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Nanostructured Titanium Oxide Platform for Application to Ascorbic Acid Detection

P.M. Chavhan, Venu Reddy, CheolGi Kim[*]

Center for nano-BioEngineering and SpinTronics (nBEST), Department of Materials Science and Engineering, Chungnam National University, 220 Gungdong, Yuseong Gu, Daejeon 305-764, Republic of Korea
[*]E-mail: cgkim@cnu.ac.kr

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Nanostructured TiO_2 films have been deposited onto indium tin oxide (ITO) films by sol–gel technique to immobilize ascorbate oxidase (AsOx) onto nanostructured TiO_2 films. The TiO_2/ITO matrix, AsOx/Nano-TiO_2/ITO bioelectrodes have been characterized using spectroscopic techniques and used to fabricate ascorbic acid biosensor. Nanostructured TiO_2 based ascorbic acid biosensor has a response time about 20 s, an apparent K_m (K_m^{app}) value of 30.80mg/dL (1.75 mM) and can be used to estimate ascorbic acid concentration up to 200 mg dL^{-1}. The AsOx/Nano-TiO_2/ITO bioelectrodes have a detection limit of 50 mg dL^{-1} with sensitivity of 0.212 mA/mg dL cm^2. The enzyme films were found to be thermally stable up to 40 °C and have a shelf-life of about 4 weeks when stored at 4 °C.

Keywords: Sol–gel; Nano-TiO_2; Ascorbate oxidase; Ascorbic acid; Electrochemical biosensor

1. INTRODUCTION

Ascorbic acid, better known as vitamin C is most important water soluble compound present in fruits and vegetables that acts as an antioxidant against a variety of diseases and is indispensable for life, health, and daily physical activities [1]. So, accurate determination of ascorbic acid concentration is essential for monitoring of food and vegetables quality in daily use. Ascorbic acid concentration in food, drugs and plants can be determined with ease of various analytical techniques such as indirect spectrophotometric, solid-phase iodine method and liquid chromatography [2–5]. However, these conventional analytical techniques suffers from vast pretreatment method, time consuming and requires costly equipments, where as electrochemical detection method have many advantages of low cost, simplicity, low detection limit, fast response time and improved sensitivity.

Electrochemical analysis has attracted considerable attention because of sensitivity may be further improved by several strategies including use of nanoporous metal oxide matrix, enzymes, redox couple mediator and catalysts [6–14]. Matos et al. [15] had measured in beers, soda, natural fruits and commercial vitamin C tablets with a palladium modified gold electrode. Aoki et al. fabricated a micro multi-band electrode and measured its response to ascorbic acid [16]. M. G. Hosseini et al. have electroanalytically determined ascorbic acid concentration in pharmaceutical tablets using titanium oxide nanotube films containing gold nanoparticles [17].

Nanoporous metal oxide nanoparticles such as titanium oxide (TiO_2), cerium oxide (CeO_2), zinc oxide (ZnO), tin oxide (SnO_2), and zirconium oxide (ZrO_2) have recently been used for fabrication of enzyme-based biosensors. Sol–gel derived nanostructured metal oxides such as TiO_2 [18,19], CeO_2 [20–21], ZnO [22,23], SnO_2 [24] and ZrO_2 [25] due to their interesting properties such as better thermal stability, low cost, biocompatibility, non-toxicity and low temperature of processing, etc. have aroused much interest for immobilization of desired biomolecules. Among these, TiO_2 nanoparticles have attracted much interest owing to their unique properties including high mechanical strength, oxygen ion conductivity, wide band gap (3.2 eV), biocompatibility and retention of biological activities [18,19]. A. Curulli et al. have reported that nanoporous TiO_2 electrodes has been employed for electron transfer mechanisms of H_2O_2, and many interesting biological molecules, such as 3,4-dihydroxyphenylacetic acid, ascorbic acid, guanine, l-tyrosine and acetaminophen, in order to assemble a new generation of chemical sensors and biosensors. [26]

In the present manuscript, we report results relating to the immobilization of ascorbate oxidase (AsOx) onto sol–gel derived nanoporous TiO_2 film deposited on ITO glass for electrochemical ascorbic acid detection.

