Effect of immobilization technique on performance ZnO nanorods based enzymatic electrochemical glucose biosensor

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Abstract: In this paper, ZnO Nanorods (ZNR) have been synthesized over Platinum (Pt) coated glass substrate with in-situ addition KMnO₄ during hydrothermal growth process. Significant variation in ZnO nanostructures was observed by KMnO₄ addition during the growth. Glucose oxidase was later immobilized over ZNRs. The as-prepared ZNRs were further utilized for glucose detection by employing amperometric electrochemical transduction method. In order to optimize the performance of the prepared biosensor two different immobilization techniques i.e. physical adsorption and cross linking have been employed and compared. Further investigations suggest that immobilization via cross linking method resulted in the improvement of the biosensor performance, thereby significantly affecting the sensitivity and linear range of the fabricated biosensor. Among the two types of biosensors fabricated using ZNR, the best performance was shown by cross linked electrodes. The sensitivity for the same was found to be 17.7 mA-cm⁻²-M⁻¹, along with a wide linear range of 0.5-8.5 mM.

Keywords – Physical Adsorption, Cross linking, ZnO Nanorods, Glucose biosensor.

1. Introduction

Biosensors have huge applications in wide range of fields from healthcare, environmental monitoring to industries. It can be defined as a device which perceives bio-recognition event for detection of analyte carried out by biomolecules immobilized over support matrix. The output signal of such events is proportional to the analyte concentration [1]. Among the different categories of biosensors, enzymatic glucose biosensor has attracted interest of researchers due to its importance in diabetes care, beverage and food industry, drug discovery and analysis [2]. The body cells utilize glucose both as a source of energy and as a metabolic intermediate in the synthesis of more complex molecules such as fat [3]. Diseases like diabetes mellitus are result of imbalance in blood glucose levels. Owing to poor lifestyle and the resulting obesity thereby, has led to tremendous increase in the number of patients suffering from diabetes in the last few decades which has led to high demand for reliable point of care testing devices. In this regard, nanostructures of semiconductor metal oxides such as ZnO, SnO₂, V₂O₅, and TiO₂ etc. have been extensively explored in development of biosensors due to their multifunctional properties [4, 5]. Morphology of these materials in reduced dimensions has strong influence over its aspect ratio and electrical characteristics which in turn are beneficial for its usage as a support matrix. Amid all these, ZnO has been proven to be a good candidate as support matrix for immobilization of enzymes because of its low toxicity, biocompatibility, fair thermal and oxidation stability, high electron mobility, high isoelectric point (IEP=9.5) and amicable tunability into wide variety of nanostructures [6-8]. ZnO is a wide band gap (E₉ 3.3 eV at 300K) II-VI compound semiconductor with high exciton binding energy of 60 meV at room temperature [9]. ZnO can be grown in a variety of morphologies viz. nanorods, nanowires, nanobelts, nanosheets, nanotubes, etc. by different physical (chemical vapor deposition, pulsed laser...
deposition, spray pyrolysis etc.) and solution based (hydrothermal, sol-gel, electrochemical etc.) fabrication techniques [10-12].

Biosensors utilizing ZnO as support matrix for enzyme immobilization have been explored exhaustively in literature for detection of glucose, urea, cancer cells, cholesterol etc. [13-17]. Enzyme immobilization refers to confinement enzymes to the support matrix with retained catalytic activity. The major components of enzyme immobilization are enzyme, support matrix and mode of attachment. The mode of attachment can be broadly classified into four categories i.e. physical adsorption, covalent Binding, co-entrapment and cross linking each having its own merits [18]. The choice of immobilization technique and nanostructure plays key role in performance of biosensors as it directly affects enzyme loading which eventually is responsible for improvement in biosensors performance. ZnO thin films and a wide variety of ZnO nanostructures have been used for glucose biosensing applications. However owing to low aspect ratio of ZnO thin films, nanostructures such as nanorods, nanocombs, nanotubes etc. are preferred over the same for improved performance of biosensor. As high aspect ratio leads to higher enzyme loading which holds key to improved biosensor performance. In literature rf-sputtered ZnO thin films have been reported where sensitivity was obtained around 0.5-1.3 mA-cm$^{-2}$-M$^{-1}$ [19]. Unlike this, ZnO nanostructures have been found to have improved sensitivities around 12-15 mA-cm$^{-2}$-M$^{-1}$ [20].

