Cholesterol biosensor based on rf sputtered zinc oxide nanoporous thin film

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Cholesterol oxidase (ChOx) has been immobilized onto zinc oxide (ZnO) nanoporous thin films grown on gold surface. A preferred *c*-axis oriented ZnO thin film with porous surface morphology has been fabricated by rf sputtering under high pressure. Optical studies and cyclic voltammetric measurements show that the ChOx/ZnO/Au bioelectrode is sensitive to the detection of cholesterol in 25–400 mg/dl range. A relatively low value of enzyme's kinetic parameter (Michaelis-Menten constant) ~2.1 mM indicates enhanced enzyme affinity of ChOx to cholesterol. The observed results show promising application of nanoporous ZnO thin film for biosensing application without any functionalization. © *2007 American Institute of Physics*. [DOI: 10.1063/1.2768302]

Biosensors are becoming essential in the field of healthcare, chemical and biological analysis, environmental monitoring, and food processing industries. Proper immobilization of enzymes on suitable matrix and their stability are important factors in the fabrication of biosensors.[1–7] Nanostructures, because of their exceptional optical and electrical properties due to electron and phonon confinement, are receiving great deal of attention as alternative matrices for enzyme immobilization to improve stability and sensitivity of biosensors.[8–10] Nanomaterials provide high surface area for higher enzyme loading and a compatible microenvironment helping enzyme to retain its bioactivity. Besides this, they provide direct electron transfer between enzyme's active site and electrode.[11–14] Among nanomaterials, zinc oxide (ZnO), a wide band gap semiconductor, has attracted much attention due to wide range of applications.[15–18] ZnO nanostructures exhibit interesting properties including high catalytic efficiency, and strong adsorption ability. Recently, the interest has been focused towards applications of ZnO in biosensors because of its high isoelectric point (9.5), biocompatibility, and abundance in nature.[19] The high isoelectric point of ZnO results in unique property to immobilize an enzyme having low isoelectric point through electrostatic interaction. Furthermore, nontoxicity, high chemical stability, and high electron transfer capability make ZnO as a promising material for immobilization of biomolecules without electron mediator and can be employed for developing implantable biosensors.[18–21]

In this letter, we are reporting the development of cholesterol biosensor based on ZnO material where the immobilization of cholesterol oxidase (ChOx) has been carried out on the surface of ZnO nanoporous thin films deposited by rf magnetron sputtering on gold surface. The ChOx/ZnO/Au bioelectrode has been found to be sensitive to cholesterol, giving a relatively low value of the Michaelis-Menten constant (K_m).

ZnO thin film has been deposited by rf magnetron sputtering technique on gold (Au) coated 7059 corning glass to serve as a matrix for cholesterol biosensor. A 6 in. diameter metallic zinc target (99.99% pure) is sputtered in the reactive gas mixture (50% O_2+50% Ar) at a power of 300 W. The film of thickness about 50 nm is deposited at room temperature under high pressure (50 mTorr) to create native defects that have a significant role in the sensing applications.[9,17] The ChOx enzyme is immobilized onto as-grown ZnO film surface by physical adsorption technique. The 1.0 cm^2 area of ZnO surface is dipped in a solution of ChOx oxidase (1 mg/ml) prepared in phosphate buffer saline (PBS) 50 mM (0.9% NaCl). The electrode is kept overnight for enzyme (ChOx) immobilization and subsequently washed with buffer solution, and dried in nitrogen environment. The prepared ChOx/ZnO/Au electrode is stored at 4 °C when not in use.

X-ray diffraction (XRD) is carried out to identify the crystal structure of ZnO films. The enzyme activity measurements on ZnO film are studied using UV-visible spectrophotometer (Model 160A, Shimadzu). Atomic force microscopy (AFM) using Veeco DICP2 instrument is employed to examine the surface morphology. Cyclic voltammetric measurement has been carried out on an Autolab potentiostat/galvanostat (Eco Chemie, Netherlands) using a three-electrode system in PBS solution containing 5 mM $Fe(CN)_6^{3-/4-}$ with Ag/AgCl as reference electrode. Fourier transform infrared (FTIR) spectra are recorded using Perkin

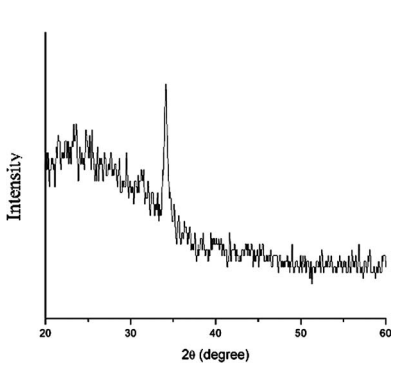

FIG. 1. X-ray diffraction pattern of rf magnetron sputtered ZnO thin films.

