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An amperometric biosensor for specific detection of glycated hemoglobin based on recombinant engineered fructosyl peptide oxidase

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Abstract

Here, we present a specific biosensor based on the detection of glycated hemoglobin (HbA1c) proteolytic digestion product, fructosyl valyl histidine (Fru-ValHis). A recombinant engineered fructosyl peptide oxidase (FPOX) enzyme with improved specificity was immobilized on the electrode surface modified by chitosan (CHIT), graphene oxide (GO) and gold nanoparticles (AuNPs). The biosensor exhibited a linear response toward different concentrations of Fru-ValHis ranging from 0.1 to 2 mM with a sensitivity of 8.45 $\mu AmM^{-1}cm^{-2}$. Detection limit of the current biosensor for Fru-ValHis was 0.3 µM as the lowest quantity required giving a signal to a background. Analytical recovery of added Fru-ValHis in whole blood was 95.1 to 98.35% for FPOX/AuNPs/GO/CHIT/FTO electrode. For Fru-ValHis determination by FPOX-AuNPs-GO-CHIT/FTO electrode, within-run coefficient of variation (CV) was between 1.3% and 2.4% and between run CV was between 2.1% and 3.5%. A significant change in electron transfer resistance after the incubation of FPOX-modified electrode with Fru-ValHis was observed, while no response was achieved with control, indicating specific measurement of Fru-ValHis. Moreover, designed biosensor measured HbA1c in human blood samples and the results were well agreed with that obtained with NORUDIA™ N HbA1c diagnostic kit. Overall, suitable specificity of the engineered FPOX made the bioelectrode responded well to the Fru-ValHis level, which demonstrates a promising application for specific detection of HbA1c biomarker.

Keywords: Biosensor, Diabetes, Fructosyl peptide oxidase (FPOX); glycated hemoglobin (HbA1c)

1. Introduction

Diabetes mellitus, a chronic metabolic disorder, is a major worldwide public health problem in the most countries of world. Diabetes disease is associated with acute complications such as cardiovascular disease, nephropathy, retinopathy, neuropathy and premature death. The determination of glycated hemoglobin (HbA1c) is considered as the gold standard of glycemic control, providing a retrospective view of diabetes control [1]. HbA1c is an important marker for clinical diagnosis and long-term blood glucose monitoring in patients. Therefore, a cost-effective measurement of HbA1c level will be highly important for the management of diabetic patients [2]. Various methods have been suggested for the HbA1c routine measurement. These methods include ion-exhange chromatography, electrophoresis, affinity chromatography and immunoassay. However, these techniques suffer from some drawbacks such as time-consuming, expensive devices, labor-intensive, complicated procedures and pretreatment of samples. Besides, the above methods are not protable and, so do not meet the requirements of routine clinical assays [3]. On the other hand, the use of electrochemical methods for clinical diagnosis has several advantages such as good sensitivity, relatively low cost and potential for miniaturization [4-5]. Electrochemical measurement of HbA1c level has been intensively studied with the aim of developing simple and point-of care methods. Hence, there is a great interest to develop electrochemical biosensors for HbA1c [6-7].

Up to now, numerous sensors such as enzyme-based biosensors, immunosensor and aptasensor have been studied for HbA1c detection [8]. The first amperometric HbA1c sensor using the membrane immobilized Hb as affinity matrix was reported by Stollner and coworkers in 2002 [9]. A flow-injection analysis enzyme sensor for HbA1c was described in 2002 [10]. This sensor was claimed to be the first sensor towards fructosyl peptide, though with insufficient sensitivity.

In 2009, Song and his coworker fabricated a sensor based on the gold electrodes modified with polyamidoamine [11]. An immunosensor consisted of anti-HbA1c antibody and gold nanoparticles was described in 2012 [12]. A disposable electrochemical biosensor using thick film screen-printed electrodes coupled with iridium nanoparticles has also been studied [13]. Jain and his coworker reported a biosensor based on FAOD immobilized on nitrogen-doped graphene and gold nanoparticles in 2017 [14]. Microfluidic systems [15], molecularly imprinted polymers [16] and aptamers [17-18] have also been applied in the development of HbA1c biosensors. However, the most electrochemical biosensors for HbA1c are enzyme-based because these methods are relatively rapid and sensitive [19]. In these sensors, fructosyl valine (Fru-Val) which is released from the proteolytic digestion of HbA1c is applied as model compound [20]. Sode and coworkers have established different types of Fru-Val sensors by immobilizing FAOD on the surface of an electrode [21-22]. However, FAOD enzyme has activity toward frutosyl-Lysine (Fru-Lys), a digestion fragment released from glycated albumin. For this reason, there is a demand for the selective measurement of HbA1c and an enzyme that reacts specifically with HbA1c will be ideal [3, 23-24]. As a result, engineered FAOD with higher specificity toward Fru-Val has been applied in some papers [25-26]. In this work, for the first time we attempted to improve the selectivity of HbA1c biosensor by using a recombinant engineered variant of fructosyl peptide oxidase (FPOX) from *Eupenicillium terrenum*. FPOX is a member of FAOD enzyme family which has a somewhat different reaction mechanism [27-28]. This enzyme that act specifically on HbA1c, has great potential to be used as a clinical diagnostic enzyme for diabetes mellitus [29-31]. The enzymatic method using FPOX consists of proteolysis of HbA1c to release Fru-ValHis, which can be detected electrochemically. As shown in Fig. 1, detection of Fru-ValHis would be important for diabetic patient management as a means for HbA1c

biosensing. Moreover, based on this method, development of a Fru-ValHis biosensor will have more specificity.

Recently, the development of nanomaterials has opened up a new direction in fabrication and improving the performance characteristics of biosensors in biomedical applications [32-33]. Among various nanomaterials, graphene possesses considerable structural, mechanical and physico-chemical features. Graphene oxide (GO), a derivative of graphene is a new class of carbon material which is an ideal component in enhancing performance of biosensor-based devices [14]. The considerable advantages of GO are high conductivity, large surface area, high mechanical strength, chemical stability, good biocompatibility, and low cost [34-35]. Furthermore, special attention has been devoted to nanoparticles such as gold nanoparticles (AuNPs) to enhance the sensitivity of biosensors. Thermal stability, irradiation resistance, electrochemical activity and high electron communication features are the advantages that expedite its potential wide applications in biosensors [36]. Chitosan (CHIT) as an immobilization matrix is commonly used to disperse nanomaterials because of its excellent capability of film formation, nontoxicity, good biocompatibility and mechanical strength [37]. In this communication, an enzyme sensor utilizing recombinant FPOX and Fru-ValHis as the base for HbA1c biosensor for the first time is reported. Recombinant purified enzyme was immobilized onto CHIT-GO-AuNPs composite and its application for the measurement of HbA1c was studied. Compared with earlier enzyme-based biosensors, the use of FPOX enzyme in designing HbA1c biosensor exhibited advantages of high specificity.

