Enzyme and Microbial Technology 82 (2016) 144–150

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Influence of immobilization strategies on biosensing response characteristics: A comparative study

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ARTICLE INFO

Article history:
Received 11 July 2015
Received in revised form
24 September 2015
Accepted 25 September 2015
Available online 30 September 2015

Keywords:
Biosensor
Immobilization technique
Physisorption
Covalent immobilization
Crosslinking

ABSTRACT

The immobilization technique plays an important role in fabrication of a biosensor. NiO based cholesterol biosensor has been used to study the effect of various immobilization techniques on the biosensing response characteristics. The biosensors were fabricated by immobilizing cholesterol oxidase on NiO thin films by three different immobilization techniques viz. physisorption, cross-linking and covalent binding. The study reveals a strong dependence of biosensing response on corresponding immobilization technique. The biosensor based on immobilization by covalent bonding shows superior response characteristics as compared to others owing to its zero length. The results highlight the significance of immobilization technique for biosensor fabrication.

1. Introduction

Biosensors have gained attention of the research community over time owing to their promising applications in healthcare industry, food industry, environmental monitoringand biological analysis. Despite commercialization of various biosensors, the research is still being propelled by the need to achieve improved sensitivity and a better stability [1]. Biosensor is an analytical device that consists of a biologically active component (receptor) in contact with an appropriate transduction element (electrical, thermal, optical, etc.) for detection of analyte bio-molecules [2]. In a biosensor, the biological element responsible for sensing is the receptor biomolecule, and the sensing relies on specific bio-chemical interaction between the analyte and receptor. Matrix provides the solid support for the receptor through which it is integrated to the biosensing system and acts as a vital link between the bio-chemical interaction and external electronics by acting as a medium of electron transfer [3]. A wide range of matrices have been used for integration of the receptor to the biosensor which includes metal oxides, conducting polymers, carbon nanotubes (CNTs) and metals in the form of thin films, nanoparticles, hydrogels, monolayers, modified metals, hybrid nano-composites, all having their own advantages and disadvantages [4–7].

Though the matrix (a physical component) and the receptor (a biological component) play an important role in the sensing mechanism but the link between the two is a vital factor governing the charge transfer and stability. There are a number of ways by which the receptor is immobilized on a matrix. Immobilization techniques like, crosslinking, covalent bonding and physical adsorption are well reported in the literature [8–14]. Physical adsorption is mainly based on the electrostatic interaction between the biomolecule and the matrix surface due to difference in iso-electric points. Crosslinking is based on employing a crosslinker molecule between the matrix and the enzyme. Covalent binding involves the formation of direct covalent bond between the matrix and receptor engaging some functional groups like amino, carboxyl, thiol, sulfhydral etc. Crosslinking and covalent binding rely on immobilization via a third molecule acting as a bridge between the matrix and the receptor. However, in the later case the bulk linking molecule is subsequently removed and a zero length bond exists between the matrix and receptor [14].

Some studies have been carried out on antibody–antigen systems where various immobilization strategies and biomolecule orientation has been compared for improvement of sensing response. Binding of antibodies via different proteins have been studied by Babacan et al. [15]. Vashist et.al. have studied the

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http://dx.doi.org/10.1016/j.enzmictec.2015.09.008
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effect of various antibody immobilization strategies on SPR sensing response [16]. Biosensing response has also been studied by carrying out antibody modification [17]. Apart from immobilization strategy the orientation of immobilized biomolecule is also known to affect the biosensing response [18]. Experimental strategies are also known to affect the sensing [19]. It is important to note that these studies are based on biosensors based antibody–antigen interactions which are affinity reaction. The immobilization strategy is expected to affect the catalytic reaction involved in enzymatic biosensors. Zhang et al. have reviewed different enzyme immobilization techniques and highlighted their effect on biosensing response [20]. Though so many reports are available but a clear inference cannot be drawn regarding the superiority of an immobilization technique from the literature. In this view, a concrete study is required where an enzyme has been immobilized using different techniques under the same working condition and a thorough sensing comparison is made.

In the present report, we attempt to study the effect of various immobilization techniques on the performance of a biosensor. Three biosensors were prepared using three different immobilization techniques keeping the matrix and the receptor fixed. NiO thin film has been chosen as the matrix owing to its inherent qualities like higher surface area, porosity, high conductivity, biocompatibility, chemical stability and electron transfer capability [21]. On the other hand, cholesterol oxidase (ChOx) has been selected as the model receptor. Three cholesterol biosensors have been designed by immobilizing ChOx on NiO thin film by physical, cross-linking and covalent immobilization techniques and their biosensing response characteristics have been studied.

2. Experiment

2.1. Chemicals and reagents

Cholesterol oxidase (ChOx) extracted from streptomyces species has been procured from SRL Pvt. Ltd., India. Triton X-100 (for the preparation of cholesterol stock solution), *N*-hydroxisuccinimide (NHS), *N*-(3-dimethylaminopropyl)-(*N*′-ethylcarbodiimide) (EDC), free cholesterol and nickel acetate tetrahydrate ($Ni(OAc)_2{\cdot}4H_2O$) were purchased from Sigma–Aldrich, USA. The chemicals were used without further purification.

ChOx solution (1 mg/ml) was freshly prepared in 50 mM Phosphate Buffer Saline (PBS) of pH 7.0 containing 0.9% NaCl. The stock solution of cholesterol was prepared in 10% Triton X-100 and stored at 4 °C.

