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IMMOBILIZATION OF MALATE DEHYDROGENASE ON CARBON NANOTUBES FOR DEVELOPMENT OF MALATE BIOSENSOR

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Abstract

An amperometric malic acid biosensor was developed by immobilizing malate dehydrogenase on multi-walled carbon nanotubes (MWCNT) coated on screen printed carbon electrode. The screen printed carbon electrode is made up of three electrodes viz., carbon as working, platinum as counter and silver as reference electrode. Detection of L-malic acid concentration provides important information about the ripening and shelf life of the fruits. The NADP specific malate dehydrogenase was immobilized on carboxylated multiwalled carbon nanotubes using cross linker EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide] on screen printed carbon electrode. An amperometric current was measured by differential pulse voltammetry (DPV) which increases with increasing concentrations of malic acid at fixed concentration of NADP. Enzyme electrode was characterized by scanning electron microscopy (SEM) and Fourier transform infrared (FTIR) spectroscopy. The detection limit of malic acid by the sensor was 60 - 120 µM and sensitivity of the sensor was 60 µM with a response time of 60s. The usual detection methods of malic acid are nonspecific, time consuming and less sensitive. However, an amperometric malic acid nanosensor is quick, specific and more sensitive for detection of malic acid in test samples.

Key words: c-MWCNT, EDC, L-malic acid, NADP specific malate dehydrogenase, Screen printed carbon electrode.

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INTRODUCTION

Biosensors are devices that combine the selectivity and specificity of a biological sensing element such as an enzyme, nucleic acid or an antibody with a suitable transducer. Based on transduction process, biosensors are classified into different categories such as electrochemical, optical, piezoelectric, thermal and colorimetric. Among these different kinds of biosensors electrochemical biosensors are most widely used and successfully commercialized (4, 24). The selection of a specific foodstuff by a consumer is largely based on sensory conception with taste, including saltiness, sweetness, bitterness and acidity. The important parameter for the selection of a foodstuff is its texture which is influenced by many factors like moisture content, fat, carbohydrate and protein levels (35). Organic acids are responsible for the taste of a fruit or vegetable along with balance of sugar and acids. Major organic acids in most fruits are L-malic acid and citric acid (3, 10). L-malic acid is an important indicator of fruit maturity (5, 18, 23). It is the predominant acid in many fruits and vegetables and second highest in concentration in various citrus fruits, berries, figs, beans and tomatoes (21). Hence, determination of L-malic acid concentration provides important information about the ripening and shelf-life of fruits than simple appearance and taste. The qualities of fruits depend on their biochemical composition. Fruit maturity is the most important factor that determines shelf life and final fruit quality (3). Life stages of fruits such as growth, maturation, senescence, color and antimicrobial activity all depend on organic acid (8).

Oxidative decarboxylation of malic acid is carried out by MDH (also called malic enzyme) using $NADP^+$ as a coenzyme (cofactor) to produce pyruvate, CO_2 and NADPH (14). Sources of NADP-malic enzymes are living organisms, including prokaryotic and eukaryotic microorganisms, plants, animals and humans (12). Three types of malic enzyme found in mammals are: (a) cytosolic $NADP^+$ dependent malic enzyme (c-NADP-ME), (b) mitochondrial $NADP^+$ dependent malic enzyme (m-NADP-ME) and (c) mitochondrial NAD $(P)^+$ dependent malic enzyme (m-NAD-ME) (9). Malic enzyme plays major role in respiration during ripening and photosynthesis. Different types of malic enzymes are differentiated by their NAD^+ or $NADP^+$ specificity, distribution and ability to decarboxylate oxaloacetate (14, 29). In immobilization, enzymes are physically confined or localized with retention of the enzyme catalytic activity and can be used repeatedly and continuously (33). Enzyme immobilization is classified into mainly three types which are binding to a support, entrapment and cross-linking. Support binding is further classified into physical, ionic and covalent. Covalent binding of enzyme to support is stronger than ionic but physical binding is generally weak to keep enzyme fixed to the support. Covalent binding prevents enzyme leakage completely. In entrapment, enzyme is packed in a polymer network such as an organic polymer or silica sol-gel and enzyme leakage is prevented entirely (17, 30). In cross-linking bifunctional reagents are used to prepare carrier-less macroparticles. For carrier free immobilization of enzymes two approaches are used viz. cross-linked enzyme crystal (CLEC) and cross-linked enzyme aggregates (CLEA). These approaches offer high enzyme activity, high stability and low production costs (28). In development of biosensor, immobilized enzyme solves several problems like loss of enzyme activity, stability and shelf life (1, 25). Performance of direct electron transfer between enzyme and electrode is enhanced by carbon nanotubes (CNT) and metal nanoparticles (11, 19, 27). CNT promotes the electron transfer between electrode enzyme and substrate due

