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Nitrate Reductase-NADH/c-MWCNT Based Electrochemical Sensor for Nitrate Determination

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Abstract
An improved and cost-effective method is described for the construction of a novel electrochemical biosensor based on covalent immobilization of Nitrate reductase (NaR-NADH) on to carboxylated multiwalled carbon nanotubes (c-MWCNT) film electrodeposited over the surface of graphite electrode. The NaR-NADH/cMWCNT/PG modified electrode was characterized by scanning electron microscopy (SEM) and cyclic voltammetry (CV). A nitrate biosensor measured current due to electrons generated at 0.6V against Ag/Agcl as reference electrode and platinum as auxillary electrode, which is produced from nitrate by immobilized nitrate reductase. The biosensor showed optimum response within 6 sec at pH 7.5 and 35°C temperature. A linear relationship was obtained between a wide nitrate concentration range (0.1-450µM/L) and current (mA) under optimum conditions. The biosensor showed high sensitivity (0.050 µA/µM/cm^2),low detection limit (0.5µM) and good storage stability(50% over 45 days). The biosensor was evaluated and employed for determination of nitrate in serum .
Key words: c-MWCNT, Nitrate Reductase, electron transfer, Nitrate biosensor, current measurement, graphite electrode, electrodeposition.

1. INTRODUCTION

Nitrate is tasteless, colourless and odourless compound. The mean concentration of nitrate in blood of healthy adults varied from 4 to 44 µmol/L with a mean of 19.7µmol/L, in plasma it is 1.22 mg/L , 0.60 mg/L in urine and 0.47 mg/L in CSF [1]. An increase in nitrate level in biological fluids due to intake of nitrate rich food stuffs (vegetables and meat) and water leads to various health problems. Nitrate determination is important for evaluation of gastric cancer, prostate cancer and urinary bladder cancer etc.[2]. Contribution of nitrate via dietary intake is 70 – 80% and via drinking water is 20 – 25% [3]. Increased nitrate level has been shown to be linked to several disorders such as thyroid dystrophy[4], methaemoglobinemia (blue baby syndrome) [5] and decrease in blood pressure in school children [6]. Furthermore the elevated level of nitrate result in formation of *N*-nitroso compounds (NOC), a class of genotoxic compounds, most of there are animal carcinogens which leads to the development of gastric cancer, prostate cancer, urinary bladder cancer etc. [7].Hence, the accurate measurement of nitrate is imperative in human physiology as it provide valuable information with regard to in vitro nitrate production and prognostic of various disease including oxidative stress. Various methods have been employed for the determination of nitrate including colorimetric assay [8], fluorometry [9], mass spectrophotometry[10], Chemiluminescence [11], differential pulse volta metric [12], and polarography [13]. All of these methods are very sensitive, reliable, and reproducible, but they are very expensive and require time consuming sample preparation ,unportable and specialized equipments ,and trained persons to operate. Recently electrochemical biosensor are proved to be a powerful tool for the measurement of nitrate by providing practical advantages such as operation stability, low expense of fabrication, suitability for real time detection, fast responsive sensitive, highly specific and accurate[25]. A number of nitrate biosensors have been reported for determination of nitrate based on immobilization of nitrate reductase amperometric as well as potentiometric onto viologen-polypyrrole film [14], polythiophene-bipyridinium [15], polyvinyl alcohol [16],polystyrene-polybutadiene[17], viologen-acrylamide[18], sol-gel matrix[19], decanethiol [20],ultrathin film composite membrane [21], cellulose acetate membrane[22], screen printed electrode [23], PPy/NR/NADH layer [24],CNT/PPy film[25],copper, zinc,superoxide dismutase and nitrate reductase based biosensor [26] and epoxy gold nanoparticles [27]. In these biosensors the immobilized nitrate reductase(NaR) enzyme brings about the conversion of nitrate into nitrite given in following reaction sequence.

