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Influence of zinc oxide nanorods on the sensitivity of a glycated hemoglobin biosensor

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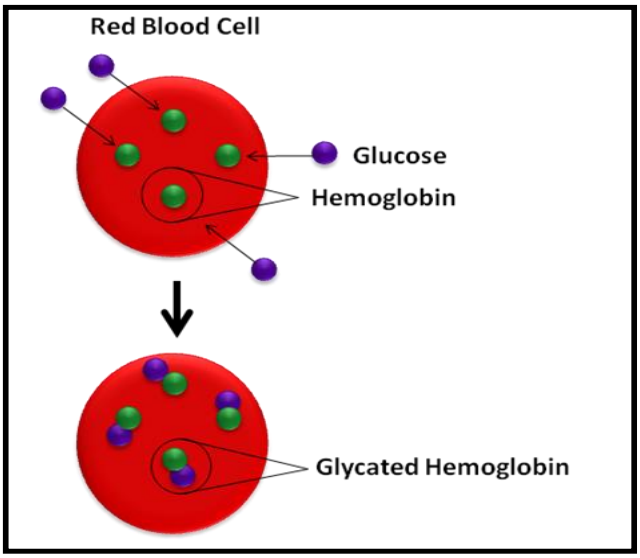
ABSTRACT

The glucose level measurement in the blood of diabetic patients without significant variations is important. The level of glycated hemoglobin (HbA1c) in the blood provides an authentic tool for glucose level measurement. In our study, a biosensing system established on properly aligned single-crystal zinc oxide (ZnO) nanorods structures grown on indium-tin oxide coated glass plate (ITO) electrode. ZnO nanorods were immobilized with fructosyl amino-acid oxidase (FAO) enzyme through physical adsorption integrated with cross linking molecules N-5-azido-2-nitro-benzoyloxysuccinimide (ANB-NOS). Whole blood samples were first hemolyzed & then properly digested with protease prior to measuring the HbA1c through the sensor. This enzyme biosensor reported an optimum response at +0.2 V. This biosensor displayed a significant sensitivity and detection limit (0.1μM), fast response time (4s) and wide linear range (from 0.1 to 2000 μM). The enzyme/working electrode is stable for about 4 months, when kept at 4 ^{o}C. This recommended biosensor method may apply for detecting HbA1c in blood samples obtained from apparently healthy as well as diabetic patients. Copyright © 2016 VBRI Press.

Keywords: Fructosyl amino-acid oxidase; ZnO nanorods; ITO electrode; glycated hemoglobin; whole blood.

Introduction

The high level of blood glucose is a result of inefficacy of human body to produce insulin causing a group of disease known as Diabetes. It inflicts many people in various part of the world [**1**]. The proper monitoring of glycaemic control (a prevailing level of glucose in diabetic patients) is requisite to decrease the risk of complications occurred during diabetic conditions [**2**]. The glycated hemoglobin (HbA1c) in the blood is a marker to indicate diabetes mellitus. HbA1c is formed once glucose binds with the β-chains of haemoglobin (with the residue of N-terminal valine) [**3**] (**Scheme 1A**). The red blood cells possess a long life span and therefore the usual % of HbA1c in overall hemoglobin indicates a standard blood glucose concentration over the couple of months preceding [**4**]. Thus HbA1c level is considered a distinct and useful diagnostic marker for detecting glucose level in the blood of diabetic persons. A typical HbA1c level appears within the range of 4%–6% [**5**]. The diverse range of methods are commercially available for determining HbA1c levels e.g. liquid chromatography [**6,7**], spectrophotometry [**8,9**], affinity and ion-exchange chromatography [**10,11**], immunoassay [**12,13**], liquid chromatography associated in tandem with mass-spectroscopy (LC-MS/MS) [**14**] and electrophoresis [**15,16**]. Although these methods unfold many disadvantages e.g. complexity, take longer time for sample preparation, labor dependent and need an expert handling. After comparing with these methods, the electrochemical techniques are precise, easy and fast. In the earlier studies, non-enzymatic sensing using ferroceneboronic acid [**17**], an oxygen electrode having poly(vinylalcohol)- Stylbazole (PVA–SbQ) membrane [**18**], iridium modified carbon electrode [**19**], non-enzymatic biosensor glassy carbon electrode (GCE) [**20**], core-shell iron oxide nanoparticles modified Au electrode [**21**] and zinc-oxide nanoparticles (ZnONPs)/polypyrrole (PPy) modified Au electrode [**22**] based biosensors are previously constructed.



