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Fabrication of multiwalled carbon nanotubes/polyaniline modified Au electrode for ascorbic acid determination

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An ascorbate oxidase (AsOx) (E.C.1.10.3.3) purified from *Lagenaria siceraria* fruit was immobilized covalently onto a carboxylated multiwalled carbon nanotubes and polyaniline (c-MWCNT/PANI) layer electrochemically deposited on the surface of an Au electrode. The diffusion coefficient of ascorbic acid was determined as 3.05×10^{-4} cm^2 s^{-1}. The behavior of different electrolytes on electrodeposition was also studied. An ascorbate biosensor was fabricated using a AsOx/c-MWCNT/PANI/Au electrode as a working electrode, Ag/AgCl (3 M/saturated KCl) as standard and Pt wire as an auxiliary electrode connected through a potentiostat. Linear range, response time and detection limit were 2–206 μM, 2 s and 0.9 μM respectively. The biosensor showed optimum response at pH 5.8 and in a broader temperature range (30–45 °C), when polarized at +0.6 V. The biosensor was employed for determination of ascorbic acid level in sera, fruit juices and vitamin C tablets. The sensor was evaluated with 91% recovery of added ascorbic acid in sera and 6.5% and 11.4% within and between batch coefficients of variation respectively for five serum samples. There was a good correlation ($r = 0.98$) between fruit juice ascorbic acid values by the standard 2,6-dichlorophenolindophenol (DCPIP) method and the present method. The enzyme electrode was used 200 times over a period of two months, when stored at 4 °C. The biosensor has advantages over earlier enzyme sensors in that it has no leakage of enzyme, due to the covalent coupling of enzyme with the support, lower response time, wider working range, higher storage stability and no interference by serum substances.

Introduction

Ascorbic acid (Vitamin C) is used as an antioxidant in various cough syrups, soft drinks and vitamin tablets. The quality of citrus fruits is judged on the basis of their vitamin C content. It plays a key role in the metabolism of cholesterol by increasing its elimination and thereby assisting lowering blood cholesterol. The plasma ascorbate concentration in a healthy individual is 61.4–80 μmol L^{-1}. Deficiency of vitamin C causes anemia, scurvy, infections, bleeding gums, muscle degeneration, poor wound healing, atherosclerotic plaques and capillary hemorrhaging, neurotic disturbances consisting of hypochondriasis, hysteria and depression followed by decreased psychomotor performances.[1] Among the various methods available for determination of ascorbic acid such as colorimetry,[2] high performance liquid chromatography,[3] spectrophotometric methods,[4] and sequential injection spectrophotometry,[5] electrochemical methods/enzyme electrodes are considered as one of the most convenient methods, because of their high sensitivity, simplicity and rapidity and no sample preparation. However, direct oxidation of ascorbic acid at bare electrodes is irreversible and requires a high over potential which may result in electrode fouling, poor reproducibility, low selectivity and low sensitivity. Various chemically modified enzyme electrodes have been reported for determination of ascorbic acid such as a β-cyclodextrin–ferrocene inclusion complex modified carbon paste electrode,[6] glassy carbon electrode modified with nickel(II) macro cycle containing dianionic tetraazaannulene ligand,[7] nylon net membrane,[8] electrochemically etched platinum microelectrode,[9] multilayer films of carbon nanotubes and redox polymer on screen-printed carbon electrodes,[10] polypyrrole nanowire modified electrode,[11] carbon nanotube-modified carbon fiber microelectrodes,[12] micelle membrane coated on aminated glassy carbon electrode and gold electrode,[13] Cu(II) zeolite-modified electrode[14] and dopamine using a poly(acriflavine)-modified electrode.[15] Carbon nanotubes (CNTs) have been proposed as an advanced and ideal material for supporting nanosized metallic particles in electrodes for electrocatalysis as well as low temperature fuel cells.[16] Various electrochemical sensors and biosensors based on CNTs have been developed to detect clinically important metabolites such as glucose, NADH and cytochrome *c*.[17–19] MWCNTs (multi-walled CNTs) consist of several concentric tubes of graphite inside one other.[20] These have been employed in biosensors as effective catalyst supports due to their

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large surface areas and unique structural and electromechanical properties, good biocompatibility, easy preparation and renewal of their surface.[21–27] In order to improve the catalytic efficiency to a reasonably high extent, these new catalyst supports were synthesized by different methods.[28–31] The immobilization of enzymes is thus an important factor in view of providing environmental stability and reusability of such a device. Polyaniline is also an attractive conducting polymer, since it exhibits two redox couples facilitating efficient enzyme-polymer charge transfer.[32,33] Various working ascorbate electrodes have been prepared by immobilizing ascorbate oxidase onto different supports either through absorption or entrapment or encapsulation. However these immobilization methods allow leakage of enzyme, resulting in a low stability of the enzyme electrode. Covalent immobilization of enzyme not only overcomes this problem but also leads to better biomolecule activity and greater stability. The present report describes the construction and application of an ascorbate biosensor by immobilizing ascorbate oxidase on a c-MWCNT/PANI modified gold electrode through glutaraldehyde coupling.

