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Construction of an amperometric glycated hemoglobin biosensor based on Au–Pt bimetallic nanoparticles and poly (indole-5-carboxylic acid) modified Au electrode

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ABSTRACT

The glucose level measurement in the diabetic patient plays a vital role in identification of the treatments going on and it also provides the control over the diabetics. A new electrochemical sensing device was constructed for determination of glycated hemoglobin (HbA1c) in whole blood samples. Fructosyl amine oxidase (FAO) was bioconjugated onto hybrid nanocomposite i.e., gold nanoparticles-platinum nanoparticles (AuNPs-PtNPs) and poly indole-5-carboxylic acid (PIN5COOH), deposited electrochemically on gold electrode. Bimetallic nanoparticles not only show their individual properties but also provides the synergistic effect between the two noble metal nanoparticles. AuNPs-PtNPs shown as an amplified sensing interface at lower voltage which makes the sensor more sensitive and specific. The FAO/AuNPs-PtNPs onto PIN5COOH/Au electrode shows a promising future in diagnosis of HbA1c and diabetes management. The novel sensor formed has good accuracy, selectivity, sensitivity, precision and reliability. In addition to these, it showed good storage stability and retained 50% of its initial activity within 12 weeks at 4 °C.

1. Introduction

By deficiency of internal secretion within the body or the defect of duct gland for insulin secretion a chronic disorder diabetes mellitus is caused [1]. High blood sugar level is observed over a prolonged period in this disorder which is analysed by glucose level or Glycaemic Index (GI). Glycated hemoglobin (HbA1c) gives a rapid analysis of the glucose level [2]. HbA1c is a very good marker for analyzing the glycine level in blood which shows reliability over a very long period of 90 days. It is observed that the HbA1c detection using electrochemical biosensing technique is highly selective, relatively low cost and have the potential for miniaturization and automation. For monitoring GI in people who are diabetic, HbA1c is the best method. After going through different experiments and literatures it has been observed that HbA1c method is easy, specific and rapid [3–5].

The development of biocompatible nanomaterials has been recently evolved for developing third generation biosensors that are based on electron transfer between biomolecule and electrode. Diverse nanomaterials for the development of HbA1c biosensor by immobilizing biomolecules for signal amplification have been reported. These nanomaterials are graphene [6,7], carbon nanotubes [8], metal nanoparticles [9,10], metal oxide nanoparticles [11] and nano structured conducting polymers [12–15]. Different polymers were employed for enzymes bioconjugation, for instance polyaniline, polythiophene, polyacetylene and polypyrrole. Among them polyindole (PIN) is the best option because it gets polymerised easily, with easy membrane preparation, chemical stable and has good conductivity. Hence, the PIN films have elevated redox activity, excellent thermal stability and less degradation speed in comparison with polyaniline and polypyrrole. It has been used in the fabrication of electrochemical biosensor with improved analytical performance [16–19].

Bimetallic nanoparticles are made of two different metal elements that attracts much more attention than monometallic nanoparticles due to potential of unique electronic, optical, catalytic or photocatalytic properties, which are not present in the monometallic nanoparticles. It has been expected that bimetallic nanoparticles in the presence of two individual metals shows not only the combination of the properties related but also new properties due to a synergy between two metals [20–22]. Shape and size of mono and bimetallic nanoparticles depends on the preparation methods and conditions provided to them and affects the physicochemical properties of resulted final nanomaterial. Combination of

