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Nitrate Determination in Drinking Water by Nitrate Reductase based Biosensor

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Abstract—*A novel electrochemical Nitrate biosensor is described based on covalent immobilization of nitrate reductase (NaR), onto carboxylated multiwalled carbon nanotubes (cMWCNT), electrodeposited on the surface of pencil graphite electrode. The NaR-NADH/cMWCNT/PG electrode was characterized by scanning electron microscopy(SEM) and cyclic voltametry (CV).The formation of optimum NaR-NADH/cMWCNT based biosensor was accompanied with an applied potential of 0.6V , 1 ml cMWCNT, 1U/ml NaR ,1ml NADH ,0.5M KCl and an optimum response was obtained at pH 7.5, temp 35°C. A good analytical recovery of 96% and 90% was obtained by adding different concentration of Nitrate in drinking water samples. A minimum detectable concentration of 0.5µM and a linear concentration range of 0.1-450 µM/L were achieved with the nitrate biosensor.*

Keywords: *c-MWCNT, Nitrate Reductase, electron transfer, Nitrate biosensor, current measurement, pencil graphite electrode.*

1. INTRODUCTION

Increasing level of nitrate in drinking water is becoming a major problem for world as it is hazardous contaminant in drinking water (primarily from ground water and wells) due to its harmful biological effects. Until 1945, nitrate in water was not considered to be as hazard. But in the same year Comely (1945) found a form of infantile cyanosis due to high level of nitrate in drinking water[1]. Then monitoring of nitrate level was started by International Standing Committee on water quality and treatment (1972). In 1974, Congress passed the Safe Drinking Water Act. State and Federal laws set the maximum allowable level of nitrate in public water as 45 mg/L and 10 mg/L according to EPA (Environmental Protection Agency). There are various sources that increase the level of nitrate in ground water e.g. disposal of sewage, industrial waste related to food processing, munitions, polyresin facilities, farming, handling and accidental spills [2-4]). A lot of health risk due to high nitrate was studied as thyroid dystrophy affecting the utilization of vitamin A [5], methaemoglobinemea (MetHb) and decrease in blood pressure in school children [6]. Studies showed that only 5% is the fresh water out of total water and this fresh water includes approximately 66% ground water (source of drinking water). According to a report published in Journal of Contaminant Hydrology, 33% of ground water samples had nitrate contents exceeding the general acceptable limit of 20 ppm and 15% of samples crossed the MPL (maximum permissible limits) of 45 mg/L[7]. Nitrate is tasteless, colorless and odorless compound. It can't be detected unless water is chemically analyzed. Nitrate level in drinking water may vary seasonally due to use of fertilizers, thus need of retesting the water every 3 – 6 months is required. As a risk of human health, determination of nitrate level in drinking water carried out with the help of chemical and analytical techniques including– Ion exchange chromatography, Ion interaction liquid chromatography, colorimetry, mass spectrophotometry, gas chromatography and differential pulse Volta metric method, polarography, three wavelength method etc. But these methods are either tedious or time consuming or not sufficiently specific. Further, many earlier nitrate biosensor has been reported based on based on, viologen-polypyrrole film [8], polythiophene-bipyridinium [9], polyvinyl alcohol [10],polystyrene-polybutadiene[11], viologen-acrylamide[12], sol-gel matrix[13], decanethiol [14],ultrathin film composite membrane [15], cellulose acetate membrane[16], screen printed electrode [17].These biosensors needs improvements either in narrow linear concentration range, or lack of stability, more interference with sample components, poor sensitivity, low detection limits. So, present research work is based on detection of nitrate as an improved electrochemical biosensor .

2. EXPERIMENTAL

2.1. Chemical and reagents

Carboxylated multiwalled carbon nanotubes (c-MWCNT) (functionalized MWCNT, 12 walls, 15–30 µm length, 90% purity, nil metal content) from Sigma-Aldrich, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), Enzyme Nitrate reductase from Aspergillus flavus from Sigma-Aldrich St. Louis , ,potassium nitrate ,NADH, tris buffer, potassium di-hydrogen phosphate and dipotassium hydrogen phosphate were purchased from

Himedia. Double distilled water (DW) was used in all experiments.