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

These biosensors need improvement in narrow linear concentration range, lack of stability, more interference with sample components, poor sensitivity, low detection limits and low reproducibility. Most of these disadvantages can be overcome by using biocompatible nanomaterials that have opened a bright field towards the development of third generation biosensors based on direct electron transfer[28]. The use of nanoparticles has led to the improvement in their stability, reusability and sensitivity.Among these, carbon nanotubes (CNTs) are the promising material for sensing applications.CNT was used as a support for immobilization of nitrate reductase in nitrate biosensor owing to their ability to

undergo fast electron transfer. Because of the novel structural and electronic properties, high chemical stability, extremely high mechanical stability and modulus, CNT have found a wide range of potential applications from structural material to catalytic support [28]. In addition of potential electrochemical application of CNTs its high conductance, tensile strength and ultra-small size make them potential nanoparticle for development of effective, low-cost and simple biosensor [29].In current study we describes the construction of improved nitrate biosensor by covalently immobilizing NaR onto carboxylated multiwalled carbon nanotubes(c-MWCNT)/PG electrode, its evaluation and its application in measurement of nitrate in serum.

2. MATERIALS AND METHODS

2.1. Chemicals and nanomaterials

NaR(from Aspergillus flavus) was obtained from Sigma-Aldrich(USA) , Carboxylated multiwalled carbon nanotubes (c-MWCNT) (functionalized MWCNT, 12 walls, 15–30 μm length, 90% purity)were obtained from Sigma-Aldrich, N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS),potassium nitrate ,NADH, sulphuric acid, hydrochloric acid, sodium carbonate, sodium bicarbonate, 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 Instruments

All electrochemical experiments were performed at 25±1°C using an autolab Potentiostat (Autolab, model:AUT83785,manufactured by Eco chemie).A conventional three electrode cell consisting of enzyme/c-MWCNT/PG, Pt wire as auxillary electrode, an Ag/AgCl electrode as reference electrode, was used for electrochemical experiments. 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) Refrigerator (LG), electronic balance (BSA223S, Sartorious). The morphological studies were carried out by using scanning electron microscopy (SEM) on commercial basis.

2.3.Construction of NaR-NADH/cMWCNT modified pencil graphite electrode

2.3.1 Electrochemical deposition of c-MWCNT on pencil 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 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 c-MWCNT in 25 ml 0.5M KCL solution were prepared in a glass cell .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 on the graphite electrode. The PG electrode coated with c-MWCNT was washed with distilled water and subsequently kept in a desiccator for 24 hr at room temperature.

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

NaR was immobilized covalently onto c-MWCNT coated PG electrode using EDC-NHS chemistry as described previously[32] with modifications. First, free and unbound –COOH groups of c-MWCNT were activated by immersing them into 0.1 M phosphate buffer (PB, pH 7.5) containing EDC and NHS of the same concentration(10mM) and then excess of EDC and NHS was removed by washing with 0.1 M PB (pH 6.8). Finally, EDC–NHS-treated electrode was incubated in 0.05 M PB (pH 7.5) containing NaR (1U) and 1 ml NADH at 4^0C for 6h and then washed with 0.05 M PB (pH 7.5).The resulting NaR-NADH/cMWCNT/PG electrode (i.e. enzyme electrode)was used as working electrode and stored at 4°C when not in use.

2.3.3 Scanning electron microscopy

The working electrode was characterized by scanning electron microscopy (SEM) at different stages of its construction.The SEM image of bare PG electrode,c-MWCNT/PG, and NaR-NADH/c-MWCNT/PG electrode were taken in a scanning electron microscopy (Zeiss EV040) at Jawaharlal Nehru university, New Delhi.

2.3.4. *Cyclic voltammetry and testing of nitrate biosensor*

Cyclic voltammetry (CV) of c-MWCNT/PG electrode with and without NaR was recorded in a potentiostat from 0.1V to 1.5V versus Ag/AgCl as reference electrode and Pt. wire as counter electrode in 25ml of

0.05 M PB (pH 7.5) containing 0.1 ml of 100 µM nitrate.

2.4 Optimization of Nitrate biosensor

To determine the optimum pH , the pH of the reaction buffer was varied between pH 5.0 to 10.0 at an interval of 0.5 pH unit 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. The current response (in mA) was recorded by applying different potentials ranging from 0.1V to 1.5V.

