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Research Article

A Novel ZnO-Methylene Blue Nanocomposite Matrix for Biosensing Application

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A novel hybrid matrix of zinc oxide-methylene blue (ZnO-MB) has been successfully developed for biosensing application. The introduction of methylene blue into the ZnO thin film leads to reduction in the charge transfer resistance and suggests an increase in the electron transfer capacity of the composite. Glucose oxidase (GOx) was chosen as the model enzyme and effectively immobilized on the surface of hybrid ZnO-MB nanocomposite matrix. Electrochemical measurements were employed to study biosensing response of the GOx/ZnO-MB/ITO bioelectrode as a function of glucose concentration. The low oxidation potential (−0.23 V) of the hybrid bioelectrode, in a mediatorless electrolyte, makes it resistant against interference from other bio-molecules. The low value of Michaelis-Menten constant (2.65 mM) indicates that immobilized GOx retains its enzymatic activity significantly on the surface of nanocomposite hybrid matrix that results in an enhanced affinity towards its substrate (glucose). The ZnO-MB nanocomposite hybrid matrix, exhibiting enhanced sensing response ($0.2\,\mu AmM^{-1}cm^{-2}$) with long shelf-life (>10 weeks), has potential for the realization of an integrated biosensing device.

1. Introduction

Though the last decade has witnessed a fast growth in the research, development, and marketing of biosensors still no clear unanimity has been made in favor of specific biomatrix [1–7]. The modern day's biosensors combine the natural sensitivity and specificity of complimentary bio-molecules with the advantages of microelectronics, through a suitable biomatrix, for realization of lab-on-chip device [1]. Zinc oxide (ZnO), a wide band-gap semiconductor, is recently grabbing attention of the scientific community for its application in the field of biosensors due to its novel properties like high isoelectric point (IEP) and biocompatibility. Owing to its high IEP the surface of ZnO matrix can adsorb the biocatalysts having low IEP (~4.2 for glucose oxidase) via electrostatic interaction. The possibility of growing large-area thin films of ZnO nanostructures on variety of materials at a relatively low cost led to a good progress in development of ZnO-based biosensor [8–12]. Recently, we have confirmed that ZnO provides direct charge transfer to immobilized enzymes and they retain their bio-activity on its surface [10]. However, due to the absence of a redox couple in ZnO, the amperometric biosensors have to depend on detecting the oxidation of H_2O_2 produced during the reactions. It is important to note that the oxidation potential of H_2O_2 is high enough to cause interference in the electrical signal, from other bio-molecules present in blood serum and thereby hinder the miniaturization of biosensor. Moreover, the ZnO-based sensors require an electron transfer reagent (mediator) in the electrolyte. Therefore, a novel biomatrix is essentially required for lab-on-chip device that can be utilized for effective detection of bio-molecules.

The excellent redox properties of methylene blue (MB), a cationic dye, and its low formal potential (E° = 0.08–−0.25 V in solution with pH 2–8) is good enough a reason for its exploitation, as a mediator, in biosensors. Therefore the synthesis of a hybrid matrix exploiting the excellent properties of ZnO along with the redox species may lead to development of an interference-free biosensor. Few biosensors have been developed, where biomolecules are

immobilized along with the mediators, on the surface of the matrix [13–15]. However, due to the low molecular weight and good solubility in water the mediators have a tendency to leach out and thereby destabilizing the bio-electrode [16]. This could be overcome by a composite matrix having the mediator embedded in it. In the present study, we have developed a nanocomposite biomatrix of ZnO with methylene blue (ZnO-MB) and successfully demonstrated the interference-free and effective glucose sensing. The low value of Michaelis Menten Constant (K_m^{app}) and a fairly interferenceless system has indicated the importance of the prepared matrix in the field of amperometric biosensors.

2. Experimental

2.1. Materials. GOx (200 U/mg), horseradish peroxidase (HRP, 200 U/mg), *o*-dianisidine, and glucose were purchased from Sigma-Aldrich. Sodium phosphate monobasic anhydrous and sodium phosphate dibasic dihydrate were obtained from Sisco chemical, India. Lithium hydroxide monohydrate and zinc acetate monohydrate were procured from Thomas Baker, India. MB was acquired from Merck & Co. Inc. All chemicals were used without further purification. Deionized water was used in the preparation of aqueous solutions.