In this work, an electrochemical enzymatic glucose biosensor has been analyzed employing two different types of immobilization methods viz. physical adsorption and cross-linking. Glucose oxidase (GO$_x$) was used for amperometric detection of glucose. ZNRs synthesized by hydrothermal growth technique were utilized as a support matrix for immobilization of GO$_x$. It is a cost effective and low temperature process which enables easy large scale synthesis of ZnO nanostructures. The high IEP feature of ZnO enables physical adsorption of GO$_x$ over it by electrostatic binding while cross linking has been performed by using glutaraldehyde and bovine serum albumin as a cross linking agent and stabilizer respectively [13, 21]. The two techniques were compared here towards optimization of biosensor performance.

2. Experimental Section

2.1 Materials

Glucose oxidase from Aspergillus niger (E.C. 1.1.3.4), D-(+)-glucose, Glutaraldehyde, Bovine serum albumin, Na$_x$ion (5 wt % in water and lower aliphatic alcohol), anhydrous Zinc Acetate, Ethanolamine, 2-methoxy ethanol, Zinc Nitrate Hexahydrate (Zn(NO$_3$)$_2$.6H$_2$O), Hexamethylenetetramine (C$_6$H$_{12}$N$_4$), KMnO$_4$, Sodium phosphate dibasic (Na$_2$HPO$_4$), Potassium phosphate monobasic (KH$_2$PO$_4$), NaCl, Potassium ferricyanide/ferrocyanide [Fe(CN)$_6$]$^{3/-4}$ were used as procured from Sigma Aldrich. All chemicals were of analytical grade and used without any further purification. 0.01M Phosphate buffered saline solution was prepared from Na$_2$HPO$_4$ and KH$_2$PO$_4$ with 0.137 mM NaCl, and pH was adjusted to 7.4. Glucose stock solution was kept overnight for mutarotation after preparation, in order to establish equilibrium concentration between $\alpha$ and $\beta$ anomers. De-ionized (DI) water (>18 MΩ) obtained from Milli-Q purifying system was used in preparation of all the solutions.

2.2 Synthesis of ZNR, enzyme immobilization and quantification

Glass samples cut into 1 cm x 1 cm size were cleaned by ultrasonication process in acetone, isopropyl alcohol and DI respectively followed by drying. After cleaning Platinum (Pt) was sputtered with a 5 nm underlying buffer layer of Cr, at base pressure of 1x10$^{-6}$ mbar. Cr buffer layer was incorporated to improve adhesion of Pt over glass [22]. Samples were rotated at a speed of 10 revolutions per minute (rpm) to ensure uniform deposition of Pt over it. During the growth process, initially seed layer was coated over Glass/Cr:Pt samples followed by hydrothermal growth of ZNRs as reported in our previous work [23]. In summary, an equimolar solution of Zinc acetate and ethanolamine in 2-methoxy ethanol has been spun coated followed by annealing for seed layer preparation. Then, seed layer deposited samples
were kept for growth of ZNR in 0.1M equimolar solution of Zinc nitrate and Hexamethylenetetramine (HMTA) with 5 mM KMnO₄ as additive. On completion of growth, samples were cleaned thoroughly with DI followed by drying to remove any residual salt present.

Finally after growth of ZNR two types of electrodes were prepared using physical adsorption and cross linking immobilization method. In physical adsorption, 5µl solution of 20 mg/ml GOx prepared in PBS was dropped over ZNR and kept in incubator for 24 h. After this GOx immobilized ZNR was washed with PBS to remove unadhered enzymes from the electrode. Further, 0.5% Nafion solution was drop casted over it and kept overnight for drying. However for employing cross linking process, 100 µL GOx solution, 50 µL (2.5 %) glutaraldehyde and 50 µL (0.5 %) Nafion solution was mixed thoroughly. After this 4 µL of the as prepared solution was drop casted over electrode and allowed to dry at room temperature. Finally, 2 µL 0.5 % Nafion solution was coated to avoid leaching of enzymes. After drying of nafion solution electrodes were cleaned for removal of unadhered GOx molecules. Both the type electrodes were kept in incubator at 4 °C when not in use [21].

An aromatic residue namely tryptophan is present in each GOx molecule which contributes to an intrinsic fluorescence in it. Therefore GOx solution when excited at 280 nm gives fluorescence emission at 330 nm. Emission intensity at 330 nm is proportional to the amount of GOx present in a solution [2]. This property of GOx has been well utilized for determining amount of immobilized enzymes over support matrix i.e. enzyme loading. The linear calibration curve for known amount of GOx with its corresponding fluorescence intensity was later obtained. PBS in which each type of electrode has been cleaned after immobilization and solution was collected separately for taking fluorescence. A known amount of GOx i.e. 100 µg has been immobilized over support matrix. Thus amount of unadhered GOx can be determined by fluorescence intensity of collected solution and calibration curve. Finally an estimate of the GOx loaded over support matrix can be made from the difference in the values of the two.