2.5 Application of nitrate biosensor in serum samples.

Nitrate content of blood serum was determined by current biosensor in a similar manner as described above for its response measurement under its optimal working conditions except that solution is replaced by blood serum samples.

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

The storage 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 enzyme electrode*

The SEM images of the surface of bare graphite electrode, c-MWCNT/PG electrode and NaR/c-MWCNT/PG electrode are shown in Fig.1A-C respectively. The stepwise modification of electrode could be seen clearly from these SEM images. The SEM image of bare PG electrode showed a smooth morphology (Fig.1A). 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(Fig.1B). After immobilization of NaR on c-MWCNT, the morphology of NaR electrode showed the sporadic appearance of globular/beaded structure due to interaction between c-MWCNT/PG electrode and NaR(Fig.1C),indicating that enzyme was successfully immobilized on surface of c-MWCNT film.

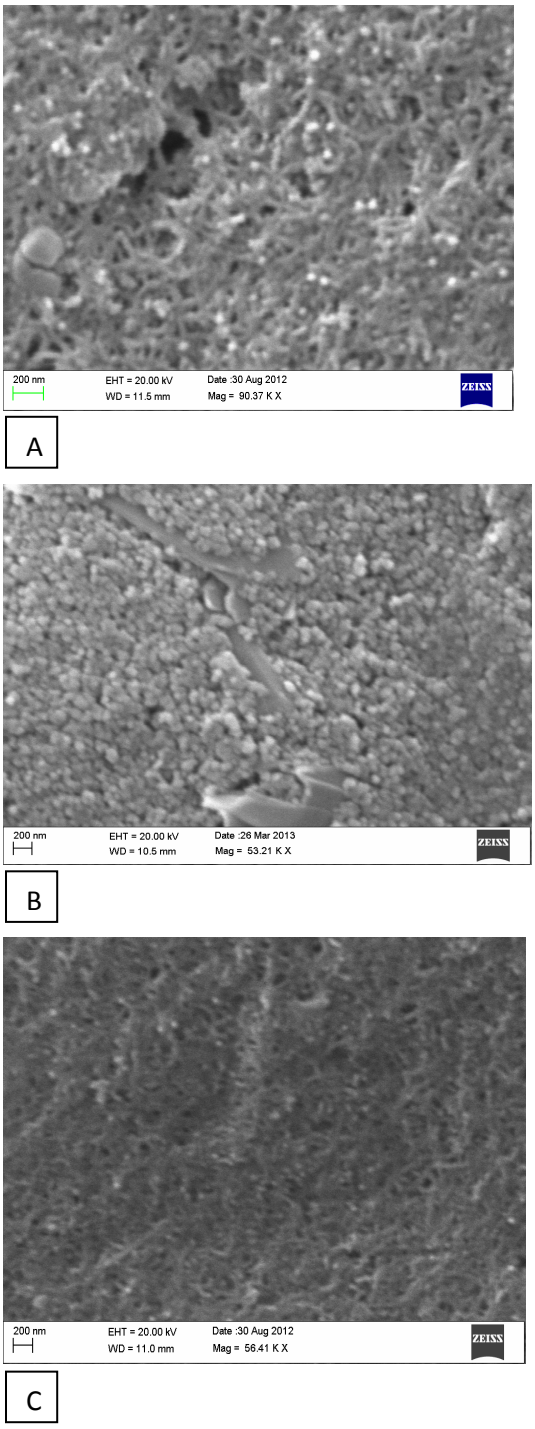

Figure 1. Scanning electron microscopy of [A] bare PG electrode [B] fabricated c-MWCNT [C] immobilized enzyme onto c-MWCNT deposited on PG electrode