Experimental

Reagents

Sephadex G-100, DEAE-Sephacel, glutaraldehyde (grade 1, 25%) and distilled aniline were from Sigma (Aldrich). Carboxylated multi-walled carbon nanotubes (c-MWCNT) (Functionalized MWCNT) (12 walls, length 15–30μm, purity 90%, metal content: nil) from Intelligent Materials Pvt. Ltd., Panchkula (Haryana), India were used. The commercial vitamin C tablets (marketed under the brand name "Lamcea and Becozyme c forte" manufactured by Bayer, EU, Turkey) and fruits (lemon, grape, orange and apple) and gold wire (1.5 × 0.05 cm^2) (23 carat) were purchased from a local market. Fresh serum samples of healthy individuals were collected from the hospital of Pt. BDS University of Health & Medical Science, Rohtak and stored at −20 °C until use. Fresh green fruits of bottle gourd (*Lagenaria siceraria*) were collected locally from a nearby village during the month of June–July (40 ± 5 °C) in an ice bath, washed in distilled water and stored at 4 °C until use. All electrochemical experiments, including cyclic voltammetry (CV) and amperometric measurements were carried out in a potentiostat/galvanostat (Autolab, Eco Chemie, The Netherlands. Model: AUT83785).

Extraction and purification of ascorbate oxidase

The extraction and purification of enzyme was carried out at 4 °C. The skin of the green fruit of bottle guard was peeled off, weighed and homogenized in a chilled pestle and mortar. The homogenate was centrifuged at 15,000 g for 30 min at 4 °C and the supernatant was collected and treated as crude enzyme. The crude enzyme was purified by 65% ammonium sulfate precipitation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-Sephacel using linear gradient of KCl (0.1 M to 0.6 M). The purity of the enzyme was checked by PAGE using $AgNO_3$ as protein stain in PAGE which showed a single band (results not shown) indicating its apparent homogeneity. The activity and protein content of various enzyme preparations were estimated as follows:

Assay of ascorbate oxidase

The assay of ascorbate oxidase was carried out as described with a slight modification.[34] The reaction mixture contained 2.9 ml phosphate/EDTA buffer (0.1 M, pH 5.6), 0.1 ml L-ascorbic acid (0.005 M) and 0.1 ml enzyme. The blank contained 3.0 ml of 0.1 M phosphate/EDTA buffer pH 5.6 and 0.1 ml of ascorbic acid (0.005 M). The change in A_{293} was read in a UV spectrophotometer (Shimagzu 1700, Japan). The activity of ascorbate oxidase was calculated as follows:

$$\text{Activity (U/ml)} = \frac{(\Delta A_{265}/\text{min}) \times 3.1 \times \text{dilution factor}}{e \times 0.1}$$

[e = 13.386 (extinction coefficient of dehydroascorbate); total volume = 3.1; enzyme volume = 0.1 ml]

Protein determination

Protein content in various enzyme preparations was determined by the Lowry method using bovine serum albumin as standard protein.[35]

Electrodeposition of aniline on the Au electrode and attachment of c-MWCNT

Aniline (50 μl) was added to 10.0 ml of 1N HCl and electrodeposited onto a Au electrode through cyclic votammetric technique using a potentiosatat-galvanostat. Prior to electrodeposition, the gold electrode (1.5 × 0.05 cm^2) was polished with 0.05 μM alumina slurry and then immersed in piranha solution (a hot mixed solution of 30% H_2O_2 and conc. H_2SO_4, 3 : 1 ratio (v/v)) for 10 min followed by ultrasonic cleaning with DW and a controlled water bath. The electrochemical polymerization of aniline onto the Au electrode (working electrode) was achieved immediately through cyclic voltammetry by applying ten polymerization cycles at 0.0 to +1.5 V as described.[26] One gram c-MWCNT was suspended in 1 ml of a mixture of concentrated H_2SO_4 and HNO_3 in 3 : 1 ratio (v/v) and ultrasonicated for 2 h to finally get a dispersed black colored solution of c-MWCNTs. The c-MWCNT solution was washed with distilled water thoroughly to remove the acid. The PANI coated Au electrode was dipped into this c-MWCNT solution for 12 h. The resulting c-MWCNT/PANI/Au electrode was washed thoroughly with distilled water to remove unbound matter and kept in a dry petri plate at 4 °C.

Immobilization of ascorbic oxidase onto the c-MWCNT/PANI/Au electrode

The c-MWCNT/PANI layer on the Au electrode was activated by spreading 0.5% glutaraldehyde solution on it and allowing it to stand at room temperature for 4 h. The excess of glutaraldehyde was removed by washing the c-MWCNT/PANI modified Au surface 4 times with distilled water.[26] Purified ascorbate oxidase (5 μl containing 11 U) was placed onto the glutaraldehyde activated surface of the c-MWCNT/PANI/Au electrode and kept overnight for immobilization. The electrode was washed 3 times with 0.1 M phosphate/EDTA buffer pH 5.6 to remove unbound enzyme from the electrode surface. Protein concentration was calculated in wash out solution to determine conjugation yield. The resulting AsOx/c-MWCNT/PANI/Au

electrode was used as a working electrode. It was stored at 4 °C, when not in use.

