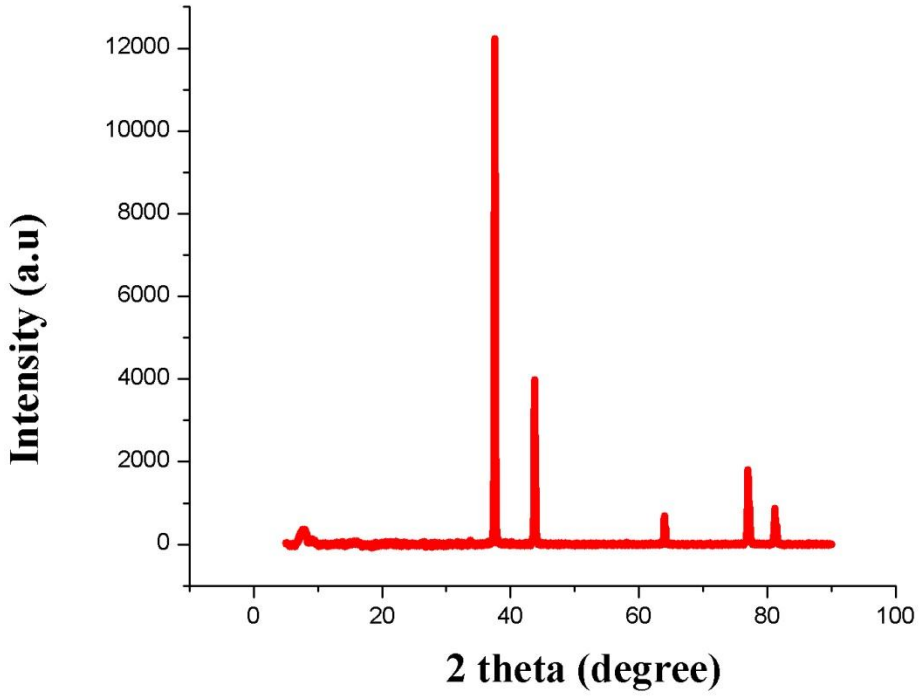

Figure 6

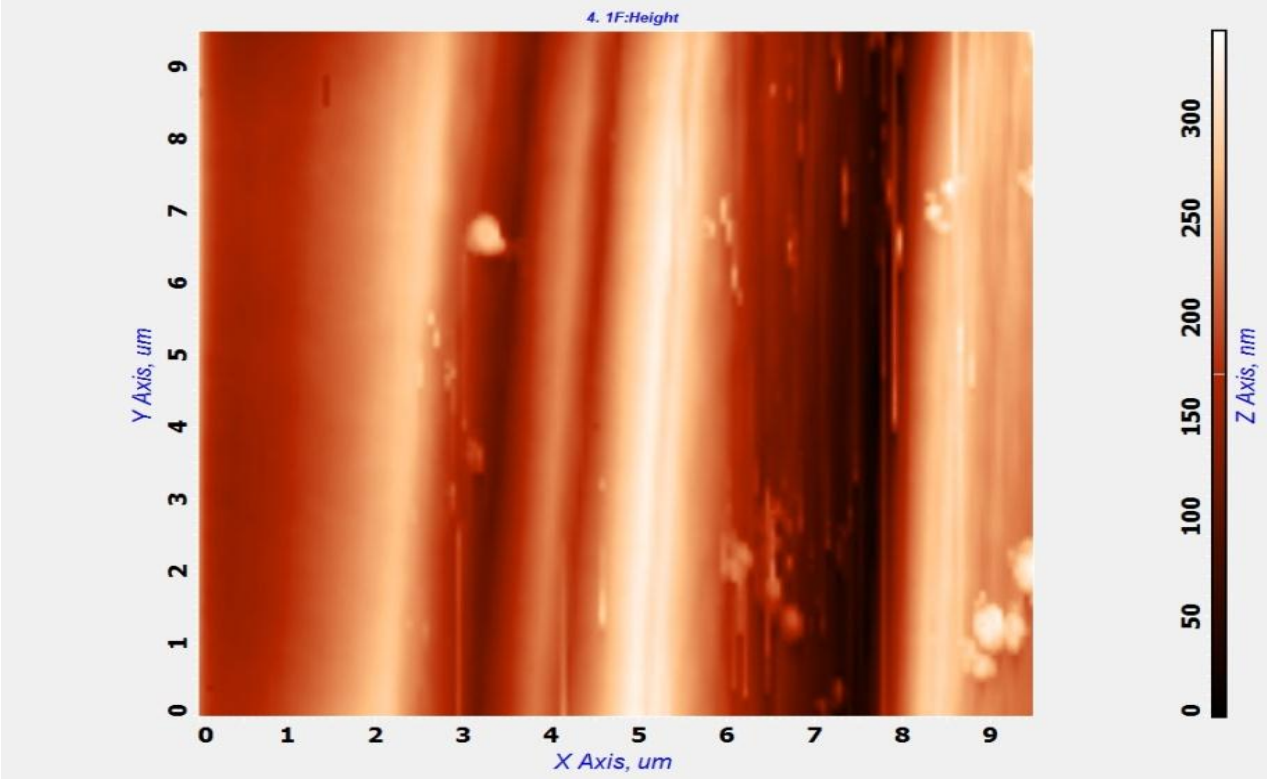

Figure 7a

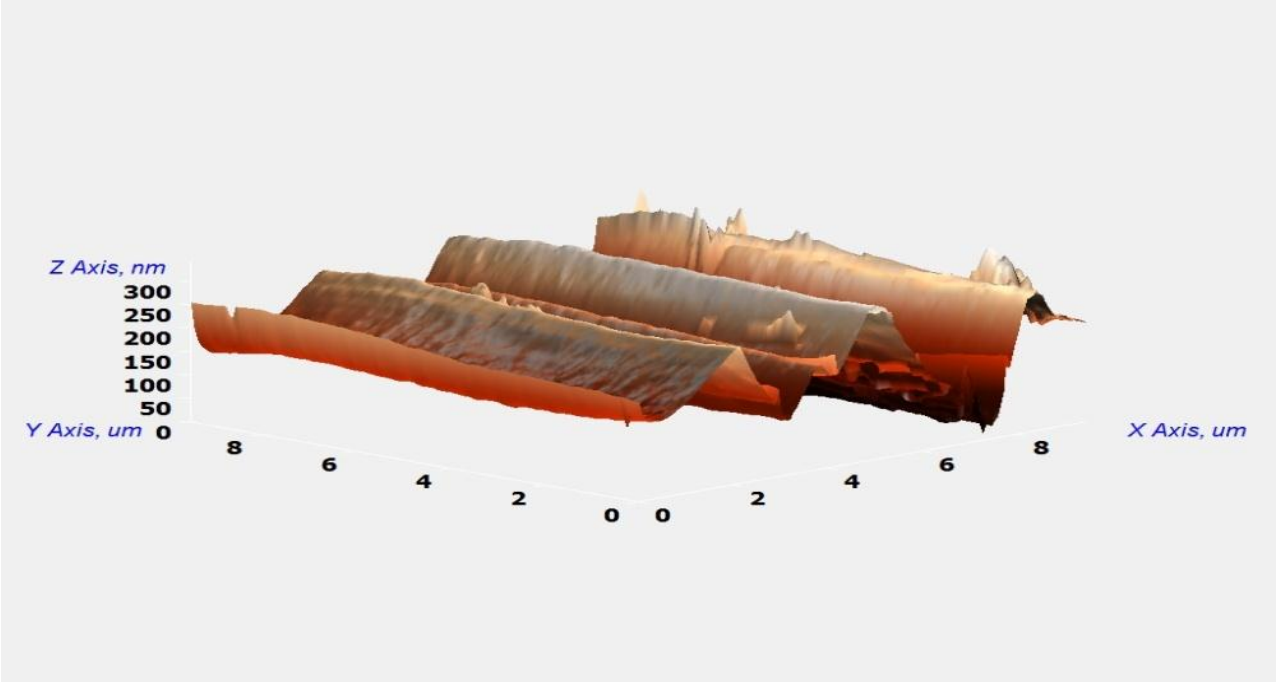

Figure 7b

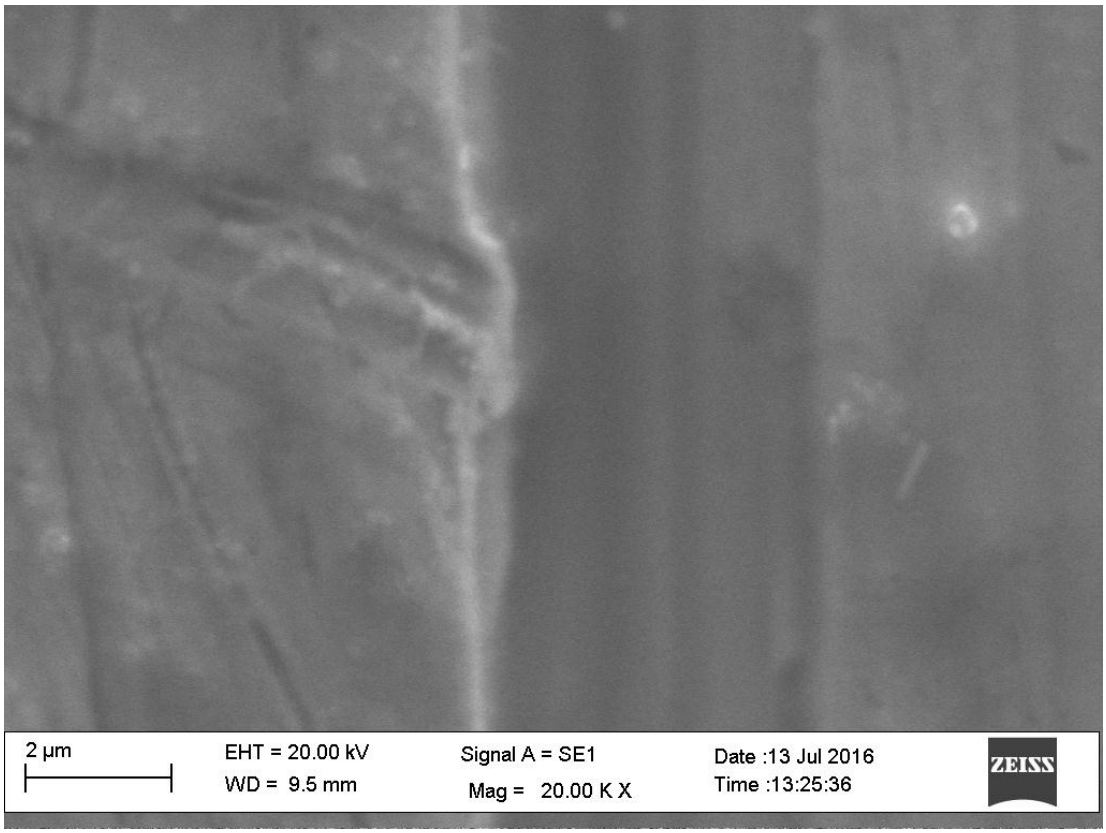

Figure 8a

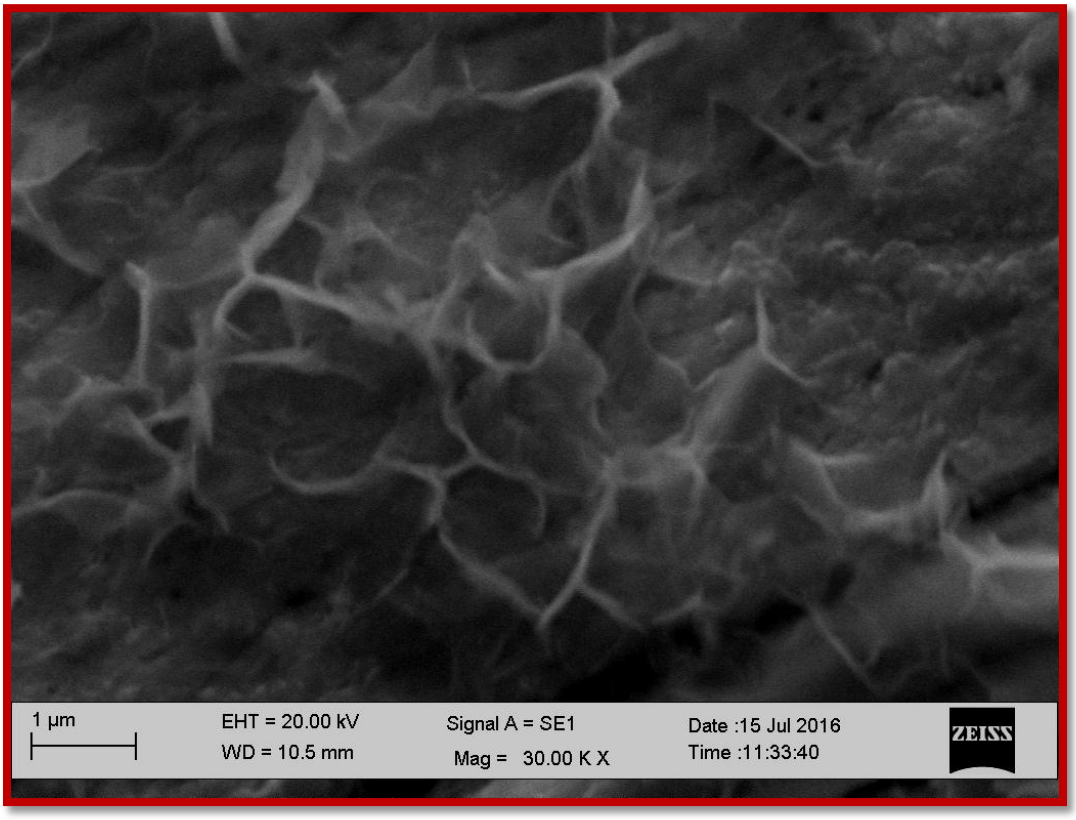

Figure 8b

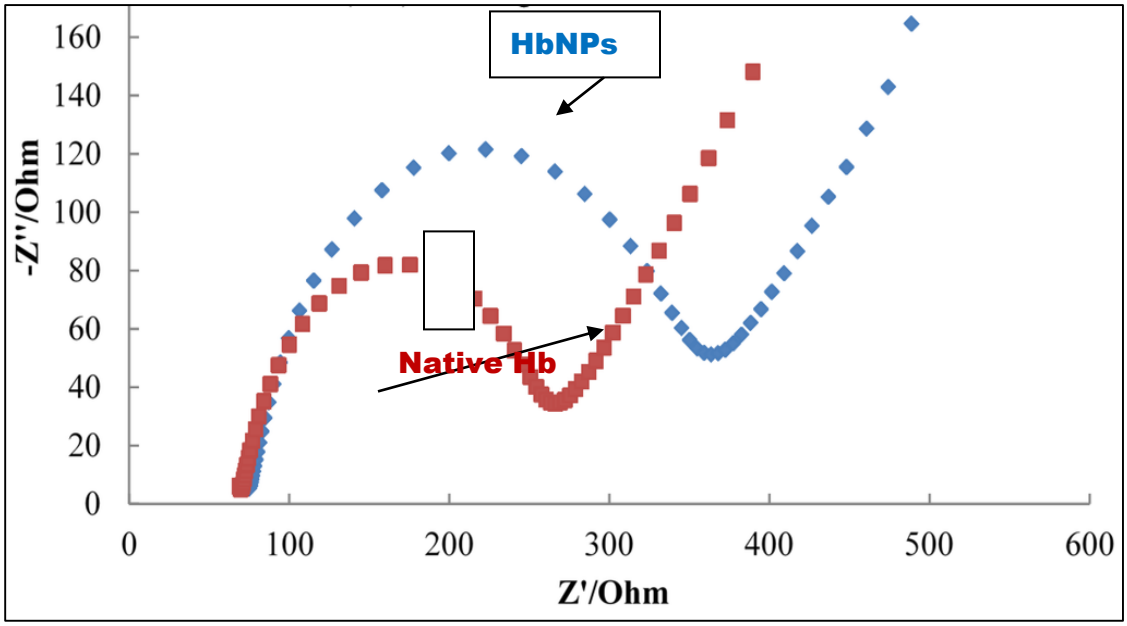