3.2.Construction of nitrate biosensor

Fig. 2 provides the schematic representation of construction of nitrate biosensor based on covalent immobilization of NaR on to the c-MWCNT, electrodeposited on the surface of graphite electrode. First, c-MWCNT was deposited onto PG electrode using cyclic voltammetry. Second, NaR enzyme was

covalently immobilized on c-MWCNT using EDC–NHS chemistry. EDC–NHS was used to activate the free –COOH groups present on c-MWCNT. Fig.3A represents the cyclic voltagramm of electrodeposition of c-MWCNT onto the PG electrode. c-MWCNT acting as electron transfer mediater, help to increase the sensor response of enzyme electrode and to increase the sensitivity of the biosensor. Fig.3B represents the cyclic voltagram of NaR immobilized onto the PG electrode showing the oxidation peak at 0.6V, at maximum current. NaR performs the chemical reaction as shown in reaction:-

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

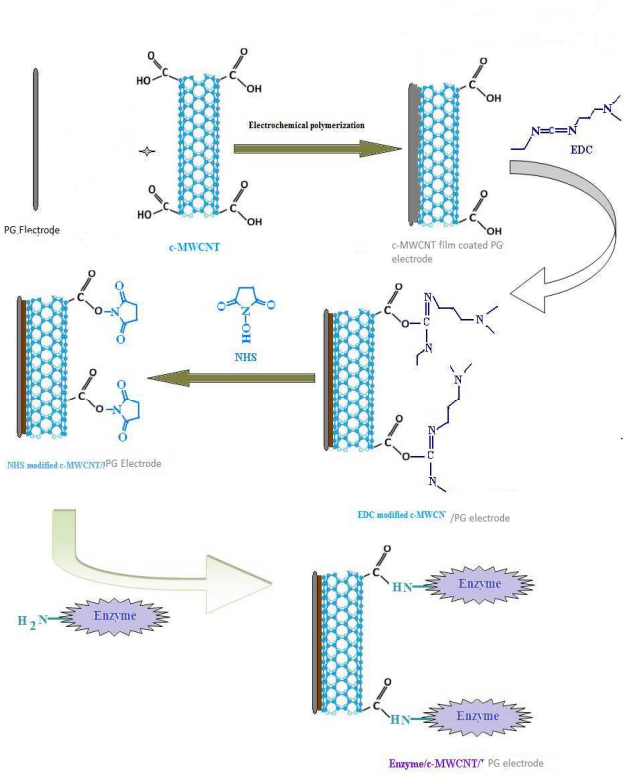

Figure2.Schematic Representation of chemical reaction involved in the fabrication of NaR-NADH/c-MWCNT/PG hybrid electrode

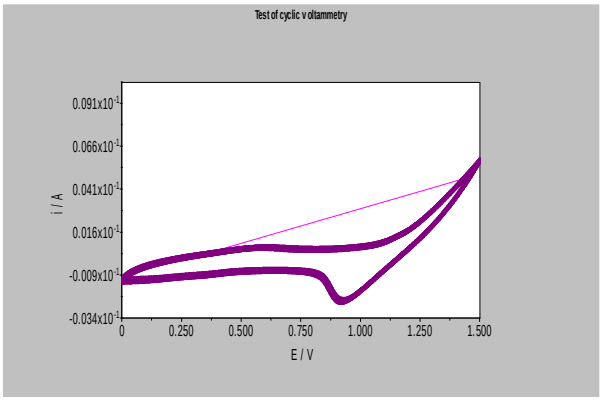

Figure 3A. Cyclic voltagram of electrodeposited cMWCNT onto the pencil graphite electrode, in 0.1M PB buffer (pH 7.5),supporting electrolyte KCL.