2. MATERIALS AND METHODS

2.1. Reagents

Ascorbic acid (Purity 98.5%) and Titanium n-tetrabutoxide (Purity 99%) were procured from Sigma-Aldrich, USA. All other chemicals were used of analytical grade. Ascorbate oxidase from cucurbita species were procured from Sigma-Aldrich USA. Ascorbate oxidase solution (1 mg/ml) was freshly prepared in phosphate buffer (50mM, pH=7.0) prior to being immobilized. The ascorbic acid solution (500mg/dL) prepared in DI water, was stored at room temperature (25ºC). This standard solution was further diluted to make different ascorbic acid concentrations.

2.2. Preparation of Nano-TiO_2/ITO electrode and AsOx/Nano-TiO_2/ITO bioelectrode

Sol–gel derived nanostructured TiO_2 film was fabricated onto ITO coated glass plate using sol–gel dip-coating technique. For TiO_2 coating, the sol–gel solution (4 wt.%) was prepared using titanium n-tetrabutoxide and 2-methoxy ethanol as precursor materials. The dip coated TiO_2 films was initially dried at an intermediate temperature ~ 110ºC and finally allowed to heat-treatment at 450ºC for 2 h in

air atmosphere. The proposed reactions involved in the syntheses of TiO_2 films fabrication onto ITO substrate using sol–gel technique are shown in Eq. 1.

These films have been utilized for immobilization of ascorbate oxidase via electrostatic interactions at pH 7.0 due to difference in IEP of TiO_2 matrix and ascorbic acid. For ascorbate oxidase immobilization, 50 µl solution of ascorbate oxidase (1 mg/ml) prepared in PBS (pH 7.0, 50mM, 0.9% NaCl) was dropped onto 0.25cm^2 area of TiO_2 electrode surface and the immobilized electrode was kept overnight at maintained temperature of 4ºC. Prior to being used, the AsOx/Nano-TiO_2/ITO bioelectrode were rinsed with buffer solution to remove any unbound or loosely bound enzyme molecules. The electrode was then dried under nitrogen flow and kept at 4ºC when not in use.

$$Ti(OC_4H_9)_4 + HO-CH_2-CH_2-O-CH_3 + H_2O \xrightarrow{HNO_3,\ Hydrolysis} \underset{Sol}{Ti(OC_4H_9)_2(OH)_2} + 2C_4H_9OH \xrightarrow{Polycondensation}$$

$$[Ti(OH)_4].\ x\,HO-CH_2-CH_2-O-CH_3 . x\,H_2O \xrightarrow{110^oC} \underset{Amorphous\ gel}{Ti(OH)_4} \xrightarrow{450^oC} \underset{Titania}{TiO_2}$$

Gel

-------(1)

2.3. Instrumentation

The Nano-TiO_2/ITO electrode and AsOx/nano-TiO_2/ITO bioelectrode have been characterized using X-ray diffractometer (CuK_α radiation, Rigaku)), Atomic force microscopy (AFM,) and cyclic voltammetric studies. Indium–tin-oxide (ITO) coated glass plates (sheet resistance: 20 Ohm/square) were used as a substrate for deposition of nanoporous TiO_2 films. Electrochemical analysis has conducted on an Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) using a three-electrode system with ITO as working electrode, platinum (Pt) sheet as the auxiliary electrode and Ag/AgCl as reference electrode in phosphate buffer saline (PBS, 50mM, pH7, 0.9%NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ as redox probe.