to its extraordinary electron transport property (32, 34). As active centers of most redox enzymes are located deeply in hydrophobic cavity of molecules, therefore electrochemistry of redox enzymes on common electrode is very difficult (16, 26). The designing of chemically modified electrode is the special feature of the electrochemical techniques for sensitive and selective analytical applications. Due to its fast electron transfer kinetics, CNT acts as an excellent transducer in electrochemical biosensors (2, 6, 22).

In the present study, immobilized NADP-specific malate dehydrogenase catalyzes the oxidative decarboxylation of L-malate to pyruvate with concomitant reduction of the cofactor $NADP^+$ (oxidized form of nicotinamide adenine dinucleotide phosphate).

$$\text{L-Malate} + NADP^+ \xrightleftharpoons{\text{Malate dehydrogenase}} \text{Pyruvate} + CO_2 + NADPH$$

The electrocatalytical property of carboxylated multiwalled carbon nanotube (c-MWCNT) modified electrode toward NADPH detection was investigated by amperometric techniques. The amperometric detection indicated that NADP specific malate dehydrogenase (MDH) could be immobilized on the surface of the c-MWCNT modified electrode. Covalent immobilization of enzyme with c-MWCNT on screen printed carbon electrode using EDC was performed for the development of malate biosensor.

MATERIALS AND METHODS

Materials and instruments

Malic enzyme (EC 1.1.1.40 from *chick liver*), malic acid and EDC were obtained from Sigma-Aldrich USA. Carboxylated multiwalled carbon nanotube (c-MWCNT) was purchased from Nanostructured and Amorphous Materials, Inc. Houston, USA. NADP and DMF were procured from Sisco Research Laboratory, India. Other chemicals were of analytical reagent (AR) grade. All electrochemical experiments were performed at room temperature (25±1ºC) using Potentiostat/Galvanostat (Model: FRA 2μ AUTOLAB). Commercially available screen printed carbon electrode (SPCE) was obtained from Dropsens, Spain. Screen printed electrode include three-electrode configuration in which carbon (working), platinum (counter) and silver (reference) electrodes are printed in close proximity. Fourier transform infrared spectroscopy (Model: IR Affinity -1 Shimadzu) was conducted at Guru Jambheshwar University of Science and Technology, Hissar. Scanning electron microscopy (Model: JEOL JSM-6510) was conducted at MD University Rohtak.

Immobilization of enzyme on SPC electrode (Enzyme/c-MWCNT/SPC electrode)