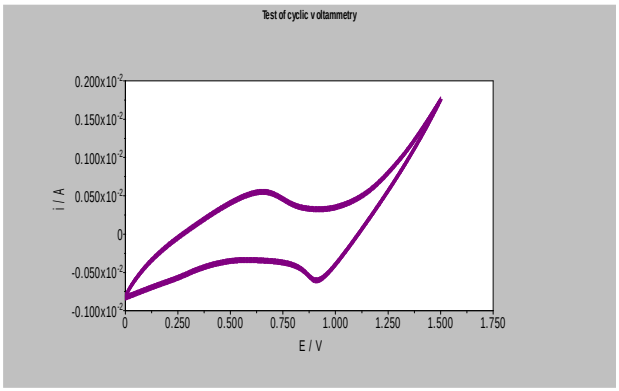

Figure 3B. Cyclic voltagram of immobilization of NaR enzyme on to the fabricated c-MWCNT PG electrode in 0.1M P.B. buffer (pH 7.5) at 50µM nitrate solution.

3.3 Response measurement of nitrate biosensor

The maximum response was obtained at 0.6V which is lower/better than earlier reported biosensors based on PPy/CNT (0.9V) [26] and poly-ortho-phenylenediamine(-0.32V) [24]. Hence, subsequent studies were carried out at this voltage. The low working potential is advantageous because it does not allow ionization of any compounds. Amperometric response of NaR-NADH/c-MWCNT/PG electrode was increased in the addition of 100 µl (0.1 mM) of nitrate at the applied potential (0.6V). When nitrate was added into the buffer solution, the redox current rose steeply to reach a stable value (Fig. 3B). The sensor responded very rapidly, producing 95% of the steady-state current within 6 s, which is less than that needed for earlier biosensors based on Poly-ortho-phenylenediamine (180s)[24], Polypyrrole (4min, 2-4s) [33,34], Redox-mediator(12s)[30].This faster response can be attributed to the influence of c-MWCNT, which provides an environment for the enhanced electrocatalytic effect and a fast electron transfer rate. The synergistic influence of c-MWCNT and other cofactor contributes to the excellent performance for the biosensor.

3.4.Optimization of nitrate biosensor

The biosensor showed the optimal response at pH 7.5 which is almost similar to earlier reported biosensor based on pyrolle- pyridinium (pH 7.5) [21] but higher than that of biosensor based on poly-ortho-phenylenediamine and PPy (pH 7.3) [24,33] and PPy/CNT (pH 7.0) [26] (Table 3). The biosensor response increases as the incubation temperature increases upto 35°C, after which it declines rapidly because of the denaturation of enzyme (NaR). The optimal temperature of the current biosensor was comparable to those of earlier nitrate biosensor (Table

3). Hence, the subsequent experiments were performed at 35°C. There was a hyperbolic relationship between biosensor response and nitrate concentration in the range of 0.1 to 1000µM, and the response was constant after 450µM. The linear plot reveals that enzyme electrode can work well in nitrate solution with a sensitivity of 0.050µA/µM/cm^2.

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 Figure 4, which is a better linear range than those of earlier reported biosensor (Table 3).

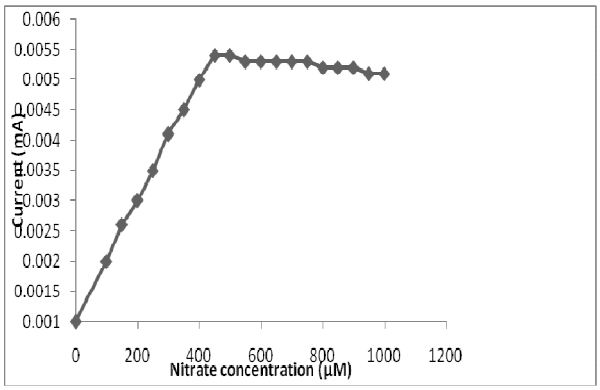

Figure 4. Linear calliberation plot corresponding to current response for different nitrate concentrations by the nitrate biosensor based on c-MWCNT electrodeposited electrode.

3.5.2. *Detection limit*

The detection limit of the current sensor was 0.5 µM at a signal to noise ration of 3 (S/N=3), which is very lower than previously reported Nitrate biosensor (Table 3).

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 and reliability of the present biosensor (Table 1).