2.2. Preparation of Solutions. Phosphate buffer saline (PBS) 50 mM of pH 7.0 (0.9% NaCl) solution was prepared by adjusting the proportion of monobasic sodium phosphate solution and dibasic sodium phosphate solution and then adding 0.9% NaCl to the solution. GOx (1 mg/mL) solution and HRP solution (1 mg/mL) were freshly prepared in PBS buffer of pH 7.0. Different concentrations of glucose solution and solution of *o*-dianisidine (1%) were freshly prepared in deionized water.

2.3. Preparation of ZnO-MB Film and Immobilization of GOx. The ZnO nanoparticles were first prepared by a wet chemical route, as reported by Yadav et al. [17]. The obtained ZnO nanoparticles are redispersed in ethanol. To obtain ZnO-MB sol, 0.1 mL of the MB solution (10 mg/mL in ethanol) is added to 1 mL of redispersed ZnO sol. The ZnO-MB/ITO electrodes were prepared by spin-coating on ITO-coated glass plates with the ZnO-MB sol.

Immobilization of GOx onto the ZnO-MB matrix was achieved via electrostatic interaction of positively charged ZnO and negatively charged GOx enzyme at 7.0 pH. For GOx immobilization, 30 μL of the freshly prepared GOx solution was dropped on the surface of ZnO-MB composite thin film and was kept at 4°C overnight followed by extensive washing with buffer to remove any unbound GOx. The bioelectrodes were dried under dry nitrogen flow and kept at 4°C when not in use.

2.4. Measurement and Apparatus. The ZnO-MB/ITO electrode was characterized by UV-visible, fourier transform infrared (FTIR), and electrochemical impedance spectroscopy techniques. FTIR studies were further carried out on the GOx/ZnO-MB/ITO bio-electrode to confirm the immobilization of GOx enzyme. The sensing response studies of the bio-electrode were carried out using cyclic voltametry (CV) techniques. The apparent enzyme activity was studied by enzymatic photometric assay. CV measurements were carried out on a Potentiostat/Galvanostat (Gamry Instruments Inc.) using a three-electrode cell configuration with Ag/AgCl electrode as a reference electrode and platinum foil as a counter electrode in 10 mL of phosphate buffer saline (PBS) solution (50 mM, pH 7.0, 0.9% NaCl). Electrochemical impedance spectroscopy (EIS) studies were made in PBS solution (pH 7.0) containing 5 mM $Fe(CN)_6^{3-/4-}$. The apparent enzyme activity of bioelectrode was studied using UV-visible spectrophotometer (Perkin Elmer lambda 35). For the photometric assay bioelectrode was dipped in the 3 mL PBS solution containing 20 μL of dye (*o*-dianisidine, 1% in H_2O), 50 μL of HRP and 100 μL of substrate (glucose). After 1 minute of incubation of bio-electrode the absorbance corresponding to the oxidation of *o*-dianisidine was noted at 500 nm for monitoring the enzyme kinetics.

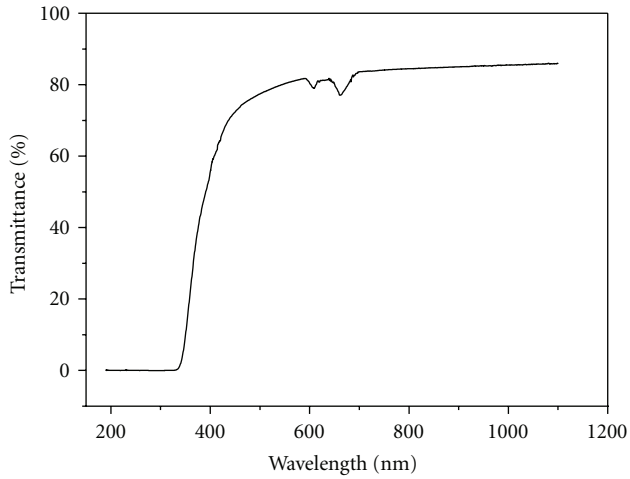


Figure 1: UV-visible spectrum of the ZnO-MB matrix.