2. **Materials and Methods**

2.1. Reagents

4-aminoantipyrine, bovine serum albumin (BSA) and horseradish peroxidase (HRP) were obtained from Sigma-Aldrich, St. Louis, USA. Sodium *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) was prepared from Santa Cruz Biotechnology. Fru-Lys (≥92.4 purity) and Fru-ValHis (≥91.2 purity) were synthesized according to the modified method of Keil et al. [20]. Purified *Bacillus* sp. protease and endoproteinase Glu-C from *Staphylococcus aureus* V8 were obtained from Sigma-Aldrich, St. Louis, USA. DNA ladder and protein marker were purchased from Thermo Fisher Scientific. Fluorine tin oxide (FTO) glass electrode with usual resistance of approximately ~ 8 Ω was prepared from Sigma-Aldrich, St. Louis, USA. HbA1c standard samples (3.0%, 5.5%, 12.5% and 16.0%) were purchased from Pishtaz Teb Zaman Diagnostics Company, Tehran, Iran. NORUDIA™ N HbA1c diagnostic kit (SEKISUI MEDICAL CO., LTD. Tokyo, Japan) was purchased from Pishtaz Teb Zaman Diagnostics Company, Tehran, Iran. Fresh whole blood samples of healthy individuals and diabetic patients were collected from the Lamarak Clinical Diagnostic Laboratory, Tehran, Iran.

2.2. Apparatus and methods

All electrochemical experiments were performed on an Autolab PGSTAT 30 electrochemical workstation (ECO Chemie, The Netherlands) with GPES software. A KCl-saturated Ag/AgCl electrode and a Pt electrode served as the reference and counter electrodes, respectively, were used in electrochemical experiments. Surface morphology was studied using a Scanning Electron Microscope (Make: TESCAN Japan, Model: VEGA2) at the University of Science and

Technology, Tehran, Iran. Electrochemical impedance spectroscopy data was analyzed with ZSimpWin (version 3.40) software.

2.3. Production of recombinant engineered FPOX enzyme

FPOX from *E. terrenum* was subjected to substrate specificity engineering for efficient recognition of HbA1c according to the procedure described in our previous work [38]. Substitution of Tyr261 with Trp resulted in a mutant enzyme with improved specificity for Fru-ValHis, a model compound of HbA1c. *Escherichia coli* strain BL-21 (DE3) cell containing expression construct of the engineered FPOX was cultivated overnight in 5 mL of Luria-Bertani (LB) medium containing 100 μg/mL of ampicillin at 37°C and 180 rpm. One mL of seed culture was inoculated into 100 mL of LB medium in culture flasks and incubated at 37°C and 250 rpm. When cell density reached an OD_{600} of 0.6–0.8, induction was done by the addition of 0.6 mM sterile isopropyl-β-d-thiogalactopyranoside (IPTG). After 24 h induction at 37°C, cells were harvested, washed twice with 0.9% NaCl solution and stored at −20°C. The cell pellet was dissolved in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 1% glycerol, pH 8.0) and disrupted by sonication [39]. To purify recombinant proteins, supernatant of cell lysis was applied to a Ni–IDA affinity column (Jena Bioscience, Jena, Germany) according to the manufacture's instruction. The purity of purified enzymes was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [40]. The activity of enzyme was determined with the HRP-coupled reaction system [41]. Enzyme concentration was determined using Bradford method with bovine serum albumin (BSA) as the standard.

2.4. Fabrication of biosensor

The fabrication process of HbA1c biosensor is presented in Fig. 2. FTO with thickness of 2.2 mm and dimensions 5.0 mm × 5.0 mm was used as the working electrode. Prior to surface modification, FTO electrode was cleaned with piranha solution (H_2SO_4/H_2O_2 in ratio 3:1, v/v) for 20 min and then rinsed thoroughly with distilled water. Then, the electrode was sonicated in ethanol and distilled water to remove the adsorbed particles. Chitosan (0.1 g) was acylated by dissolving in 10 ml distilled water containing 2.0% (v/v) acetic acid under continuous stirring for 4 h. To form a GO solution, GO were dispersed in potassium phosphate buffer (PBB, 0.1 M, pH 7.4) with the concentration of 5.0 mg/mL. The GO solution was sonicated in bath sonication for 20 min to form a stable GO suspension. For the preparation of CHIT-GO-AuNPs nanocomposite, 1 ml of GO solution was mixed with 2 ml of chitosan solution and 2 ml of colloidal gold aqueous solution. The obtained mixture was bathsonicated for 15 min to obtain a uniformly dispersed mixture. About 30 µl of prepared nanocomposite was drop casted onto the prepared FTO electrode and kept at room temperature to dry. The resulting CHIT-GO-AuNPs modified FTO electrode was washed thoroughly with PBB buffer to remove unbound matter and kept in a dry Petri plate at 4°C. Purified FPOX was immobilized onto CHIT-GO-AuNPs modified FTO electrode surface by physical adsorption. The enzymatic FPOX biosensor was constructed by dropping 10 µl of FPOX enzyme solution (10.0 IU/ml) containing 1.5 % BSA onto nanocomposite modified electrode and kept for 24 h at 4°C. The resulting enzyme electrode was washed completely with PBB buffer to remove unbound enzymes and stored at 4°C prior to measurement. The different parameters such as mixing procedure, solution homogeneity, amount of solution, evaporation temperature, and evaporation time were controlled to obtain the desired thickness and uniformity of modified electrode.

2.5. Optimization of fabricated biosensor

To determine the optimal working conditions of biosensor, the pH of reaction buffer (0.1 M) was varied from pH 5.0 to 9.0 using potassium phosphate buffer. To study the effect of substrate concentration, different Fru-ValHis concentrations ranging from 0.1 to 2.0 mM, corresponding to the physiological concentration of HbA1c were used.

2.6. *Electrochemical measurements*

All amperometric experiments were performed using a conventional three-electrode system with FPOX/CHIT-GO-AuNPs/FTO electrode as a working electrode, a platinum wire as a counter electrode, and an Ag/AgCl electrode as a reference electrode. All measurements were carried out at 25°C in 15 ml of PBB (0.1 M, pH 7.4) containing Fru-ValHis and supporting electrolyte [2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 1:1]. The cyclic voltammetry tests were done at potential -0.2 to 0.8 V at scan rate 50 mVs^{-1} for 10 successive cycles and the response current was marked with the change value (ΔI) between the steady-state current and background current.