2.2. Preparation and characterizations of NiO thin film

For the preparation of nanostructured NiO thin film, 4.35 g of $Ni(OAc)_2{\cdot}4H_2O$ was dissolved in 30 ml of de-ionised (DI) water. A separate solution was prepared by dissolving 1.5 g of NaOH in 75 ml de-ionised water containing 0.5 g PVP as a surfactant. The two solutions were mixed and spin coated onto the surface of an ITO coated corning glass substrate of area 2 cm × 1 cm of which 1 cm × 1 cm area was masked for electrical contacts. This deposition was repeated five times and the films were dried at 150 °C after each coat. Finally, the films were annealed in air at 400 °C for 2 h. All the NiO films for different immobilizations were made in the same batch.

The prepared NiO thin film has been characterized by X-Ray diffraction (XRD, Bruker D8 Discover). All electrochemical measurements were performed on Gamry Reference 600 Potentiostat/Galvanostat using a three-electrode cell in PBS (pH 7.0) with Ag/AgCl as the reference electrode and platinum as the counter electrode. Photometric studies were carried out using PerkinElmer UV–vis spectrophotometer.

2.3. Immobilization of ChOx on NiO thin film

Physical adsorption was carried out by spreading 10 μl of ChOx solution over one of the NiO thin films and incubating at 27 °C for 2 h. The bio-electrode (ph-ChOx/NiO/ITO/Glass) was kept overnight at 4 °C and washed with PBS to remove any unbound enzyme molecules. The enzyme physically adsorbs on NiO film through electrostatic interaction due to the high isoelectric point of NiO [21].

For crosslinking, 10 μl of ChOx solution was mixed with 1 μl of 1% aqueous solution of glutaraldehyde which is a widely used crosslinking agent [10,22]. It was then spread over the NiO film and incubated at 27 °C for 2 h. The bio-electrode thus obtained (cr-ChOx/NiO/ITO/Glass) was rinsed with DI water before use and stored at 4 °C when not in use.

ChOx was covalently immobilized on the NiO thin film via EDC-NHS [13]. The film was subjected to hydrolysis and silanization to activate the surface for covalent bonding. The film was immersed in a solution of $H_2O_2/NH_4OH/H_2O$ for 30 min at 80 °C for hydrolysis, followed by through rinsing with DI water and drying. The hydrolyzed film was immersed in 1% solution of 3-aminopropyl triethoxysilane (APTES) in toluene overnight at room temperature for silanization. After the coupling reaction, the electrode was rinsed with toluene and DI water to remove the unbound silanes from the surface. 10 μl of ChOx solution containing 0.4 M EDC

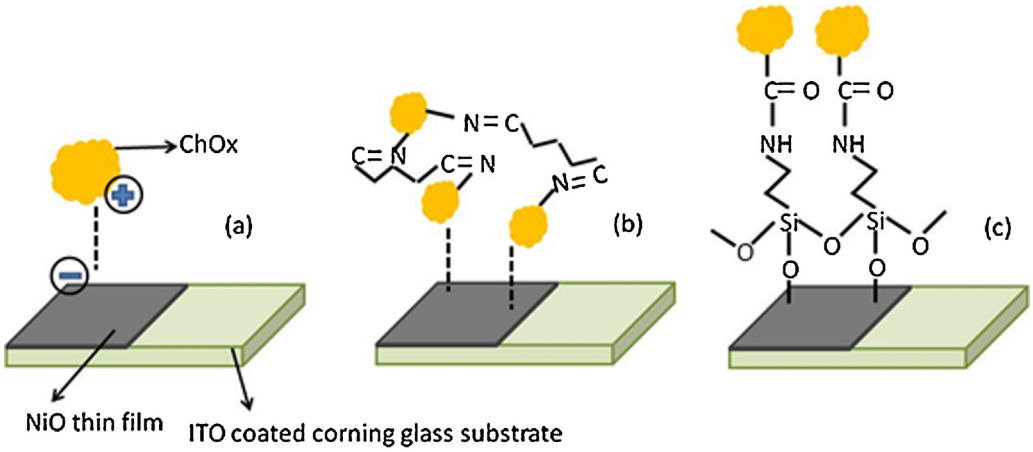

Fig. 1. Schematic diagram of enzyme immobilization by (a) physical adsorption (b) crosslinking and (c) covalent bonding.

and 0.1 M NHS was then spread on the film and incubated for 2 h at 27 °C followed by rinsing with DI water to remove unbound enzyme molecules present on the surface of the bio-electrode (co-ChOx/NiO/ITO/Glass). The bio-electrode was stored at 4 °C when not in use. In EDC-NHS immobilization, the carboxyl group present in the enzyme links to the amino group of silanized film surface through a stable amide bond [14]. A schematic showing different immobilization techniques on NiO thin film is shown in Fig. 1.

3. Results

The XRD pattern of the NiO film is shown in Fig. 2. Two peaks at $2\theta = 36.84°$ and 43.10°, corresponding to the (1 1 1) and (2 0 0) planes of NiO, indicate the formation of face centered cubic (FCC) structure of NiO. The broadness of the XRD peak may be attributed to the small crystallite size. The crystallite size was determined using the Scherrer's formula and was found to be 8 nm [23].