Scanning electron microscopy

Scanning electron microscopy (SEM) of bare gold and AsOx/cMWCNT/PANI/Au electrode were carried out at the Department of Anatomy, AIIMS, New Delhi to confirm immobilization of ascorbic oxidase. The electrodes were cut into small pieces (1 cm) and placed on a copper mount of 2 cm diameter using a spray gun and micrographs were taken with a scanning electron microscope.

Construction and response measurement of the AsOx/c-MWCNT/PANI/Au electrode

For cyclic voltammetric studies, different electrolytes were tested such as H_2SO_4 (0.5 M), HCl (1 N) and KCl (1 N). A solution (20 mL) containing 15 mL electrolyte and 5 mL phosphate/EDTA buffer (0.1 M, pH 5.8.) was pipetted into the electrochemical cell. The modified electrode (AsOx/c-MWCNT/PANI/Au electrode) together with the reference electrode (Ag/AgCl) (3 M/saturated KCl) and the auxiliary electrode (Pt wire) were immersed into this solution, the potential was scanned from 0.0 to +1.5V *vs.* Ag/AgCl using cyclic voltammetry. Then different concentrations of ascorbic acid were added to the voltammetric cell and the potential was scanned from 0.0 to +1.5V *vs.* Ag/AgCl. For the amperometric response of the AsOx/c-MWCNT/PANI/Au electrode, the current (mA) was measured by applying a potential of +0.6V. All electrochemical measurements were conducted at room temperature. The following electrochemical reactions occur during the response measurement:

$$\text{L}\cdot\textbf{A}\text{scorbic acid} + \frac{1}{2}O_2 \xrightarrow{\text{AsOx}} \textbf{D}\text{ehydroascorbic acid} + H_2O$$

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

Optimization of the AsOx/c-MWCNT/PANI/Au electrode

To determine the optimum working conditions of the working electrode, the pH of phosphate/EDTA buffer (0.1 M) was varied from pH 5.0 to 8.0 at intervals of pH 0.5. Similarly, to determine optimum temperature, the reaction mixture was incubated at 20 °C to 50 °C at an interval of 5 °C. To determine the optimum response time, the current response was studied from 1 s to 25 s. To study the effect of substrate concentration, different ascorbic acid concentrations, ranging from 0.002 mM to 0.206 mM were used. K_m for ascorbic acid was calculated from a Lineweaver Burk plot.

Amperometric measurement of ascorbate in fruits, Vit-C tablet and serum

The average sized fruits (orange, lemon, apple and grapefruit) were peeled and their juice was prepared using a blender. The juice was centrifuged at 2000 g for 10 min at 4 °C and the supernatant was collected and diluted suitably. To measure L-ascorbic acid in vitamin C tablets, Lamcea 1000 mg (L-ascorbic acid tablet) and Becozyme c forte 500 mg (L-ascorbic acid tablet) were dissolved in the phosphate/EDTA buffer (0.1 M, pH 5.6) separately after grinding it in a pestle and mortar. The contents of L-ascorbic acid in both vitamin C tablet solutions and fruit juices were determined by the present biosensor as described for its testing under optimal working conditions except that ascorbic acid was replaced by fruit juice/dissolved tablet/serum. The content of ascorbic acid was determined from a standard curve between ascorbic acid concentrations *vs.* current in mA.

Comparison of the present method with standard DCPIP titrimetric method

The ascorbic acid content was determined in clear fresh juice by the standard 2,6-dichlorophenolindophenol (DCPIP) method[37] (x) and the present method (y). To determine ascorbic acid by the standard DCPIP method, the juice was diluted by 10 times with 6% metaphosphoric acid and then titrated against the DCPIP until a pink tint persisting for about 30 s appeared. A standard of ascorbic acid was also run in a similar manner and the ascorbic acid content in fruit juice was calculated. The results of both methods were compared and correlated using a regression equation.

Results and discussion

Cyclic voltammetric and FT-IR characterization of c-MWCNT/PANI/Au and AsOx/c-MWCNT/PANI/Au electrode

Fig. 1 reveals the cyclic voltammograms (CV) of the c-MWCNT/PANI modified Au electrode before and after immobilization of enzyme recorded in phosphate/EDTA buffer 0.1 M, pH 5.8 at 30 mV s^{-1}. CV of c-MWCNT/PANI/Au revealed one anodic and one cathodic peak due to grouping of c-MWCNT/PANI. The absence of the oxidation peaks in the cyclic voltammogram of AsOx/c-MWCNT/PANI indicated the immobilization of ascorbate oxidase onto the c-MWCNT/PANI surface. These results indicated that a slow redox process occurred at the electrode surface due to slow electron transfer from the surface showing the immobilization of ascorbate oxidase.[22] Fig. 2 summarizes the chemical reactions involved in covalent immobilization of the enzyme (ascorbate oxidase) onto the c-MWCNT/PANI layer through glutaraldehyde coupling. Fig. 3 shows FT-IR spectra for the PANI/Au electrode (Curve i), c-MWCNT/PANI/Au electrode (Curve ii) and AsOx/c-MWCNT/PANI/Au electrode

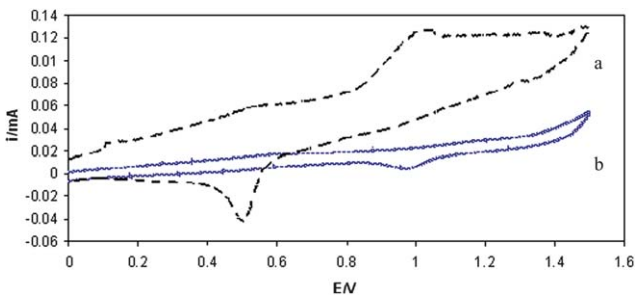

Fig. 1 Cyclic voltammograms of c-MWCNT/PANI Au electrode biosensor: (a) unmodified and (b) modified with AsOx/c-MWCNT/PANI in 0.1 M phosphate/EDTA buffer, pH 5.8.