2.7. Detection of HbA1c in real samples

The whole blood samples of apparently healthy individuals and diabetic patients were collected with EDTA as anticoagulant from Lamarak Clinical Diagnostic Laboratory (Tehran, Iran). Because HbA1c assay requires whole blood, no centrifugation steps were involved in separating blood cells from plasma. The samples were subjected to proteolytic digestion on the addition of 50 mM ammonium acetate (pH 4.0) containing 4.0 kU/ml purified *Bacillus* sp. proteases, and endoproteinase Glu-C from *S. aureus* V8 1. The proteolytic digestion led to a release of Fru-

ValHis, which then serve as a substrate for FPOX. The electrochemical signal produced in the reaction of Fru-ValHis, which is released from HbA1c digestion, is used to report the %HbA1c. The *ΔI* produced for diagnosis is based upon splitting of H_2O_2 released by the oxidation of Fru-ValHis by immobilized FPOX which is proportional to the HbA1c concentration. A linear calibration curve expressed in %HbA1c was prepared by plotting current (*ΔI*) against different percentages of HbA1c standard samples (%3.0, %5.5, %12.5 and %16.0). Then, the concentration of unknown sample was obtained based on the calibration curve. The accuracy of the current method was evaluated by comparing HbA1c levels in 46 whole blood samples by the fabricated biosensor with those obtained by the NORUDIA™ N HbA1c diagnostic kit. The HbA1c measurements with NORUDIA kit were performed by an auto-analyzer laboratory instrument (Roche Hitachi 912 chemistry analyzer). In the assay procedure of NORUDIA kit, protease cleaves Fru-ValHis from the N-terminal beta-chains of HbA1c. In the second reaction, Fru-ValHis reacts with FPOX and generates hydrogen peroxide. Hydrogen peroxide in the presence of HRP reacts with 10-(carboxymethyl aminocarbonyl) -3,7-bis (dimethylamino) phenothiazine sodium salt (DA-67) to develop color . The change in absorbance is measured at 660 nm and 800 nm to determine the concentration of HbA1c. Statistical analysis including paired samples t-test, and correlation coefficient were performed using GraphPad Prism 6 (GraphPad Software, La Jolla California USA).

3. Results and Discussion

3.1. Production and purification of engineered FPOX

The results of this section have been published in our earlier work [38]. Briefly, Tyr261Trp mutant were expressed in active form in *E. coli* BL21 (DE3). The purified engineered enzyme

had a specific activity of 145.2 ± 3.2 U/mg for Fru-ValHis. The specific activity of engineered variant with Fru-Lys was 1.3 ± 0.9 U/mg. The engineered FPOX indicated improved substrate specificity for Fru-ValHis than for Fru-Lys (relative activity Fru-ValHis/Fru-Lys: 111.7). The kinetics properties were studied using Fru-Lys and Fru-ValHis as substrates. The K_m of engineered enzyme toward Fru-ValHis was reduced to 0.43 ± 0.5 mM. On the contrary, K_m value for Fru-Lys was increased to 74.8 ± 1.4 mM. Tyr261Trp substitution caused an 11.7-fold increase in K_{cat}/K_m for Fru-ValHis while K_{cat}/K_m for Fru-Lys decreased by 22.4-fold. Accordingly, the engineered variant had less activity toward Fru-Lys and greater activity toward Fru-ValHis than the wild-type enzyme.

3.2. Morphological characterization and electrochemical impedance spectroscopy

The modification steps were characterized using SEM and electrochemical impedance spectroscopy (EIS). The morphologies of bare FTO electrode, CHIT/FTO electrode, GO-CHIT /FTO electrode, AuNPs-GO-CHIT/FTO electrode and FPOX immobilized on AuNPs-GO-CHIT/FTO electrode were investigated using SEM (Fig. 3). The figures show changes in surface morphology of FTO electrode after deposition of CHIT, GO, AuNPs and then immobilization of FPOX. The bare FTO electrode showed a smooth and featureless surface (Fig. 3A), whereas the electrode modified by CHIT displayed a polymer network covering on the FTO surface (Fig. 3B). The granular porous morphology of AuNPs was shown in Fig. 3D. The micrograph of FPOX-AuNPs-GO-CHIT/FTO (Fig. 3E) showed a regular globular morphology, confirming the immobilization of FPOX in the surface of modified electrode. EIS is an important method to provide useful information on the impedance changes during the modification process [17]. Fig. 4A shows Nyquist plots of EIS for the different electrodes in 2.5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$.

The value of electron transfer resistance (R_{CT}) for bare FTO was 9154.0 Ω. The values of R_{CT} for GO-CHIT/FTO and AuNPs-GO-CHIT/FTO electrodes were decreased to 5042.0 Ω and 2967.0 Ω, respectively. This indicates high electron transfer nature of nanocomposite that can be attributed to the conducting nature of GO and AuNPs. R_{CT} value was increased to 7279.0 Ω for FPOX-AuNPs-GO-CHIT/FTO. This increase in R_{CT} can be attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors and cause hindrance to electron transfer [14, 32]. This result also confirmed the immobilization of FPOX on nanocomposite. Moreover, a comparative analysis was also performed for FPOX-modified electrode after binding with Fru-ValHis (Fig. 4B). A significant change in R_{CT} after the incubation of FPOX-modified electrode with Fru-ValHis was observed (Rct = 933.8 Ω), while no response was achieved with control, indicating specific measurement of Fru-ValHis.

3.3. Cyclic voltammetric experiments

Electrochemical properties of each modified surfaces were studied using cyclic voltammetry. Fig. 5A shows the comparative cyclic voltammograms of bare FTO electrode, GO-CHIT/FTO electrode, AuNPs-GO-CHIT/FTO electrode and FPOX-AuNPs-GO-CHIT/FTO. The bare FTO electrode shows no oxidation and reduction peaks in forward and reverse scan. The addition of GO and AuNPs on electrode enhanced the current response of CVs with well-defined oxidation and reduction peaks. It demonstrated that AuNPs-GO modified FTO electrode resulted in an improvement of electric conductivity of enzyme electrode. The increase in peak current for AuNPs-GO-CHIT/FTO electrode is attributed to the GO and AuNPs in CHIT network that provides high electro active area and electron mobility [42]. The FPOX-AuNPs-GO-CHIT/FTO exhibited a less current, which was related to the hindrance of electron transfer by immobilized

FPOX. The bare FTO electrode and FPOX-AuNPs-GO-CHIT/FTO showed current intensity of 3.2 µA and 17.3 µA, respectively. To evaluate the bio-catalytic activity of FPOX at the fabricated biosensor, the modified electrode was characterized by CV studies in presence of Fru-ValHis. Fig. 5B shows CVs of the FPOX-AuNPs-GO-CHIT/FTO with and without Fru-ValHis at a scan rate scan rate 50 mVs^{-1}. The current increased with the addition of 1.0 mM Fru-ValHis showing the catalytic performance of modified electrode to the oxidation of Fru-ValHis.