The FTIR spectra of the NiO thin film, shown in Fig. 3a, exhibits characteristic peaks at 460 and 678 cm^{-1} corresponding to the stretching vibration modes of Ni—O bond [21]. FTIR spectra of the NiO films after enzyme immobilization through different techniques are shown in Fig. 3b (i–iii). Vibrational modes at 1100 and 1660 cm^{-1} are observed in the FTIR spectra of all the three bio-electrodes. These modes are due to carbon oxygen bond stretching of the amide I band which is a characteristic bond of cholesterol oxidase thus confirming its immobilization. It is important to point out that a mode at 1420 cm^{-1} corresponding to bending vibrations of amide II band of the enzyme is seen in cr-

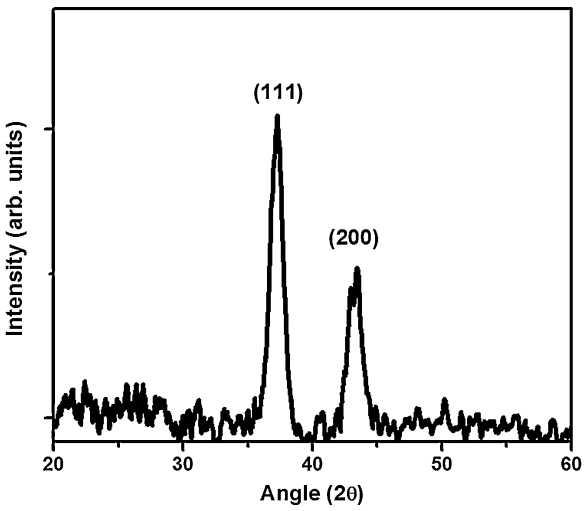

Fig. 2. XRD of NiO thin film.

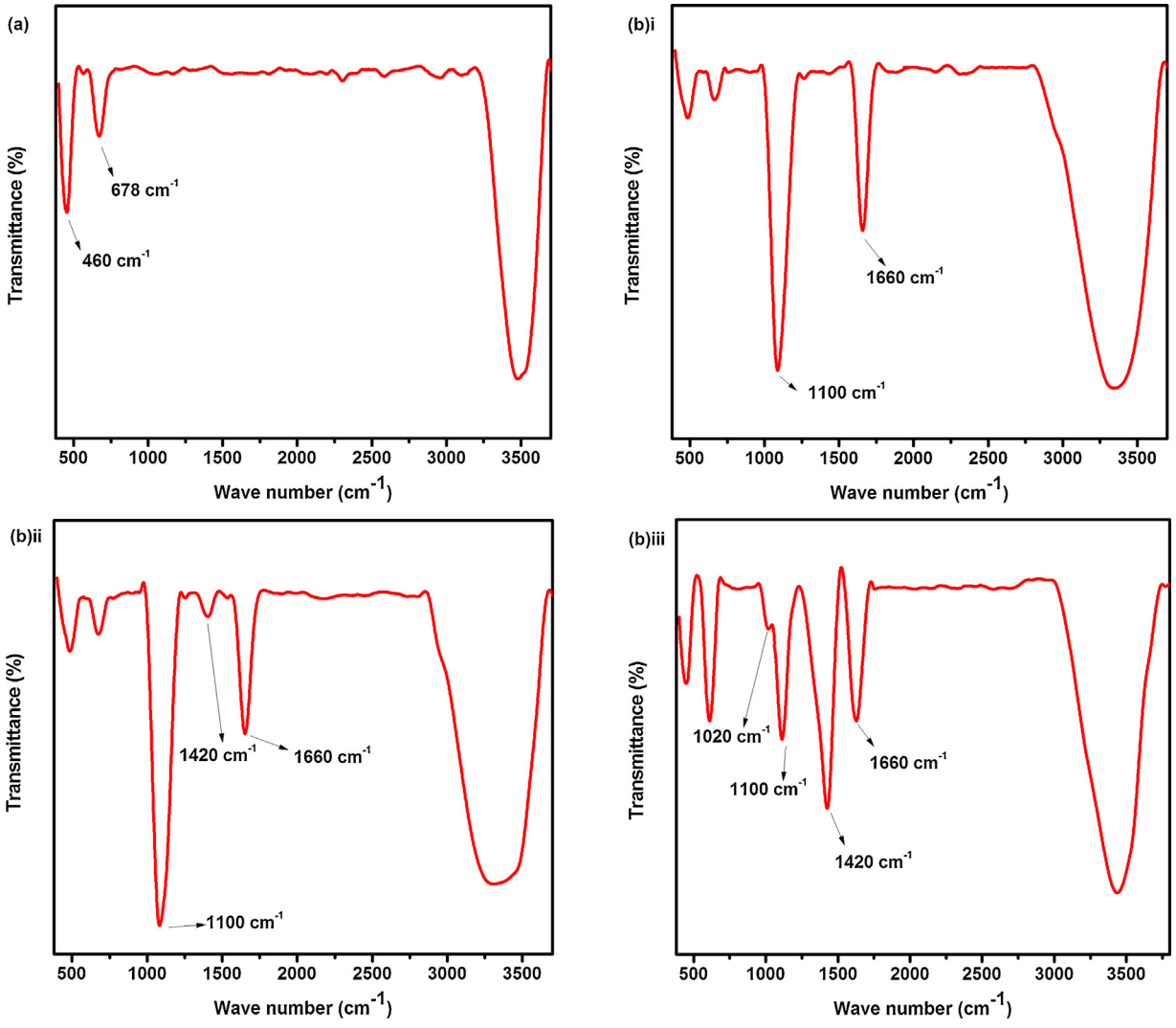

Fig. 3. FTIR spectra of (a) NiO thin film (b) (i) ph-ChOx/NiO/ITO (ii) cr-ChOx/NiO/ITO and (iii) co-ChOx/NiO/ITO bio-electrodes.