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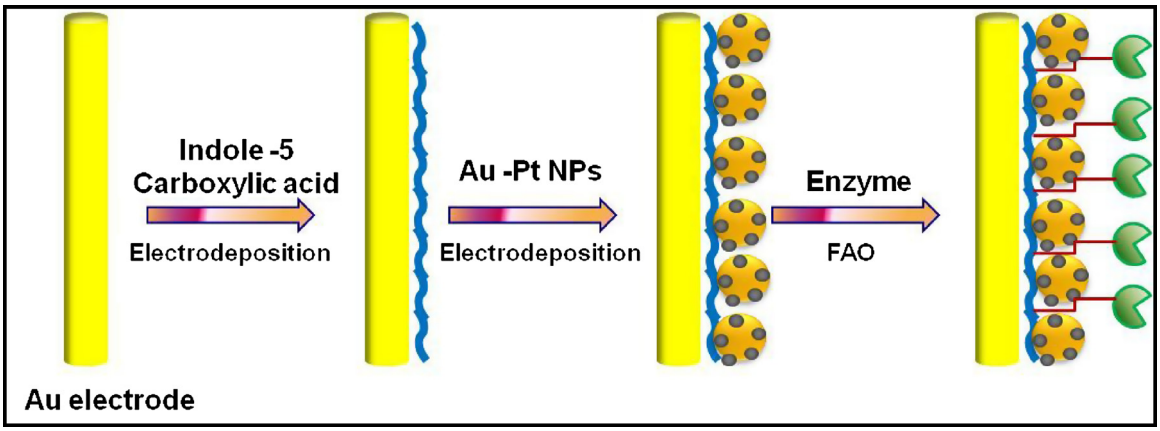

Scheme 1. Graphical model for the preparation of working electrode.

two metal nanoparticles results in possibilities of various shape and structure due to miscellaneous distribution of each metal within a particle and their various organization [23]. Among various noble metal nanoparticles gold (AuNPs) and platinum (PtNPs) nanoparticles are widely used for the reasons like biocompatibility, always available for conjugation with biomolecules for example proteins, enzymes, DNA, and amino acids [24]. Because of their small size and uniform dispersity they can easily reach to the targeted site with the flow. The early finding suggests that AuNPs-PtNPs supported on carbon demonstrates single-phase alloy properties [25] represented the first example indicating the difference of nanoscale materials from the bulk counterparts. This finding was supported by a theoretical study based on thermodynamic model and analytically embedded atom method [26], which showed a negative heat of formation for AuNPs-PtNPs smaller than 6 nm, and several experimental studies of different AuNPs and PtNPs systems [27–30].

In our present research work, we have electropolymerized polyindole-5-carboxylic acid (PIN5COOH) film onto the gold electrode with bimettalic AuNPs and PtNPs and further used it for the biosensing of HbA1c. Surface morphological studies by Scanning Electron Microscope (SEM) were obtained before and after enzyme immobilization. Cyclic voltammetry (CV), square wave voltammetry and electrochemical impedance spectroscopy (EIS) studies were performed. Results obtained through this work demonstrated that FAO/AuNPs-PtNPs/PIN5COOH nanocomposites film provides an effective electrochemical response for the selective detection of HbA1c.

2. Experimental

2.1. Chemicals and reagents

Fructosyl-amino acid oxidase (FAO, EC:1.5.3, extracted from recombinant *E. coli*), Bovine serum albumin (BSA), protease, glutaraldehyde (GA), poly (indole-5-carboxylic acid) were procured from Sigma–Aldrich, St. Louis, USA. Hexachloroplatinic(IV) acid hydrate ($H_2PtCl_6{\cdot}xH_2O$), $HAuCl_4$, Phenol, $NaNO_3$, H_2SO_4, $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ were obtained from Sisco Research Laboratory, Mumbai, India and gold wire (1.5 cm × 0.05 cm) (23 carat) were purchased from nearby market. Deionized water (DI) was used for all the experiments.

Potentiostat/galvanostat (Biologics SP-150) was employed for all electrochemical measurements. Scanning electron microscopy (SEM; ZEISS EVO® HD) of prepared modified electrodes was performed at Amity Institute of Advanced Research and Studies (AIARS), Amity University, Noida, Uttar Pradesh.

2.2. Preparation of substrate (fructosyl valine synthesis)

Because substrate for HbA1c i.e. fructosyl valine (FV) was not available commercially so it was synthesized in our laboratory [31]. 40 mL pyridine and 40 mL acetic acid were mixed together with L-valine (0.16 M, 1.88 g) and then stirred for 30 min at 25 °C. Then 4 g glucose (0.25 M) was added and purged by using nitrogen gas for 10 min and again stirred for 3 days at 25 °C. The dark colored product was separated out by filtering and the solvent was left for evaporation. By the process of re-crystallization in methanol, a yellowish amorphous product was obtained [32].