3.4. Effects of scan rate and pH

The experimental conditions affecting the biosensor response were studied in terms of the effect of pH, and scan rate. The maximum current was observed at pH 7.4 and 25°C (Fig. 6A). Changing the pH from neutral to acidic or basic can change the surface charge of target enzyme and lead to the deformation of catalytic site [38]. The influence of scan rate from 5 to 100 mVs^{-1} on the biosensor response using 1.0 mM Fru-ValHis in 0.1 M potassium phosphate buffer solution (pH 7.4) was also investigated (Fig. 6B). A potential scan rate 50 mVs^{-1} was selected because with this experimental condition, the highest analytical signal and good cyclic voltammogram profile were obtained.

3.5. Performance characteristics

The influence of substrate concentrations on the response of fabricated biosensor has been shown in Fig. 7A. The electrode system had a reasonable linearity (R^2=0.998) toward different concentrations of Fru-ValHis ranging from 0.1 to 2 mM (Fig. 7B). To ensure the sensitivity of the biosensor, differential pulse voltammetric (DPV) response curves were recorded in the presence of four concentrations of Fru-ValHis (2 µM, 4 µM, 6 µM, 8 µM) (Fig. 7C). As shown

in Fig. 7D, a linear response to current with R^2 of 0.999 is apparent for Fru-ValHis concentrations. The sensitivity of the biosensor was determined by the slope of the linear least-squares calibration plot over the range of 1.5 to 6 µM. Based on the calculated slope of the calibration curve, the fabricated biosensor exhibits a sensitivity of 8.45 µA $mM^{-1}cm^{-2}$, which is comparable with that reported for Fru-ValHis by Fang and coworkers (0.41 µA mM^{-1} $cm^{-2)}$ [13]. The detection limit of current biosensor for Fru-ValHis was found to be 0.3 µM with signal to noise of 3.0 using the equation 3(SB/S) [43], where SB is the standard deviation of the blank signal (0.84 µA) and S is the sensitivity or the slope of the calibration cruve. For investigation the selectivity of the fabricated biosensor, the effect of interfering substances e.g. ascorbic acid 12 mg/dL, uric acid 7.0 mg/dL, bilirubin 15.0 mg/dL, urea 80.0 mg/dL, triglycerides 500.0 mg/dL and glucose 180.0 mg/dL were studied. As shown in Fig. 7E, high response signal was observed when the biosensor was incubated with Fru-ValHis, while no response was obtained with addition of these substances. To study analytical recovery, known concentrations of Fru-ValHis were added to the whole blood sample. The concentration of HbA1c was measured in whole blood before and after the addition of exogenous Fru-ValHis. The analytical recovery of added Fru-ValHis in whole blood was 95.1 to 98.35% for FPOX-AuNPs-GO-CHIT/FTO electrode, indicating good efficiency of the biosensor (Table 1). The assay precision was determined by calculation of coefficient of variation (%, CV) (Table 2). For Fru-ValHis determination in whole blood by FPOX-AuNPs-GO-CHIT/FTO electrode, within-run CV was between 1.3% and 2.4% and between run CV was between 2.1% and 3.5%., revealing the reproducibility and reliability of the method. A comparison on the analytical performance of the proposed biosensor with previously reported enzyme-based HbA1c biosensors is provided in Table 3. This comparison indicated that fabricated bioelectrode had an improvement in detection

limit. As a matter of fact, the observed improvement in bioelectrode detection limit is attributed to the specificity of engineered FPOX toward Fru-valHis and the biocompatibility of nanocomposite for enhancing immobilization and electron transfer.

3.6. Determination of HbA1c in real samples

The accuracy of the current method was tested by comparing HbA1c levels in 46 whole blood samples by the fabricated biosensor with those obtained by the NORUDIA™ N HbA1c diagnostic kit used in clinical laboratory. A linear calibration curve expressed in %HbA1c was prepared by plotting current (ΔI) against four different percentages of HbA1c standard samples (Fig. 8A). The concentration of unknown sample was obtained based on the calibration curve. The analysis of HbA1c was performed three times for each sample, and the percentage of HbA1c was calculated. The percentages of HbA1C using the proposed sensor were compared with the results from the NORUDIA™ N HbA1c diagnostic kit. The HbA1c results obtained by both methods showed a very good correlation (R^2=0.996) with regression equation y=0.987x + 0.063 (Fig. 8B). *P*-value was 0.128 indicating that the two methods agreed with 95% confidence. These results indicate a very good analytical performance of the current biosensor in biological samples.

4. Conclusion

Collectively, an amperometric biosensor for the quantitative determination of Fru-ValHis, a model compound of HbA1c was fabricated using the immobilized engineered recombinant FPOX on AuNPs/GO/CHIT nanocomposite. The engineered enzyme showed a specific bioelectrocatalytic reaction toward Fru-ValHis. The AuNPs/GO/CHIT nanocomposite provided

a good microenvironment for the immobilization of FPOX, thus facilitating the electrocatalysis of Fru-ValHis. Moreover, the biosensor was successfully employed to analysis real blood samples as compared with the NORUDIA™ N HbA1c diagnostic. Briefly, since the engineered FPOX possessed high sensitivity and selectivity for the detection of Fru-ValHis in blood samples, the fabricated biosensor can be effectively applied for HbA1c determination in medical management.

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Highlights

- An enzyme sensor utilizing recombinant FPOX and Fru-ValHis as the base for HbA1c biosensor is reported
- An engineered FPOX with improved specificity for Fru-ValHis was used for biosensor fabrication.
- FPOX enzyme was immobilized on chitosan, graphene oxide and AuNPs nanocomposite.
- The suitable specificity of the engineered FPOX made the biosensor responded well to the HbA1c in blood.