2.3 Characterization and measurement

Morphology and structures of ZnO dumbbells and ZNR were studied by FESEM (Sigma Supra™ 55; Carl Zeiss). The crystalline structure and phase transformations were analyzed by powder XRD (Rigaku Smart Lab® System) with Cu Kα radiation operating at 40 kV and 40 mA, using Cu Kα radiation (λ = 1.5418 Å) in 2θ range from 20° to 80°. The PL spectrometer (Dongwoo Optron DM 500i) having an excitation source consisting of a continuous wave He-Cd laser (excitation wavelength, 325 nm, PMT detector) was used to measure the PL emission from these samples. Fluorescence emissions were obtained using FluoroMax-4p Spectrofluorometer from Horiba Jobin Yvon (Model: FM-100). Excitation and emission slit widths were set at 2 nm each and the samples were excited at 280 nm. Image processing toolbox of MATLAB has been used to study length and diameter distribution of as prepared ZNR. Electrochemical measurements were performed by electrochemical work station (Autolab PGSTAT 302N) using three electrode cell configuration with Ag/AgCl and Pt foil electrode as reference and counter electrode respectively.

3. Results and discussion

3.1 Surface Morphology and Structural Characterization –

The morphology of obtained ZnO nanostructures were analyzed by FESEM which is a non-contact mode technique generally used for surface characterization of films. Figure 1(a) shows the image of ZnO microstructures which were grown in pristine solution i.e. without addition of KMnO₄. The microstructures in form of dumbbells were observed with the growth in all the directions. Apart from this grain boundary was observed to be in the middle of microstructures which were ranging from 3 µm to 6 µm [25]. Figure 1 (b-c) shows the top view and cross sectional view of ZNR grown with the addition of 5
mM KMnO₄. The as prepared nanorods were found to have average diameter and length distribution to be around 130 nm and 530 nm, respectively. The nanorods were uniformly distributed over the entire substrate. The mechanism of growth of ZNR with KMnO₄ addition has been discussed in detail in our previous report [23]. Briefly, presence of additive has strongly influenced hydrolysis of HMTA and ceased formation of dumbbells, while the oxygen rich environment in the nutrient solution has led to the passivation of the defects.

**Figure 1.** FESEM images of (a) ZnO dumbbells, (b) top view and (c) cross sectional view of ZNR.

The XRD pattern for as prepared ZnO dumbbells and ZNR grown with 5 mM KMnO₄ is shown in Fig. 2. Both ZnO dumbbells and ZNR were found to consist of all the diffraction peaks corresponding to ZnO wurtzite phase (space group P63mc, JCPDS – 36 - 1451). An extra peak indexed to Pt coated over glass substrate at 2θ = 40° indexed. The c-axis (002) peak intensity was observed to enhance on addition of KMnO₄ since presence of strong oxidizing agent acts as a driving force for anisotropic growth which provides an oxygen rich environment. There was no impurity or intermediate formation during growth in the sample as no peak other than ZnO was present within the detection limit of the instrument. The as prepared dumbbells and ZNR were well crystalline owing to sharp and intense diffraction peaks.

**Figure 2.** XRD pattern for ZnO dumbbells and ZNR.

**Figure 3.** Room temperature PL spectra for ZnO dumbbells and ZNR.

### 3.2 Optical Characterization

The optical characterization of ZnO dumbbells and ZNR was performed by the room temperature Photoluminescence (PL) measurements. Generally PL of ZnO consists of two peaks i.e. Near band edge emission (NBE) in ultra violet region and defect level emission (DLE) in visible region. The NBE corresponds to excitonic transitions around band gap of ZnO. The broad peak associated with DLE originates by presence of more than one type of defects [26, 27]. The actual reason behind DLE is still not fully understood. The normalized PL spectra for the two types of nanostructures obtained with and
without addition of KMnO$_4$ have been shown in Fig. 3. It consists of two peaks; a sharp NBE around 385 nm and a broad DLE ranging from 450 nm to 750 nm centered around 580 nm. It is interesting to note that DLE got suppressed by incorporation of KMnO$_4$ during the growth which creates an oxygen rich environment [23]. Besides this a blue shift in UV peak was observed in samples grown with additive due to Burstein-Moss effect. In n-type semiconductors, when carrier concentration reaches beyond a limit, Fermi level moves into the conduction band due to filling of conduction band by electrons [28-30]. PL study has revealed a clear repression of defects by KMnO$_4$ addition.