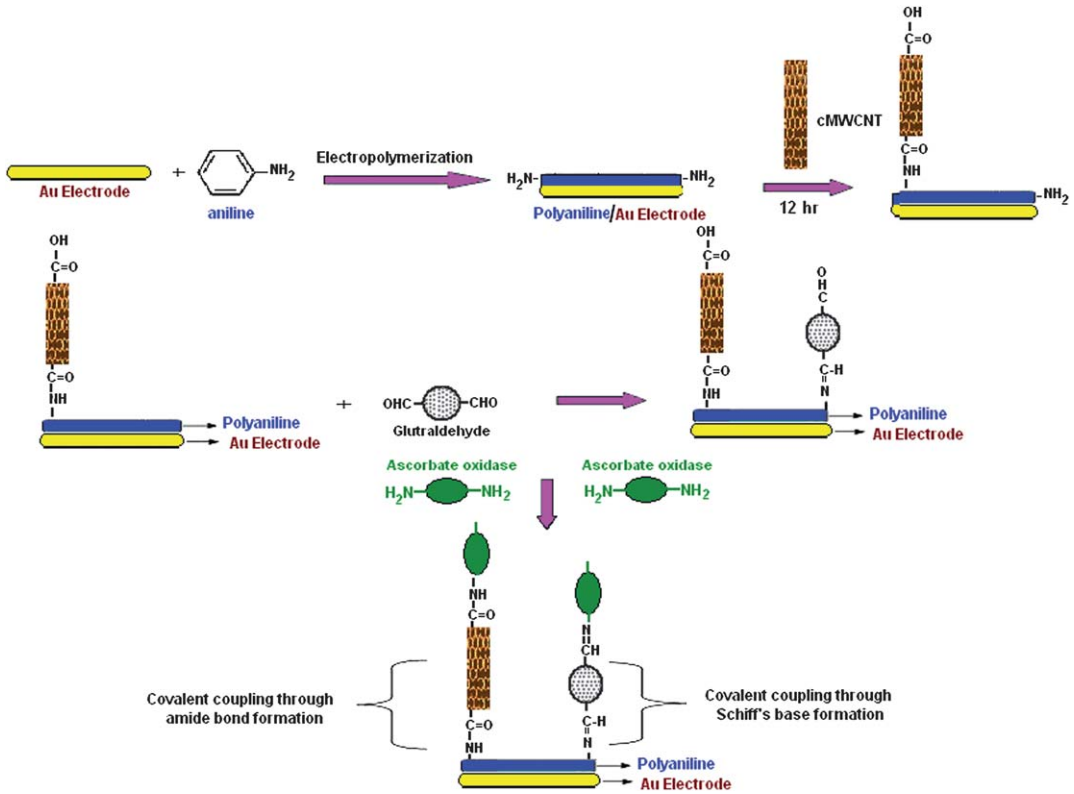

Fig. 2 Scheme of the chemical sequence of electropolymerization of aniline on Au electrode, attachment of c-MWCNT to PANI and immobilization of the enzyme (ascorbate oxidase) on c-MWCNT/PANI layer on modified Au electrode.

(Curve iii). Curve i shows peaks of quinoid and benzenoid structures of PANI at 1577 and 1485 cm^{-1} and bands at 1319 cm^{-1} assigned to –C–N stretching of 2° amines. It shows that the aniline was polymerized to form PANI chain linked through –NH– bonds at the Au electrode with two free $–NH_2$ groups at its ends.[38] Curve ii shows the peak of C–O bond of free –COOH groups present at 1294.99 and 1343.93 cm^{-1} and C–N bonds of c-MWCNT immobilized on PANI at 1045.63 and 1164.13 cm^{-1}. It reveals that the –COOH group at one end of c-MWCNT gets attached to $–NH_2$ groups of PANI through –CO–NH– bonds while it is free at another end.[39] Curve iii shows the peak of the C═N bond of immobilized enzyme through

