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Multiwalled carbonnanotubes enhance the response and sensitivity of the ammonium biosensor based on alanine dehydrogenase

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ABSTRACT

An amperometric ammonium biosensor based on alanine dehydrogenase of *Bacillus subtilis* and functionalized multiwalled carbonnanotubes was developed by using screen printed electrodes. The output current of the biosensor was increased around 2.4 times at 100 mM NH_4^+ after the addition of functionalized multiwalled carbonnanotubes at the concentration of 0.1 μg/ml along with the enzyme. The biosensor showed much broader linearity range (0.05–500 mM NH_4^+), better detection limit (0.001 mM NH_4^+) and response time (30 s) due to the incorporation of the functionalized multiwalled carbonnanotubes. The cyclic voltammogram of enzyme-carbonnanotubes/carbon working electrode showed the output current at the oxidation peak, which was >2 times higher compared to that of enzyme/carbon working electrode. This suggests the promotion of electron transfer from the enzyme to the working electrode by carbonnanotubes, which may act as electron transducers and rapidly transfer the electrons from the redox centre of the enzyme to the electrode, thereby improving the signal, response time and sensitivity. The sensor retained 89.93% of its initial output current after 119 days. The concentrations of NH_4^+ detected by the biosensor were in close agreement with that of standard Nessler's reagent method suggesting the suitability of the present biosensor for the testing of water samples.

1. Introduction

High concentrations of ammonium in drinking water and water resources can lead to adverse effects on man and environment [1,2]. Ammonium can lead to direct toxicity to plants [3,4] and loss of plant diversity [5]. Toxicity of ammonium is believed to be a source of losses in fish hatcheries. Inhalation of ammonium causes irritation to eyes, nose, throat and lungs in humans. Higher concentrations of ammonium can cause permanent blindness, lung disease, disturbances in brain function or death. Therefore, real-time monitors are necessary to allow continuous determination of ammonium, so that appropriate decisions can be taken to control the levels of ammonium.

Ammonia sensors based on metal oxide used SnO_2 [6] and they operate based on the change of conductance due to the chemisorption of ammonia gas molecules on the sensing layer. The major drawback with these sensors is they are not selective to the particular gas. Another kind of ammonia sensors are based on the change of charge carrier concentration in the catalytic metal in response to the concentration of the ammonia gas [7,8]. Change in charge carriers was quantified by using the field effect devices like capacitor or transistor [8]. However, this kind of sensor is not selective and response depends on the different parameters like catalytic metal, morphology of the metal layer and the operating temperature.

Potentiometric ammonia sensor based on zirconium titanium phosphate ion exchanger was developed. This sensor showed potentiometric response to other cations, even though it has long life [9]. A fluorosensor for ammonia detection was constructed by using rhodamine incorporated poly vinyl chloride (PVC) [10]. An ammonium electrode based on PVC membrane containing palmitic acid and nonactin as an ammonium ionophore for the determination of ammonia was constructed [11]. A conductometric sensor for ammonium detection was constructed by using PVC membrane containing nonactin [12]. However, this sensor is not specific for the ammonium. Multiwalled carbonnanotube/copper composite paste electrode was developed for the detection of ammonia [13]. But, reproducibility was very poor with this electrode.

Colorimetric and optical based approaches were extensively used for the detection of ammonium [14,15,16,17]. Classical colorimetric

Abbreviations: SPE, screen printed electrode; f-MWCNT, functionalized multiwalled carbon nanotubes; p-MWCNT, pristine multiwalled carbon nanotubes; AlaDH, alanine dehydrogenase; WE, working electrode; NADH, nicotinamide adenine dinucleotide-reduced; GlDH, glutamate dehydrogenase.

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methods were based on Nessler's reagent [18] and Berthelot's indophenol reaction [15]. However, in the colorimetric methods the phenolic reagent and its by-product generated during the reaction were highly toxic [18]. High-performance liquid chromatogram coupled with fluorescence detector was used for the ammonium detection [19,20]. But, in the chromatographic methods samples should be subjected to pre-column derivatization for detecting the fluorescence [21]. Ammonium was detected by a system with flow-injection analysis and it contains immobilized enzyme reactor and chemiluminescence detector [22]. Here, ammonium will be converted to glutamic acid by GlDH in the presence of 2-oxoglutarate and NADH. Then glutamic acid will be oxidized by glutamate oxidase to produce hydrogen peroxide which will be detected by a chemiluminometric method using luminol and hexacyanoferrrate (III). An optical and dual enzyme based biosensor was developed by using GlDH and diaphorase for the detection of ammonia [23]. But, this sensor didn't work well at higher concentrations of ammonia.