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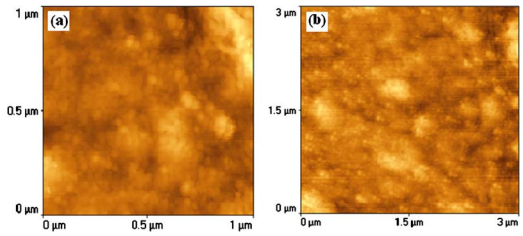

FIG. 2. AFM micrographs of (a) ZnO thin film (1×1 μm) and (b) ChOx/ZnO thin film (3×3 μm).

Elmer (Model Spectrum) spectroscopy to check the binding of ChOx enzyme with ZnO films.

Figure 1 shows the XRD pattern of the ZnO thin film deposited on corning glass substrate. Only (002) reflection at 34.14° is observed in the XRD spectra of ZnO thin film indicating the growth of preferred oriented crystallites with *c* axis normal to the substrate. The grain size of the as-grown film, estimated using Scherer relation was about 36 nm. It may be noted that the position of (002) peak is lower in comparison to reported value for bulk ZnO (34.43°) and is attributed to the presence of stress in the deposited film.[22] The origin of stress may be attributed to the embedded oxygen at the interstitial sites in the ZnO thin film deposited under high sputtering pressure (50 mTorr). The estimated value of the lattice parameter *c* has been found to be 5.2482 Å and is large in comparison to the corresponding bulk value (5.2066 Å), revealing that the unit cell is slightly elongated along the growth direction.[22]

The AFM image of as-grown ZnO thin film [Fig. 2(a)] reveals the formation of rough microstructure having uniformly distributed nano pores. The formation of rough and porous microstructure is attributed to the processing of ZnO thin film under high sputtering pressure. The intensive *in situ* bombardment of energetic species under high pressure is likely to embed large concentration of oxygen at the grain boundaries or interstitial sites, thereby changing the microstructure of deposited film. The average rms roughness of the film surface has been found to be about 4 nm. The presence of uniformly distributed globular structures over the ZnO porous surface can be clearly seen [Fig. 2(b)] in the AFM image and is attributed to the functionalization of oxide layer by cholesterol oxidase. Figures 3(a) and 3(b) shows the FTIR spectra for ZnO/Au and ChOx/ZnO/Au layered structures. The presence of a sharp and intense band at 578 cm^{-1} confirms the formation of ZnO thin film on gold surface [Fig. 3(a)]. The appearance of additional absorption bands at 1561, 1637, and 3330 cm^{-1} in the FTIR spectra of ChOx/ZnO/Au has been attributed to the amide bond present in ChOx enzyme [Fig. 3(b)].

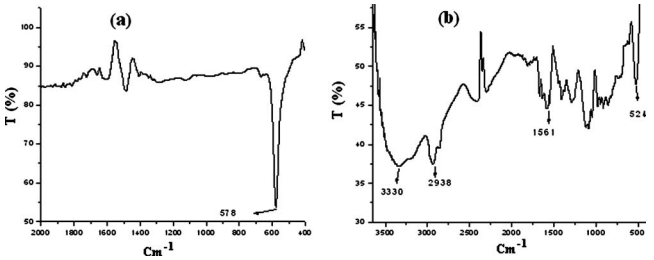

FIG. 3. FTIR spectra of (a) ZnO/Au and (b) ChOx/ZnO/Au films.

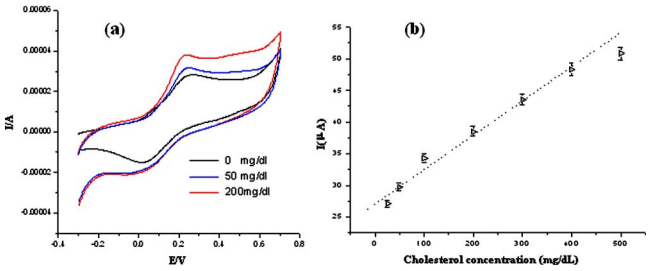

FIG. 4. (a). Amperometric response, (b) Responce curve of ChOx/ZnO/Au bioelectrode with cholesterol concentration.

Cyclic voltammetry (CV) in PBS buffer in the range of −0.3–0.8 V has been used to investigate the enzymatic activity. A peak at around 0.5 V is observed in the *CV* spectra of the prepared electrode with addition of cholesterol [Fig. 4(a)] corresponding to the oxidation of H_2O_2. The magnitude of the peak is found to increase linearly with an increase in the cholesterol concentration [Fig. 4(a)]. The variation in the current measured at a fixed voltage of 0.5 V is shown in Fig. 4(b) as a function of cholesterol concentration (25–500 mg/dl). The measured current increases linearly with an increase in cholesterol concentration. The response of bioelectrode is found to be relatively fast ~15 s and is attributed to the high electron communication feature of the ZnO. The observed oxidation peak current for lower concentration of cholesterol suggests the enhanced electron transfer rate and highlights the advantage of rf sputtered ZnO based ChOx/ZnO/Au electrode for cholesterol sensing.