3. Results and Discussion

3.1. UV-Visible Studies. ZnO-MB film was deposited on a fused quartz slide for optical characterization. UV-Visible spectra (Figure 1) confirms the deposition of a thin film having high transmission (>80%) in the visible region. The band gap estimated from the fundamental absorption edge (using Tauc plot) was found to be 3.61 eV. The estimated value of the bandgap is much higher than the corresponding value (~3.3 eV) reported for bulk ZnO [18] and may be attributed to the quantum confinement effect which comes into the picture due to the small size of the synthesized ZnO nanoparticles (~4 nm). It is important to point out that two small dips in the transmittance spectra are seen at 610 nm and 660 nm and are attributed to the presence of methylene blue in the prepared composite matrix [16].

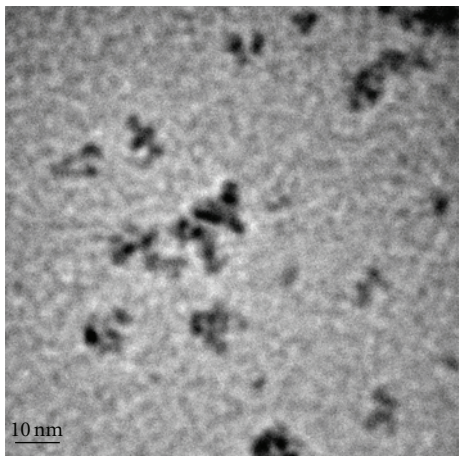


Figure 2: TEM image of the ZnO-MB nanoparticles.

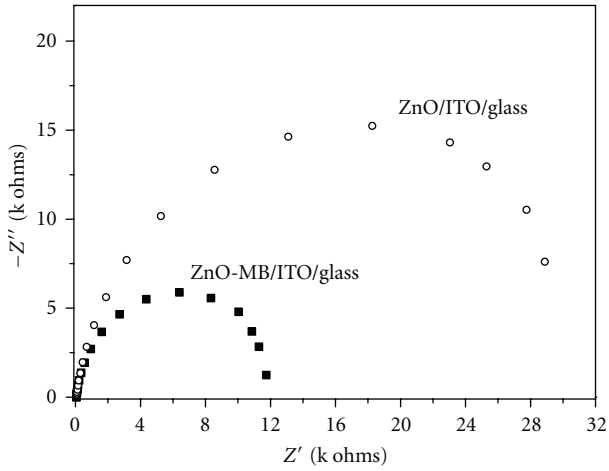


Figure 3: Impedance spectra of the pure ZnO and composite ZnO-MB thin films.

3.2. TEM Analysis of the ZnO-MB Sol. The transmission electron microscope (TEM) image (Figure 2) of the ZnO-MB nanoparticles shows the synthesis of spherical shape and monodispersed nanoparticles with average size of about 4 nm. MB can be seen congregated on the ZnO nanoparticles.

3.3. Electrochemical Impedance Studies. Electrochemical impedance spectroscopy (EIS) study was carried out, on both ZnO-MB thin film and ZnO thin film, to have an insight into the effect of methylene blue on the charge transfer behavior of ZnO in nanocomposite biomatrix. Figure 3 shows the impedance spectra of ZnO/ITO/glass and ZnO-MB/ITO/glass electrodes. The impedance plots are semicircular in shape for both the electrodes. The diameter of the semicircle was found to reduce with incorporation of MB in the ZnO matrix. The plot can be modeled by an equivalent electrical circuit consisting of a resistance (R_S) in series with parallel combination of a capacitance (C_p) and a resistance (R_{CT}) [19]. The value of R_{CT} for ZnO/ITO electrode was found to be 30.6 kΩ which decreased to 12.1 kΩ for the composite ZnO-MB/ITO electrode. The reduction in the value of R_{CT} clearly indicates that the presence of mediator (MB) in the ZnO matrix plays an important role in increasing the charge transfer capacity of the ZnO biomatrix.