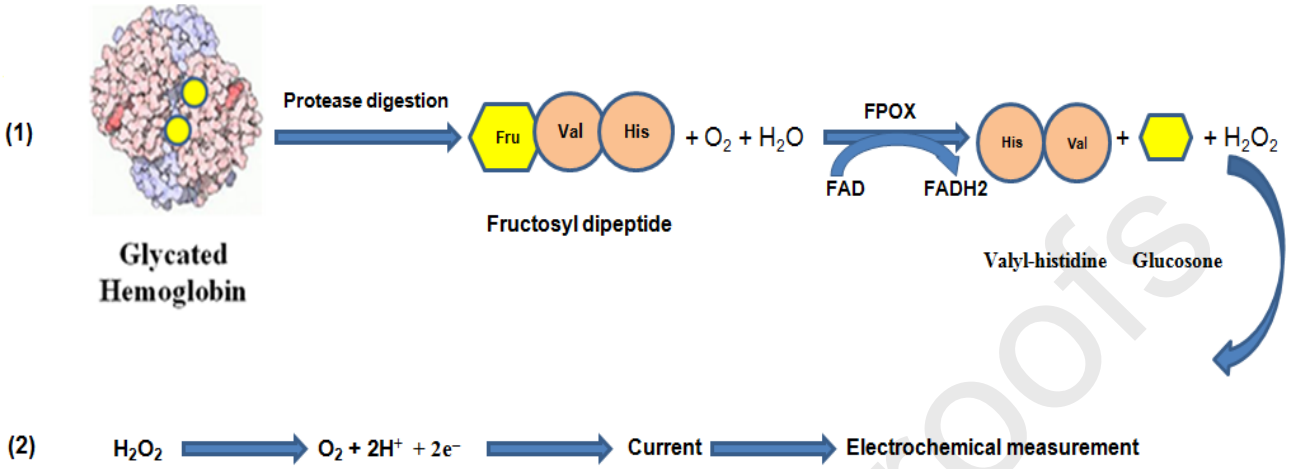


Fig. 1. Schematic representation of HbA1c detection by designed biosensor.

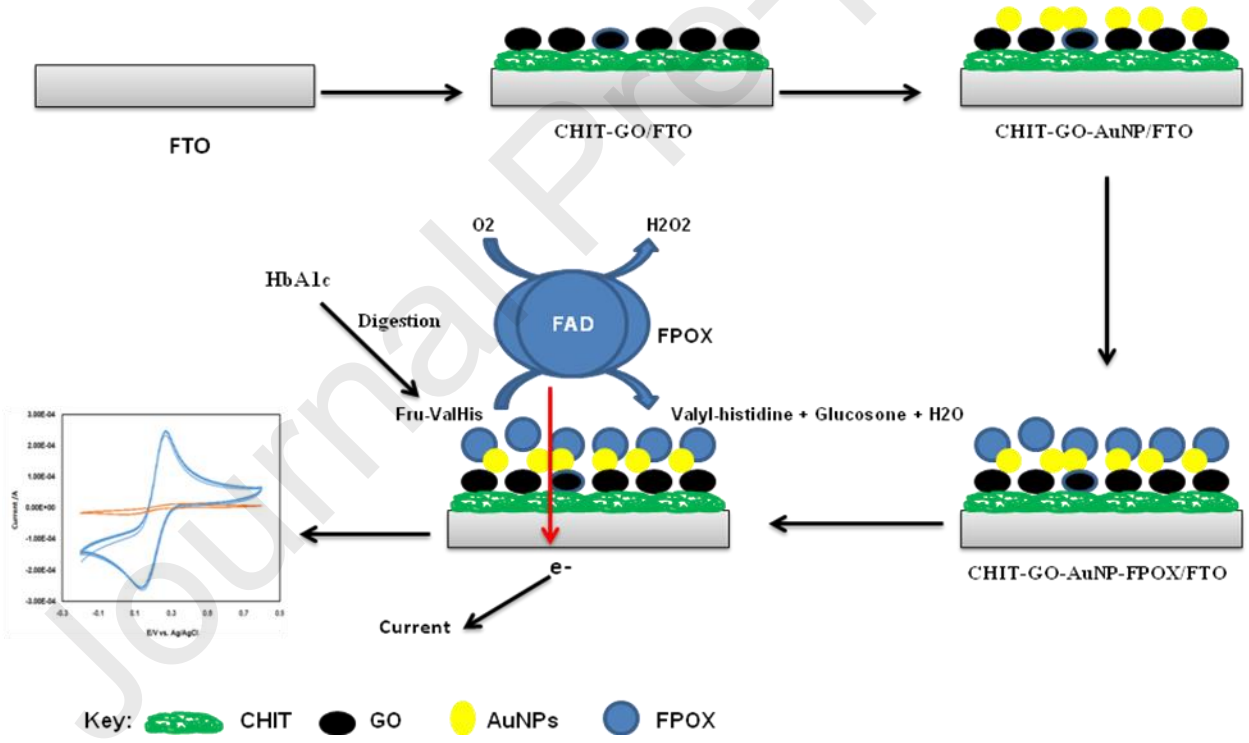


Fig. 2. Schematic illustration of biosensor fabrication and its application in HbA1c detection.