Figure9

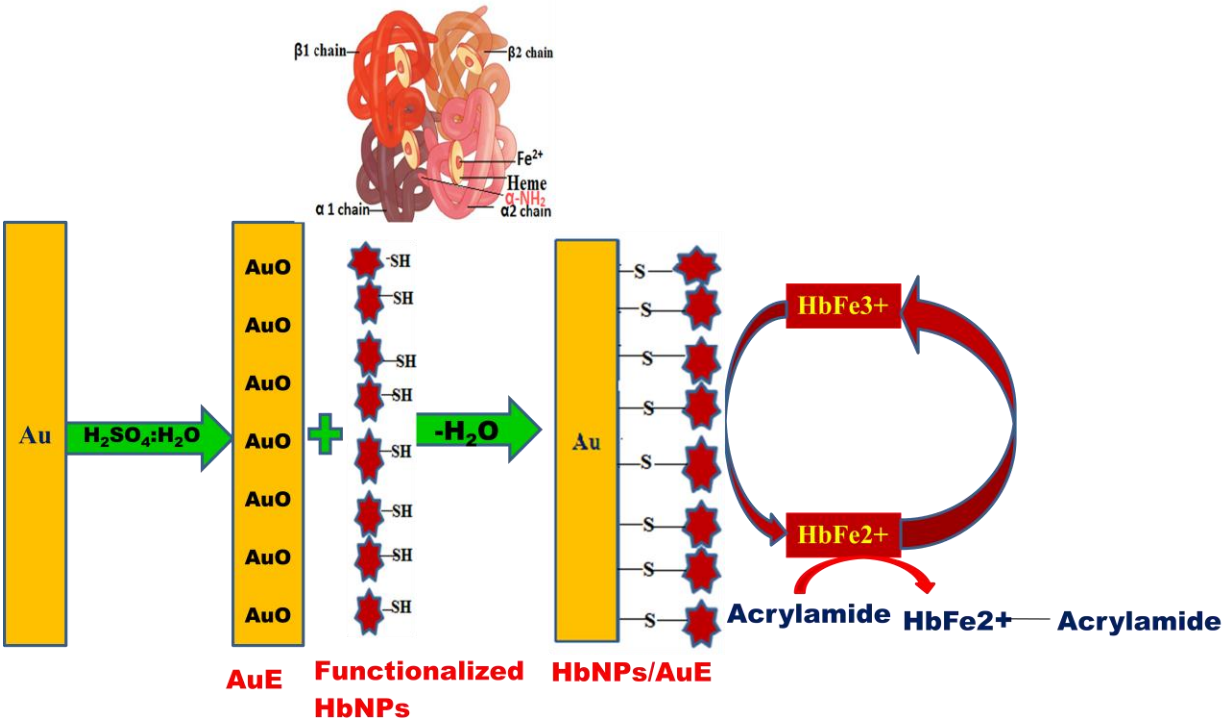

Figure 10

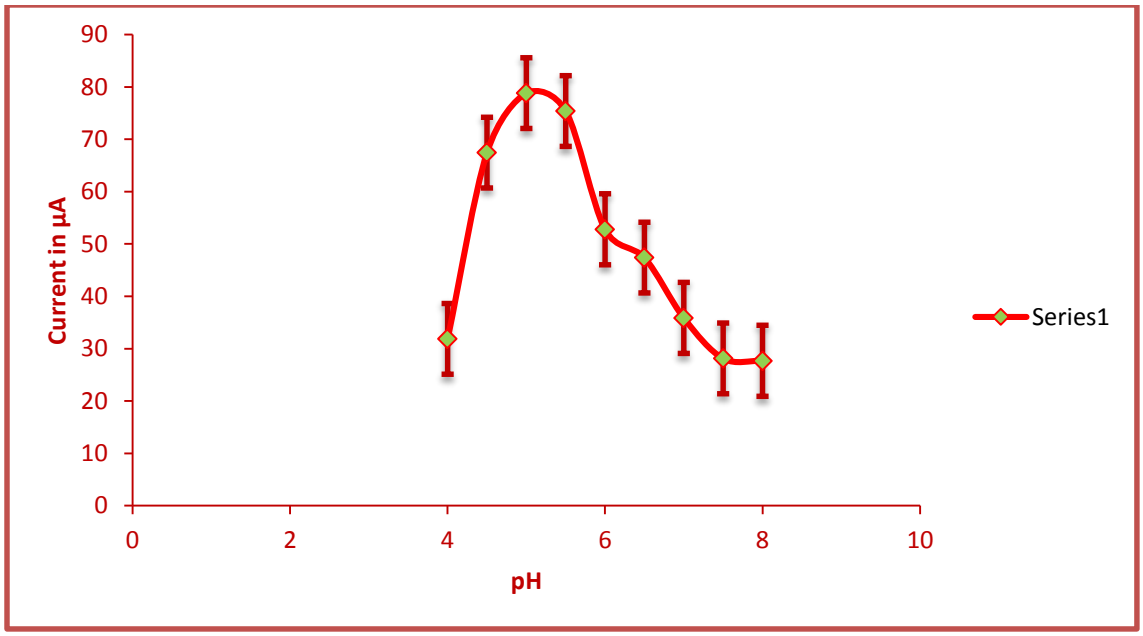

Figure 11

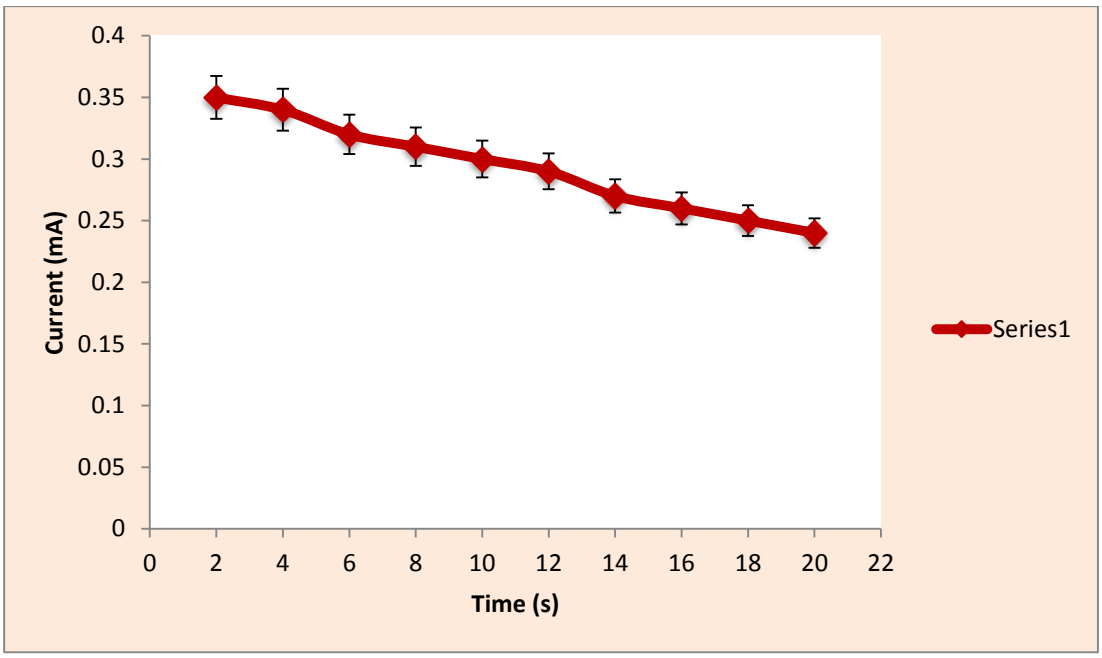

Figure 12

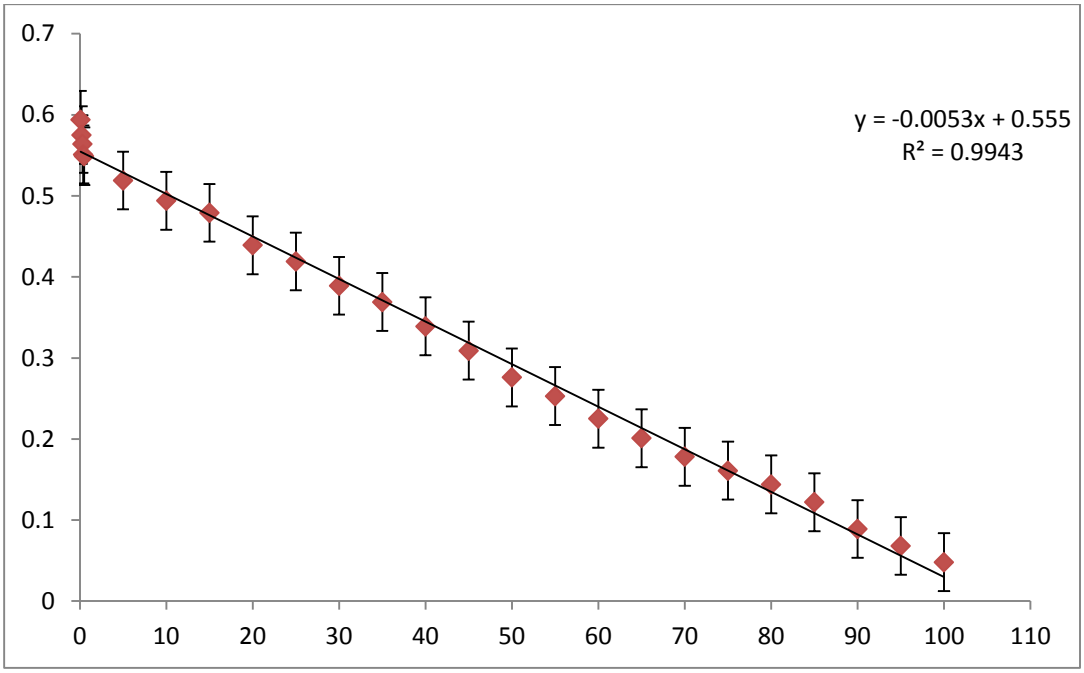

Figure 13

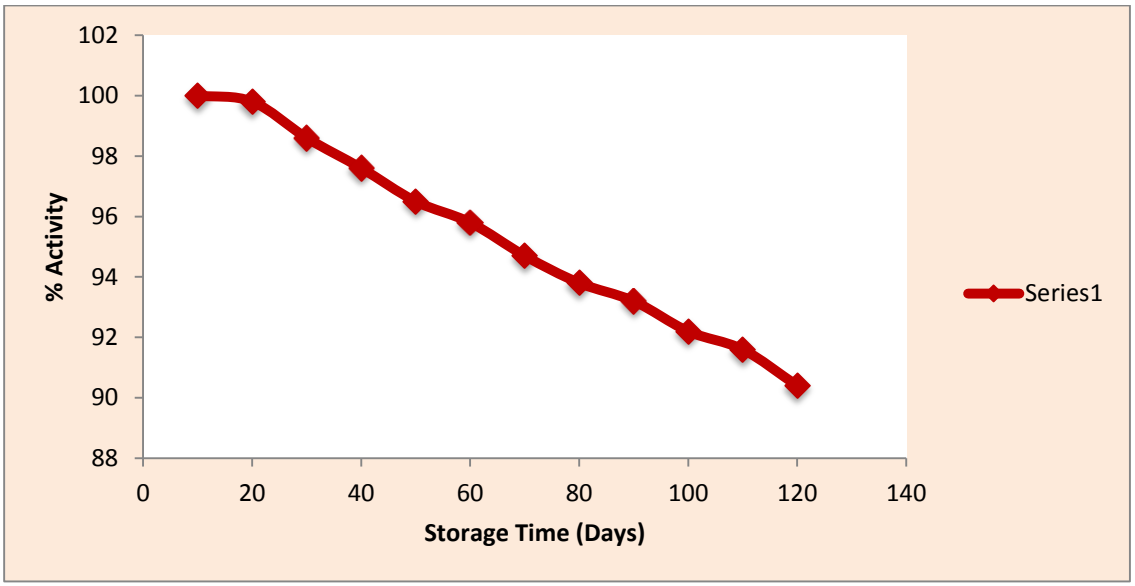

Figure 14