Scheme. 1A. Process for the glycation of hemoglobin, when glucose molecules attach to the hemoglobin in red blood cells.

However, these biosensors also bear certain drawbacks including improper electron communication, complex immobilization process and fast instability of enzyme. Therefore, an electrochemical bio sensing interface to

construct a direct and sensitive method for detection of HbA1c to get better response is utmost important which will meet the requirements of various clinical applications for Diabetes patients.

For many years, semiconducting nanomaterials are implied in nano –biomedical research which provide a unique way to construct and develop various functional nanodevices [**23, 24**]. These nanomaterials based devices for instance comprising zinc oxide (ZnO) nanomaterials, nanorods and nanowires have many valuable properties which give a lot of attentions to the researchers. A large number of shapes of ZnO nanostructures (NSs) are comparatively easy to synthesize by various methods [**25,26**]. The ZnO materials also have a large family of NSs. It is noted that NSs will participate in a very significant role for the variety of practical applications which includes optoelectronic procedure included UV sensors, solar cells based sensors, biosensors and light emitting diodes (LEDs) [**27-42**]. In the field of biosensors ZnO nanorods possess a tremendous application potential because of its good biocompatibility, optical similarity, non-toxicity, electrochemical stability, high electron transfers capability and large surface area. Commercially they are very simple to fabricate and grow [**43-54**].

In our current research work, we have described the precise synthesis of ZnO nanorods (ZnONRs) using zinc nitrate and hexamethylenetetramine (HMT) precursor on indium-tin-oxide coated glass plate (ZnONRs/ITO) & further been used for fructosyl amino acid oxidase (FAO) immobilization. This biosensor was then successfully applied for the detection of HbA1c in blood samples collected from healthy and diabetic patients. In comparison with the other amperometric biosensors, this sensor which is constructed by immobilizing FAO onto the ZnONRs modified ITO exhibited various advantages over a wide range of measurement with high sensitivity.

Experimental

Chemicals and reagents

Fructosyl-amino acid oxidase (FAO, 0.45 Umg^{-1} from recombinant *Escherichia coli*) and indium tin oxide coated glass plate (ITO) (resistivity: 70-100 Ω/sq; L × W × thickness: 75 mm × 25 mm × 1.1 mm) from Sigma-Aldrich, USA and L-valine, pyridine, CHES (2-(Cyclohexyl-amino) ethane sulfonic acid), Triton X-100, SDS (Sodium dodecyl sulfate), dess martin periodinane, MES(4-Morpholinoethanesulfonic acid), 4 aminoantipyrine, purified *Bacillus sp*. Protease, zinc nitrate hexahydrate $Zn(NO_3)_2{\cdot}6H_2O$ & hexamethylenetetramine (HMT), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOS) from SRL, Mumbai, India were bought. All the solutions were prepared in Milli-Q ultrapure water. All other chemicals used in the experiments were of analytical reagent grade (AR). Blood samples of apparently healthy persons and diabetes patients were taken in Bio Diagnostics Laboratory, Rohini, New Delhi and further stored at 4 ^oC.

Electrochemical measurements were taken by a potentiostat (model; Autolab AUT83785, Ecochemie, The Netherlands). Scanning electron microscopy (SEM) studies were conducted at Amity Institute of Advanced Research and Studies (AIARS), Amity University, Noida. The process of Ultrasonication was accomplished on Misonix Ultrasonic Processors (mode; XL-2000). X-ray diffraction (XRD) study of ZnONRs is achieved at Department of Physics, G. J. University, Hisar by using X-ray diffractometer (Make: Rigaku MiniFlex-II).