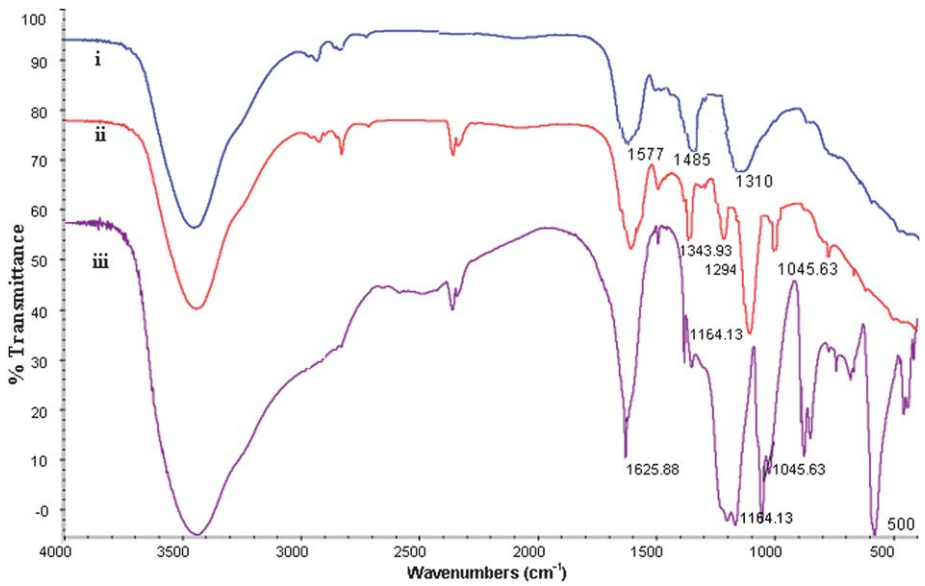

Fig. 3 FT-IR spectra of PANI (i), c-MWCNT/PANI/Au electrode (ii) and AsOx/c-MWCNT/PANI/Au electrode (iii).

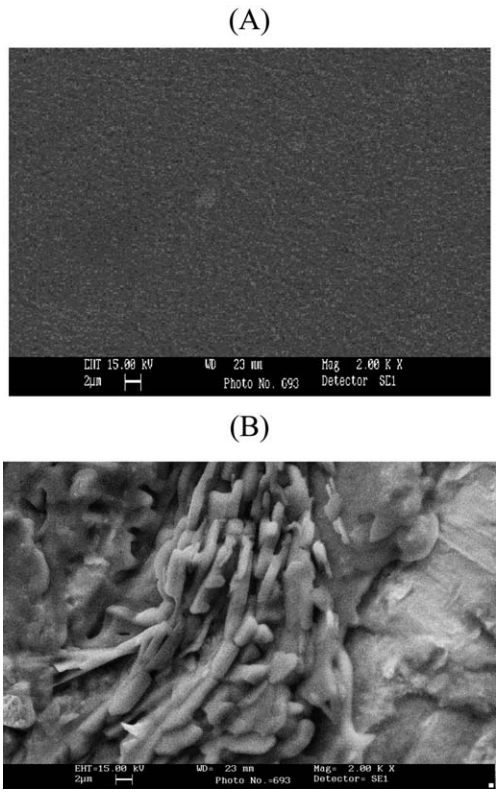

Fig. 4 (A) SEM of Au electrode, (B) SEM of Au electrode with electrodeposited AsOx/MWCNT/PANI layer.

glutaraldehyde at 1625.88 cm^{-1} beside the peak of the C–N bond. These studies confirmed that the enzyme was immobilized covalently onto the free –COOH group of MWCNT through –CONH– bonds and free $–NH_2$ groups at one end of PANI through Schiff's base (glutaraldehyde coupling). The disappearance of the peak of free –COOH at 1294 cm^{-1} in this curve proves it. There was one more peak at wavelength 500.00 cm^{-1}. The electrodeposition of PANI over the Au electrode and simple immobilization of enzyme on c-MWCNT/PANI/Au electrode was also confirmed by SEM images (Fig. 4 A and B).

Optimization of the biosensor

Fig. 5 shows that the CV voltammetric peak height of the modified electrode consisting of immobilized enzyme depends strongly on the type of supporting electrolyte. Among different electrolytes tested such as H_2SO_4 (0.5 M), HCl (1 N) and KCl (1 N), KCl gave a well-defined oxidation and reduction peak. The current was increased as the potential reached the reduction potential of the analyte, but then fell down, as the concentration of the analyte was depleted close to the electrode surface. When the applied potential was reversed, it reached the potential that reoxidized the product formed in the first reduction reaction, and produced a current of reverse polarity from the forward scan. The addition of H_2SO_4/HCl caused a decrease in the pH of the reaction buffer from 5.8 to 4.0, which affected the activity of AsOx adversely. When KCl was used it did not alter the pH, hence it was used as an electrolyte in the subsequent amperometric studies. The optimal pH of the biosensor/immobilized

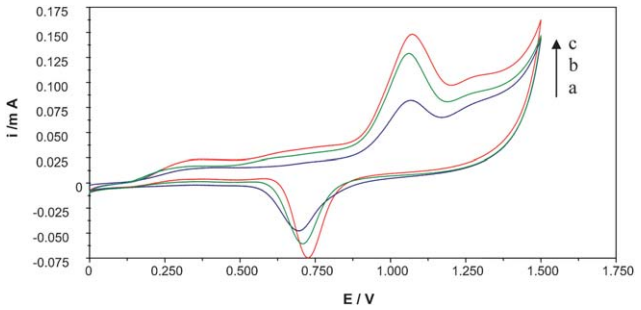

Fig. 5 Cyclic voltammograms of AsOx/c-MWCNT/PANI/Au electrode in 0.1 M phosphate/EDTA buffer (pH 5.8) containing: (a) H_2SO_4 (0.5 M); (b) HCl (1 N); (c) KCl (1 N).