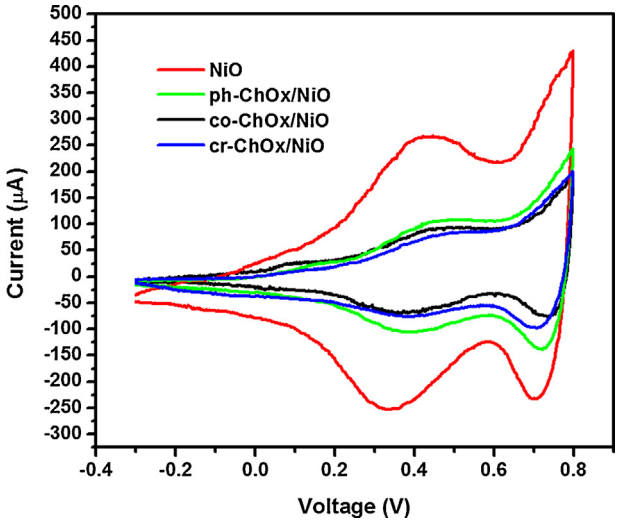

Fig. 4. Cyclic voltammograms of NiO/ITO electrode, ph-ChOx/NiO/ITO, cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO bio-electrodes.

ChOx/NiO/ITO and co-ChOx/NiO/ITO bio-electrodes. However, the said mode is not observed in the ph-ChOx/NiO/ITO bio-electrode. It is important to note that when an enzyme is attached on a matrix it immobilizes in a particular orientation (called conformation) and it is an important factor governing the activity of the immobilized enzyme. A favorable conformational orientation results in an enhanced enzyme activity leading to a better biosensing response. The observed amide II band in cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO bio-electrodes may be an indication of the fact that the conformation of the enzyme is fully maintained in the said bio-electrodes. An extra mode at 1020 cm^{-1} is observed in the FTIR spectra of co-ChOx/NiO/ITO bio-electrode which is due to the Si—O stretching. This validates the formation of siloxane bond between AEAPTS and hydrolyzed ITO surface in co-ChOx/NiO/ITO bio-electrode. The broad mode at around 3400 cm^{-1} in FTIR spectra of all the bio-electrodes is related to stretching of O—H bond of water molecule [24].

Fig. 4 shows cyclic voltammograms (CV) obtained for NiO/ITO, ph-ChOx/NiO/ITO, cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO electrodes in the potential range of −0.3 V to 0.8 V. A well-defined redox peak is observed in all the voltammograms which is due to the redox couple ($Ni^{3+} \leftrightarrow Ni^{2+}$ species) present in the matrix. The magnitude of oxidation and reductioncurrent decreased upon enzyme immobilization owing to the insulating nature of the bulk molecular structure of cholesterol oxidase in all the three bio-electrodes [25].

CVs of all the bio-electrodes at various scan-rates (70–160 mV/s) were recorded (data not shown). The variations of the peak current, for all the three bio-electrodes, show a linear variation with square root of scan rate indicating that the reaction is governed by a quasi-reversible diffusion electron process [26]. Surface coverage (Γ) of ionic species for all the bio-electrodes were computed using Brown–Anson model which is given by the following equation [21]:

$$I_p = \frac{n^2 F^2 \Gamma A v}{4RT}$$

where, I_p is the peak current obtained at a scan rate of v, F is the Faraday's constant (96,485 C mol^{-1}), R is the gas constant (8.314 J mol^{-1} K^{-1}), T is the absolute temperature, A is the area of the prepared electrode and n is the number of electrons taking part in the redox reaction which is '1' in our case, since only one electron is involved in the oxidation/reduction of Ni ($Ni^{2+} \leftrightarrow Ni^{3+}$). The surface coverage (Γ) is an indicative of the amount of redox species available on the surface of the working electrode. The surface coverage of ph-ChOx/NiO/ITO bio-electrode was found to be 9.0×10^{-7} mol cm^{-2}, while that of cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO were evaluated to be 9.5×10^{-7} mol cm^{-2} and 1.1×10^{-6} mol cm^{-2} respectively. A higher value of surface coverage indicates higher concentration of active redox species which in turn may be attributed to the better conformational orientation of ChOx enzyme on the bio-electrode. This indicates that the enzyme immobilized by covalent bonding might have a better orientation as compared to the other two.

Fig. 5 shows the CVs of the bio-electrodes recorded over a range of cholesterol concentrations (0.12–10.23 mM). The studied concentration range has been chosen such that it includes the physiological range of cholesterol. Both the oxidation and reduction peak current increase with cholesterol concentration. The cholesterol added in the solution is spontaneously oxidized in presence of ChOx thus releasing excess electrons. During the potential sweep of cyclic voltammetry these excess electrons are responsible for the increased current. As the cholesterol concentration increases there is a growth in the number of released electrons, which leads to an enhancement in the redox current [26]. The calibration curves show a linear increase in the peak oxidation current with increasing cholesterol concentration for all the three bio-electrodes (Fig. 6). The sensitivity of the ph-ChOx/NiO/ITO, cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO bio-electrodes calculated from the slope of the calibration curves were