Synthesis of fructosyl valine (FV)

Fructosyl valine (FV) is not easily available commercially. Synthesis of FV was performed in our laboratory as described [**55, 56**]. In order to prepare FV, L-valine (0.16 mol, 18.8 g) were mixed in pyridine (400 mL) & CH_3COOH (400 mL) and further mixed at room temperature for 30 min by stirring. Then (0.22 mol, 40 g) of glucose was mixed and purged with N_2 for about 5 min and then stirred at 35 ^oC for continuous 4 days. The dark colored mixture was appeared which was then filtered and the solvent was allowed to evaporate. Furthermore, re-crystallization in methanol was carried out when a slightly yellowish amorphous product was found. This product was then lyophilized to obtain a colorless residue.

Preparation of nanomaterials and working electrode

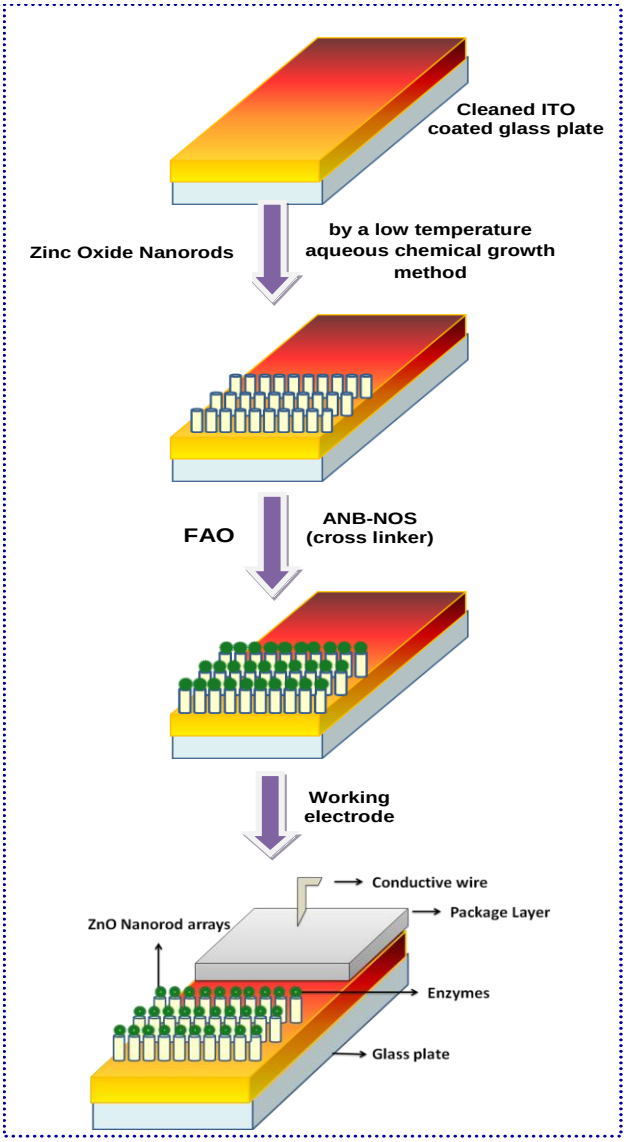
The deposition of ZnO seeds was carried out by cleaning the ITO substrates with sonication in acetone, 2-propanol and deionized water. Once the deposition is completed, a vertical well aligned ZnONRs were synthesized onto the ITO coated plate with the use of a low temperature aqueous chemical growth method (ACG). In this process, a homogeneous layer of $Zn(CH_3COO)_2 \cdot 2H_2O$ seed solution is incorporated by spin coater at 3000 rpm for 60 s. Later on the modified ITO plate were annealed for 10 min at 150 °C. These annealed samples were fastening in the holders and kept in the $Zn(NO_3)_2{\bullet}6H_2O$ and HMT mixture (1:1 equimolar) & heated in oven which is preheated at 90 °C for 6 h. Once this process is finished, this ITO plate was removed out from oven and washed with distilled water to further eliminate the residual particles of salt & then dried with N_2 gas.