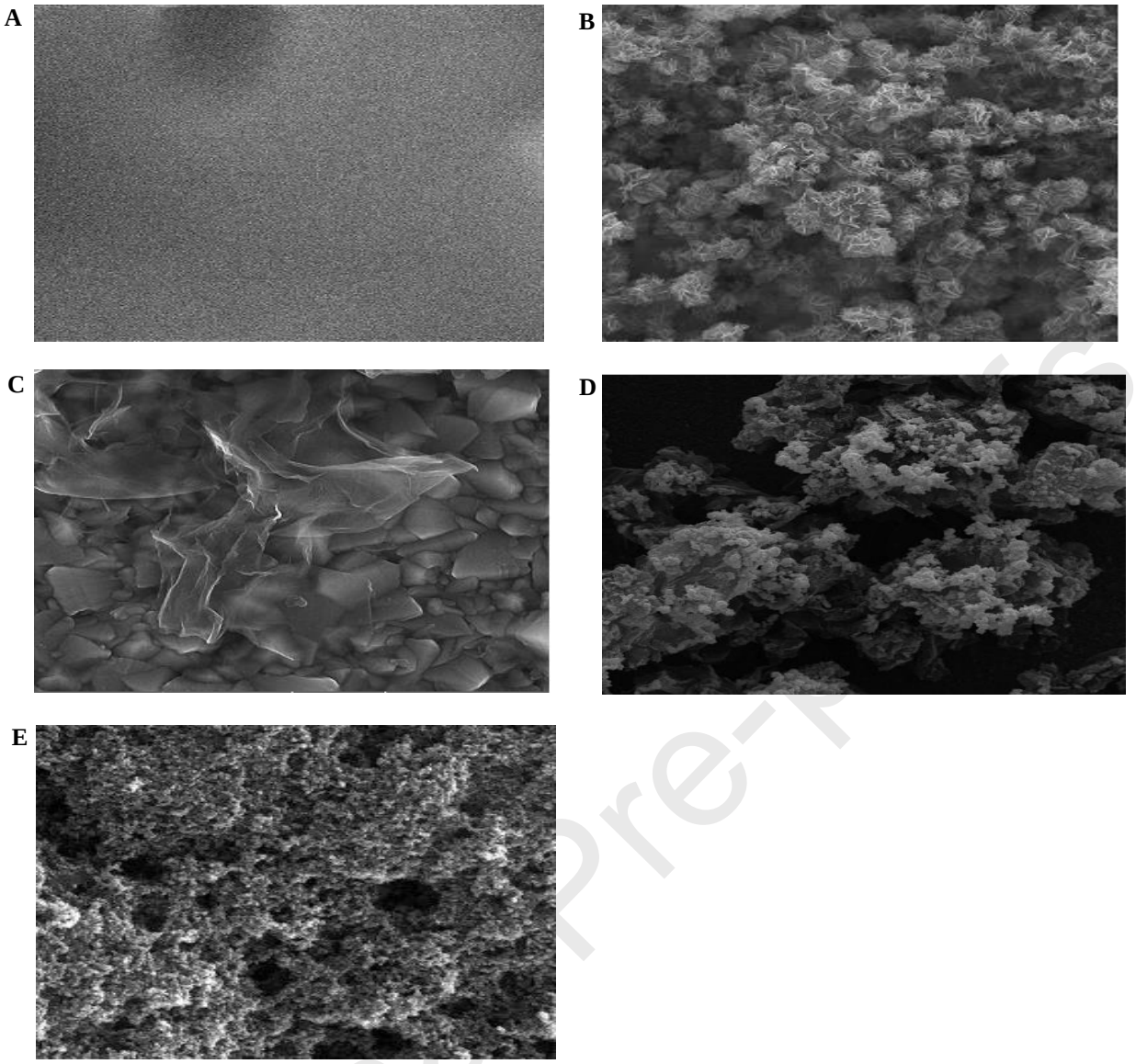
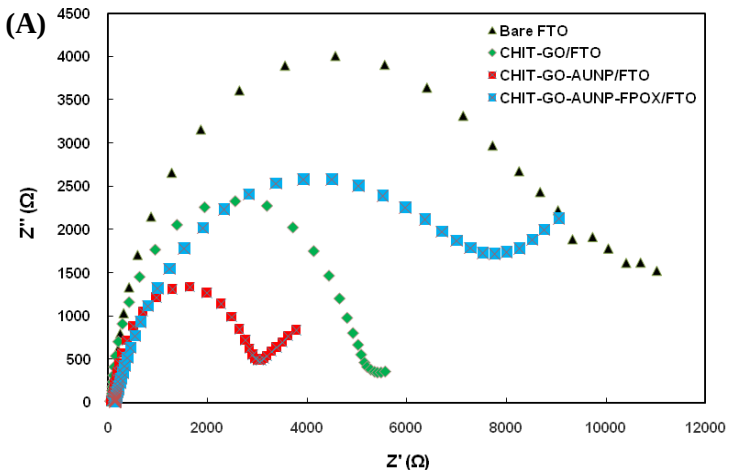


Fig. 3. SEM images. (A) Bare FTO electrode. (B) CHIT/FTO electrode. (C) GO-CHIT/FTO electrode. (D) AuNPs-GO-CHIT/FTO electrode. (E) FPOX-AuNPs-GO-CHIT/FTO electrode.



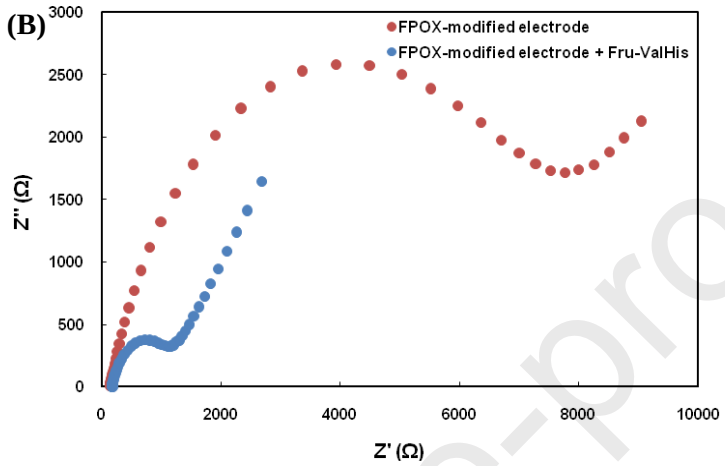
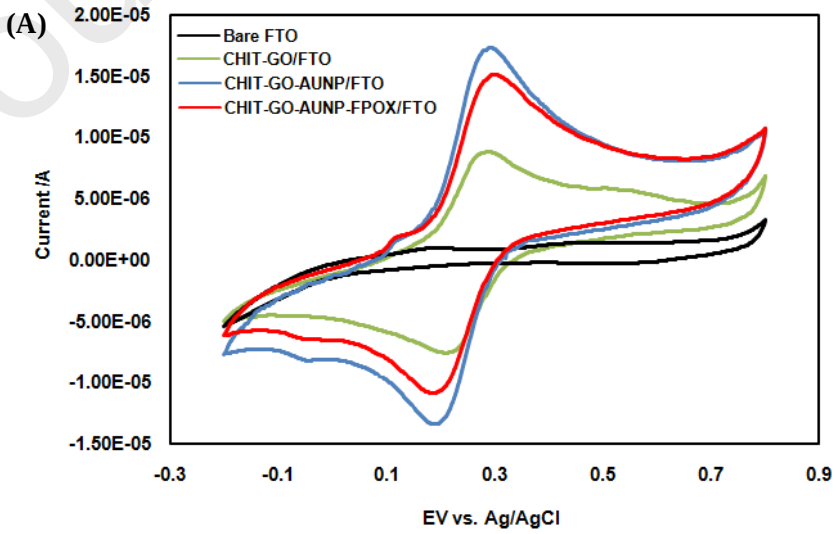


Fig. 4. (A) Nyquist plots of EIS of bare FTO electrode, GO-CHIT/FTO electrode, AuNPs-GO-CHIT/FTO electrode, and FPOX-AuNPs-GO-CHIT/FTO. (B) Nyquist plots of FPOX-modified electrode before and after incubation with 1.0 mM Fru-ValHis.