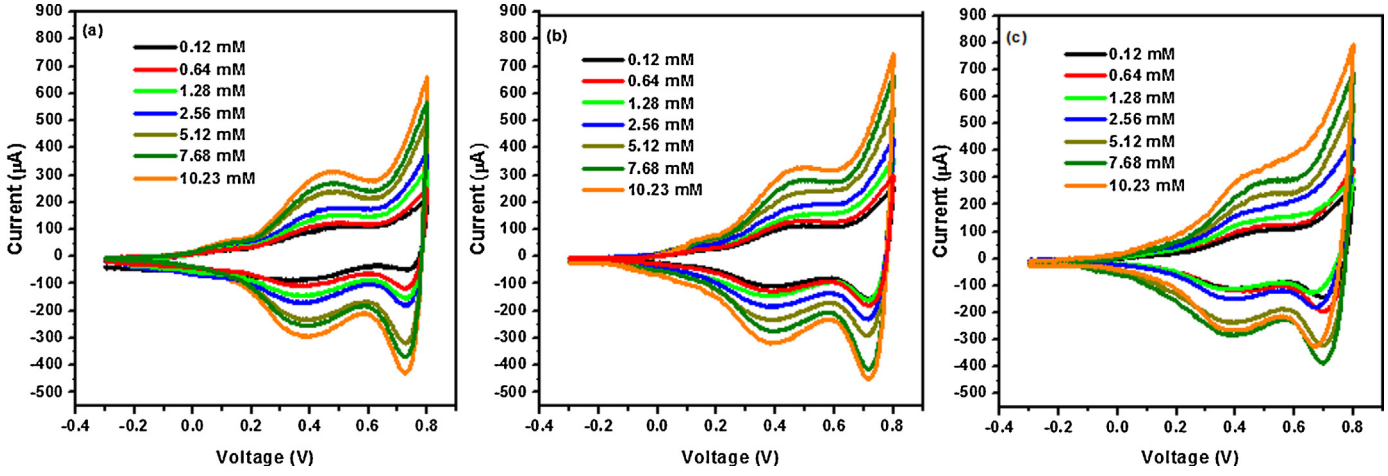

Fig. 5. Variation in CV with cholesterol concentration for (a) ph-ChOx/NiO/ITO, (b) cr-ChOx/NiO/ITO and (c) co-ChOx/NiO/ITO bio-electrodes.

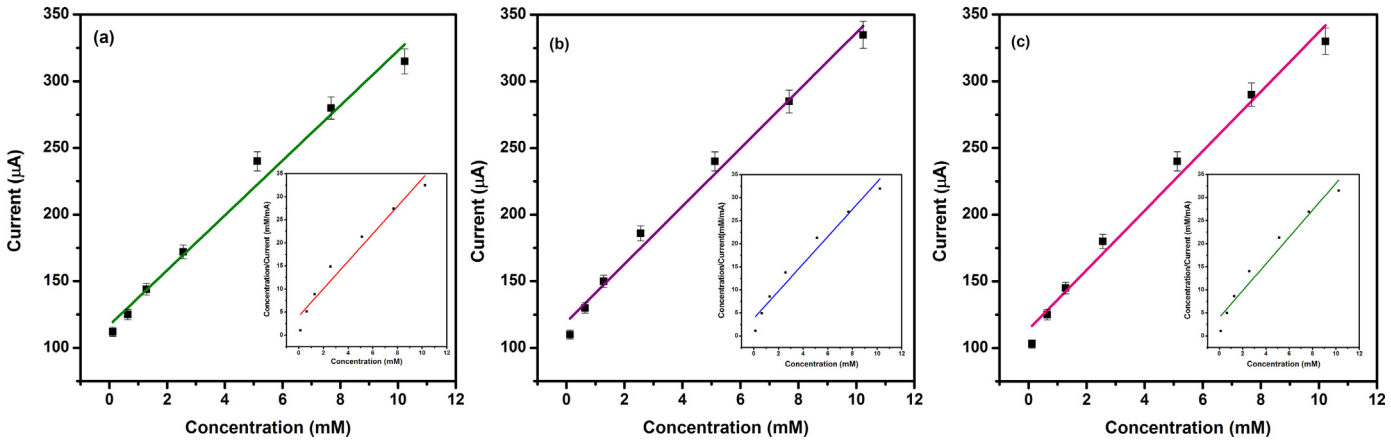

Fig. 6. Variation of peak oxidation current with cholesterol concentration for (a) ph-ChOx/NiO/ITO, (b) cr-ChOx/NiO/ITO and (c) co-ChOx/NiO/ITO bio-electrodes. Insets show the Hanes plots for respective bio-electrodes.

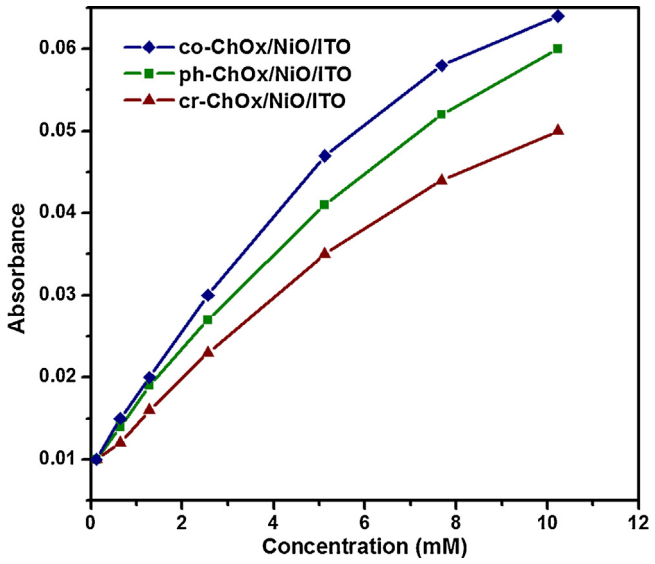

Fig. 7. Photometric assay of ph-ChOx/NiO/ITO, cr-ChOx/NiO/ITO and co-ChOx/NiO/ITO bio-electrodes.

found to be 20.59 μA/mM, 21.17 μA/mM and 22.29 μA/mM respectively. It may be noted that the bio-electrodes were fabricated in triplicate and repeated measurements were taken. The observations were within ±5% error limit denoted by the error-bars in the graph (Fig. 6).