After immobilization of FAO onto the surface of grown ZnONRs with a cross linker ANB-NOS, electrodes were prepared. The immobilization process consists of 2 steps; in the first step, a 10 mM ANB-NOS solution was prepared in phosphate buffer at pH 7.4 and then ZnONRs modified electrode was dipped in the above solution for about 1 h. In second step, this electrode was further kept into the enzyme solution for 20 min. Furthermore, every enzyme immobilized electrode was placed at 4 °C temperature for about 16 h. Once the procedure is completed electrochemical measurements were carried out to set up the experiment, an enzyme immobilized ZnONRs is used as working electrode & Ag/AgCl as a standard/reference electrode (**Scheme 1B**).

Response measurement of FAO/ZnONRs onto the ITO electrode

In order to measure the response for electrochemical characterizations, a conventional 3 electrode system using FAO/ZnONRs onto the ITO plate as working electrode, a platinum wire as the counter electrode and an Ag/AgCl

electrode as the standard/reference electrode were configured. Cyclic voltammetric studies were taken in 3 electrode cell consisting 20 mL electrolyte [2.5 mM $K3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ (1:1)], 5 mL phosphate buffer 0.1 M, pH 7.0) and 0.1 mL of FV.



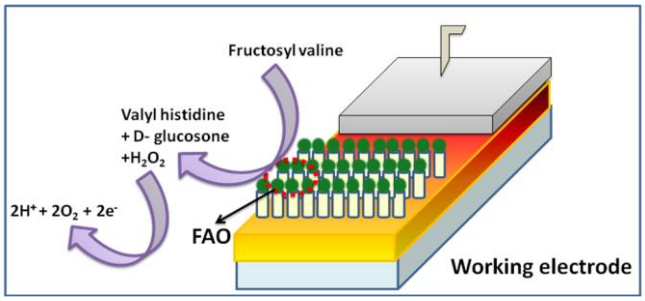
Scheme 1B. Schematic representation for preparation of working electrode. ZnONRs were immobilized with FAO enzyme using the physical adsorption approach in combination with N-5-azido-2-nitro-benzoyloxysuccinimide (ANB-NOS) as cross linking molecules.

Optimization of FAO/ZnONRs modified ITO electrode

Enzyme electrode was first employed to determine its optimum working conditions considering various factors. The reaction buffer pH (0.1 M) was used in the range of pH 5.0 to 9.0 with pH 0.5 intervals. Phosphate buffers in pH range 5.0 - 7.0 and Tris buffer in pH range 7.5 - 9.0 were used. Likewise, for optimum temperature the reaction mixture was incubated at 20 ºC to 50 ºC with the temperature difference of 5 ºC. Furthermore, the optimum response time was determined considering the current response studied from 2s to 20s at an increase of every 1s. In the last, the effect of substrate concentration was ascertained with different FV concentration ranging from 0.1 µM to 2000 µM.

Measurement of HbA1c in whole blood by enzyme/ZnONRs modified ITO electrode

The whole blood samples of diabetes patients as well as healthy individuals were taken in EDTA as anticoagulating agent from Biodiagnostics Laboratory, Rohini, New Delhi. Since HbA1c assay is carried out in whole blood directly, without centrifugation methods were performed for separation of blood cells from plasma. After collection and before testing, the whole blood samples were first mixed by the vortex to re-suspend the settled erythrocytes. The sample was hemolysed by mixing lysis buffer (consist of 100 mM CHES (pH 8.7), SDS (0.45%), Triton X-100 (1%) and 0.5 mM of oxidizing agent) in the ratio of 20 µL whole blood: 250 µL of lysis buffer and then incubated for minimum 10 min at 30 ^{o}C. These lysed whole blood samples were then employed for proteolytic digestion by adding MES (5 mM); pH 7.0, 4 kU/mL purified *Bacillus sp.* proteases and oxidizing agent (1 mM). This proteolytic digestion allows liberation of amino acids (release of glycated valines from the beta chains of hemoglobin) and these amino acids suffice as substrates for the FAO. However, this substrate is with other mixtures including 0.1 M phosphate buffer (pH 8.0), 90 U/mL peroxidase & 0.8 mM of 4-amino antipyrine cleaving N-terminal amino acids either histidine or valine. Cleaving results into formation of H_2O_2 [**22, 57**]. Since the sensing mechanism is based upon measurement of e- or the current supplied through splitting of H_2O_2 which is produced during the process of oxidation of FV. This current is proportional directly to HbA1c content (**Scheme 1C**).