To carry out the photometric enzyme assay of immobilized ChOx, the ChOx/ZnO/Au electrode is dipped in 3 ml PBS solution containing 20 μl horseradish peroxidase (HRP), 20 μl *o*-dianisidine dye and 100 μl of biosubstrate (cholesterol). The difference between the initial and final absorbance value at 405 nm after 5 min incubation of biosubstrate is recorded and is plotted in Fig. 5 as a function of cholesterol concentration. The ChOx enzyme activity increases with an increase in the concentration of cholesterol (Fig. 5). The amount of bound enzyme is calculated using equation $a_{\mathrm{app}}^{\mathrm{enz}}\ (U\ \mathrm{cm}^{-2}) = AV/\varepsilon ts$, where *A* is the difference in absorbance before and after incubation, *V* is the total volume, ε is the millimolar extinction coefficient for *o*-dianisidine, *t* is the reaction time, and *s* is the surface area of the electrode.[23] The estimated value of immobilized ChOx is found to be about 2.32×10^{-3} unit/cm^2.

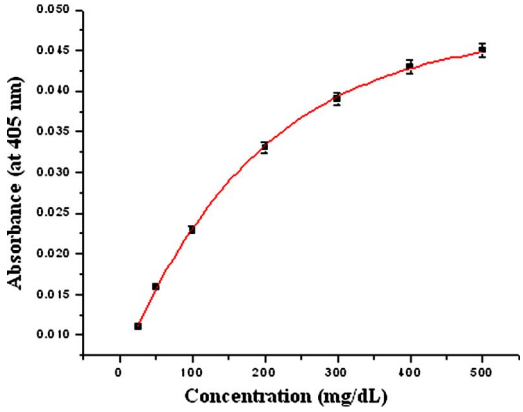

FIG. 5. UV-vis response of ChOx/ZnO/Au electrode as a function of cholesterol concentration.

TABLE I. Characteristics of rf sputtered ZnO based ChOx/ZnO/Au electrode with those reported in the literature.

Immobilization matrix	Sensing element	Method of Immobilization	Linearity	Transducer used	K_m	Shelf life	References
Modified ODT	ChOx	Covalent	1.29–12.93 mM	Optical	⋯	2 months	1
Polyaniline	ChOx	Electrochemical	0.05–0.5 mM	⋯	2.27 mM	11 days	2
Polymeric film	ChOx/HRP	Entrapment	0.07–0.27 mM	Amperometry	6 mM	1 week	3
3-Mercaptopropionic acid	ChOx	Covalent	1.29–5.17 mM	Amperometry	⋯	4 days	4
Tetraethylorthosilcate	ChOx/HRP	Covalent	2–12 mM	Amperometry	21.2 mM	8 weeks	5
Polypyrrole	ChOx,ChEt	Entrapment	1–8 mM	Amperometric	9.8 mM	4 weeks	6
Iron nanoparticles	Chox	Covalent	1.3–5.2 mM	Spectrophotometric	0.45 mM	15 days	7
ZnO	ChOx	Physical	0.65–10.34 mM	Electrochemical	2.1 mM	10 weeks	Present work

The value of K_m determined from Lineweaver-Burke plot was found to be 2.1 mM and is relatively lower than the value reported with other matrices.[1–6] The conformational changes are known to affect an enzyme reaction which in turn may be influenced by the surface morphology and the nature of immobilization matrix. The activity of enzyme increases with favorable conformational changes and leads to an increased interaction between the biosubstrate and the active site of enzyme and is reflected by lower value of K_m. The observed higher affinity of ChOx/ZnO/Au electrode could be attributed to the favorable conformation of enzyme and an efficient ChOx loading provided by the microenvironment of nanoporous ZnO film surface.

The effect of *p*H on the ChOx activity has been studied in the range of 6–8. The study shows that ChOx/ZnO/Au electrode has best enzymatic activity in the range of 7.0–7.5. The value of the absorbance obtained at different time intervals (2, 7, 14, 30, 45, 60, and 75 days) is compared with those in the initial stage (0 day). For a fixed concentration of cholesterol the activity of bioelectrode was >90% even after 75 days, indicating better stability of ZnO based electrode. The results obtained in the present study are summarized in Table I along with those reported in literature. Table I indicates that the nature of matrix not only affects the catalytic property of ChOx but also the storage stability and the range over which bound enzyme shows a linear response. The advantage of nanoporous ZnO film over other matrices is clearly visible from Table I, as it shows linearity over a broad range of 0.65–10.34 mM for ChOx along with relatively low value of K_m, and exhibit better shelf life of about 10 weeks.

In summary, we have fabricated a cholesterol biosensor by immobilizing ChOx on rf sputtered nanoporous ZnO thin films without any additional mediator. The fabricated ChOx/ZnO/Au electrode exhibits high sensitivity and linear response in the range 25–400 mg/dl of cholesterol concentration with high stability (~10 weeks). The low value of Michaelis-Menton constant (~2.1 mM) indicates enhanced enzyme affinity of ChOx. The results clearly suggest that a nanoporous ZnO thin film provides an attractive matrix for effective immobilization of biomolecules, and high electron communication feature of ZnO can be exploited by functionalizing with various biomolecules leading to realization of inexpensive lab-on chip for healthcare.

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