3.4. FTIR Studies. The FTIR studies on the ZnO-MB film (Figure 4(a)) confirm the formation of a hybrid composite matrix. In Figure 4(a) the absorption peak observed at 452 cm^{-1} corresponds to the E2 mode of wurtzite ZnO [20]. The appearance of absorption bands at 1574 cm^{-1}, 1413 cm^{-1}, and 1340 cm^{-1} are due to the presence of MB in matrix [16]. The presence of modes corresponding to both the ZnO and MB, in the FTIR spectra, clearly shows that the MB has formed a composite with ZnO without disturbing its lattice structure. FTIR of the GOx/ZnO-MB bio-electrode (Figure 4(b)) shows additional absorption mode corresponding to the characteristic band of protein at 1655 cm^{-1}, assigned to amide II, confirming the immobilization of GOx on the surface of composite (ZnO-MB) matrix [21].

3.5. CV Studies. Cyclic voltammograms of the ZnO-MB electrode at different scan rates (0.01 V/s to 0.08 V/s) are shown in Figure 5(a). A well-defined redox peak was obtained in reagentless phosphate buffer saline (PBS) solution using ZnO-MB/ITO electrode. The oxidation peak is seen at −0.23 V and can be attributed to the oxidation of MB in the nanocomposite biomatrix. However the peak is slightly shifted to negative potential as compared to the oxidation potential of MB (−0.20 V) suggesting good catalytic behavior of the system [9]. The CVs remained essentially unchanged on consecutive scanning thereby ruling out the possibility of any leaching effect of mediator (MB) from the composite (ZnO-MB) matrix as reported previously by other workers [16]. The anodic ($I_p{}^a$) and the cathodic ($I_p{}^c$) peak current are found to vary linearly with potential scan rate (inset of Figure 5(a)) indicating improved electrocatalytic behavior of the prepared system and demonstrating that the hybrid composite matrix is suitable for biosensing applications.

Immobilization of GOx enzyme onto the ZnO-MB/ITO electrode resulted in the decrease of oxidation current in CV (inset of Figure 5(b)) and is attributed to the effective binding of GOx, which is a protein having macromolecular structure of nonconducting nature. Brown-Anson model (1) has been used to calculate the value of surface concentration of redox species for ZnO-MB/ITO and GOx/ZnO-MB/ITO electrodes

$$I_p = \frac{n^2F^2I^*Av}{4RT}. \quad (1)$$

I_p is the peak current, n is the number of electrons transferred, F is the faraday constant, I^* is the surface concentration, A is the surface area of the electrode, v is the scan rate, R is the gas constant, and T is the absolute temperature [22]. The surface concentration of redox species in the GOx/ZnO-MB/ITO bio-electrode (3.46×10^{-11} mol/cm^2) was found to be higher than the corresponding value obtained for ZnO-MB/ITO electrode (2.93×10^{-11} mol/cm^2). An increment