Scheme 1C. Chemical reaction of immobilization of FAO on ZnONRs modified ITO electrode.

Fru-Val- His- Leu-Thr- His- Pro- Glu- Glu- Lys- Ser.... (N terminal residue of HbA1c β-chain)

↓ enzyme (protease)

Fructosyl-valine + (amino acid) $_{n-1}$
(FV) (Thr, His, Leu)

FV + O_2 +H_2O

↓

Valine + D-glucosone + H_2O_2

The procedure depicted as above for HbA1c detection from whole blood sample was similar to that of the response measurement via electrode, excluding when FV was changed by whole blood. Since FV concentration corresponds to HbA1c, therefore HbA1c concentration was determined forming the standard plot between different FV concentrations (mM) versus respective current (μA). Final level of HbA1c is interpreted as the ratio between glycosylated hemoglobin (mM) & overall hemoglobin (mM).

$$\%\text{HbA1c} = \frac{\text{Glycosylated hemoglobin (mM)}}{\text{Total hemoglobin (mM)}} \times 100$$

Results were evaluated by various statistical criterions & correlation with the gold reference auto analyser method. This measurement for overall Hb value by the regular method was performed in Biodiagnostics Laboratory, Rohini, New Delhi.

Evaluation

There were various factors which were considered for the performance of biosensor including analytical recovery, detection limit, repeatability (precision), linearity, reproducibility and storage stability.

Results and discussion

Structure analysis

The XRD pattern showed in **Fig. 1**(a), for ZnONR/ITO surface in the range of $30^o < 2\theta < 60^o$. The polycrystalline ZnO was formed in wurzite crystalline structure indicated by diffraction peaks from planes (100), (002), (101), (102) and (110) [**58**]. There was high similarity of diffraction peaks obtained from different planes and d- values with the reported JCPDS data of nano-ZnO [**58**]. The entire peak positions and relative peak intensities of ZnO product were analogous with standard JCPDS. There were not any impurities for instance Zn, Zn $(OH)_2$ or HMT were detected representing the high purity of synthesized nano ZnO by optimized temperature and chemical growth method (ACG). The designated phase structure of nano ZnO corresponds to wurtzite structure (hexagonal phase). Furthermore, a high level of crystallinity of prepared ZnO nanorods was observed via the diffraction peaks of the product which shows strong peak intensities indicating that have high. An average size was calculated using Scherrer equation which indicated a size of 32.3+0.4 nm. The hexagonal phase ZnO formed in the prepared samples were fortified by FT-IR spectra shown in **Fig. 1(b)**. A broad band at 3380 cm^{-1} emulates to vibrational mode of OH group of water. This is an indication that some water was adsorbed on ZnO crystal surface. Band near to 1650 cm^{-1} represents asymmetric stretching of zinc acetates or oxalates [**59**]. Band at 1533 cm^{-1} shows a C=O stretching vibration of small fractions of unreacted COO-Zn. An intense band at 500 cm^{-1} confirms the Zn-O vibration of ZnO.