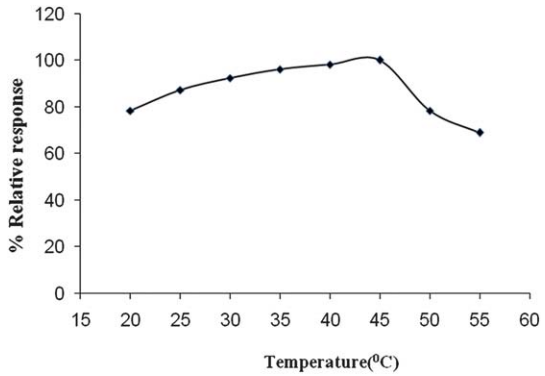

Fig. 6 Effect of temperature on the response of the ascorbate biosensor based on AsOx/c-MWCNT/PANI/Au electrode (the optimum biosensor response at 45 °C was considered as 100% relative activity. The biosensor response at other temperatures as % relative activity was in comparison to that at 45 °C).

ascorbate oxidase was 5.8, which is slightly higher than that of the free enzyme (pH 5.5).[36] Although the present biosensor exhibited optimum temperature at 45 °C, it has a broader temperature range for activity, as it showed 98% activity at 40 °C, 97% at 35 °C and 94% at 30 °C (Fig. 6). Hence it can be used effectively between the temperature range 30 °C to 45 °C. This optimum temperature of the present biosensor/AsOx immobilized on MWCNT/PANI/Au is higher than that of the free enzyme (40 °C)[36] and earlier biosensors (25–35 °C).[13,14,36] The polyaniline matrix might protect the enzyme from denaturation at 45 °C as well as thermal variations, which favors the repeated use of the PANI-AsOx electrode.[23,24] The rate of reaction of immobilized ascorbate oxidase was linear up to 2 s, after which it was constant.

Effect of ascorbic acid concentration on AsOx/MWCNT/PANI/Au electrode response

Fig. 7(A) shows the cyclic voltammogram (CV) of the AsOx/MWCNT/PANI/Au electrode response at different ascorbic acid concentrations (2–206 μM or 0.352–36.25 mg L^{-1}). There was a linear increase in oxidation current with the increase in ascorbic acid concentration in the range 2–206 μM (0.352–36.25 mg L^{-1}) after which it was constant. K_m for ascorbic acid was found to be 16.6 μM (2.92 mg L^{-1}). Chronoamperometry (CA) was

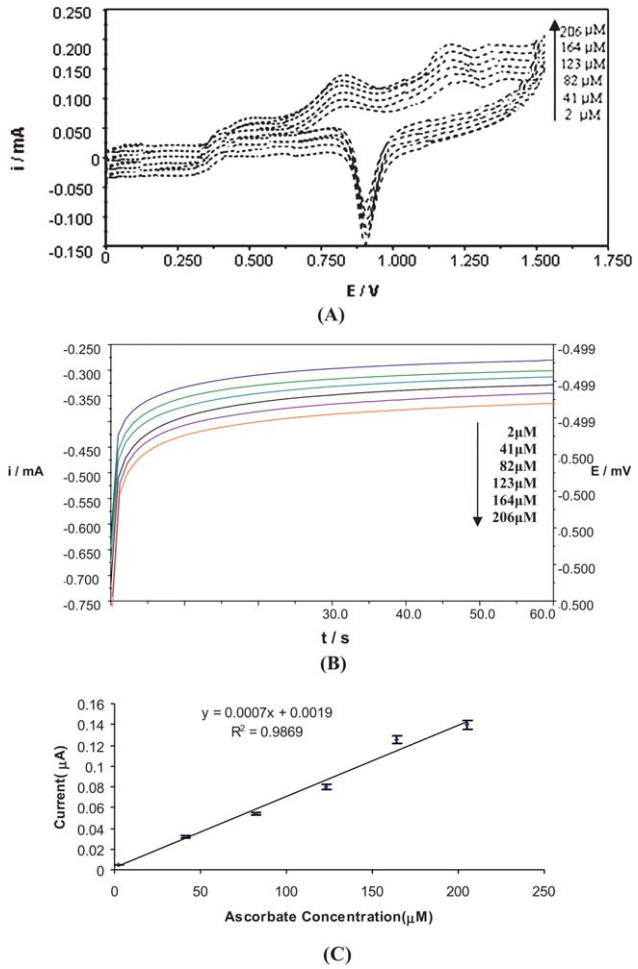

Fig. 7 (A) Cyclic voltammograms, (B) Chronoamperograms of AsOx/MWCNT/PANI Au electrode at +0.6V in 0.1 M phosphate/EDTA buffer pH 5.8 containing different substrate (ascorbic acid) concentrations in the range 2–206 µM and (C) the linear relationship between the amperometric response of the biosensor (µA) and the concentration of ascorbic acid (µM).

employed for the investigation of electrochemical processes at the chemically modified electrode. Fig. 7(B) shows chronoamperometric measurements of AsOx/c-MWCNT/PANI/Au at different concentrations of ascorbic acid. It provides the current–time profiles obtained by setting the working electrode potential at +0.6V for different concentrations of ascorbic acid.