3. RESULTS AND DISCUSSION

3.1. X-ray diffraction analysis

Fig. 1 shows results of X-ray diffraction pattern of sol–gel derived TiO_2 film deposited onto ITO coated glass substrate via dip-coating technique. X-ray diffraction pattern of TiO_2 film shows typical diffraction peaks at 2θ= 37.94, 48.12, 53.97, 55.15, 62.64, 68.94 corresponding to 101, 004, 200, 105, 211, 204, 220 reflection plane, respectively confirming tetragonal crystal planes of anatase phase of TiO_2 [27]. The average crystallite size of the film obtained by Scherrer's formula ($D=k\lambda/\beta\cos\theta$) is calculated to be ~ 14 nm. It is evident that the smaller crystallite size is related to the broadening of the reflection planes, which in turn responsible for higher surface area of Nano-TiO_2/ITO electrode.

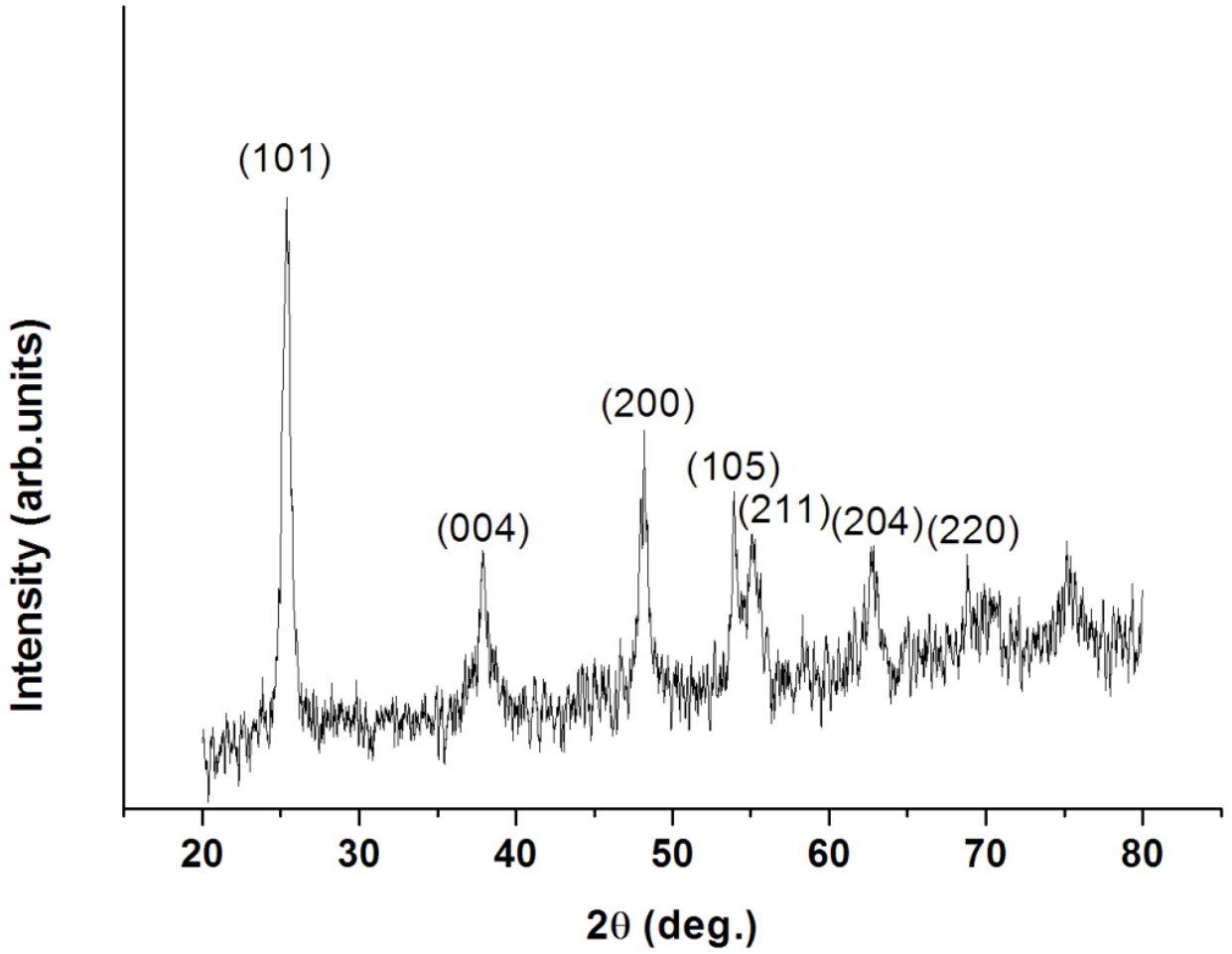

Figure 1. X-ray diffraction pattern of Nano-TiO_2/ITO electrode

3.2. AsOx immobilization studies

Atomic force microscopy (AFM) has been used to investigate surface morphologies of the Nano-TiO_2/ITO electrode (Fig. 2, image a,c) and AsOx/Nano-TiO_2/ITO bioelectrode (Fig. 2, image b,d), respectively. The AFM image of Nano-TiO_2/ITO electrode exhibits nanoporous granular morphology with average grain size in the range of about 100–150 nm.

The granular and porous morphology of Nano-TiO_2/ITO electrode changes into well-arranged regular smooth morphology after immobilization of AsOx onto Nano-TiO_2 electrode surface reveal uniform immobilization of AsOx onto Nano-TiO_2/ITO electrode.

It may be mentioned that appearance of hill-valley like naoporous topography of Nano-TiO_2/ITO electrode is less predominant due to immobilization of enzymes onto Nano-TiO_2/ITO electrode via electrostatic interactions. Nanostructured TiO_2 matrix perhaps may provide nanoporous surface resulting in enhanced enzyme loading at the AsOx/Nano-TiO_2/ITO bioelectrode surface.