List of Tables for HbNPs Based Acrylamide Biosensor

Table 1:- A comparison of analytical parameters of methods for detection of acrylamide in processed foods (Source:- Qinqin et al., 2015)

Method		Sample	Linear Range	LOD (µg/kg)	LOQ (µg/kg)	Recovery (% age)	RSD (%)	Reference
Standard methods	LC-MS/MS (HPLC, UPLC)	Potato chips	1~200	1	3	81.6-99.0	0.4-4.5	Zhang et al., 2007
		Coffee	2~100	5	16	92-95	<5	Bortolomeazzi et al., 2012
		Cereal-based foods	1~2,000	6	18	90.6-98.5	1.8	Senyuua 2006
		Tea	1~20	1	5	74-79	1.6-8.3	Liu et al., 2008
		Infant foods	0.1~200	1	3	87-96	<6.5	Zhang et al., 2005
	GC-MS (GC based)	Potato chips	10~1,000	5	-	81.9-95.7	5.3-13.4	Yamazaki et al., 2012
		Coffee	0~1,500	5	10	84-97	2-10	Soares et al., 2010
		Cereal-based foods	5~50,000	2	36	91-99	<4	De Vleeschouwer et al., 2007
Rapid methods	Electrochemical Biosensors	Potato crisps	$9.2x10^{-4}$ ~$3.4x10^{3}$	$8.5x10^{-3}$	-	-	-	Stobiecka et al., 2007
		Potato crisps	$5.3x10^{6}$	$1.4x10^{-2}$	-	95.40-97.56	-	Batra et al., 2013

	ELISA	Pringles crisps	51.76-3,311.5	65.7	-	-	-	Preston et al., 2008
		Mashed potatoes	50-1,280	50	350	92.6-95.5	-	Fu et al., 2011
	Fluorescence	Potato chips	35-350,000	35	-	-	-	Hu et al., 2014
		French fries, fried puffs, fried chicken roll, bread, biscuits	50-20,000	15	-	66.0-110.6	-	Liu et al., 2014
			Correlation coefficient		**Prediction accuracy**			
	Computer vision	Potato chips	0.989		98% at a threshold of 1000µg/kg			Gokmen et al., 2006
		Cookies	0.946		100% at a threshold of			Gokmen et al.,