2.3. Fabrication of working gold electrode

A polycrystalline gold electrode (1.5 cm × 0.05 cm) was first polished with alumina slurry and then cleaned ultrasonically with ethanol and DI. It was then cleaned electrochemically by cycling the electrode with potential between +1.6 and −0.4 V in 0.5 mol L^{-1} H_2SO_4 until a stable voltammogram was obtained.

For polymer nanocomposite film deposition, electropolymerization was carried out onto the gold electrode by immersing it in the solution of 1.0 mL PIN5COOH and then applying 10 polymerization cycles from potential range 0.0 V to 0.6 V at 20 mV/s. After electropolymerization the electrode was washed with DI water. Then the modified electrode was dipped into 0.5 M H_2SO_4 and the solution containing 1 mM $HAuCl_4$ and 1 mM H_2PtCl_6. AuNPs-PtNPs were then electrodeposited with chronoamperometric method at −0.2 V vs Ag/AgCl for 400 s at room temperature (30 ± 5 °C). After the electrodeposition, the electrode was washed carefully with DI water and then kept for drying [33].

2.4. Preparation of biosensor

One Unit is defined as the amount of the enzyme that catalyzes the conversion of 1 μM of substrate per minute. Onto the dried modified gold electrode FAO enzyme was immobilized; BSA (bovine serum albumin 0.5 mg mL^{-1}) and FAO (Fructosyl Amino acid oxidase 30 U per 100 μL) were assorted properly, then the mixture of 100 μL of total amount was dropped over the AuNPs-PtNPs/PIN5COOH modified gold electrode. The biosensor was achieved once the enzyme immobilization occurs during a period of stillness at 4 °C and kept for overnight drying. The enzyme molecules was covalently bind with the polymer modified electrode through amide bond formation between COOH group of PIN and amino group of the enzyme. After drying of the electrode it can be further used for the measurements [34] (Scheme 1).

All electrochemical studies were done using a conventional three-electrode system with the gold electrode (sensor) as working electrode, Pt as auxiliary electrode, and an Ag/AgCl as reference electrode. We optimized all of parameters that robustly influenced the electrochemical performance of HbA1c. Various kinetic parameters for example pH, temperature and substrate concentration were optimized. Simultaneously, the detection conditions like upper/lower limiting potential were also optimized. The detection principle is as follows:

$$\underset{\text{(N-terminal residue of } \beta\text{-chain in HbA}_{1c})}{\text{Fru-Val-His-Leu-Thr-Pro-Glu-Glu-Lys-ser...}} \xrightarrow{\text{Protease}} \underset{\text{and/or Fructosyl valine (His, Leu, Thr)}}{\text{Fructosyl valyl histidine + amino acid}}$$

$$\underset{\text{Fructosyl valine}}{\text{Fructosyl valyl histidine and/or} + H_2O} \xrightarrow{\text{FAO}} \underset{\text{or valine}}{\text{valyl histidine}} + \text{D-glucosome} + H_2O_2$$

2.5. Analytical procedure for HbA1c measurement in whole blood samples

The apparently healthy individuals and diabetes patients provided blood samples which were collected with EDTA as anticoagulant from Biodiagnostics Laboratory, Rohini, New Delhi. In order to mix the blood cells, a mild inversion was applied. The mixing of 30 μL of whole blood in 300 μL of lysis buffer was carried out to check proper hemolysis and afterwards incubated for 10 min. Furthermore, the cells were lysed by using lysis buffer consisting 100 mM CHES (pH 8.7), 0.45% SDS, 1% Triton X-100 and 0.5 mM of dessmartin *peri*- odinane. A proteolytic digestion was performed of human blood samples by mixing of a solution including oxidizing agent (1 mM dess martin periodinane), 4 U/mL purified *Bacillus sp.* proteases and 5 mM MES. This prosteolytic digestion leads to the discharge of certain amino-acids that includes glycated valines from the hemoglobin beta chains which then serves as substrates for the FAO and consequently produces H_2O_2 [35]. The numbers of electrons produced for detection is principally based upon splitting of H_2O_2 released by the oxidation of substrate by immobilized enzyme (FAO) which is evidently proportional to the HbA1c concentration.