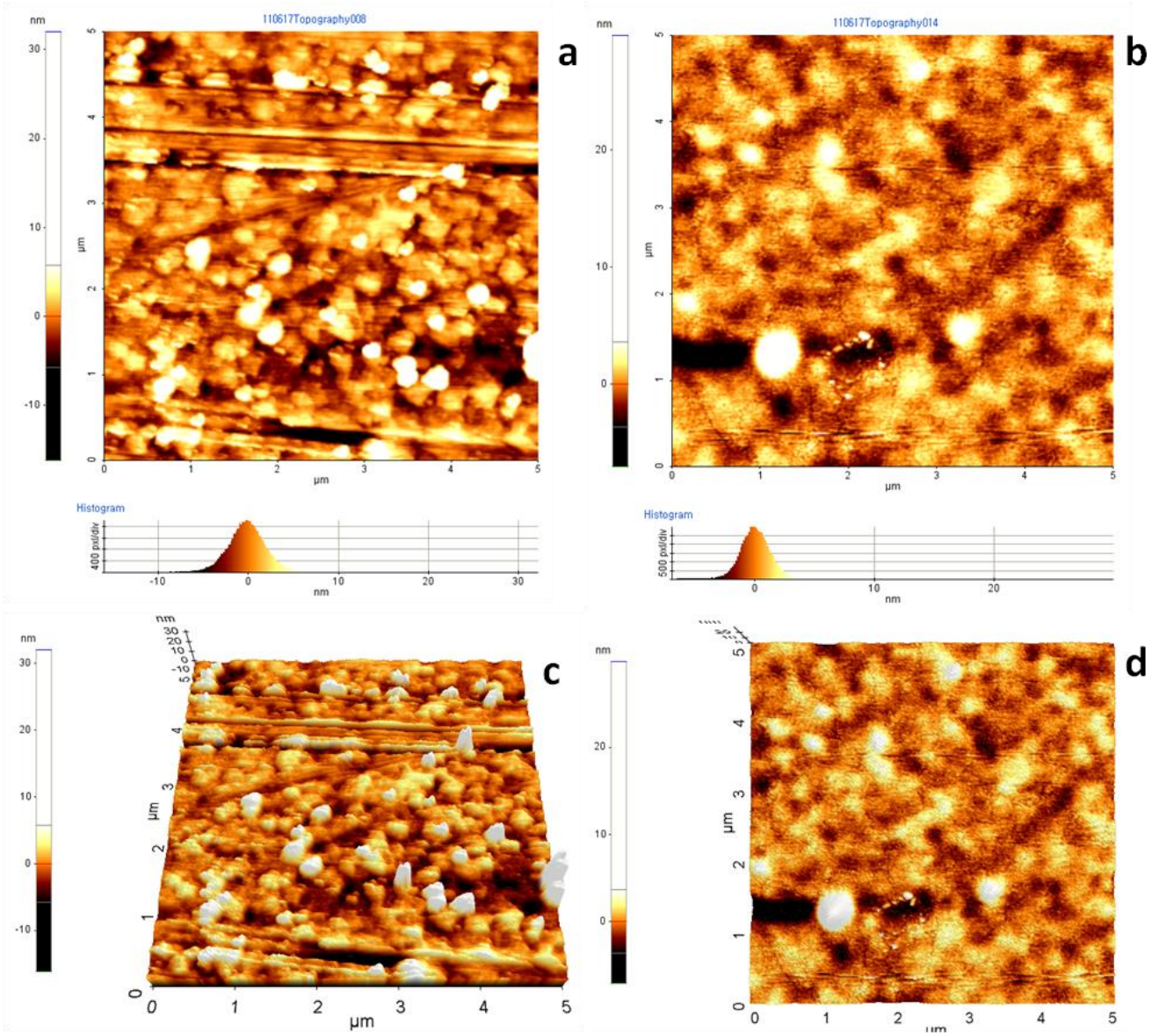

Figure 2. AFM (2D) and (3D) images of Nano-TiO_2/ITO electrode (a,c); AsOx/Nano-TiO_2/ITO bioelectrode (b,d), respectively

3.3. Electrochemical studies

Cyclic voltammetric (CV) studies (Fig. 3A) of Nano-TiO_2/ITO electrode (curve b) and AsOx/Nano-TiO_2/ITO bioelectrode (curve c) have been carried out in PBS (50mM, pH 7, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$ at scan rate of 50 mV/s. It has been observed that the current magnitude response of Nano-TiO_2/ITO electrode is lowered than that of bare ITO (curve a) electrode revealing that hindrance of electron transport between ITO and the Nano-TiO_2 electrode interface. However, magnitude of the current response increases after immobilization of ascorbate oxidase onto Nano-TiO_2/ITO electrode (curve b). This may perhaps be due to that Nano-TiO_2 film provides increased surface area for ascorbate oxidase immobilization resulting in enhanced electron kinetics. In addition to that the presence of some of non-binding sites on enzyme may enhance the magnitude of

current response. Moreover, sol–gel derived Nano-TiO_2 matrix provides a three-dimensional structure in ordered orientations which may also favors direct and faster electron communication between ascorbate oxidase and the electrode surface. These results indicate the immobilization ascorbate oxidase on to the Nano-TiO_2/ITO electrode surface (Fig. 3A).