Enzyme based amperometric biosensors gained lot of importance in the last decade due to high selectivity of the bioreceptor and the sensitivity of electrochemical signal transduction. This led to the specific detection and quantification of target analyte in the complex matrices. In this kind of sensors, the enzyme acts as bioreceptor and electrons will be generated when the target analyte comes in contact with the enzyme. These electrons will be transduced to the WE faster since the enzyme was coated on the working electrode and electrons are generated in close vicinity of the WE. As a result of early transduction of electrons the sensitivity of the sensor improves. The selectivity of the sensor improves since the enzyme is specific to the targeted analyte. Gwent sensors Ltd. has developed an amperometric biosensor for the detection of ammonium and it was based on the GlDH. But, its working range is 1–10 ppm which is limited. Another sensor was constructed for the detection of ammonium by immobilizing the two enzymes, GlDH and glutamate oxidase on a Clark-type oxygen electrode [24]. But, this sensor lost around 60% of its response after 18 days. Yet another amperometric ammonia sensor based on the two enzymes, GlDH and diaphorase was developed [25]. But, it shows linear response only at lower concentrations of ammonium (2.5–500 μM). The sensor based on two enzymes is difficult to manage since each enzyme needs different optimum conditions and it's difficult to maintain both the conditions simultaneously and it affects the stability of the sensor. Hence, single enzyme system is advantageous. By using the enzyme, AlaDH a reagent less amperometric ammonia biosensor was developed [26]. This enzyme converts pyruvate to alanine and generates 2 electrons in the presence of ammonium and NADH. However, this sensor response is linear only at higher levels of ammonium (10–100 mM) and the signal level is very low in response to the different concentrations of ammonium. In the present work, signal, sensitivity and response time was improved by using multiwalled carbonnanotubes.

In the earlier decades, a considerable amount of analytical research has been done for the electrochemistry of the NAD^+/NADH redox reactions coupled to various electrodes [27]. However, only a limited number of electrochemical sensors based on dehydrogenases were reported due to large overvoltage encountered for NADH oxidation at electrodes [28] and surface fouling associated with the accumulation of reaction products [27]. This problem can be addressed by applying carbonnanotubes to the electrodes since they offer an accelerated electron transfer along with minimization of surface fouling [29,30].

In the present work, we have explored the possibility of immobilizing the enzyme, AlaDH along with f-MWCNT on the carbon electrode in order to acquire the enhanced sensitivity and the higher signal output from the ammonium sensor.

2. Experimental

2.1. Materials and reagents

The enzyme, AlaDH was purified from *Bacillus subtilis* (ATCC 6633) by adopting standard procedures. The NADH, ammonia solution and sodium pyruvate were purchased from Sisco Research Laboratories, India. Polyacrylamide was obtained from Sigma-Aldrich, India. Multiwalled carbonnanotubes were procured from Reinste nano ventures, India. The screen printed electrodes (SPE) were designed in our laboratory and manufactured by SILTECH Corporation, India. We have used two different kinds of SPE out of which one has carbon based WE and another has silver epoxy-carbon WE. The reference and counter electrodes were made from silver and the diameter of WE was 3 mm and the dimensions of the SPE were 5 × 1.5 × 0.1 cm in both the cases.

2.2. Functionalization of multiwalled carbonnanotubes

Functionalization of multiwalled carbonnanotubes is required for better interfacial bonding and dispersion. Therefore, 0.5 g of multiwalled carbonnanotubes were weighed and mixed with 50% aqueous solution of acid mixture containing concentrated HNO_3 and H_2SO_4 in the 1:3 ratio. The mixture was sonicated for 4 h at 65 °C, 42 KHz and 500 W. Further, this suspension was diluted with 1.5 L of deionized water, filtered and carbonnanotubes were washed until the pH of the filtrate was neutral. The retained carbonnanotubes were dried in the hot air oven at 110 °C for 12 h. Finally, they were washed with acetone, dried and stored in the desiccator until used. The percentage of recovery was 97.26% and they were used as f-MWCNTs.

2.3. Characterization of f-MWCNTs

The f-MWCNTs were characterized by FT-IR, Raman and Transmission electron microscopic methods. The f-MWCNTs were mixed with KBr in the ratio of 1:100, pelletized and analyzed by FT-IR (Spectrum

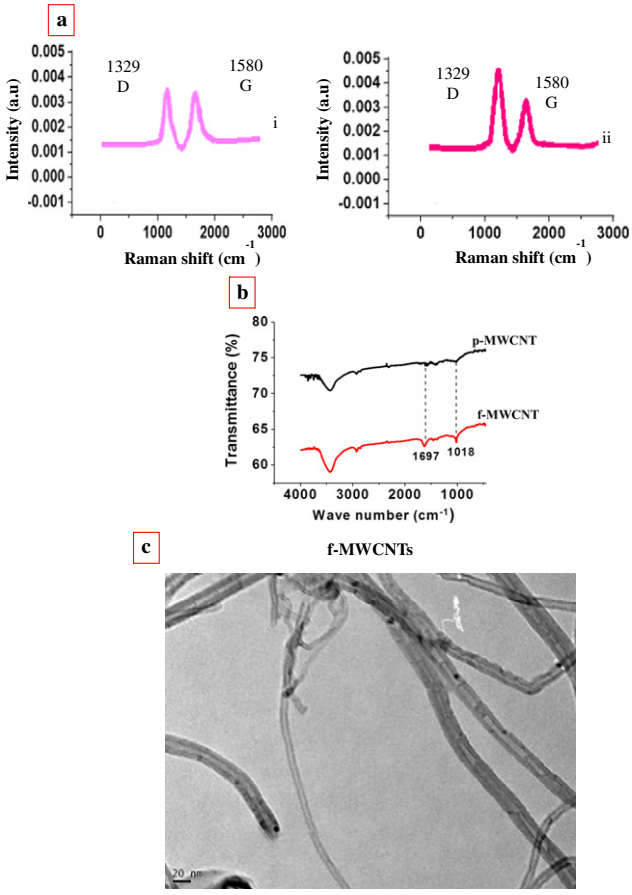

Fig. 1. Characterization of pristine (i) and functionalized (ii) MWCNTs by Raman spectroscopy (a) and FT-IR (b). (c) Transmission electron microscopic image of f-MWCNTs.