Carboxylated multiwalled carbon nanotubes (6.0 mg) were dispersed in 10 ml of Dimethylformamide (DMF) and ultrasonicated at room temperature for 6 h to obtain a completely homogenized solution. c-MWCNT solution (5 μl) was coated on the surface of working carbon electrode of SPCE and kept for 12 h at room temperature. The excess unbound c-MWCNTs were removed by washing with DMF: Water (1:1) 2-3 times. Then, c-MWCNT/SPCE was dried completely at room temperature. The working electrode of SPCE was treated with 5 μl of 0.4 M EDC, pH 4.6 for 15 min. The electrode was washed with PBS buffer (50 mM Sodium phosphate buffer, pH 7.4; 0.9% NaCl) and dried before immobilization of enzyme. An enzymatic solution of NADP specific malate dehydrogenase with an activity of 0.4 units in PBS buffer, pH 7.4 was prepared and coated 5 μL on the surface of working electrode and left for 1 h at room temperature. The immobilized enzyme SPC electrode was stored in the refrigerator at 4^0C. The enzyme electrode was characterized by scanning electron micrograph (SEM) and Fourier transform infrared spectroscopy (FTIR).

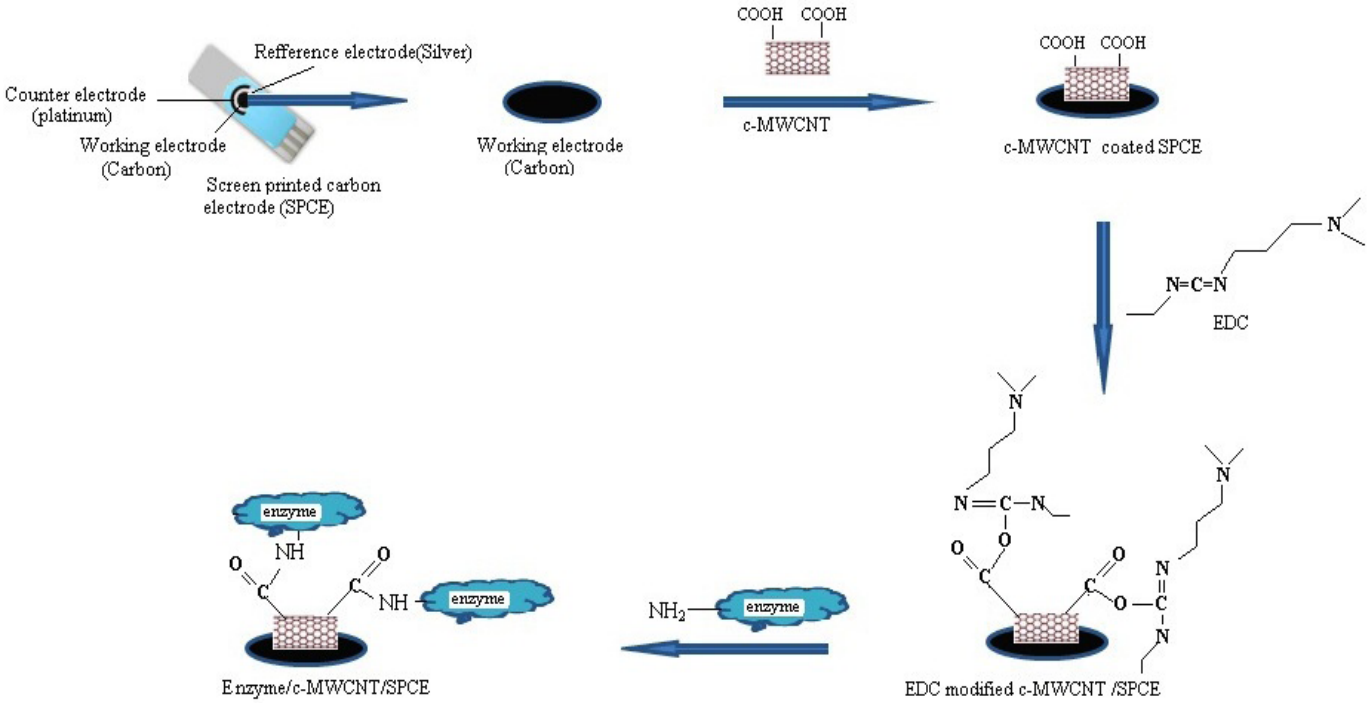

Figure 1. Schematic representation of chemical reaction involved in the fabrication of Enzyme/c- MWCNT / SPCE for malate biosensor.