Under the ideal conditions, the HbA1c test was brought forward with FV as the calibrated standard. Along these conditions the HbA1c% was figured by the proportion between HbA1c (mM) and aggregate hemoglobin (mM), which was acquired from the laboratory. The results obtained from this method (biosensor) was compared with the standard HPLC method.

2.6. Stability and reproducibility of enzyme electrode

For reusability of FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode was washed with 0.1 M sodium phosphate buffer (pH 7.0). The storage stability of the enzyme electrode was studied for 3 months, when it was kept at 4 °C. The activity of FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode was checked once in a week.

3. Results and discussion

3.1. Characterization study of various stages for the fabrication of working electrode

Surface morphology of the bimetallic nanoparticles and modified gold electrode was observed by SEM in Fig. 1. The morphologies of bare gold electrode (image a), AuNPs-PtNPs onto PIN5COOH/gold electrode (image b) and FAO/AuNPs-PtNPs onto PIN5COOH/gold electrode (image c) were characterized by SEM. A smooth and uniform surface was visualized on the bare Au electrode (image a). In image b, AuNPs-PtNPs were uniformly distributed onto PIN5COOH/gold electrode. This bimetallic nanoparticles AuNPs-PtNPS and PIN5COOH nanocomposite not only could increase the current response [33,36], but also provides more surface area for the nucleation. Moreover, presence of globular morphology as indicated by circle in image c shows immobilization of FAO on the surface of AuNPs-PtNPs/PIN5COOH/gold electrode. The spherical, rough and folded surface of AuNPs-PtNPs/PIN5COOH/gold electrode nanocomposites provides a large rough surface area for elevated loading of enzyme.

3.2. Electrochemical studies

In this sensor, enzyme (FAO) was immobilized covalently on AuNPs-PtNPs/PIN5COOH modified gold electrode. The cyclic voltammogram (CV) study was done for evaluation of various steps of electrode fabrication. Fig. 2 illustrated the CV of bare electrode, hybrid nanocomposite modified gold electrode and enzyme modified gold electrode in presence of 5 mM potassium ferricyanide at scan rate of 20 mV/s.

There was no well-defined oxidation and reduction peaks for bare gold electrode (curve a) in PBS after 20 mL of potassium ferricyanide was added into electrochemical cell. The CV of AuNPs-PtNPs/PIN5COOH modified gold electrode showed an oxidation peak and reduction peak at 0.2 V and 0.09 V, respectively (curve b). There was increase in the signal in the form of current due to the presence of AuNPs-PtNPs/PIN5COOH on the surface of gold electrode, which possessed surface area with large specificity and an inherent, conducting ability and high electricity. The CV of FAO/AuNPs-PtNPs/PIN5COOH modified electrode also identified a highest oxidation and reduction peak (curve c) due to the enzyme catalysis.

3.3. Impedance study

Electrochemical impedance spectroscopy (EIS) was exploited for the biosensing response studies. EIS is a powerful technique that gives a wealth of information regarding internal resistance of the electrode material and resistance between the electrode and the electrolyte. We therefore performed electrochemical impedance measurements, Nyquist plots for the three different stages of the electrodes were plotted and analysed. In EIS, the semicircle diameter equals the electron transfer resistance (Ret). Fig. 3 represents the bare gold electrode which has maximum Ret, AuNPs-PtNPs/PIN5COOH and FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode. The Ret gradually decreases with the fabrication of the biosensor. The reason was that biocomplex layer on the electrode act as electron transfer and mass transfer layer and further gives conductive support and access to redox probe towards the electrode's surface [37]. The electron transfer resistance (Rct) of bare gold electrode was 3500 Ω. As shown in Fig. 3, the value of Rct was decreased to 1500 Ω for AuNPs-PtNPs/PIN5COOH modified gold electrode. This indicates relatively fast electron transfer kinetics of nanocomposites between the electrolyte and electrode surface. In the FAO/AuNPs-PtNPs/PIN5COOH modified gold