Amperometric determination of ascorbic acid

An amperometric method was developed for determination of ascorbic acid employing the present biosensor. The method was evaluated as follows:

Evaluation of the biosensor

Linearity. There was a linear relationship between current (mA) and ascorbic acid concentration ranging from 2 to 206 µM (0.352–36.25 mg L^{-1}) (Fig. 7C), in the reaction mixture as established by CV at 0.0 to +1.5 V, which is better than a micelle

Table 1 A comparison of various types of ascorbate biosensors.[a]

Type	Type of support for immobilization	Type of electrode	Method of immobilization	Optimum pH	Potential (V) for max. current	Detection limit (10^{-6} mol L^{-1})	Linear range (10^{-6} mol L^{-1})	Response time (seconds)	Storage stability (months)	Reference
Amperometric	Teflon membrane	DO probe	Adsorption	7.5	0.8	400 and 1000	50 to 200	4.5	2	Erol Akyilmaz *et al.* (1999)38
Potentiometric	Ethylene-vinyl acetate membrane	Graphite/epoxy electrode	Covalent	5.0	NR	4.2	8 to 450	300	3	Julio Cesar B. *et al.* (1999)39
Amperometric	b-Cyclodextrin–ferrocene inclusion complex	Carbon paste electrode	Absorption	10.0	0.20	0.1	50	NR	12	Zhang Guorong *et al.* (2000)6
Potentiometric	Ferrocene-l-cysteine self-assembled supramolecular film	Gold electrode	Electrostatic adsorption	5.8	0.0 to 0.5	0.2	1.0 to 5.0	0.10	NR	Shengfu Wang *et al.* (2004)36
Potentiometric	poly(3,4-ethyl-endioxythiophene) modified	Pt disk electrode	NR	7.0	–0.5 to +0.5	4.41	NR	4	NR	A. Bello *et al.* (2007)37
Amperometric	Micelle membrane	GC ectrode and Au electrode	Absorption	5.5	NR	NR	5 to 400	NR	NR	Xiuyun *et al.* Wang (2008)13
Amperometric	Graphite zeolite-modified	Pt electrode	NR	4.5	NR	0.276	0.003 to 6.00	NR	NR	Tahereh Rohani *et al.* (2009)14
Amperometric	Multiwalled carbon nanotube/ Polyaniline modified	Au electrode	Covalent	5.6	0.6	0.9	2 to 206	3	2	Present work

[a] NR = Not reported.

Table 2 Ascorbic acid levels in sera of apparently healthy adults, as determined by the biosensor based on AsOx/MWCNT/PANI/Au electrode

Serum sample	Sex	Ascorbate (μmol L^{-1})[a] Mean ± S D
S1	F	29.0 ± 1.0
S2	F	84.0 ± 0.70
S3	M	86.8 ± 0.44
S4	F	78.8 ± 0.44
S5	M	80.0 ± 0.70
S6	M	71.3 ± 0.30
S7	M	74.5 ± 0.44
S8	M	74.7 ± 0.45
S9	F	63.8 ± 0.80
S10	F	33.8 ± 0.90

[a] Mean value of five replications.

Table 3 Determination of ascorbic acid in fruit juices by the ascorbate biosensor based on AsOx/MWCNT/PANI/Au electrode

Fruit juice	Ascorbic acid by biosensor (mg dl^{-1})[a] Mean ± S D	Ascorbic acid by DCPIP(mg dl^{-1})[a] Mean ± S D	Relative SD (%) of proposed method
Lemon	51.33 ± 1.15	50.23 ± 2.04	2.24
Grape	34.75 ± 1.38	38.00 ± 3.00	3.97
Orange	48.33 ± 2.88	48.24 ± 1.37	5.95
Apple	7.00 ± 1.00	6.89 ± 1.80	14.2

[a] Mean value of three replications.

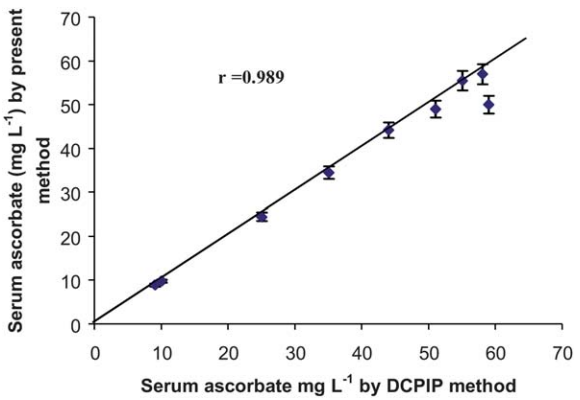

Fig. 8 Correlation between fruit ascorbic acid value as determined by the DCPIP method (*x*-axis) and the present biosensor (*y*-axis) based on AsOx/MWCNT/PANI Au electrode.

membrane based biosensor (5 μM to 400 μM or 0.88 to 70.40 mg L^{-1}) (Table 1).[10,12,17]

Detection limit. The detection limit of the present method was 0.9 μM (0.158 mg L^{-1}), which is lower than/comparable to earlier reported biosensors[10,12,13,40–43] (Table 1).