RESULTS AND DISCUSSION

Construction of Enzyme/c- MWCNT / SPC electrode

The fabrication of malic acid biosensor based on NADP-specific malate dehydrogenase immobilized enzyme/c-MWCNT/SPC electrode is summarized in Fig.1. The c-MWCNT was first deposited on the central surface of screen printed carbon electrode and then NADP specific malate dehydrogenase enzyme was immobilized on c-MWCNT/SPCE by cross-linking through EDC. The enzyme malate dehydrogenase was covalently linked to the c-MWCNT and unbound enzyme was removed by several washing with PBS, pH 7.4.

Differential pulse voltammetry study

To evaluate the catalytic activity of immobilized enzyme/c-MWCNT/SPCE, the modified electrode was characterized by differential pulse voltammogram (DPV) in the presence of different concentrations of malic acid at the potential range from -0.6V to +0.3V. (Fig.2 A) shows differential pulse voltammograms of the enzyme/c-MWCNT/SPCE in PBS, pH 7.4 with malic acid at different concentrations ranging from 0.06 mM to 0.5 mM. DPV was also measured without malic acid (0.0 mM). The maximum response was observed at -0.15 V and hence subsequent studies were carried out at this potential. The DPV was measured in a microcell containing 1.0 µl NADP (4mM) and 1.0 µl of different concentration of malic acid in 48 µl of PBS, pH 7.4 on enzyme/c-MWCNT/ SPCE. DPV peaks increase with increasing concentration of malic acid due to increased oxidation of malic acid and the maximum current generated was recorded at -0.15V. The principle of the malate biosensor is the oxidative decarboxylation of malic acid which is carried out by NADP- specific malate dehydrogenase using $NADP^+$ as a cofactor to produce pyruvate, CO_2 and NADPH. The c-MWCNT increases the surface area for enzyme to bind and also acts as an electron transfer mediator helping in enhancing the sensor response of enzyme electrode and thus increasing the sensitivity of the biosensor. The sensitivity was 0.06 mM malic acid and response time of the sensor was 60 s which is lower than of earlier report of response time 6 min and sensitivity 0.028- 0.7 mM (3).