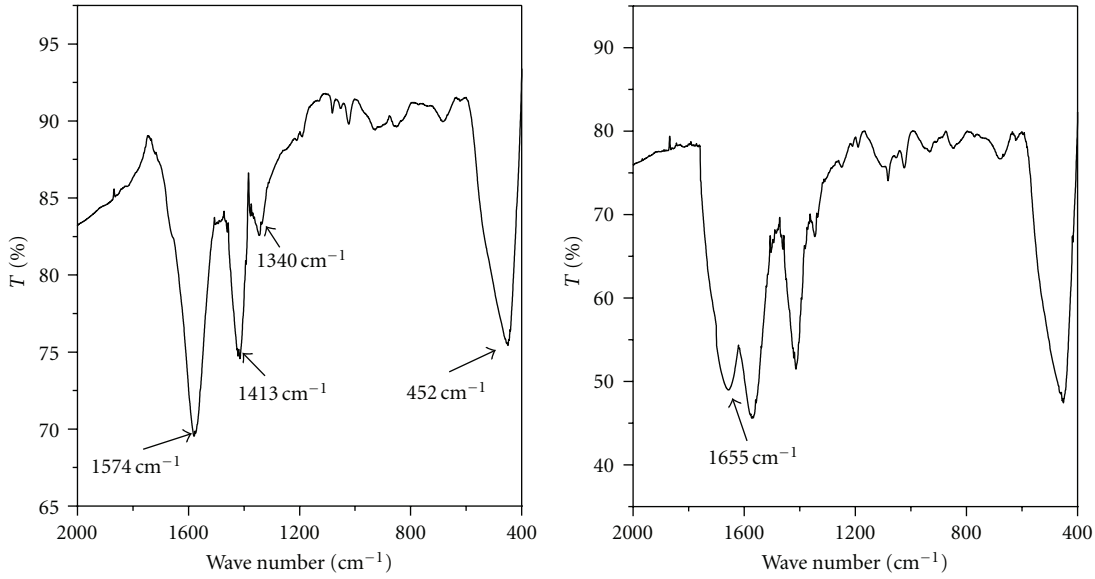


Figure 4: FTIR spectra of (a) ZnO-MB film and (b) GOx/ZnO-MB electrode.

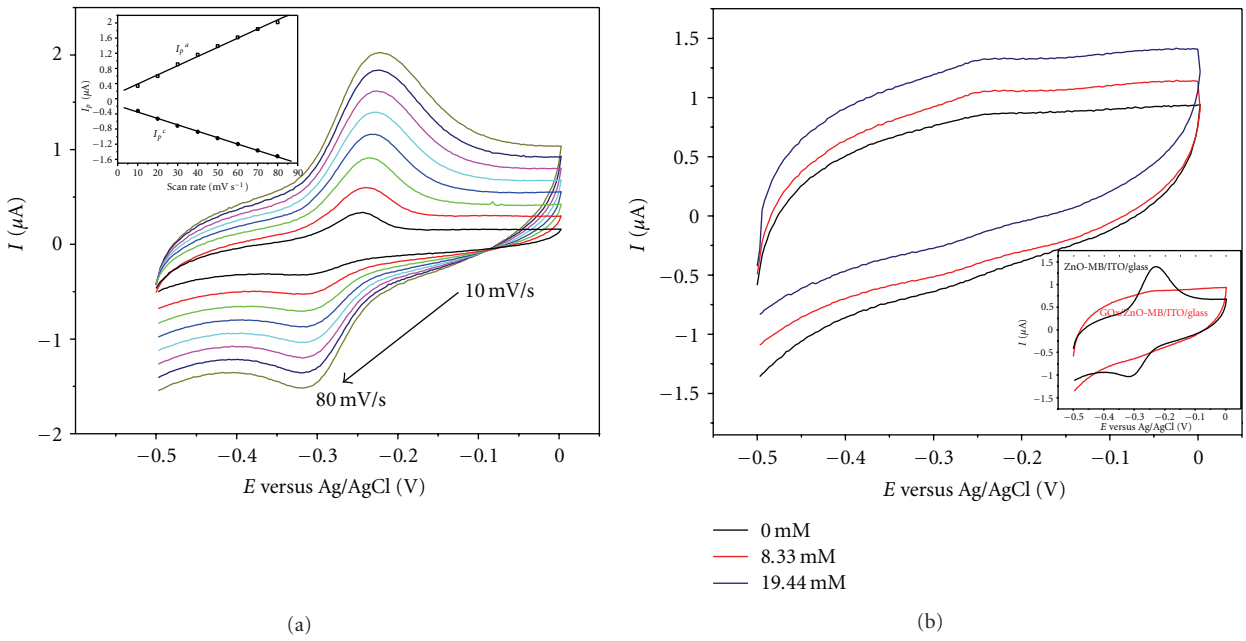
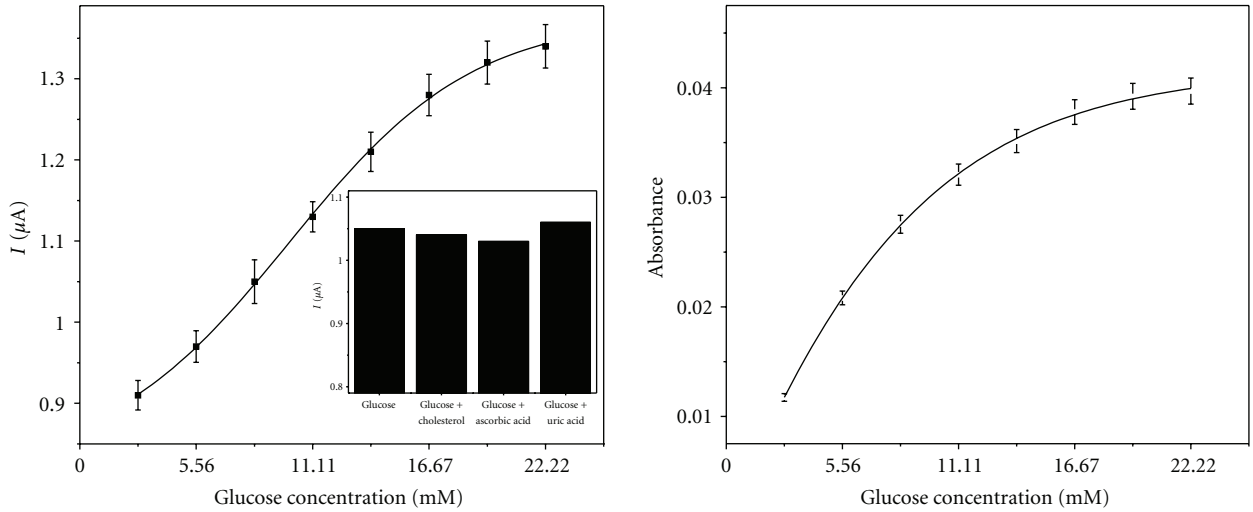