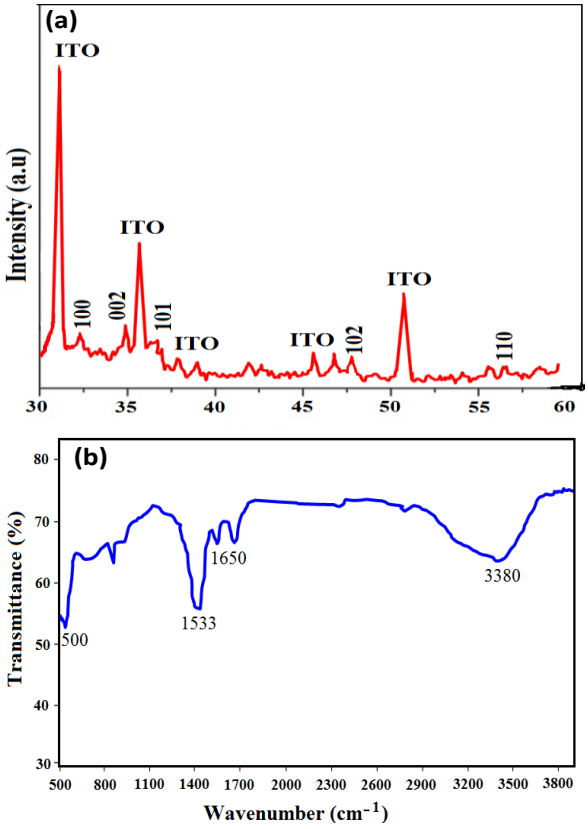


Fig. 1. (a) XRD spectra of ZnONRs/ITO electrode and (b) FT-IR spectra of synthesized ZnONRs.

Surface characterization of working electrode by SEM and EIS

Bare ITO electrode, ZnONRs/ITO electrode and FAO immobilized ZnONRs/ITO electrode were morphologically characterized using SEM technique (**Fig. 2**). The morphology of the bare Au electrode (image a) is a smooth and featureless after visualization in SEM. The fabricated ZnONRs film in **Fig. 2(b)** clearly indicates that there is a formation of vertically-aligned nanorods having dimension of approximately 33 nm diameter and 100 nm lengths.

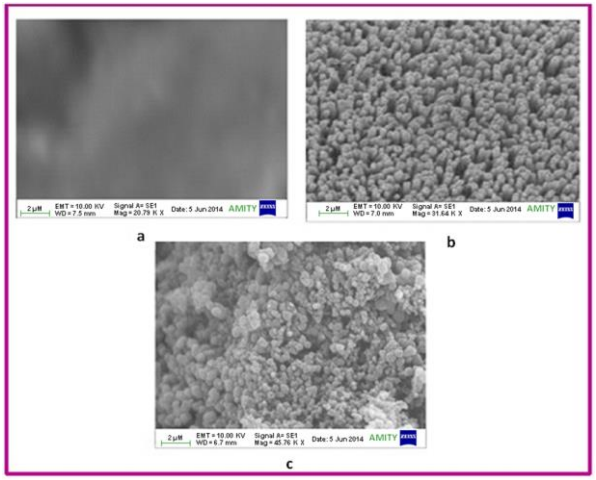


Fig. 2. Scanning electron microscopic (SEM) images of (a) bare ITO electrode, (b) ZnONRs modified ITO electrode (A higher-magnification image is presented in the upper right hand corner) and (c) FAO/ZnONRs modified ITO electrode.

The growth of ZnONRs initiated on ZnO seeds by heating zinc nitrate hexahydrate and hexamethylenetetramine; during the heating process, hexamethylenetetramine hydrolyzes and releases OH− ions into solution, which then reacts with Zn^{2+} to form ZnO_2^{-2}, which in turn results in ZnO nanorod crystallization from homogeneous precipitation under these mild conditions. The SEM of FAO/ZnONRs/ITO (c) clearly indicated a uniform globular structural morphology, showing the enzyme immobilization onto the surface of ITO electrode.

EIS is a splendid technique for investigating the alterations in the interface properties of modification process steps on the electrode surface. Nyquist plots for various electrodes were acquired by adding 0.1 M KCl containing 10 mM $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ as the probe (**Fig. 3**). The charge transfers resistance (Ret) was obtained 1027 Ω for bare ITO electrode. As shown, the value of Rct was decreased to 207 Ω for ZnONRs modified ITO electrode (curve b) indicating a relatively fast electron transfer properties of ZnONRs between the electrolyte and electrode surface. For the FAO/ZnONRs modified ITO electrode (curve c), the R_{et} value increased to 324 Ω due to the presence of enzyme molecules, which work as a barrier layer.