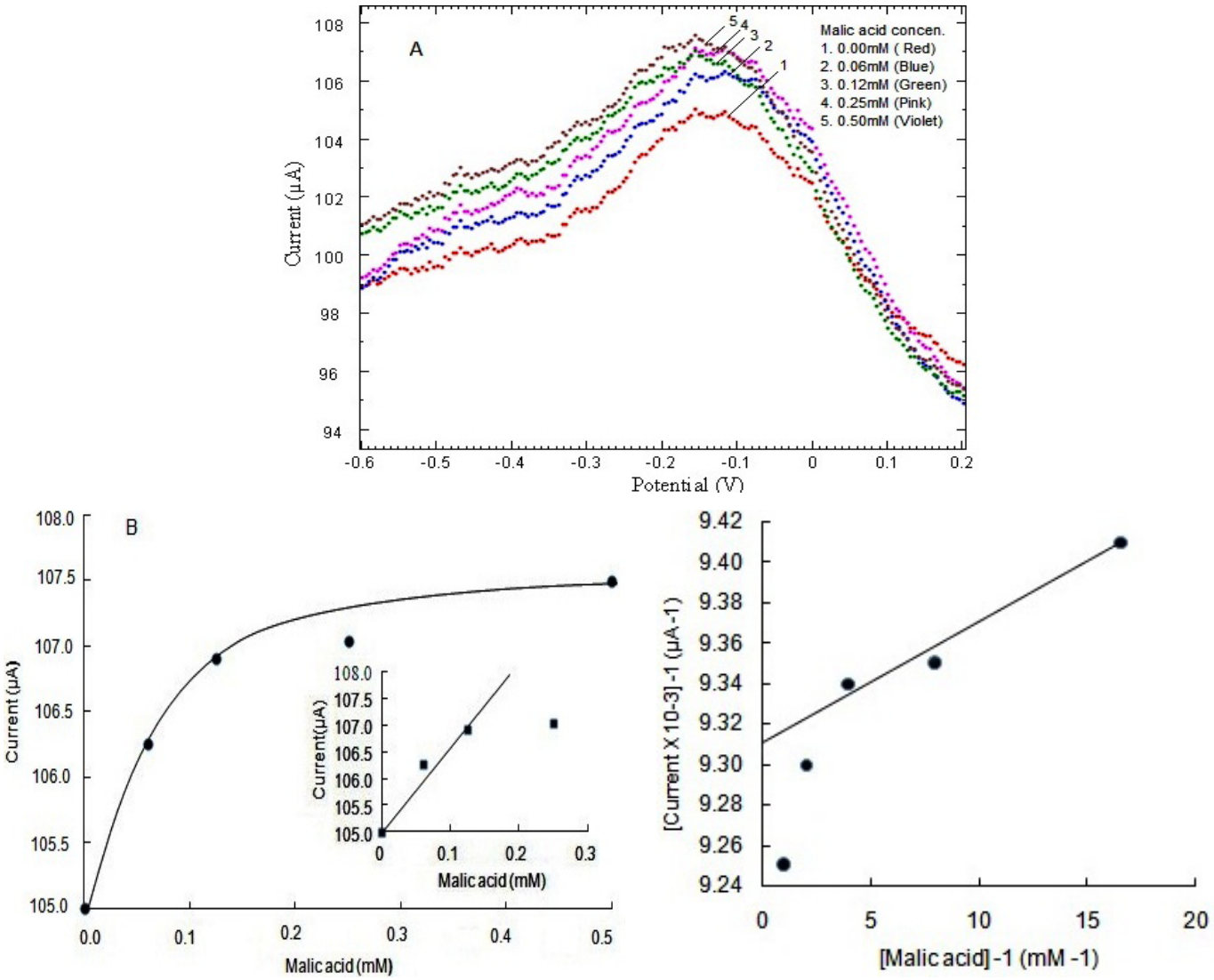

Figure 2. (A) Differential pulse voltammogram of amperometric response studies as a function of Enzyme/c-MWCNT/SPCE in malic acid concentration from (0.0 to 0.5 mM) in PBS, pH 7.4. (B) Effect of substrate concentration study (hyperbolic curve) and calibration plot (inset) of current (µA) responses at different malic acid concentrations (mM) by malate biosensor based on Enzyme/c-MWCNT/SPCE (C) Lineweaver-Burk plot for effect of malic acid concentration on response of malate biosensor (K_m) based on NADP-specific malate dehydrogenase.

Effect of substrate concentration on biosensor (K_m)

To study the effect of substrate concentration on biosensor, the concentration of malic acid was varied from 0.0 to 0.5 mM in PBS, pH 7.4. A hyperbolic relationship was found between malic acid concentrations versus current (Fig.2 B). K_m value for malic acid as calculated from Lineweaver-Burke plot was 0.08 mM (Fig.2 C), which is lower than 0.6 mM reported earlier (31). The V_{max} was found to be 107.4 µA. The standard calibration curve of the sensor response at different concentration of malic acid showed that the sensor response was linear from 0 to 0.12 mM malic acid (Fig.2 B inset) which is lower than 0.01 to 0.4mM (13), 0.1 to 1mM (20) 0.028 to 0.7mM reported earlier (3).

Effect of pH

The DPV was measured at different pH of PBS (pH 6.0 to pH 8.0) in the presence of 0.5mM malic acid and 4 mM NADP. Maximum current was obtained at pH 7.4 (data not shown). Therefore, pH 7.4 was used throughout the experiment.