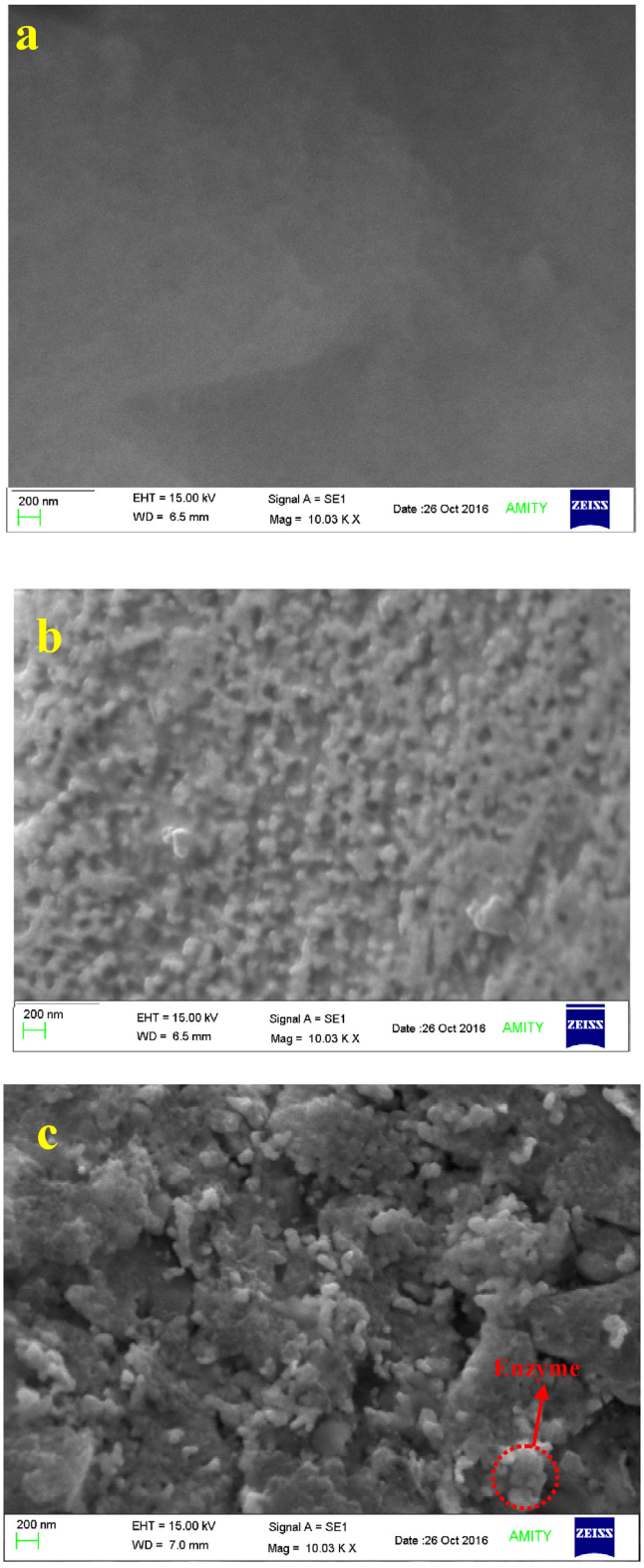

Fig. 1. Surface morphological studies by SEM image of the (a) bare gold electrode, (b) AuNPs-PtNPs/PIN5COOH modified gold electrode and (c) FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode.

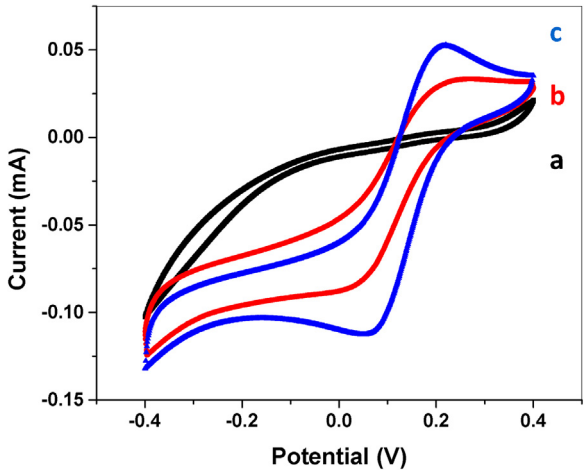

Fig. 2. Biosensing response studies (CV) of (a) bare gold electrode, (b) AuNPs-PtNPs/PIN5COOH modified gold electrode and (c) FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode in 5 mM $[Fe(CN)_6]^{3-/4-}$ at a scan rate of 20 mVs^{-1} between −0.4 and +0.4 V.