Recovery. The analytical recovery of a known amount of added ascorbic acid into serum was determined by the present biosensor. The mean analytic recoveries of added 1.0 mg dl^{-1} and 2.0 mg dl^{-1} (56.8 μM to 113.6 μM) (final concentration in the reaction mixture) were 91.3% and 91% respectively, which were non-significantly different ($p > 1$) than that by the o-phenylenediamine mediated ascorbate oxidase assay (95%).[44]

Precision. To test the reproducibility and reliability of the present ascorbate biosensor ascorbic acid content in six serum samples was estimated on a single day (within batch) and five times again after storage at −20 °C for one week (between batch). The results showed that determinations were consistent and within and between coefficients of variation (CVs) were 6.5% and 11.4% respectively. The high precision indicated the reproducibility and consistency of the present method.

Study of interferences. Efforts were made to study the effects of individual interferants at 10 mM concentration. Among the interferants tested in fruits were compounds such as: oxalic acid, glucose, fructose, citric acid, lactose, starch, sucrose, tartaric acid and sodium chloride. The value of I_{max} does not vary significantly in the presence of interferants indicating non-influence of the individual interferants.

Applications. The biosensor was applied for determination of level of ascorbic acid in serum, fruit juices and vitamin C tablets. However the working range of the biosensor is specific for serum and vitamin C tablets.

Ascorbic acid determination in serum. The serum ascorbic acid level in healthy adults as measured by the present biosensor ranged from 71.3–86.8 μmol L^{-1} (12.54–15.27 mg L^{-1}) with a mean of 77.4 μmol L^{-1} (13.62 mg L^{-1})($n = 10$) in males and 33.8–84.0 μmol L^{-1} (5.94–14.78 mg L^{-1}) with a mean of 65.1 μmol L^{-1} (11.45 mg L^{-1}) ($n = 10$) in females (Table 2), which is in the normal established range (5.1–80 μmol L^{-1} or 0.89–14.08 mg L^{-1}).

Ascorbic acid determination in fruit juices. The content of L-ascorbic acid in fruit juices was determined by the present ascorbate biosensor and found to be 51.33 mg dl^{-1} (2910 μM) in

Table 4 Determination of ascorbic acid in pharmaceutical vitamin C tablets by the ascorbate biosensor based on AsOx/MWCNT/PANI/Au electrode

Tablet	L-Ascorbic acid conc. (mg/tablet)	L-Ascorbic acid conc. by biosensor (mg/tablet)[a] Mean ± S D	RSD (%)	L-Ascorbic acid conc. by DCPIP method (mg/tablet)[a] Mean ± S D	RSD (%)
Lamcea	500	496.3 ± 1.52	0.306	495.2 ± 1.48	0.30
Becozyme c forte (multivitamin)	200	192.7 ± 1.52	0.78	190.0 ± 1.73	0.91

[a] Average value of three replications.

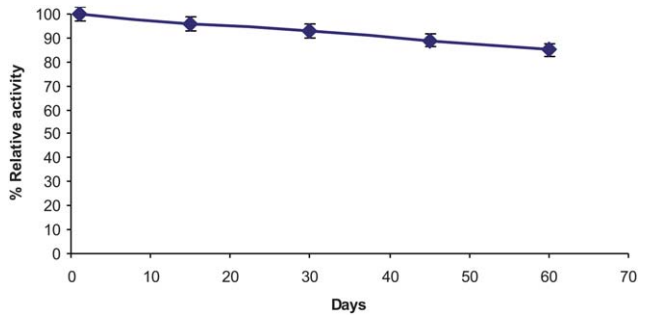

Fig. 9 Effect of storage at 4 °C on the response of the ascorbate biosensor based on AsOx/c-MWCNT/PANI/Au electrode.

lemon, 48.33 mg dl^{-1} (2740 μM) in orange, 34.75 mg dl^{-1} (1974 μM) in grapes and 7.0 mg dl^{-1} (397.7 μM) in apple. The ascorbic acid values of fruit juices measured by our method were in good agreement with those by the standard DCPIP method (Table 3) with a good regression coefficient ($r = 0.989$) (Fig. 8).

Ascorbic acid determination in vitamin C tablets. Ascorbic acid content in vitamin C tablets as measured by the present sensor was 496.3 mg/tablet in Lamcea and 192.7 mg/tablet in Becozyme C forte (multivitamin tablet) (Table 4). These results were also in good agreement with those by the DCPIP method. This shows the reliability of the present method.

Stability and reusability. The biosensor was stored at 4 °C when not in use. To determine the biosensor storage stability, the amperometric measurements were carried out periodically every fifth day for 2 months. The enzyme electrode lost only 15% of its initial activity during its storage over a period of 2 months (Fig. 9). Polyaniline being a good thermal insulator for enzymes provided the microenvironment to protect the enzyme from environmental and thermal variations resulting in the repeated use of PANI-ascorbate electrodes.[23,24] A comparison of various types of ascorbate biosensors with the present biosensor is summarized in Table 1.

Conclusions

The use of a c-MWCNT/PANI modified Au electrode has resulted in an improved analytical performance of the ascorbate biosensor in terms of low response time (2 s), wider working range (2–206 μM), higher storage stability (>2 months) and no interference by serum substances compared to earlier biosensors. However the detection limit of the present sensor is not better than earlier sensors[14] and could be improved further, by modifying the matrix.

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