Effect of interference to malate sensor

Acetyl-CoA (0.5 mM), Glyoxylate (2 mM), NADH (0.3 mM) and succinic acid (0.5mM) were used in PBS, pH 7.4 in the presence of 0.5 mM malic acid and 4 mM NADP. None of them were found inhibitory to the sensor whereas succinic acid acts as inhibitor in tomato fruits (15). The effect of these substances have been reported only on enzyme MDH and found insignificant. 1.5 mM ADP acts as inhibitor in our study which is lower concentration than 2mM reported in *Trypanosoma* cruzi (7).

The FTIR spectrum of c-MWCNT shows a peak at 2366 cm^{-1} associated with O-H stretch from strongly hydrogen bonded –COOH (Fig. 3A). Peak obtained at 1637 cm^{-1} is associated with the stretching of CNT backbone. Increased strength of signal at 1170 cm^{-1} may be associated with C-O stretching in same functionalities. Peak at 3207 cm^{-1} shows the O-H stretching. An FTIR spectrum of immobilized enzyme with peaks at 1610 cm^{-1} and 1109 cm^{-1} corresponds to N-H bending and C-N stretching, respectively (Fig. 3 B).

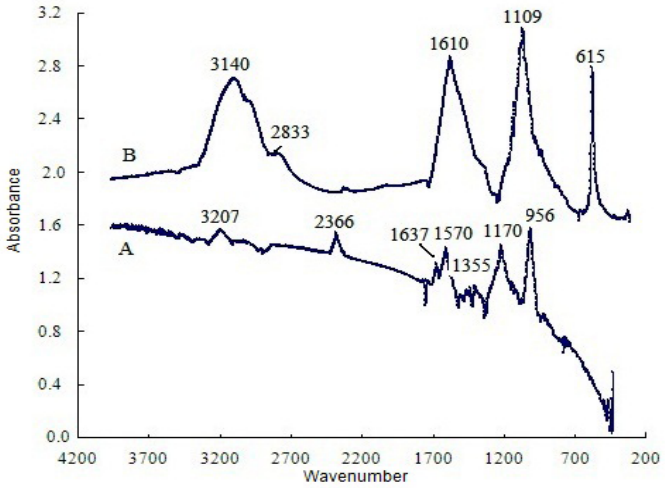

Figure 3. FTIR Spectra obtained for (A) c-MWCNT/SPCE (without enzyme) (B) Enzyme/c-MWCNT/SPCE (after immobilization of malate dehydrogenase).

SEM studies

The morphology of c-MWCNT/SPC electrode and enzyme/c-MWCNT/SPC electrode were characterized by SEM studies (Fig.4 A) shows the presence of c-MWCNT on SPCE at resolution of 1 µm and beam energy 15 KV whereas globular structure indicates the presence of immobilized enzyme on c-MWCNT/SPCE at the same resolution and beam energy (Fig.4 B), which was not observed in c-MWCNT/SPCE. Hence, change in the surface morphology of electrode after immobilization process is the evidence of immobilization of enzyme on electrode.

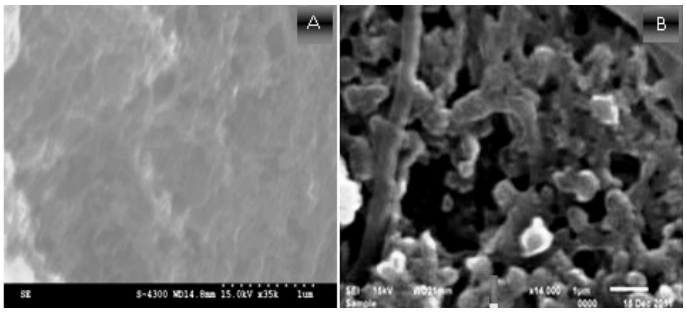

Figure 4. SEM Images at resolution of 1 µm and beam energy 15KV of (A) c-MWCNT/SPCE (without enzyme) (B) SEM Images of Enzyme/c-MWCNT/SPCE (after immobilization of malate dehydrogenase).

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Other articles in this theme issue include references (36-63).

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