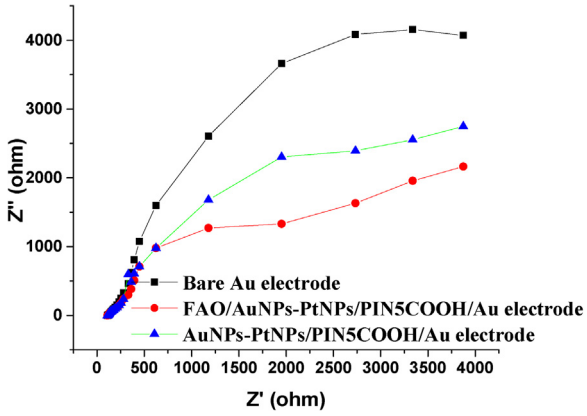

Fig. 3. The electrochemical impedance spectroscopy of bare gold electrode, AuNPs-PtNPs/PIN5COOH modified gold electrode and FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode in 5 mM $[Fe(CN)_6]^{3-/4-}$. The frequency varied from 0.01 Hz–10 kHz, and 5 mV amplitude of sine voltage signal was used.

electrode, Rct value was increased to 2200 Ω. This increase was occurred by the formation of barrier layer of the FAO molecules resulting a pathway of electron transfer by enzyme was hindered. The change in EIS of the modified process proves that the immobilization has taken place successfully which was in good agreement with the CV results.

3.4. Optimization

The maximum response of biosensor was observed at 7.5 pH; hence, subsequent studies were done at the same pH. The optimum amount of enzyme for the preparation of working electrode was 30 IU. To observe the effect of different concentration of FAO's substrate FV was added and CV curves were obtained. As shown in Fig. 4 with the subsequent addition of the substrate the current intensity gradually increases with the increase in the concentration of FV which indicates that modified gold electrode was highly conductive. The sensing surface demonstrated wide linear range (0.1–1000 μM) and a low detection limit of 0.2 μM. The limit of detection of the present biosensor was calculated as the lowest quantity of FV required to give a signal to a background (blank) +3 times SD of blank. The apparent K*m* value was found to be 1.6 μM. The present biosensor showed a maximum response (comparatively faster) within 4 s. The percent recovery of added substrate

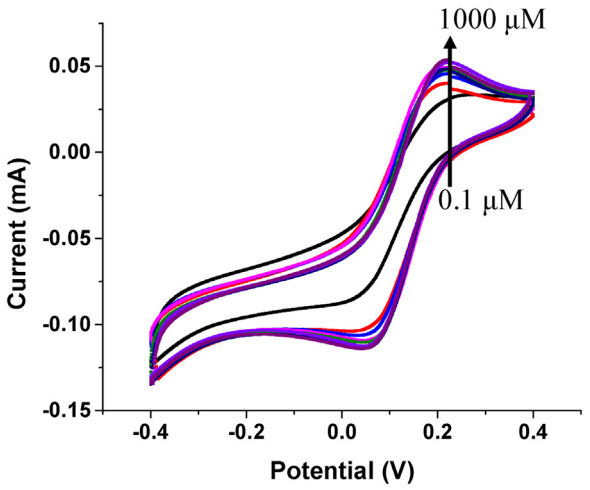

Fig. 4. Response study of FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode with different concentrations of fructosyl valine (FV) at a scan rate of 20 mVs^{-1} between −0.4 and +0.4 V.