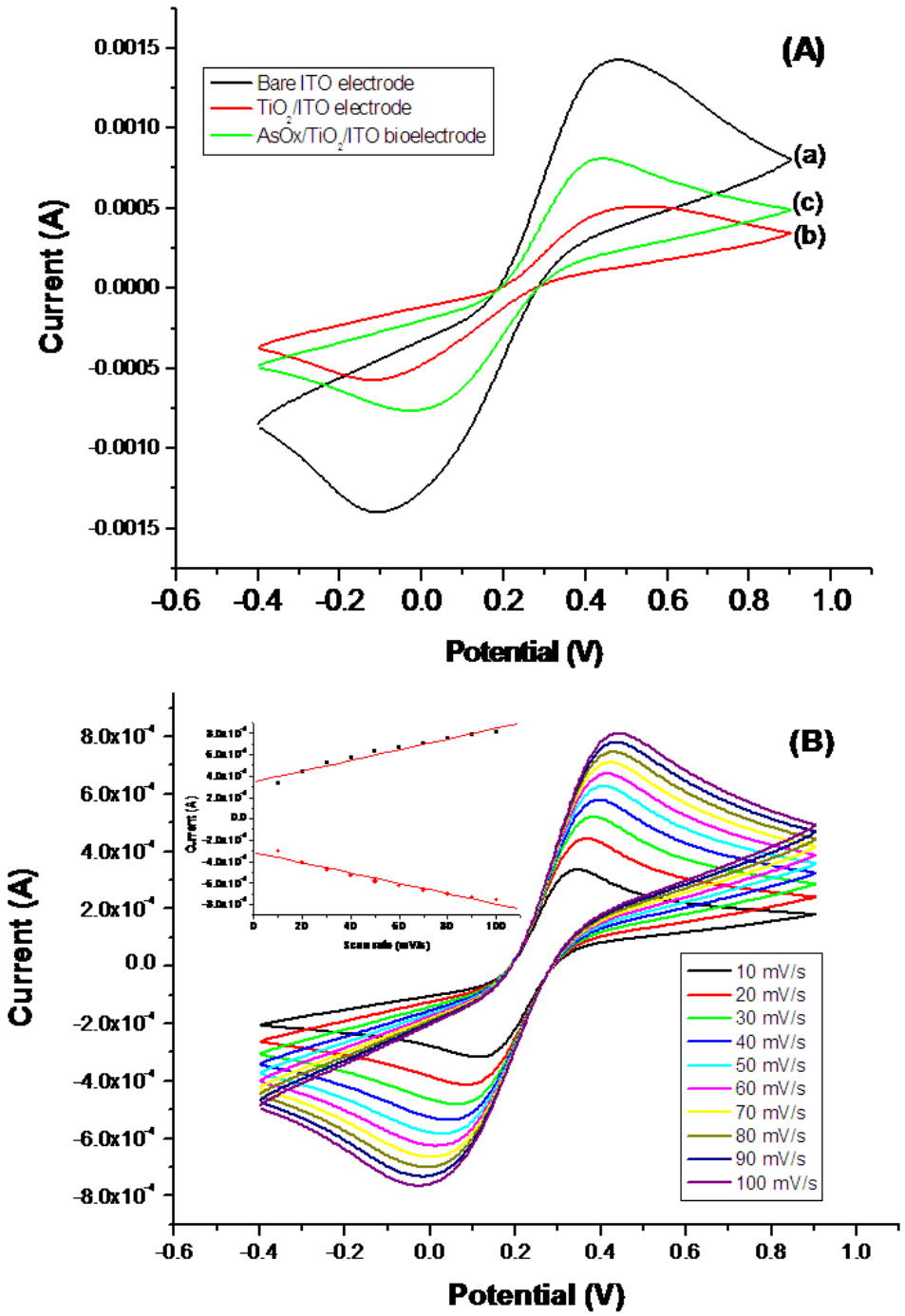

Figure 3. Cyclic voltammogram of (A) bare ITO electrode (a); Nano-TiO_2/ITO electrode (b); AsOx/Nano-TiO_2/ITO bioelectrode (c) at 50 mV/s in PBS containing 50mM $Fe[(CN)_6]^{3-/4-}$ buffer; (B) CV of AsOx/Nano-TiO_2/ITO bioelectrode at different scan rate (10–100 mV/s), inset: magnitude of current as function of scan rate (10–100 mV/s).