The Michaelis–Menten constant (K_m) was evaluated using Hanes plot (inset of Fig. 6) [27]. The K_m value was estimated to be 1.39 mM for the ph-ChOx/NiO/ITO bio-electrode. However, a lower value of K_m was found for cr-ChOx/NiO/ITO (1.30 mM) and co-ChOx/NiO/ITO (1.27 mM) bio-electrodes. The lower K_m value signifies an increased affinity of the enzyme towards its analyte (cholesterol).

Fig. 7 shows the photometric assays of the three bio-electrodes which wereused to calculate the apparent enzyme activity (a_{enz}), i.e., the amount of enzyme bound on the surface of the matrices [28]. For carrying out the photometric assay, the bio-electrode was dipped in 3 ml PBS solution containing 20 μl horseradish peroxidase (HRP), 20 μl *o*-dianisidine dye and 100 μl of cholesterol and incubated for three minutes. The difference between the values of initial absorbance and final absorbance measured at a wavelength of 500 nm is recorded as a function of cholesterol concentration.The apparent enzyme activity, i.e., the amount of enzyme bound on the surface of the thin film matrix, has been calculated using the equation

$$\alpha = \frac{AV}{\varepsilon t s}$$

where A is the difference in absorbance before and after incubation, V is the total volume of the reactant (3.17 cm^3), ϵ is the millimolar extinction coefficient (7.5 for *o*-dianisidine at 500 nm), t is the reaction time (3 min) and s is the surface area of the prepared bioelectrode (1 cm^2). The values of apparent enzyme activity are tabulated in Table 1. The apparent enzyme activity of the co-ChOx/NiO/ITO was found to be maximum, while that of cr-ChOx/NiO/ITOwas found to be minimum. The results indicate that maximum amount of enzyme immobilization takes place on the co-ChOx/NiO/ITO bio-electrode.

The stability and reusability of the biosensors were investigated by recording the current response of the same bio-electrode for a fixed cholesterol concentration (2.56 mM) at regular intervals over a period of 10 weeks (Fig. 8). The biosensors degrade with time owing to loss of activity of enzyme. The stability of a bio-electrode relies on the matrix as well as immobilization procedure adopted. All the prepared bio-electrodes retained more than 90% of activity even after ten weeks. The ph-ChOx/NiO/ITO bio-electrode retained 92% of activity after 10 weeks whereas cr-ChOx/NiO/ITO bio-electrodesustained 93% of activity. The co-ChOx/NiO/ITO bio-electrodedemonstrated the highest storage stability by retaining 95% of activity after 10 weeks. All the biosensing characteristic parameters are tabulated in Table 1.

4. Discussion

The immobilization technique is seen to play an important role in the biosensing response characteristic of a bio-electrode. The biosensing characteristics of the studied bio-electrodes compiled in Table 1 clearly indicates the superiority of the covalent immobilization technique over the other two. It may be observed from Table 1 that the co-ChOx/NiO/ITO bio-electrode exhibits the highest sensitivity among the three bio-electrodes under study. Sensitivity of a bio-electrode is the change in current generated on addition of unit amount of reagent (cholesterol in the present case). Sensitivity is directly related to the availability of active sites of the enzyme immobilized on it. Moreover, it may also be affected by the amount of enzyme loaded on the bio-electrode which is quantified by the apparent enzyme activity. Both the factors seem to favor the co-ChOx/NiO/ITO bio-electrodes. On one hand, the photometric assay confirms a higher enzyme loading

Table 1
Characteristic biosensing characteristic parameters of the three bio-electrodes based on different immobilization techniques.

	Physical adsorption (ph-ChOx/NiO/ITO)	Crosslinking (cr-ChOx/NiO/ITO)	Covalent bonding (co-ChOx/NiO/ITO)
Sensitivity	20.59 μA/mM	21.17 μA/mM	22.29 μA/mM
Michaelis–Menten constant (K_m)	1.39 mM	1.30 mM	1.27 mM
Surface coverage	9.0×10^{-7} mol cm^{-2}	9.5×10^{-7} mol cm^{-2}	1.1×10^{-6} mol cm^{-2}
Apparent enzyme activity	1.6×10^{-2} U cm^{-2}	1.4×10^{-2} U cm^{-2}	1.8×10^{-2} U cm^{-2}
Stability (amperometric activity after 10 weeks)	92%	93%	95%