2.2. Apparatus

Scanning electron microscope (SEM), Spectronic-20D (Systronics, VIS Double Beam Spectro 1203), Cyclo mixer (Remi equipment, Mumbai), Temperature controlled water bath shaker and oven (Remi equipment, Mumbai), Digital pH meter (335D, Systronics, Ahemdabad), Potentiostat (Autolab, model:AUT83785,manufactured by Eco chemie) with a three electrode system composed of pt wire as auxillary electrode, an Ag/AgCl electrode as reference electrode and NaR-NADH/cMWCNT/PG as working electrode, Double distilled water (DW) was used in all experiments.

2.3. Fabrication of modified electrode

2.3.1. Electrodeposition of c-MWCNT on to graphite electrode

One milligram of c-MWCNT was suspended in a 4.0 ml mixture of concentrated H_2SO_4 and HNO_3 in a 3:1 ratio and ultrasonicated for 2 hr to obtain a homogeneous black colored Solution. The dispersed cMWCNT solution (0.1ml) was mixed into a mixture of 0.5ml EDC (0.2M) and 0.5ml NHS (0.2ml).The pH was adjusted to 7.5 and it was kept at RT for 1 min. The c-MWCNT were electrodeposited onto PG electrode through electropolymerization using a potentiostat/galvanostat. Prior to electrodeposition, the graphite electrode (1.95 cm × 1 mm) (length × diameter) were ultrasonicated in 5.0 M HNO_3 and acetone for 15 min and then rinsed with DW. For electrodeposition of c-MWCNT , solution of 1ml dispersed c-MWCNT in 25 ml 0.5M KCL solution were prepared in a glass cell.

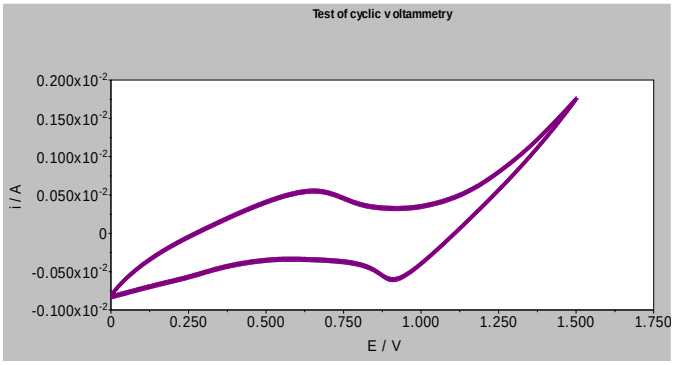


Fig. 1: Cyclic voltammograms for electrochemical deposition of NaR/c-MWCNT onto surface PG electrode. Supporting electrolyte: 1M KCl solution; scan rate: 50 mV/s.

The three-electrode system (Graphite as working electrode, Platinum as counter current electrode and Ag/AgCl as reference electrode) were immersed in the electrodepositing solutions, and the potential scan was cycled 20 times between 0.1V to 1.5V v/s Ag/AgCl at a scan rate of 50 mV/s. During the electrochemical polymerization, the surface of PG electrode gradually became black, indicating the deposition of c-MWCNT onto the graphite electrode. The PG electrodes coated with c-MWCNT was washed with Distilled Water and subsequently kept in a desiccator for 24 h at room temperature.

2.3.2. Immobilization of NaR on to c-MWCNT fabricated graphite electrode.

The enzyme, NaR was immobilized covalently onto c-MWCNT coated PG electrode . The electrode was incubated in 1ml 0.1 M PB (pH 7.5) containing 1ml enzyme NaR and 1 ml NADH at 4 °C for 3 hr and then washed with 0.05 M Potassium phosphate Buffer (pH 7.5). The resulting bioelectrode (NaR-NADH/c-MWCNT/PG) was dried and stored in a refrigerator at 4 °C.

2.3.3. Characterization of NaR-NADH/c-MWCNT/PG electrode by SEM

The electrode was cut into small pieces (1 cm) and placed on a specimen chamber of 2 cm diameter using a spray gun, generally mounted rigidly on a specimen holder called a specimen stub and micrographs were taken with a scanning electron microscope. The samples were coated with an ultrathin coating of electrically conducting material gold. In SEM, data were collected over a selected area of the surface of the sample and 2-dimensional images were generated that displayed spatial variations in these properties.