Fig. 3 B exhibits results of the CV studies of AsOx/Nano-TiO_2/ITO bioelectrode as a function of scan rate in the range of 10 to100 mV/s carried out in PBS (50 mM, pH =7.0, 0.9% NaCl). It is observed that magnitudes of both anodic and cathodic peak currents response of AsOx/Nano-TiO_2/ITO bioelectrode increase linearly with the increasing scan rate (inset, Fig. 3B) suggesting that electrochemical reaction is a diffusion-controlled process. Moreover, cathodic and anodic peak potentials separation also increases linearly indicating facile charge transfer kinetics in the 10–100 mV/s range of scan rates. These results reveal that sol–gel derived Nano-TiO_2/ITO electrode provides a suitable microenvironment for immobilization of ascorbate oxidase, which perhaps may accelerates electrons transport between electrode and electrolyte medium.

3.4. Electrochemical response studies

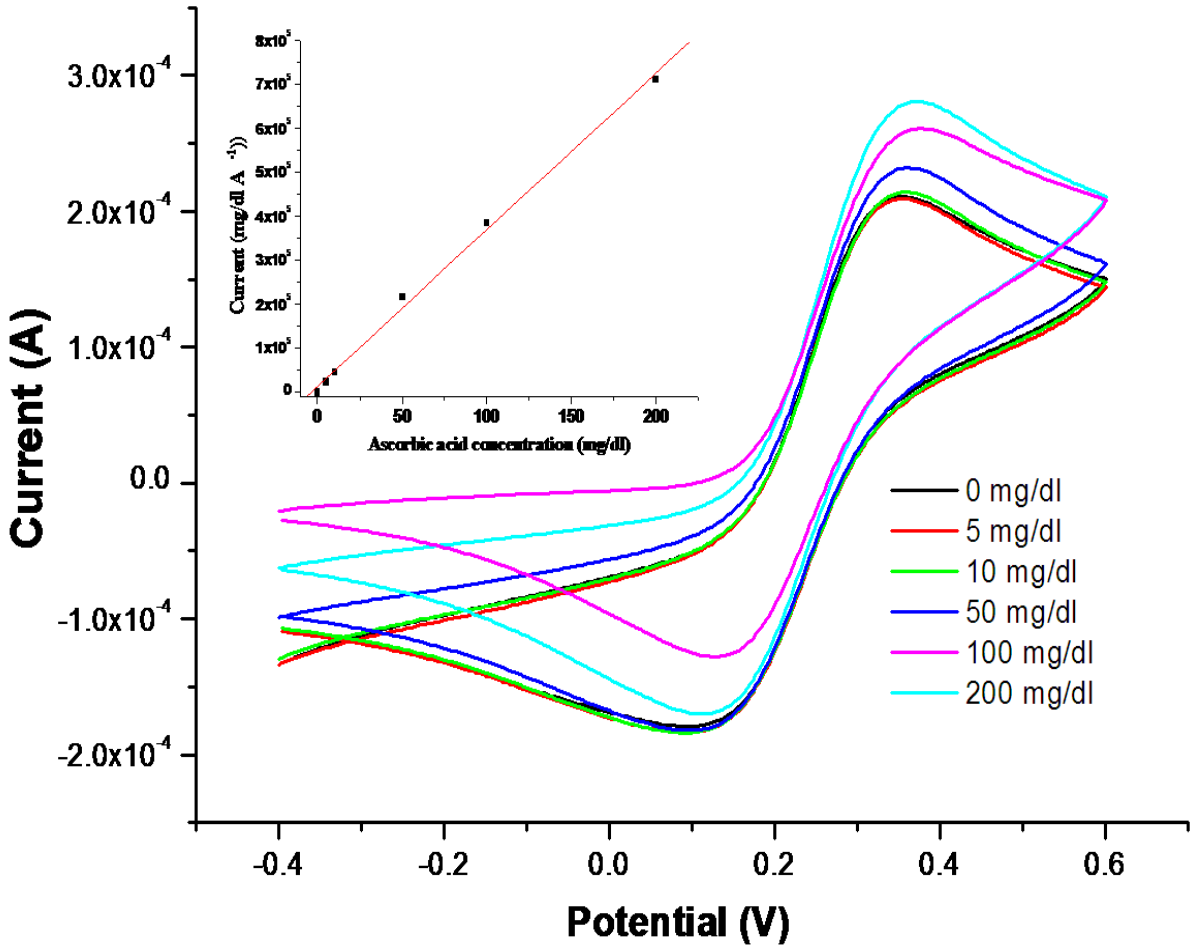

Figure 4. Electrochemical response studies of AsOx/Nano-TiO_2/ITO bioelectrode as function of ascorbic acid concentration using CV, Inset: calibration curve between magnitude of concentration/current and ascorbic acid concentration (5–200 mg/dL).

The electrochemical response of the AsOx/Nano-TiO_2/ITO bioelectrode has been investigated as a function of ascorbic acid concentration (10–200 mg/dL) using CV technique at 50 mV/s scan rate

(Fig. 4) in PBS (50mM, pH 7, 0.9% NaCl) containing 5mM $[Fe(CN)_6]^{3-/4-}$. During response studies of the AsOx/Nano-TiO_2/ITO bioelectrode, it has been observed that magnitude of the response current increases with increasing concentration of ascorbic acid. The increase in electrochemical current response can be attributed to pH sensitive behavior of the AsOx/Nano-TiO_2/ITO. It may be noted that during biochemical reaction mild ascorbic acid undergoes oxidation and release proton in presence of immobilized ascorbate oxidase. This generated protons decrease pH of buffer medium resulting in change of the electrochemical signal of the AsOx/Nano-TiO_2/ITO bioelectrode with increasing concentration of ascorbic acid. Inset in Fig. 4 shows that variation of peak response current with ascorbic