3.5.4 *Precision*

To check the reproducibility and reliability of the present Nitrate biosensor, the Nitrate content of the sample in one run (Within batch) and after storage at -20°C for one week (Between batch) were determined. The results showed that the nitrate value of these determination agreed with each other and within batch and between batch coefficient of variation (CVs) were 3.3 % and 2.9 % respectively (Table 2), showing the good reproducibility and reliability of the method.

Table 1. Analytical recovery of added Nitrate in the serum samples, as measured by c- MWCNT based Nitrate biosensor

Nitrate added(µM/L)	Nitrate found(µM/L)	Recovery%
5	34.7	94%
10	69.8	98%

Table 2. Within batch and between batch coefficient of variation for determination of Nitrate in serum samples as measured by c-MWCNT based nitrate biosensor.

N	Nitrate (µM/L)	CV %
Within assay (5)		
0.4		
0.42		
0.46	0.434	3.3%
0.48		
0.41		
Between assay *	**Nitrate (µM/L)**	**CV%**
0.5		
0.6		
0.55	0.574	2.9 %
0.63		
0.59		

* Samples were assayed after storage at -20 °C for one week

3.5.5. Application of Nitrate biosensor

To study the accuracy of the current method, nitrate level in serum sample was determined by the present method (y) with those obtained by standard spectrophotometric method (x) [35]. 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 (Fig. 5). These results show high accuracy of the current method. It proved that the modified electrode ascertained the practical application of the biosensor in the routine quantitative analysis of nitrate.

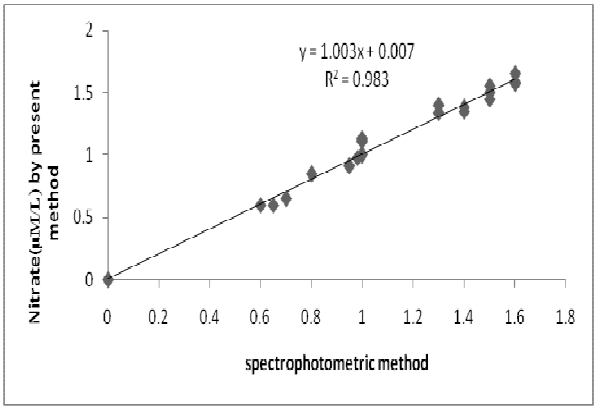

Figure 5. Correlation between serum nitrate values measured by chemical spectrophotometric method (x axis) and the current method (y axis) employing the nitrate biosensor based on c-MWCNT electrodeposited.

Table 3 Comparison of analytical properties of NaR-NADH/c-MWCNT/PG biosensor with earlier reported biosensor.

Property	Ref.[14]	Ref.[21]	Ref.[30]	Ref.[31].	Ref.[23]	Ref.[24]	Ref.[25]	Ref.[26]	Ref.[33]	Ref.[34]	Current work
Source of NaR	-	Aspergillus niger	Pseudomonas stutzeri	-	E.coli	Aspergillus niger	-	Aspergillus niger	yeast	Aspergillus niger	Aspergillus flavus
Immobilization support	Polypyrrole-viologen film	Pyrolle-pyridinium	Redox - mediator	Polyvinyl/pyridinium/saffaranin.	-	Poly-ortho-phenylenediamine(o-PDA)	PPy/CNT	PPy/CNT	PPy	PPy	c-MWCNT
Type of Transduce	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric	Amperometric	potentiometric	potentiometric	Amperometric
Working Optimal pH	NR	7.5	NR	NR	NR	7.3	NR	7.0	7.3	7.3	7.5
Working temperature	NR	35°C	NR	NR	NR	32°C	NR	30°C	32°C	32°C	32°C
Working electrode	Glassy carbon electrode	Glassy carbon electrod	Graphite electrode	Gold electrode	Screen printed electrode	platinum	graphite	Platinum	platinum	platinum	Graphite
Response time	NR	NR	12s	NR	NR	180s	NR	NR	4min	2-4s	6s
Linear concentration range	NR	100µM	NR	NR	0.5-10mM	10-500µM	NR	500nM-10mM	100-5000µM	50-5000µM	0.1-450 µM

3.5.6.Storage and reusability of NaR electrode

The stability of enzyme electrode was investigating by measuring the current response of the biosensor every week under its storage at 4°C. The enzyme electrode maintained 50% of its initial activity even after 45 days of regular 50 uses (Fig. 6), indicating the better stability of current enzyme electrode than earlier enzyme electrodes (stability 1-45 days)[14-34].This can be attributed to covalent immobilization of NaR onto c-MWCNT/PG electrode.