2.3.4. Construction, Cyclic voltametry measurement and testing of nitrate biosensor

The NaR biosensor was constructed using a three-electrode electrochemical cell system, consisting of NaR-NADH/c-MWCNT/PG as a working electrode, a silver/silver chloride (Ag/AgCl) as reference electrode and Pt wire as auxillary electrode**.** The electrode system was dipped into a reaction mixture containing 25 ml 0.1 M PB, pH 7.5 and 1 ml nitrate solution .The electrode response was measured in terms of milliampere (mA) applying a potential range of 0.1V-1.5V vs Ag/AgCl. The maximum response was observed at 0.6V as depicted in Fig. 1. The reaction involved reduction of nitrate and ultimately generation of nitrite, which is broken by applied potential between working electrode and counter electrode as shown in Fig. 2. The electrons released were transferred to the working electrode to be relayed to potentiometer, in which it was read as current in mA.

$$NO_3^- + NADH + H^+ + 2e^- \xrightarrow{NaR} NO_2^- + NAD^+ + H_2O$$

2.4. Optimization of Nitrate biosensor

To determine the optimum pH of the enzyme electrode, the pH of the reaction buffer was varied from pH 5.0 to 10.0 using the following buffer, each at a final conc. of 0.1M; pH 5.0 to 8.0 potassium phosphate buffer, pH 8.5 to 9.0 Tris-HCl buffer and

9.5 to 10.0 sodium carbonate/bicarbonate buffer. The response current in terms of mA was measured. Similarly, to determine the optimum incubation temperature for maximum response of the enzyme electrode, the reaction mixture was incubated at different temperatures ranging from 20 to 50 ºC at 5 ºC interval and incubation time is studied from 1 to 20s at an interval of 2 sec.The effect of nitrate concentration on biosensor response was determined by varying its concentration from 0.1-1000µM/L.

2.5. Determination of Nitrate in Drinking Water.

Drinking water samples from different locations of Rohtak city were collected and tested for Nitrate concentration. Samples were collected in a vials and tested for nitrate.

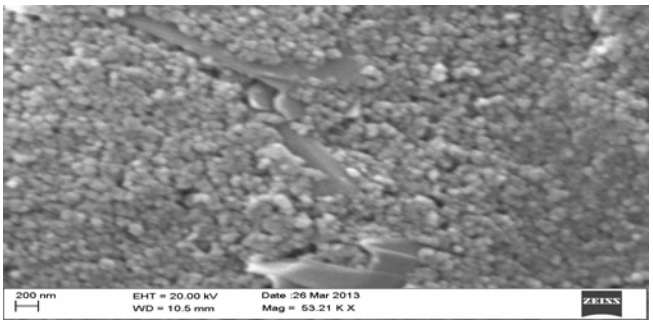
2.6. Storage stability of NaR-NADH/cMWCNT/PG electrode

The stability of working electrode was studied for 45 days by performing the assay on weekly basis. The present electrode was stored in dried condition at 4°C when not in use.

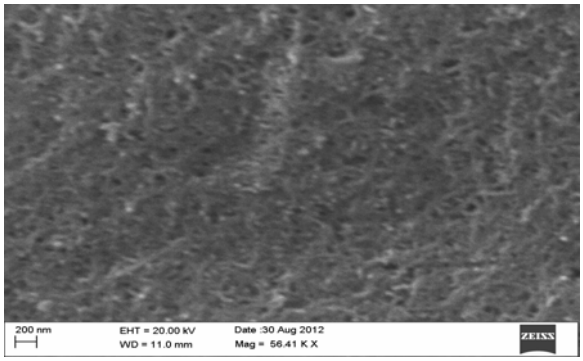
3. RESULTS AND DISCUSSION

3.1. SEM studies of graphite electrode with modification

The SEM images of the surface of bare graphite electrode, c-MWCNT/PG electrode and NaR/c-MWCNT/PG electrode are shown in Fig. 3A-C. The stepwise modification of electrode could be seen clearly from these SEM images.