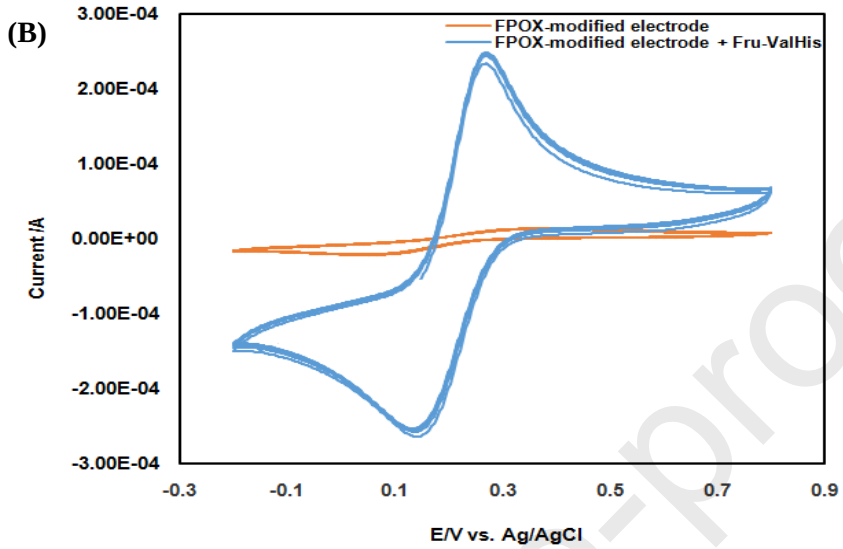


Fig. 5. (A) Comparative voltammogram curves of bare FTO electrode, CHIT-GO/FTO electrode, CHIT-GO-AuNPs/FTO electrode, and CHIT-GO-AuNPs-FPOX/FTO at scan rate 50 mVs^{-1}. (B) Voltammogram curves of FPOX-modified electrode without and with 1.0 mM Fru-ValHis.

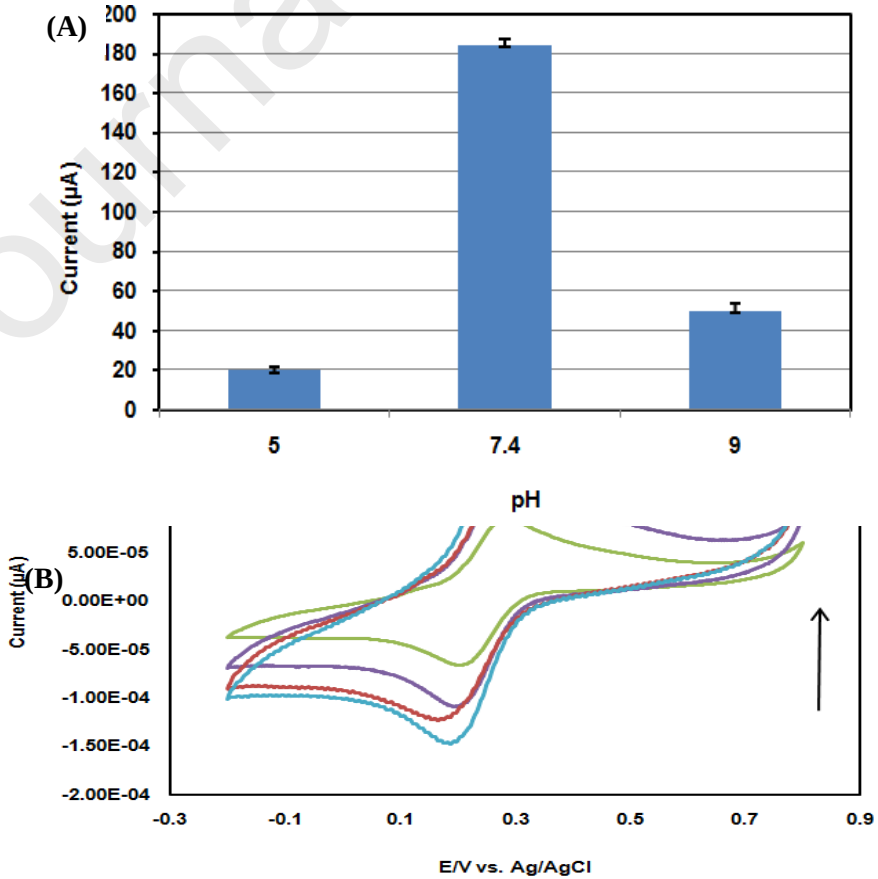
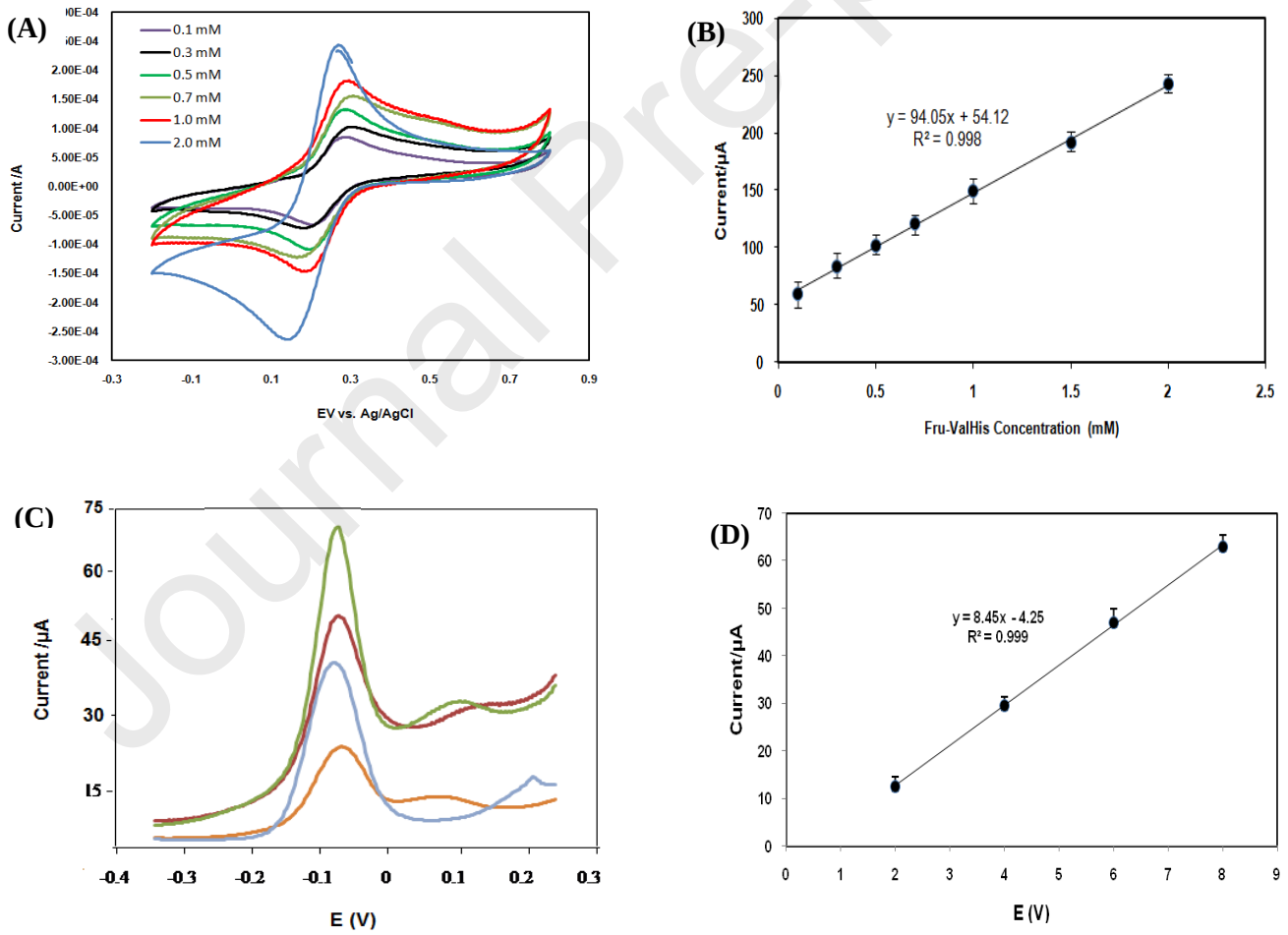


Fig. 6. Optimization of pH and scan rate. (A) pH of sensing solution. (B) Cyclic voltammogram of FPOX-AuNPS-GO-CHIT/FTO at different scan rates (5, 25, 50 and 100 mVs^{-1}).