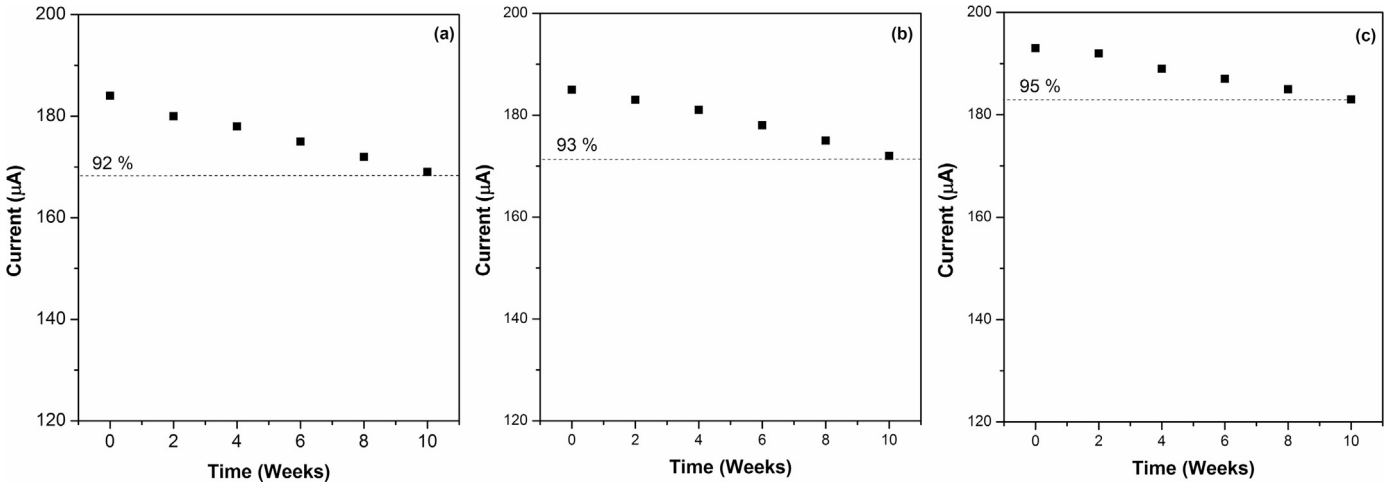

Fig. 8. Variation in amperometric response with time for a fixed cholesterol concentration (2.56 mM) for (a) ph-ChOx/NiO/ITO, (b) cr-ChOx/NiO/ITO and (c) co-ChOx/NiO/ITO bio-electrodes.

in co-ChOx/NiO/ITO bio-electrode as compared to the other two bio-electrodes. The higher enzyme loading in co-ChOx/NiO/ITO may be due to the fact that more enzymes would remain stable on the bio-electrode where the linking is through some agent which keeps the molecules close to the electrode surfaceas a zero bond-length is achieved in covalent immobilization [14]. Besides, a lower value of K_m suggests a better conformational orientation of the immobilized enzyme on cr-and co-ChOx/NiO/ITO as compared to that on ph-ChOx/NiO/ITO bioelectrode. Proper conformational orientation of enzyme on surface of the matrix ensures a higher affinity towards the analyte (cholesterol in our case). The higher value of surface coverage and FTIR data independently support the inference of a better conformational orientation of enzyme immobilized on co- and cr-ChOx/NiO/ITO bio-electrodes as compared to that on ph-ChOx/NiO/ITO. The response characteristics (Table 1) further shows that co-ChOx/NiO/ITO bio-electrode has an inherent edge over the cr-ChOx/NiO/ITO in sensitivity. This may be attributed to the zero length of the covalent linking via EDC-NHS chemistry as the linker is removed during the immobilization process [14]. On the other hand, the presence of cross-linking molecule, which acts as a spacer between the enzyme and the matrix, in case of cr-ChOx/NiO/ITO bio-electrode, may act as a hindrance to the charge transfer process thus degrading the sensitivity in comparison to that in co-ChOx/NiO/ITO bio-electrode. A slightly higher K_m value for the cr-ChOx/NiO/ITO bio-electrode as compared to that of co-ChOx/NiO/ITO indicates an inferior conformational orientation of enzyme immobilized by cross-linking and may be due to the formation of a rigid assembly due to crosslinking. The co-ChOx/NiO/ITO bio-electrode is also observed to have a better stability as compared to the other two as indicated by the superior shelf-life (Table 1). Physical adsorption does not involve chemical treatment of the enzyme and thus it is loosely bound on the surface which can be easily desorbed on repeated use, resulting in poor stability. Crosslinking results in formation of bonded structure that prevents enzyme leakage. However, covalent immobilization owes its superior stability to the zero length.

The characteristics of the ph-ChOx/NiO/ITO bio-electrodeare observed to be inferior due to unstable immobilization and a poor conformational orientation. The cr-ChOx/NiO/ITO bio-electrode shows better performance as compared to ph-ChOx/NiO/ITO bio-electrode but the spacing between the enzyme and the matrix along with its rigid structure results in its reduced activity. Covalent binding, in contrast, is direct stable chemical binding which enhances the overall performance of the biosensor. Thus, the enzyme covalently immobilized on the surface of the bio-electrode shows better biosensing characteristics in form of higher sensitivity and stability.

5. Conclusion

Various enzyme immobilization techniques (physical adsorption, croslinking and covalent bonding) were compared by preparing three different bio-electrodes using NiO thin film as the matrix and cholesterol as the analyte. The performance of the biosensors was studied by evaluating factors such as sensitivity, surface coverage, Michaelis–Menten constant, apparent enzyme activity and stability. Bio-electrode based on covalent binding exhibited enhanced results as compared to the other two owing to a stable zero length immobilization. The study highlights the significance of the immobilization technique on thecharacteristic parameters of a biosensor.

Ethics

The work presented in the manuscript titled “Influence of Immobilization Strategies on Biosensing Response Characteristics: A Comparative Study” has been drafted from the original experiments carried out by the authors.

Acknowledgements

Authors are grateful to University of Delhi and Department of Science and Technology, India for financial support. One of the authors (GK) is thankful to University Grants Commission for research fellowship.