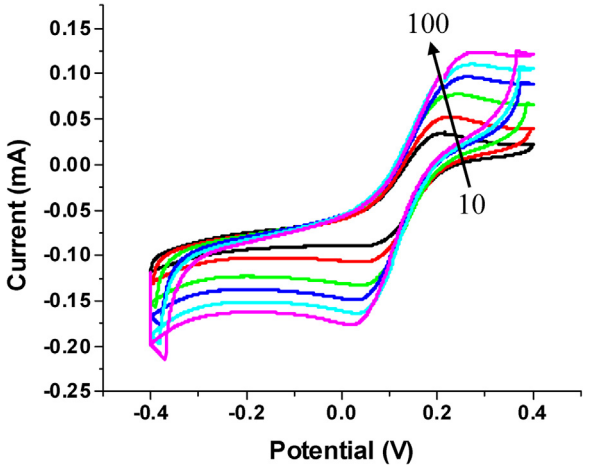

Fig. 5. Cyclic voltammogram recorded at different scan rates of FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode (10–100 mV/s) in 5 mM $[Fe(CN)_6]^{3-/4-}$.

in blood was calculated. Analytical recovery of added FV in blood (10 mM and 20 mM) was 97.2% and 98.5%, respectively, indicating good competence of the electrode. The within and between batch coefficient of variation for FV determination in blood by the present biosensor were 2.44% and 5.26%, respectively, indicating the good reproducibility and reliability of the method. These results indicate a very good analytical performance of the present biosensor as compared to earlier reported biosensors [38–40].

The typical CV curves of the modified electrode in 5 mM potassium ferricyanide at different scan rates ranging from 10 to 100 mV/s were studied in Fig. 5, which suggesting a qausi reversible diffusion with electron transfer process. It was observed a pair of roughly symmetrical anodic and cathodic peaks were formed with almost equal peak currents in the studied scan rate range. Also peak to peak separation increases with increase in the scan rate.

Fig. 6 showing high stability of the present sensor. No change in potential at different scan rates on the fabricated sensor was noticed. As indicated in Fig. 6, a low current was obtained after evaluating varying concentrations of solution at relatively negative potentials however a high current was observed at positive potentials. Highest response was recorded at scan rate of 100. This curve shows a relationship between the response current and scan rate for the prepared sensor. As clearly seen in the 3D graph, when scan rate increases consequently the response current also increases.

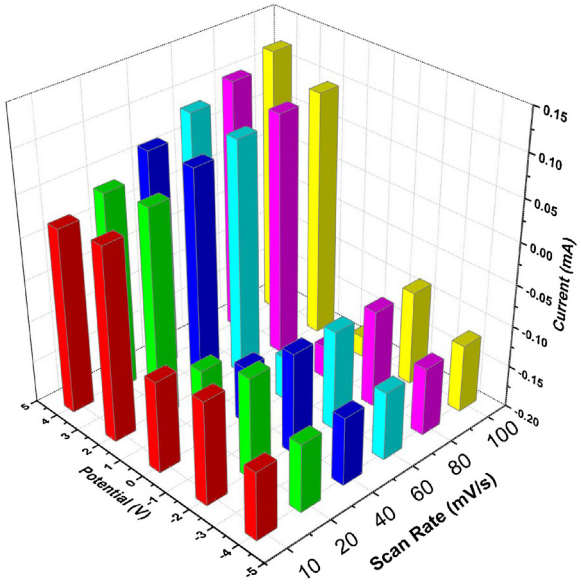

Fig. 6. 3D representation of the cyclic voltammogram data at FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode for different scan rate.