[A]



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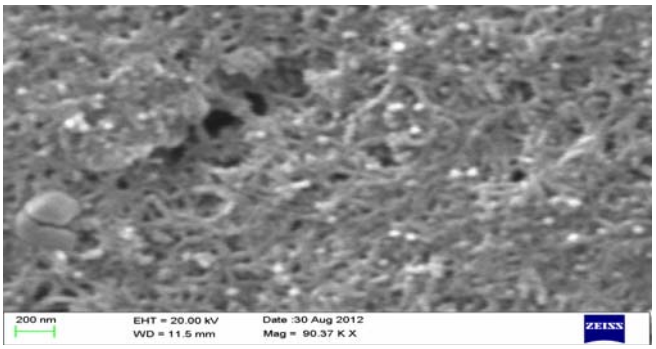

[C]

Fig. 2: Scanning electron microscopy of [A] bare PG electrode [B] fabricated c-MWCNT [C] immobilized enzyme onto c-MWCNT deposited on PG electrode.

The SEM image of bare PG electrode showed a smooth morphology. c-MWCNT/PG electrode that reveal the uniform, homogenous and cage like morphology of the nanostructure of c-MWCNT, which showed the pore is smaller and denser in c-MWCNT. On immobilization of NaR, the morphology of hybrid electrode shows the sporadic appearance of globular/beaded structure due to interaction between c-MWCNT/PG electrode and NaR.

3.2. Construction of Nitrate biosensor

The enzyme NaR was immobilized covalently onto c-MWCNT electrodeposited on surface of a PG electrode using EDC-NHS chemistry through amide bond formation between the free and unbound –COOH groups of c-MWCNT and the –NH_2 groups on the surface of enzyme. EDC and NHS were used to activate the free –COOH groups of c-MWCNT layer. EDC was used to conjugate the free carboxyl (-COOH) groups of c-MWCNT to amine (-NH_2) groups of the enzyme, using NHS as a catalyst. .This covalent coupling of the enzyme onto c-MWCNT does not allow the leaching of the enzyme during the repeated washing of enzyme electrode for its reuse. These observation also suggest that the cMWCNT provide a large surface area for immobilization of NaR.

3.3. Response measurement of Nitrate biosensor

To evaluate the catalytic activity of NaR at NaR-NADH/cMWCNT/PG electrode, the modified electrode was characterized by a cyclic voltagramm 25ml 0.1M PB buffer (pH 7.5) containing 1ml nitrate .A redox peak is observed in case of cMWCNT modified PG electrode due to deposition of cMWCNT, which facilitate the electron transfer appeared that the cMWCNT provided a biocompatible environment to the enzyme, cMWCNT act as electron mediator resulting in an accelerated electron transfer between enzyme and electrode.

3.4. Optimization of nitrate biosensor

The biosensor response was affected by the experimental conditions which were studied in terms of effect of pH, incubation temperature, substrate concentration (Nitrate), the optimum current was obtained at pH 7.5,which is almost

similar to that of earlier biosensors, the optimum temperature was 35°C. There was a straight relationship between sensor response and nitrate concentration in the range of 0.1-1000µM. The response was constant after 450µM.

3.5. Evaluation of Biosensor

3.5.1. Linearity

There was a linear relationship between current (mA) and Nitrate concentration ranging from 0.1 to 450µM in 0.1M PB pH 7.5 for NaR enzyme bound electrode as depicted in Fig. 5, which is a better linear range than those of earlier reported biosensor.