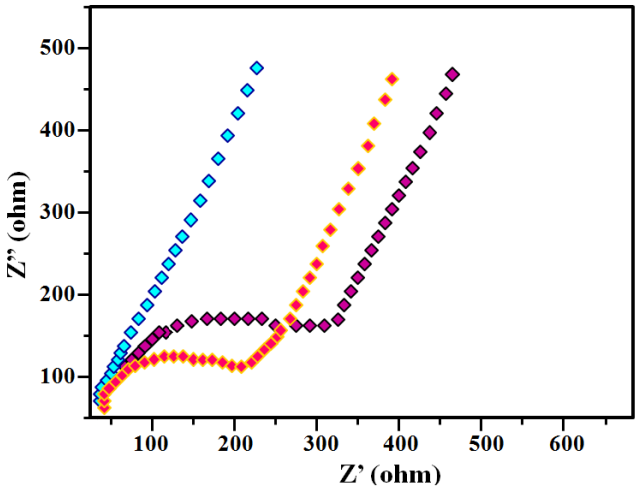


Fig. 3. Nyquist plots of 0.1 M KCl containing 10 mM $[Fe(CN)_6]^{3/4}$ as supporting electrolyte from 0.1 MHz to 0.1 Hz at ac amplitude of 5 mV under open-circuit potential conditions, obtained at the (a) bare ITO-coated glass plate, (b) ZnONRs onto the ITO-coated glass plate and (c) FAO/ZnONRs onto the ITO-coated glass plate incubated with FV.

Electrochemical response towards fructosyl valine (FV) at FAO modified ZnONRs/ITO plate

In order to analyze the catalytic/enzymatic activity of FAO on the ZnONRs/ITO electrode, a modified electrode was assembled and then characterized using cyclic voltammogram (CV) by applying potential from 0.0V to +0.6V in the presence of the substrate. **Fig. 4** shows CV of the ZnONRs/ITO electrode in a buffer solution (pH 7.0) which is not stirred and without [curve (a)] and with [curve (b)] FV solution at a regular scans rate 20 mVs^{-1}. Both oxidation and reduction current were deliberately increased while adding 2 mM FV. This indicates the catalytic properties of enzyme electrode for FV oxidation.

The cyclic voltammogram of the FAO/ZnONRs/ITO electrode recognized an oxidation peak at 220 m V (curve b). This voltammogram also exhibited a single major reduction peak. The ZnONRs used for modification of the electrode conductivity resulting in a higher Ip and smaller DEp to increase an effective surface. The Ip of FAO/ZnONRs modified ITO electrode was even higher, illustrating the oxidation of FV, which is catalyzed by the immobilized FAO on the ZnONRs/ITO surface.

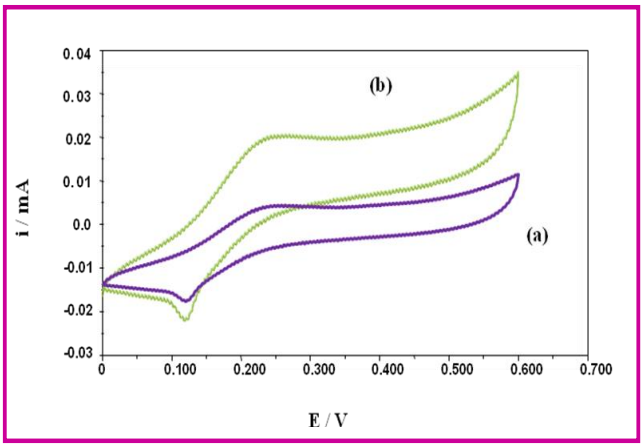


Fig. 4. Amperometric analysis of the enzyme electrode: Cyclic voltammogram of FAO/ZnONRs modified ITO electrode without (curve a) and with 0.5 ml fructosyl valine (curve b) solution (2 mM) in 0.1 M sodium phosphate buffer pH 7.0, at a potential scan rate of 20 mV/s. A constant surface area of the ITO electrode, *i.e.*, 0.5 cm^2, was used throughout the study.