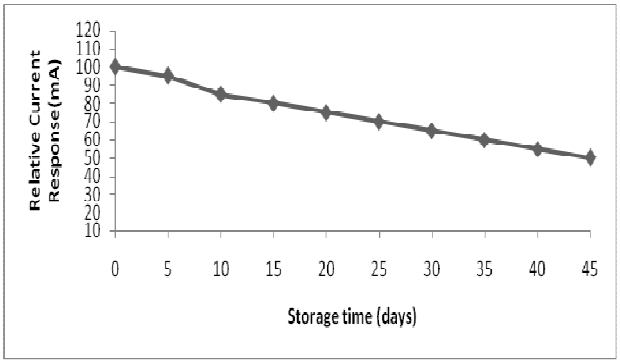

Figure 6. Storage of Nitrate biosensor at 4 °C

4. CONCLUSION

An improved amperometric nitrate biosensor was constructed by covalent immobilization of NaR-NADH onto c-MWCNT modified PG electrode. The biosensor was highly specific, more rapid (6s response time) and sensitive (0.050 μA/μM/cm^2), with a detection limit of 0.5μM, good reproducibility and high storage stability (45 days). It is concluded that c-MWCNT improves the analytical performance of nitrate biosensor. Based on these observations, it indicates that the proposed biosensor has high selectivity to nitrate detection.

5. ACKNOWLEDGEMENT

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REFERENCES

[1] H. Moshage, B. Kok, R. Johannes, Huizenga and L.M. peter jansen, Nitrite and Nitrate determinations in Plasma:A Critical Evaluation. Clin.Chem 41(1995) 892-896.

[2] M. Ikeda, I. Sato, T. Yuasa, T. Miyatake, S. Murota, Nitrite, nitrate and cGMP in the cerebrospinal fluid in degenerative neurologic diseases. Journal of Neural Transmission / General Section JNT,100(1995) 263-267.

[3] JL. hirondel, Dietary Nitrate: Where Is the Risk?,Environ Health Perspect. Aug 2006; 114(8): A458–A459.

[4] I. Guevara, J. Iwanejko, A. Dembińska-Kieć et al., "Determination of nitrite/nitrate in human biological material by the simple Griess reaction," Clinica Chimica Acta, vol. 274(1998), pp. 177–188.

[5] Fang, Y.I., Ohata, H., Honda, K., 2009. Journal of Pharmacological and Toxicological Methods 59, 153–155

[6] A. George , Sythe,G. Metanovie ,Analysis of N03-gas chromatography-mass spectroscopy (1999).

[7] Nagababu, E., Rifkind, J.M., 2010. Methods in Molecular Biology 610, 41–49.

[8] Z. Navratilova, M. Kopanica, Differential-pulse voltammetric determination of molybdenum in nitrate medium in the presence of 8-hydroxyquinoline. Analytical chemical Acta, 244(1991) 193–196.

[9] A. Walter , Lawrance, M. Richard, Briggs, Polarographic Determination of Nitrate, Analytical chemistry, (1953) 964-966.

[10] S. Cosnier, B. Galland, C. Innocent, New electropolymerizable amphiphilic viologens for the immobilization and electrical wiring of a nitrate reductase, J. Electroanal. Chem. 433 (1997) 113–119.

[11] Willner, E. Katz, N. Lapidot, Bioelectrocatalysed reduction of nitrate utilizing polythiophene bipyridinium enzyme electrodes, Bioelectrochem. Bioenerg. 29 (1992) 29–45.