				150µg/kg	2008

Table 2:- Analytical recovery of added acrylamide in processed foods measured by HbNPs/Au electrode

Sr. No.	Acrylamide added (mM)	Acrylamide found (mM)	% Recovery
1	-	35.2	100
2	5	39.8	99
3	10	44.4	98.2

Table 3:- Within and between assays of coefficients of variations for the detection of acrylamide in processed foods using HbNPs/Au electrode

N=5	Acrylamide (mM)	CV%
Within assay (7)		
98	**66.8**	**3.85**
74		
63		
72		
27		
Between assay (7)		
98	**61.8**	**4.67**
83		
58		
27		
43		

Table 4:- Acrylamide level in different kinds of processed food items of various brands measured by HbNPs/AuE based acrylamide biosensor

Sample No.	Name of food items	Brands of food items	Acrylamide Concentration in mM (Mean ± SD)
1	Biscuits(A)	A_1	44.69 ±35.68
		A_2	
		A_3	
		A_4	
		A_5	
		A_6	
		A_7	
		A_8	
		A_9	
		A_{10}	
		A_{11}	
2	Cakes(B)	B_1	20.11 ±10.44
		B_2	
		B_3	
		B_4	
		B_5	
		B_6	
		B_7	
		B_8	
		C_9	
3	Chips(C)	C_1	59.8 ±0.021
		C_2	
		C_3	
		C_4	

4	**Fried cereals(D)**	**D_1**	**23.5±10.82**
		D_2	
		D_3	
		D_4	
5	**Nuts(E)**	**E_1**	**12.33±7.1**
		E_2	
		E_3	
6	**Kurkure(F)**	**F_1**	**47.25±26.6**
		F_2	
		F_3	
		F_4	

Table 5:- A comparison of various electrochemical biosensors for detection of acrylamide in processed foods

Support for immobilization	Methods of immobilization	Optimum pH	Detection limit (µM)	Linear range in (µM)	Response time (s)	Interfering compound	Storage stability at 4°C in days	Applications	Reference
MIP/Au NPs-MWCNTs-CS/GCE, NIP/Au NPs-MWCNT-CS/GCE, MIP/MWCNTs-CS/GCE and NIP/MWCNTs-CS/GCE	Noncovalently/ covalently interaction	7.4	0.39	.70-70	ND	ND	9	Detection of acrylamide in potato chips	Xia et al., 2015
cMWCNT/CuNP/PANI	Covalent immobilization	5.5	0.0002	0.05-0.07	<2	ND	100	Detection of acrylamide in potato chips	Batra et al., 2012
Glassy carbon electrode	ND	4.8	$1.2x10^{-10}$ M	$1.3x10^{-11}$-$5.6\text{-}10^{-3}$	ND	ND	ND	ND	Stobiecka et al., 2007
Glassy carbon electrode	ND	5.0	$1.2x10^{-10}$	$1.ox10^{-11}$-$1.0x10^{-9}$	ND	ND	ND	ND	Krajewask et al., 2008
Ammonium ion selective	ND	ND	6.31x10-4	ND	3.5 min.	ND	$T_{i/2}$-27	ND	Silva et al.,2011
Gold electrode	ND	5.0	$0.2x10^{-6}$	$3x10^{-6}$ to $90x10^{-6}$	8	ND	ND	ND	Batra et al., 2013
Gold electrode	ND	ND	$4x10^{-5}$	$1x10^{-4}$ to $5x10^{-3}$	ND	ND	ND	ND	Sun et al., 2013

Glassy carbon electrode	ND	ND	ND	$5x10^{-8}$ to $1x10^{-3}$	ND	ND	ND	ND	Li et al., 2014
DDAB/Hb/GCE	sND	ND	ND	ND	ND	ND	ND	ND	Hai Wu et al., 2015
Gold electrode	ND	ND	ND	ND	ND	ND	ND	ND	Huang et al., 2016

ND= Not detected,MIP = Molecular imprinting, CS= Chitosan, MWCNT= Multiwalled carbon nano tubes, GC= Glassy carbon, DDAB= Dimethyldioctadecyle ammonium bromide