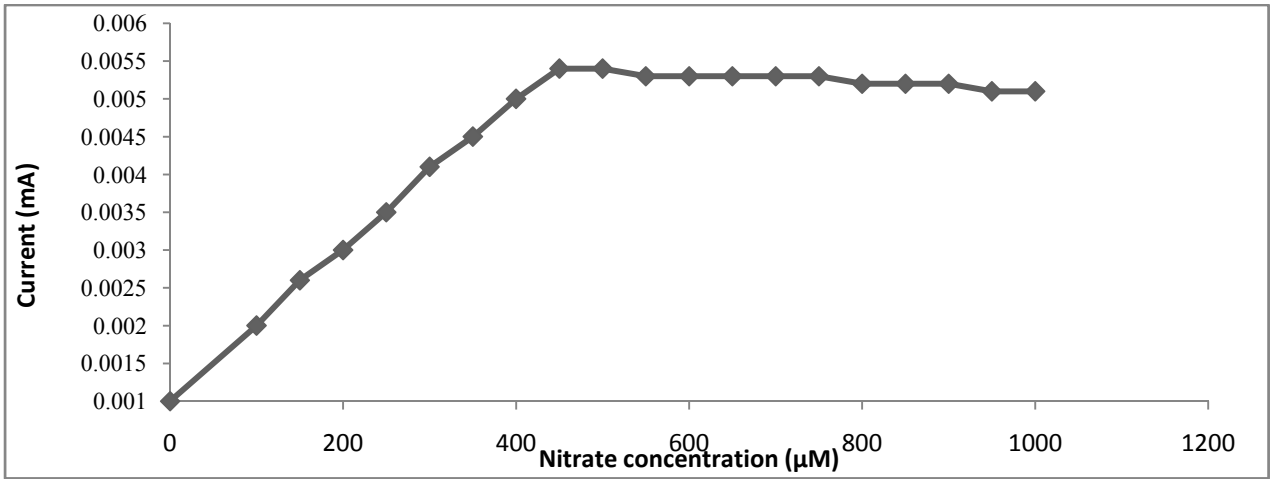

Fig. 3: Effect of substrate (Nitrate) concentration on current response of c-MWCNT based Nitrate biosensor.

3.5.2. Detection limit

The detection limit of the present method was 0.5 µM at a signal to noise ration of 3 (S/N=3),which is better than previously reported electrochemical Nitrate biosensor.

3.5.3. Analytical recovery

The mean analytic recoveries of exogenously added 5 µM/L and 10 µM/L Nitrate in serum (final conc. in reaction mixture) were 94% and 98 % respectively demonstrating the satisfactory accuracy of the present biosensor as shown in Table 1.

Table 1: Analytical recovery of added nitrate in drinking water sample, as measured by c-MWCNT based Nitrate biosensor

Nitrate added (mg/dl)	Nitrate found (mg/dl)	Recovery %
5	37.8	96%
10	74.0	90%

3.5.4. Correlation

The accuracy of the present method was tested by comparing Nitrate level in serum and water sample by the present method (y) with those obtained by standard spectrophotometric method (x) (Zatar.A *etal;*1999). The value obtained by both methods showed a very good correlation of serum nitrate (r=0.983) with the following regression equation: y = 1.033x +0.007 as shown in Fig. 6. These results showed that the data obtained by present method and previously reported standard method are comparable. It proved that the modified electrode ascertained the practical application of the biosensor in the routine quantitative analysis of nitrate.

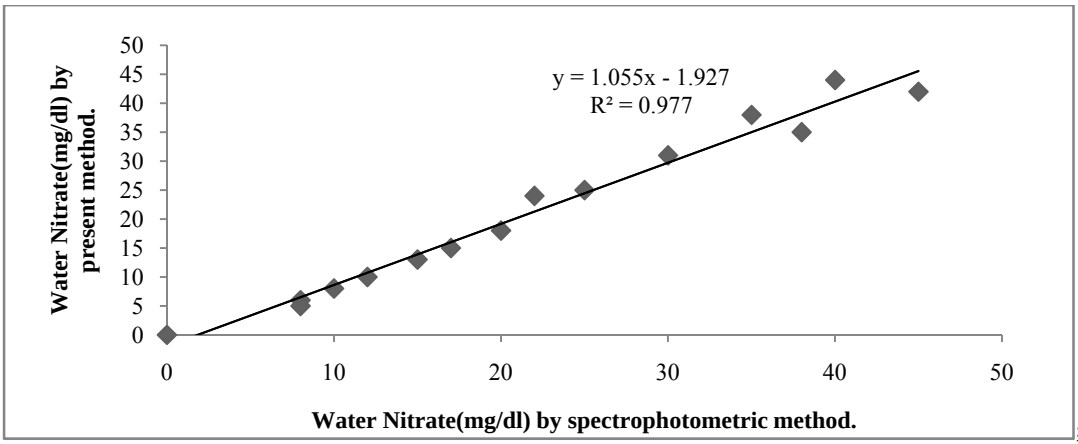

Fig. 4: Correlation between water nitrate values as determined by standard spectrophotometric method (x-axis) and present nitrate biosensor (y-axis) based on c-MWCNT

3.5.5. Sample analysis

Water samples collected from different location of rohtak city, were tested for nitrate concentration.EPA set a upper permissible limit of 45mg/L for nitrate level. Nitrate level above 45 mg/L cause methaemoglobinimea, nitrate poisioning etc.Water nitrate content was ranged from 41.00 to 86.30 mg/L with a mean of 55.16±02.362mg/L (Table-2).