Figure 5: (a) Cyclic voltammograms of ZnO-MB/ITO film at different scan rate (inset shows the variation of anodic and cathodic peak current with potential scan rate) (b) CV of GOx/ZnO-MB/ITO bio-electrode with different glucose concentration (Inset shows CV of ZnO-MB/ITO and GOx/ZnO-MB/ITO electrodes).

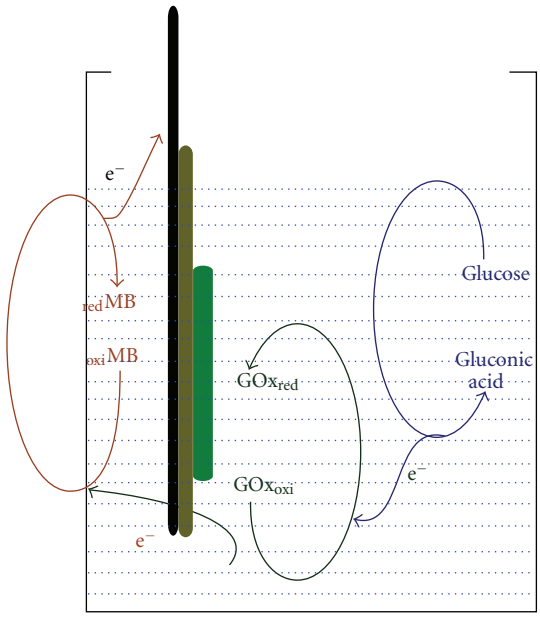
in the number of redox species indicates the activation of the redox centers present in the enzyme being immobilized on the composite hybrid matrix. The oxidation current was found to increase continuously, in the CV, with an increase in the glucose concentration (Figure 5(b)). The GOx oxidizes the glucose in the solution and in the process it gets reduced. The reduced GOx donates the excess electron to the ZnO-MB nanocomposite matrix to reduce the redox species. The MB reoxidizes by transferring the electron to the external circuit due to efficient electron transfer and good redox property of prepared nanocomposite biomatrix (Scheme 1). The increase in current with increasing concentration of glucose is attributed to the increase in the number of released electrons during oxidation of glucose.