acid concentration (linear range as 50–200 mg/dL) follows the linear regression with the value of correlation coefficient as 0.981. This bioelectrode shows fast response time (20 s), high sensitivity (0.212 mA/mg dL cm^2) and low detection limit as 50 mg/dL. J.C.B. Fernandes et al. [28] reported fabrication of a potentiometric biosensor for L-ascorbic acid based on ascorbate oxidase where enzyme is immobilized in a graphite/epoxy electrode by occlusion in a poly(ethyleneco-vinyl acetate) matrix. The comparative lower detection limit value is about 73.9 mg/dL.

In order to confirm affinity of enzyme (ascorbate oxidase) for the substrate (Ascorbic acid), enzyme substrate kinetics parameters have been estimated. The value of apparent Michaelis–Menten constant (K_m^{app}) for the AsOx/Nano-TiO_2/ITO bioelectrode, estimated by Hanes plot (Fig. 4, inset) has been found to be 30.80 mg/dL (1.75 mM). The low value of K_m^{app} indicates enhanced affinity of ascorbate oxidase from the ascorbic acid.

The shelf life of the AsOx/Nano-TiO_2/ITO bioelectrode has been investigated by measuring electrochemical current response with regular interval of 1week. It is observed that AsOx/Nano-TiO_2/ITO bioelectrode preserve ~ 78% of the ascorbate oxidase activity even after 28 days when stored in refrigerated conditions (4ºC) and thereafter the current response decreases.

4. CONCLUSIONS

In the present work, sol–gel derived Nano-TiO_2 film has been utilized to immobilize ascorbate oxidase from Cucurbita species by physiosorption for ascorbic acid detection. The AsOx/Nano-TiO_2/ITO bioelectrode shows improved biosensing performance like linearity as 50–200mg/dL, low detection limit of 50 mg/dL, response time of 20 s, shelf life of 4 weeks, and sensitivity of 0.212 mA/mg dL cm^2 with linear regression coefficient as 0.981. The low value of K_m^{app} obtained as 30.80mg/dL (1.75 mM) indicates high affinity of AsOx/Nano-TiO_2/ITO bioelectrode for ascorbic acid. It should be encouraging to utilize this electrode for detection of other biomarkers such as lipoproteins and uric acid in serum samples.

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