3.3 Enzyme quantification

Enzyme loading was analyzed by optical characteristic of GO$_x$ molecules due to the presence of tryptophan which is responsible for intrinsic fluorescence in it. Aspect ratio has been calculated as ratio of average length to average diameter and was found to be 4.2 for as prepared ZNR. Enzyme loading depends on the surface to volume ratio available for immobilization of biomolecules and mode of immobilization. Enzyme loading was measured to be 425 µg-cm$^{-2}$ and 575 µg-cm$^{-2}$ for physical adsorption and cross linking immobilization method respectively. Physical adsorption utilizes electrostatic attraction between GO$_x$ molecules (IEP=4.5) and ZnO (IEP=9.5) at neutral pH value while in case of cross linking, enzymes join in 3D structure by covalent bond formation between enzyme (GO$_x$) and bi-functional cross linking agent (Glutaraldehyde) [2]. Thus, cross linking has resulted in higher enzyme loading as compared to physical adsorption.

3.4 Biosensor Characterization

3.4.1 Cyclic Voltammetry Studies

All the electrochemical experiments were performed using three electrode cell configuration. Glass/Cr:Pt/ZNR/GOx/Nafion, Ag/AgCl and Pt foil were utilized as working, reference and counter electrode respectively. PBS with 0.01 M concentration and pH=7.4 was used as electrolyte solution. The electrochemical characterization of different biosensors was investigated by Cyclic Voltammograms (CV) to study catalytic oxidation of glucose. CV was recorded in an un-stirred PBS solution at scan rate of 100 mV/s swept from -0.2V to 0.8V. Figure 4 (a) shows CV for the cross linked ZNR electrodes in absence and on subsequent addition of glucose. As compared to the background in absence of glucose a sharp increase in the anodic current for cross linked electrode in presence of glucose has been observed. It was clearly evident from the CV that anodic oxidation current has increased significantly and cathodic reduction current was suppressed indicating catalytic oxidation of glucose. For cross linked GO$_x$ electrodes equal amount of glucose was injected from 3 mM to 12 mM with an increment of 3 mM. It was noticeable from it that almost same increment in anodic current was observed till 9 mM glucose injection which suggests that the biosensor exhibits extended linear range of operation. Further CV was obtained for the two types of electrodes in presence of 3 mM glucose in PBS as shown in Fig. 4 (b). The anodic current was significantly enhanced in case of cross linked electrodes as compared to physically adsorbed electrodes owing to the fact that enzyme loading has increased. Further to explore findings obtained by CV, amperometric biosensor response was recorded.
3.4.2 Amperometric response

Amperometric detection of glucose was used because of its simplicity and accuracy. Current response measurement was performed for the two types of electrodes which were prepared by employing different immobilization techniques. In the catalysis mediated by GOx glucose gets oxidized into gluconic acid and the enzyme itself gets reduced. Each GOx molecule has an active site i.e. Flavine adenine dinucleotide (FAD) buried into its protein shell which gets reduced into FADH2 during oxidation of glucose. The enzyme is re-oxidized by the molecular oxygen resulting in evolution of H2O2. The released H2O2 is quantitatively detected by application of potential to the working electrode as shown in Eqs. (1) - (3). Thus, oxidation of H2O2 by applied potential leads to flow of current in the electrochemical cell proportional to the glucose concentration [31].

\[
\begin{align*}
C_6H_{12}O_6 + Gox(oxd) + H_2O &\rightarrow C_6H_{12}O_7 + Gox(red) \\
Gox(red) + O_2 &\rightarrow Gox(oxd) + H_2O_2 \\
H_2O_2 &\xrightarrow{0.8V} 2H^+ + 2e^- + O_2
\end{align*}
\]

Current density increases on injection of glucose due to generation of electrons. After this for each response calibration curve was obtained which relates the current density to the amount of glucose concentration injected into electrolyte solution. It is hyperbolic in nature which mainly consists of two parts i.e. linear and saturation region. Initially active sites of enzymes were unoccupied and an instantaneous rise in current on addition of glucose results in linear region of calibration curve. While slowly all the active sites were occupied by glucose molecules eventually heading towards saturation with the increase in glucose concentration in the solution. No further increment in the current was observed upon injection of glucose into the electrolyte solution, once saturation was reached.
Figure 5. Amperometric response showing current density variation \textit{viz} time for biosensors fabricated via physical adsorption of GO\textsubscript{x} and cross linking of GO\textsubscript{x}.

Figure 6. Calibration curves corresponding to as prepared biosensors.