Table 2: Nitrate content in drinking water of various sites of Rohtak, Haryana

Sample collected sites (n=20)	Drinking water nitrate (mg/L)(Mean±SD)
University campus	43.01±01.247
Industrial site	86.30±06.265
PGI campus	41.00±01.837
Taliyar site	68.70±05.850
Domestic site	36.83±02.349
Mean	55.16±02.362

3.5.6. Stability

The peak steady state current at each concentration level is plotted against time (in days). The NaR-NADH/c-MWCNT/PG electrode showed gradual ageing and deterioration in current response, decreasing up to 50% in 45 days. This may be due to polymerization and loss of chemical activity in NaR or due to extended exposure to light (Fig. 5).

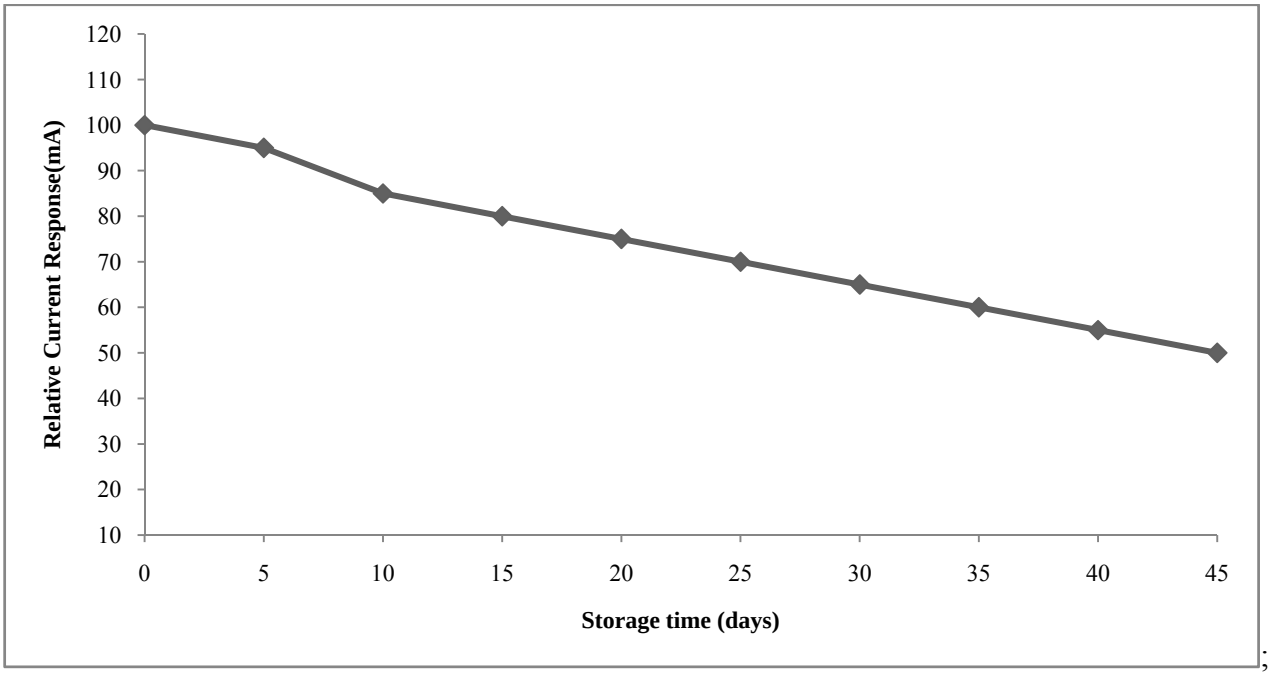

Fig. 5: Effect of storage at 4 °C on the response of Nitrate biosensor based on c-MWCNT

4. CONCLUSION

A novel, simple, cost-effective biosensor has been constructed by immobilizing nitrate reductase along with NADH as a cofactor onto the multiwalled carbon nanotubes electrodeposited on to the graphite electrode. The use of cMWCNT has improved the performance of nitrate biosensor in terms of low working potential (0.6V), short response time (6 sec), high sensitivity (0.050 μA/μM/cm^2), minimum detection limit (0.5μM) and good storage stability compared to earlier biosensors. Based on these observations, this sensor could be best biosensor for nitrate determination in drinking water compare to earlier reported biosensors.

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