Figure 6(a) shows the variation of the current measured for the GOx/ZnO-MB/ITO bio-electrode at a fixed potential of −0.23 V as a function of glucose concentration. The observed linear response upto 16.67 mM indicates that



(a) (b)

Figure 6: (a) Response curve: variation of current at a fixed potential of −0.23 V for GOx/ZnO-MB/ITO bio-electrode with glucose concentration (inset shows the effect of various interferants on the response of the biosensor), (b) Photometric assay of GOx/ZnO-MB bio-electrode.



Scheme 1: The electron transfer process of the matrix.

the prepared bio-electrode can be efficiently used for glucose sensing. The sensitivity estimated from the linearity curve is found to be 0.2 μAmM^{-1}cm^{-2}. The results of triplicate sets indicated by error bars reveal the reproducibility of measurements within ±2% and show the higher reliability of prepared bio-electrode based on ZnO-MB nanocomposite matrix for glucose detection.

3.6. Estimation of Michaelis-Menten Kinetic Parameters (K_m^{app}). Hanes plot, that is, a graph between (substrate concentration) and (substrate concentration/current), has been employed to estimate the Michaelis-Menten kinetic parameter (K_m^{app}) of enzymatic reaction [3]. The value of K_m^{app} is found to be 2.65 mM for the GOx immobilized on the nanocomposite matrix. The low K_m^{app} value, as compared to free GOx (27 mM), indicates that the immobilized GOx attains favourable conformation for enzymatic reaction on the ZnO-MB/ITO nanocomposite biomatrix that result in enhanced affinity of GOx towards glucose.

3.7. Interference Study. Upon addition of similar concentration of interferants such as cholesterol, ascorbic acid, and uric acid with glucose (8.33 mM) in PBS, the value of the currents at the peak potential varies insignificantly within the error limits only as is shown in the bar graph (inset of Figure 6(a)). The low working potential of the prepared hybrid biomatrix prevents the effect of interferants on the sensing response characteristics and thereby result in an interference-free biosensor.

3.8. Photometric Assay. The photometric assay was carried out to estimate the apparent enzyme activity (Figure 6(b)) and has been calculated using the equation a_{app}^{enz} (Units cm^{-2}) = $AV/\varepsilon ts$, where a_{app}^{enz} is the apparent enzyme activity, A is the difference in absorbance before and after incubation, V is the total volume (3.17 cm^3), ε is the millimolar extinction coefficient (7.5 for *o*-dianisidine at 500 nm), t is the reaction time (min), and s is the surface area (cm^2) of the electrode [3]. The estimated value of apparent enzyme activity of the prepared hybrid biomatrix was about 1.65 × 10^{-2} U cm^{-2}. The photometric assay was further used to carry out the shelf life studies, at regular interval for 10 weeks with 8.33 mM glucose concentration (data not shown). The study indicates that the prepared bioelectrode retain more than 80% of activity even after 10 weeks.

4. Conclusion

The present study shows that the ZnO thin film matrix incorporated with methylene blue is a promising nanocomposite hybrid matrix for biosensor application. The ZnO nanoparticles offer a suitable microenvironment for enzyme immobilization and enhanced electron transfer between the enzyme's active site and the electrode. Due to low oxidation potential (−0.23 V), the hybrid composite matrix is practically immune from interferants in the biological system. Glucose oxidase enzyme has been successfully immobilized on the ZnO-MB nanocomposite films deposited onto ITO coated glass. The CV studies of the prepared bioelectrode (GOx/MB-ZnO/ITO) shows linearity up to 16.67 mM indicating the potential of nanocomposite biomatrix for realization of a interference-free biosensor. The GOx immobilized on the ZnO-MB/ITO electrode displays excellent catalytic property towards glucose which is confirmed by the relatively low value of Michaelis-Menten constant (2.65 mM). The shelf life of the prepared bioelectrode is more than 10 weeks suggesting that the hybrid composite matrix provides an attractive option for fabrication of a stable lab-on-chip biosensing device.

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