The typical amperometric responses were obtained at an applied potential of 0.8 V for the two types of electrodes prepared by physical adsorption and cross linking of enzymes. The electrolyte solution was continuously stirred to provide convective transport during electrochemical measurement. Figure 5 shows the typical amperometric responses obtained for the same. 0.5 mM glucose has been introduced into the cell at every 25 s for both the electrodes. It has been clearly observed that upon successive injection of glucose current density increases very quickly and saturates resulting in a clear step like response which corresponds to the typical enzyme kinetics behavior as evident from Fig. 5. This indicates that enzymes have been immobilized properly with retained activity over ZNR support matrix. Response time has been defined as the time required by current density to increase by 90 % after addition of glucose [31]. Fast and immediate rise was observed with response time to be around 5-6 s for the two. Moreover calibration curves for two electrodes have been compared and shown in Fig. 6. Sensitivity and linear range was computed from the slope of calibration curves obtained from amperometric responses. Linear range of operation was calculated by linear regression analysis of calibration curves. Sensitivity was calculated to be 12.5 mA-cm\textsuperscript{-2}-mM\textsuperscript{-1} with a poor linear range of operation of 0.5-3 mM for electrodes prepared using physical adsorption mode of immobilization. In contrast, sensitivity was enhanced with a wider linear range of operation for cross linking immobilization method which was computed to be 17.7 mA-cm\textsuperscript{-2}-mM\textsuperscript{-1} and 0.5 – 8.5 mM respectively. This improvement in figure of merits of biosensor has been attributed to increased enzyme loading due to cross linking mode of immobilization. Cross linking increases amount of enzyme by covalent bond formation with cross linking agent. Table 1 illustrates performance of the two types of biosensors examined.

| Electrode          | Sensitivity (mA-cm\textsuperscript{-2}-M\textsuperscript{-1}) | Response Time (s) | Linear Range (mM) |
|--------------------|---------------------------------------------------------------|-------------------|-------------------|
| Physically adsorbed GO\textsubscript{x} | 12.5                                                        | 5-6               | 0.5-3              |
| Cross linked GO\textsubscript{x}    | 17.7                                                        | 5-6               | 0.5-8.5            |

Table 1. Comparison of the two types of electrodes.
3.4.3 Selectivity

Selectivity of a sensor has been defined as the ability of a sensor to detect a specific analyte of interest. High selectivity is necessary for accurate measurement of analyte. Enzymatic reaction has been widely described by lock and key model in literature and thus enzymes are highly specific towards the substrate \[6, 7\]. Due to this, it has been utilized widely for biosensing applications.

3.4.4 Interference Study

The human serum contains many electroactive species other than glucose such as Uric acid, Ascorbic acid etc. which may interfere with the biosensor performance while detecting glucose. Therefore it is necessary to evaluate anti interference ability of biosensor. Here anti interference capability of as fabricated cross linked GOx biosensor was examined by initially injecting 5 mM glucose in the electrochemical cell setup followed by addition of 0.4 mM Uric acid and 0.1 mM Ascorbic acid sequentially. The amount of the three species added into the cell was kept same as the physiological concentration of it in the human body. Figure 7 shows the corresponding amperometric current response of the electrode. As visible from Fig. 7 biosensor almost immediately acquired the steady state after glucose injection while it does not respond to uric acid addition and 4.2 % increment with respect to the current density for 5 mM concentration of glucose was observed for ascorbic acid addition. This anti interference capability of biosensor can be attributed to selectivity of enzymes and presence of nafion as a semipermeable membrane.

![Figure 7. Interference effect study for cross linked GOx biosensor](image)

4. Conclusions

In summary a simple method involving in situ addition of KMnO4 during hydrothermal growth process was utilized to cease formation of dumbbells in nutrient solution and for growth of ZNR. Highly crystalline ZNR with largely suppressed defects was obtained for studying the biosensor performance. The two types of immobilization techniques viz physical adsorption and cross linking have been analyzed and compared. The biosensor figures of merit were found to improve by incorporation of cross linking as immobilization method. Sensitivity of biosensor has increased from 12.5 mA-cm\(^{-2}\)-M\(^{-1}\) for physically adsorbed GOx to 17.7 mA-cm\(^{-2}\)-M\(^{-1}\) for cross linked GOx. Apart from this linear range has enhanced significantly from 0.5-3 mM to 0.5-8.5 mM for physically absorbed and cross linked GOx respectively. Since the support matrix (ZNR) utilized was same for both the electrodes response time, was determined to be equal i.e. 5-6 s. The electrode fabricated employing cross linking mode of immobilization has been found to outperform over physically adsorbed GOx electrode in terms of sensitivity and linear range along with good anti interference capability. Further improvement in aspect ratio of ZNR can be utilized for achieving even better figure of merits for the as prepared biosensor.
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