Optimization of the biosensor

For evaluating the optimum conditions of our biosensor (having FAO/ZnONRs/ITO electrode), the parameters including pH, temperature, time and scan rate were contemplated. Direct effect of pH ranging from 5.0 to 9.0 in 0.1 M FV solution was probed. A maximum value of current was obtained for enzyme-catalyzed reaction at optimal value of pH 7.0. In next experiments, pH 7.0 was applied. The optimum temperature of biosensor was 25 ◦C. This biosensor provided a maximum response (comparatively faster) within 4 s. Furthermore, an effect of the scan rate in the range of 25 to 200 mVs^{-1} using 0.1 M FV in 0.1 M potassium phosphate buffer solution (pH 7.0) for biosensor performance was analyzed. The highest analytical signal & significant CV curves were achieved at potential scan rate of 50 mVs^{-1}.

Evaluation of biosensor

The biosensor was thoroughly evaluated after its construction. Linearity between current and FV concentration was perceived which was ranging from 0.1 to 2000 μM in most reaction mixtures. This biosensor was able to detection (limit - S/N = 3) upto 0.1 μM concentration. An analytical recovery procedure was carried out by using exogenous known substrate concentration once added to the blood samples. HbA1c concentration in the blood was carefully measured before and after addition of exogenous FV. The added FV in blood was quantified in the form of % recovery. A good

efficiency of analytical recovery of added FV in blood in concentrations ranging from 5 mM and 10 mM were 96.4% and 97.2%. The batch coefficient of variation (CV) of FV determination in blood samples by constructed biosensor were 4.23% and 5.22% (within and between). This reveals the reproducibility and reliability of the method developed. In order to determine the accuracy of the developed method, a comparison of HbA1c values (in %) in 20 blood samples of current method (y) with standard auto analyzer method (x) were performed. A very good correlation with R_2 = 0.98 was achieved after comparison. These results stipulate a remarkable analytical performance of our biosensor for biological samples. The electrode vanished almost 50% of its initial activity after more than 100 experiments over the period of 4 months not withstanding this is even better than previously reported electrodes [**17-22**].

The good sensitivity, stability and reproducibility detected for this biosensor could be attributed to the FAO immobilization onto ZnONRs deposited ITO electrode.

Glycated hemoglobin assessment in whole blood employing enzyme/ZnONRs/ITO electrode

The normal HbA1c levels measured in whole blood specimens of apparently healthy individuals are ranged in between 3.9–5.7% (**Table 1**). The value of HbA1c by present biosensor obtained in whole blood samples of diabetic patients are found in the range of 6.4–9.8 % (**Table 2**). Comparatively this is particularly significant and more than those of healthy individuals (p value<0.001). The increased levels of HbA1c in diabetic persons were occurred because of the presence of higher glycation of b-chain of hemoglobin once blood glucose level is elevated over a long period of time [**60**].

Table 1. HbA1c levels of apparently healthy persons, as measured by FAO/ZnONRs /ITO based biosensor.

S.No.	Age (years)	Sex[a]	% HbA1c by FAO/ZnONRs/ITO electrode
1.	32	M	3.9
2.	34	F	4.3
3.	52	F	5.4
4.	35	M	5.1
5.	39	F	4.4
6.	52	M	5.7
7.	47	M	5.4
8.	41	F	4.2
9.	36	F	4.3
10.	54	F	5.6

*M: Male; F: Female

Table 2. HbA1c levels of diabetic patients, as measured by FAO/ZnONRs/ITO based biosensor.

S.No.	Age (years)	Sex[a]	% HbA1c by FAO/ZnONRs/ITO electrode
1.	46	F	9.4
2.	41	F	7.3
3.	45	F	7.5
4.	38	M	7.9
5.	49	F	8.6
6.	45	M	7.9
7.	36	F	6.4
8.	54	F	9.8
9.	53	M	9.2
10.	46	F	7.5

Conclusion

The precise assessment of fructosyl valine (FV) was determined by a novel amperometric sensor thoroughly based on FAO immobilization onto ZnONRs. The FV is a prototype compound for the HbA1c detection. After construction of this biosensor, it was used for evaluation and the biosensor represented a higher sensitivity, proper linear range, high reproducibility & a very fast response time. A relatively strong electrostatic interaction between ZnONRs & FAO leads to provide sustained enzymatic activities for a long time. After evaluation of this novel amperometric biosensor, we propose an effective and convenient method for HbA1c detection in healthy and diabetic patient's medical treatments.

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