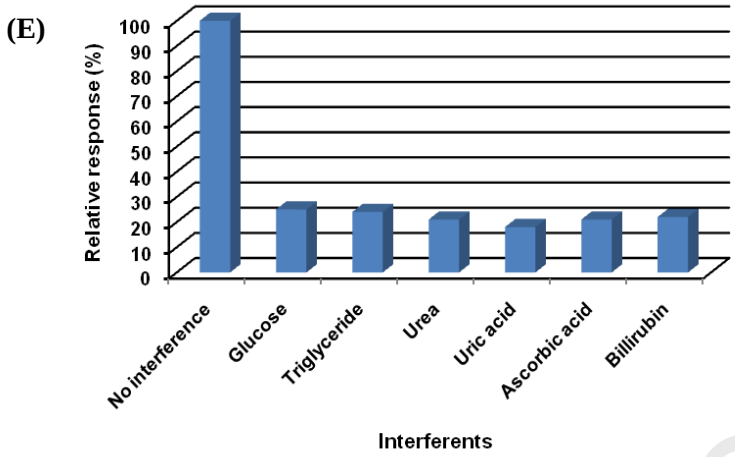
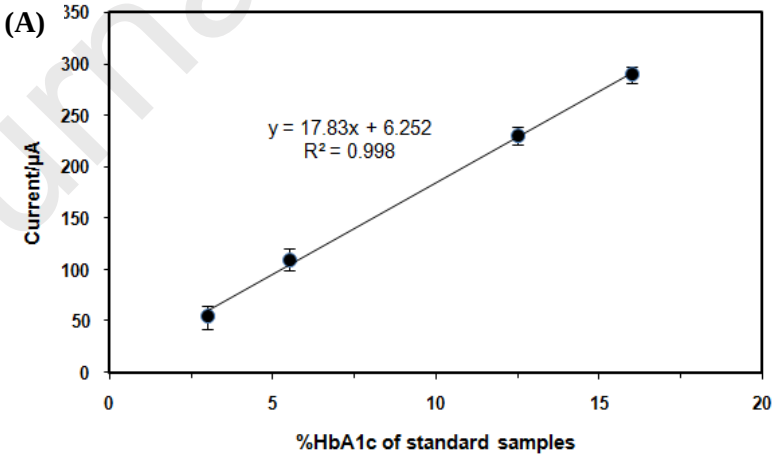


Fig. 7. (A) Influence of substrate concentrations on the response of fabricated biosensor. (B) The linearity of reaction curve of biosensor. (C) DPV plots of biosensor at different concentrations of Fru-ValHis. (D) Calibration curve for different concentrations of Fru-ValHis. (E) Influence of potential interference on biosensor response. Error bars show standard deviation for three different experiments.



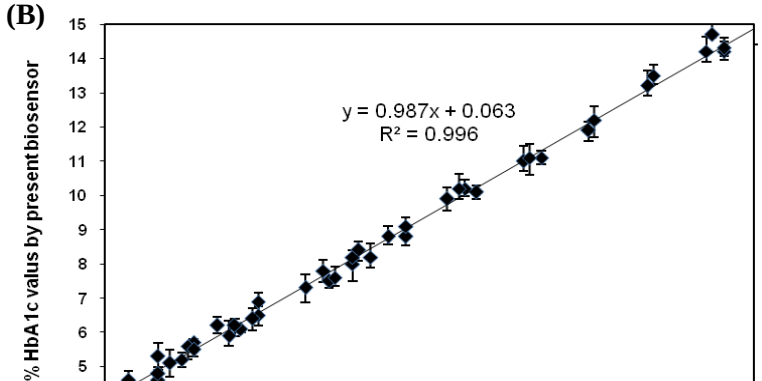


Fig. 8. (A) Calibration curve between current (ΔI) and %HbA1c using standard blood samples. (B) Correlation between HbA1c levels in blood samples measured by the fabricated biosensor and standard method (NORUDIA™ N HbA1c diagnostic kit). Error bars show standard deviation for three different experiments.

Table 1 Recovery of Fru-ValHis added to blood samples.

Fru-ValHis concentration (μmol/L)			Recovery (%)
Added	Expected	Observed	
0	_	0.6	_
200.0	530.7	521.9	98.35
400.0	730.7	710.38	97.22
800.0	1130.7	1085.47	96.0
1600.0	1930.7	1836.1	95.1

Table 2 Within-run and between run precision of the fabricated biosensor.

Standard	Concentration (mM)	Wthin-run (n=10)		Between-run (n=5)	
		(mean±SD)	CV%	(mean±SD)	CV%
Fru-ValHis	0.05	0.06 ± 0.14	2.4	0.06 ± 0.21	3.5
	0.6	0.71 ± 1.34	1.9	0.71 ± 2.2	3.1
	1.2	1.25 ± 2.0	1.6	1.25 ± 3.1	2.5
	2.5	2.65 ± 3.44	1.3	2.65 ± 5.56	2.1

SD Standard Deviation, CV coefficient of variation.

Table 3 Comparison of analytical performance of the presented biosensor with other enzyme-based electrodes reported previously.

Modified electrode	Sensitivity ($\mu AmM^{-1}cm^{-2}$)	Detection limit (μM)	Linear range	Reference
FPOX/AuNPs/GO/CHIT/FTO	8.45	0.3	0.1-2 mM	This paper
FAO/MNPs[a]/Au	_	100	0.1-2 mM	[33]
FAO/AuNPs/GNs[b] modified FTO	_	200	0.3-2 mM	[14]
FAO/iridium-modified electrode	21.5	_	_	[13]
FAO/ZnONPs/PPy[c]/Au	38.42	50	0.1-3 mM	[32]

[a] Magnetic bionanoparticles
[b] Graphene nanosheet
[c] Polypyrrole
¯ Not determined