References

[1] P.T. Kissinger, Biosensors—a perspective, Biosens. Bioelectron. 20 (2005) 2512.
[2] S.K. Arya, S. Saha, J.E.R. Vick, V. Gupta, S. Bhansali, S.P. Singh, Recent advances in ZnO nanostructures and thin films for biosensor applications, Anal. Chim. Acta 737 (2012) 1.
[3] Z. Xu, X. Chen, S. Dong, Electrochemical biosensors based on advanced bioimmobilization matrices, Trends Anal. Chem. 25 (2006) 899.
[4] M. Gerard, A. Chaubey, B.D. Malhotra, Application of conducting polymers to biosensors, Biosens. Bioelectron. 17 (2002) 345.
[5] S. Sotiropoulou, V. Gavalas, V. Vamvakaki, N.A. Chaniotakis, Novel carbon materials in biosensor systems, Biosens. Bioelectron. 18 (2003) 211.
[6] P. Yáñez-Sedeño, J.M. Pingarrón, Gold nanoparticle-based electrochemical biosensors, Anal. Bioanal. Chem. 382 (2005) 884.
[7] S. Saha, V. Gupta, Influence of surface defects in ZnO thin films on its biosensing response characteristic, J. Appl. Phys. 110 (2011) 064904.
[8] A.K. Basu, P. Chattopadhyay, U. Roychoudhuri, R. Chakraborty, Development of cholesterol biosensor based on immobilized cholesterol esterase and cholesterol oxidase on oxygen electrode for the determination of total cholesterol in food samples, Bioelectrochemistry 70 (2007) 375.
[9] C. Dhand, S.K. Arya, M. Datta, B.D. Malhotra, Polyaniline–carbon nanotube composite film for cholesterol biosensor, Anal. Biochem. 383 (2008) 194.
[10] H.B. Nguyen, V.C. Nguyen, V.T. Nguyen, H.D. Le, V.Q. Nguyen, T.T.T. Ngo, Q.P. Do, X.N. Nguyen, N.M. Phan, D.L. Tran, Development of the layer-by-layer biosensor using graphene films: application for cholesterol determination, Adv. Nat. Sci: Nanosci. Nanotechnol. 4 (2013) 015013.
[11] C. Wang, X. Tan, S. Chen, R. Yuan, F. Hu, D. Yuan, Y. Xiang, Highly-sensitive cholesterol biosensor based on platinum–gold hybrid functionalized ZnO nanorods, Talanta 94 (2012) 263.
[12] J. Singh, P. Kalita, M.K. Singh, B.D. Malhotra, Nanostructured nickel oxide-chitosan film for application to cholesterol sensor, Appl. Phys. Lett. 98 (2011) 123702.
[13] S.K. Arya, A.K. Prusty, S.P. Singh, P.R. Solanki, M.K. Pandey, M. Datta, B.D. Malhotra, Cholesterol biosensor based on *N*-(2-aminoethyl)-3-aminopropyl-trimethoxysilane self-assembled monolayer, Anal. Biochem. 363 (2007) 210.
[14] Z. Matharu, P.R. Solanki, V. Gupta, B.D. Malhotra, Mediator free cholesterol biosensor based on self-assembled monolayer platform, Analyst 137 (2012) 747.
[15] S. Babacan, P. Pivarnik, S. Letcher, A.G. Rand, Evaluation of antibody immobilization methods for piezoelectric biosensor application, Biosens. Bioelectron. 15 (2000) 615.
[16] S.K. Vashist, C.K. Dixit, B.D. MacCraithad, R. O'Kennedy, Effect of antibody immobilization strategies on the analytical performance of a surface plasmon resonance-based immunoassay, Analyst 136 (2011) 4431.
[17] S.K. Vashist, Effect of antibody modifications on its biomolecular binding as determined by surface plasmon resonance, Anal. Biochem. 421 (2012) 336.
[18] P. Peluso, D.S. Wilson, D. Do, H. Tran, M. Venkatasubbaiah, D. Quincy, B. Heidecker, K. Poindexter, N. Tolani, M. Phelan, K. Witte, L.S. Jung, P. Wagner, S. Nock, Optimizing antibody immobilization strategies for the construction of protein microarrays, Anal. Biochem. 312 (2003) 113.
[19] C.K. Dixit, S.K. Vashist, B.D. MacCraithad, R. O'Kennedy, Evaluation of apparent non-specific protein loss due to adsorption on sample tube surfaces and/or altered immunogenicity, Analyst 136 (2011) 1406.
[20] D.-H. Zhang, L.-X. Yuwen, L.-J. Peng, Parameters affecting the performance of immobilized enzyme, J. Chem. 2013 (2013) 946248.
[21] M. Tyagi, M. Tomar, V. Gupta, NiO nanoparticle-based urea biosensor, Biosens. Bioelectron. 41 (2013) 110.
[22] D.R. Walt, V.I. Agayn, The chemistry of enzyme and protein immobilization with glutaraldehyde, Trends Anal. Chem. 13 (1994) 425.
[23] S.R. Cullity, Elements of X-Ray Diffraction, Pearson, USA, 2001.
[24] S. Aravamudhan, A. Kumar, S. Mohapatra, S. Bhansali, Sensitive estimation of total cholesterol in blood using Au nanowires based micro-fluidic platform, Biosens. Bioelectron. 22 (2007) 2289.
[25] A.A. Ansari, A. Kaushik, P.R. Solanki, B.D. Malhotra, Electrochemical cholesterol sensor based on tin oxide-chitosan nanobiocomposite film, Electroanalysis 21 (No. 8) (2009) 965.
[26] S. Srivastava, Md. Azahar Ali, P.R. Solanki, P.M. Chavhan, M.K. Pandey, A. Mulchandani, A. Srivastava, B.D. Malhotra, Mediator-free microfluidics biosensor based on titania–zirconia nanocomposite for urea detection, RSC Adv. 3 (2013) 228.
[27] K. Arora, M. Tomar, V. Gupta, Reagentless uric acid biosensor based on Ni microdiscs-loaded NiO thin film matrix, Analyst 139 (2014) 4606.
[28] S. Saha, S.K. Arya, S.P. Singh, K. Sreenivas, B.D. Malhotra, V. Gupta, Nanoporous cerium oxide thin film for glucose biosensor, Biosens. Bioelectron. 24 (2009) 2040.