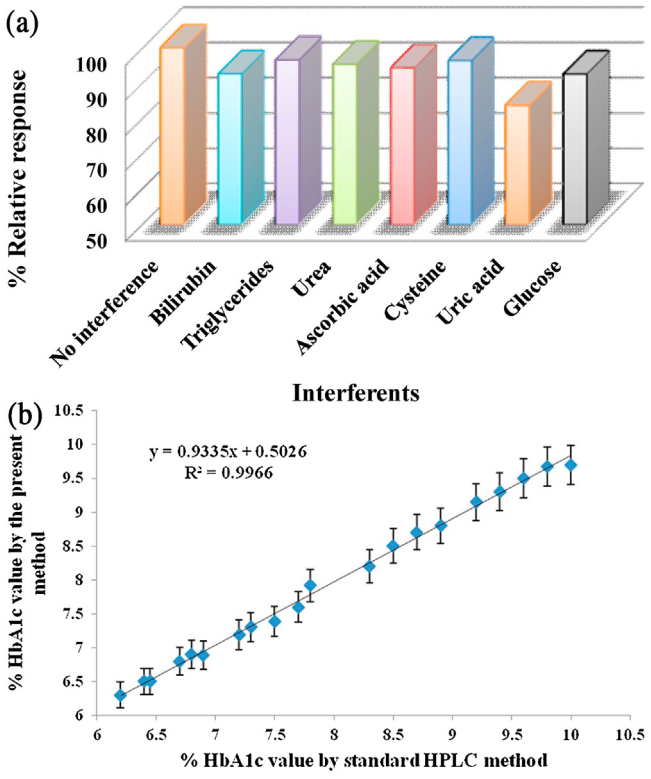

Fig. 7. (A) Effect of potential interferents on FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode with 0.1 mM FV in 0.1 M sodium phosphate buffer (pH 7.5). (B) Correlations between HbA1c values determined by standard HPLC method (x-axis) and present method (FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode) (y-axis). The analysis was done in the triplicate.

The effects of numerous possible interfering substances e.g. ascorbic acid, uric acid, bilirubin, urea, triglycerides, cysteine and glucose on this biosensor were investigated at +0.2 V versus Ag/AgCl in sodium phosphate buffer (pH 7.5). The results obtained after comparing ascorbic acid, bilirubin, urea, cysteine, uric acid, triglycerides and glucose indicated that there is no interference of these substances (Fig. 7A).

3.5. Application of the biosensor in whole blood samples

To estimate HbA1c from clinical sample using FAO/AuNPs-PtNPs/Pin5COOH/gold electrode, human whole blood samples were collected. The modified electrode was dipped into the electrolyte containing sodium phosphate buffer (0.5 mM, pH 7.5) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ with 100 μL of human whole blood sample and the current response was observed. The result outcomes of HbA1c in blood samples as calculated from the calibration curve using FAO/AuNPs-PtNPs/PIN5COOH/gold electrode were almost similar with clinical values. In order to evaluate the accuracy of this HbA1c biosensing method, after acquiring the results in whole blood samples, the results were compared with HPLC method. After comparing these values, it was noticed that both the methods showed a very good correlation $R^2 = 0.996$ with regression equation, $y = 0.9335x + 0.5026$ (Fig. 7B). The analysis was done in the triplicate. Error bars represent standard error from triplicate measurements.

3.6. Stability and reusability of the modified electrode

The shelf life of the FAO/AuNPs-PtNPs/PIN5COOH modified gold electrode was checked by the current response in the presence of 100 μM standard FV solution dissolved in PBS at a regular interval of 7 days. It showed good storage stability and retained 50% of its initial activity within 12 weeks at 4 °C. In earlier reports, the initial activity was lesser as compared to prepared FAO biosensor. In these FAO biosensors, iridium nano-particles incorporated in the carbon-based working electrode [38], boronic acid modified gold substrate [39] and core shell magnetic bionanoparticles modified gold electrode [40] were used.

4. Conclusion

The present work shows that the electrochemical signal in HbA1c biosensing by using bimettalic nanoparticles and PIN5COOH modified gold electrode is significantly enhanced. The present FAO biosensor showed a good analytical response for the electrochemical detection of HbA1c, and provides lower working potential (+0.2 V vs. Ag/AgCl), wider linear range (0.1–1000 μM for FV), higher sensitivity (0.316 μM/mA) and lower detection limit (0.2 μM). Nevertheless, the result that has been achieved by application of the proposed sensor for determining HbA1c in whole blood samples confirmed very good accuracy, selectivity, sensitivity, precision and reliability of our approach. The present electrode showed a shelf life of about 12 weeks. Further, this biosensor was successfully used for the detection of the HbA1c in the whole blood samples. It can be concluded that FAO/AuNPs-Pt NPs onto PIN5COOH/gold electrode has a promising future in diagnosis of HbA1c and diabetes management.

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