[12] D. Quan, J.H. Shim, J.D. Kim, H.S. Park, G.S. Cha, H. Nam, Electrochemical determination of nitrate with nitrate reductase-immobilized electrodes under ambient air, Anal. Chem. 77 (2005) 4467–4473.

[13] T.L. Goff, J. Braven, L. Ebdon, D. Scholefield, High-performance nitrate- selective electrodes containing immobilized amino acid betaines as sensors, Anal. Chem.74 (2002) 2596–2602.

[14] Willner, A. Riklin, N. Lapidot, Electron-transfer communication between a redox polymermatrix and an immobilized enzyme: activity of nitrate reductase in a

viologen-acrylamide copolymer, J. Am. Chem. Soc. 112 (1990) 6438–6439.

[15] J.W. Aylotta, D.J. Richardson, D.A. Russell, Optical biosensing of nitrate ions using a sol–gel immobilized nitrate reductase, Analyst 122 (1997) 77–80.

[16] V. Reipa, S.-M.L. Yeh, H.G. Monbouquette, V.L. Vilker, Reorientation of tetradecylmethyl viologen on gold upon coadsorption of decanethiol and itsmediation of electron transfer to nitrate reductase, Langmuir 15 (1999) 8126–8132.

[17] L.M. Moretto, P.Vgo,M. Zanata, Nitrate biosensor based on ultrathin film composite membrane.Anal.Chem,70 (1998)2159-2163.

[18] M. Bodea, platinum electrode modified with cellulose acetate membrane.2001.

[19] D. Albanese, M. Di Matteo, , C. Alessio, Screen printed biosensors for detection of nitrates in drinking water. ESCAPE20,(2010).

[20] M. Sohail and S.B. Adeloju, Polupyrrole-based bilayer nitrate amperometric biosensor with an integrated permselective poly-ortho-phenylenediamine layer for exclusion of inorganic interferences.Biosensor and Bioelectronics 26(2011) 4270-4275.

[21] F. Can, K.Ozoner, S. Ergenekon , P.Erhan, Amperometric nitrate biosensor based on carbon nanotubes/polypyrrole/nitrate reductase biofilm electrode.Material science and Engineering C,32(2012) 18-23.

[22] T. Madasamy, M. Pandiraj, M. Balamurugan ,Copper,Zinc superoxide dismutase and nitrate reductase coimmobilized bienzymatic biosensor for the simultaneous determination of nitrite and Nitrate, Biosensor and Bioelect.52 (2014) 209-215.

[23] V. Sachdeva and V. Hooda, A New Immobilization And Sensing Platform For Nitrate Quantification, Talanta,124(2014)52-59.

[24] Lu, W. Schmidt, Tatar, B. Spliethoff, J. Popp, W. Kiefer, & F. Schüth ,Formation of amorphous carbon nanotubes on ordered mesoporous silica support, Carbon 43(2005)1811-1814.

[25] W. Xuejiang and S. Dzyadevych, conductometric nitrate biosensor based on methyl viologen/nafion/NR interdigited electrodes.Talanta 69 (2004) 450-455.

[26] Kirstein, D., Kirstein, L., Scheller, F., Borcherding, H., Ronnenberg, J., Diekmann, S., & Steinrucke, P., Amperometric nitrate biosensors on the basis of Pseudomonas stutzeri nitrate reductase. Journal of Electroanalytical Chemistry, 474(1999), 43-51.

[27] Ferreya N.F., Solis Velia M.,An amperometric nitrate-reductade-phenosafranin electrode:kinetic aspects and analytical applications, bioelectrochemistry 64(2004)61-70.

[28] M. M. Rahman, A.Umar, K.Sawada, development of amperometric glucose biosensor based on glucose oxidase co-immobilized with multi-walled carbon nanotubes at low potential,sens.Actuat.B 137(2009) 327-333.

[29] M..Sohail,,B.Adeloju,,Electroimmobilization of nitrate reductase and nicotinamide adenine dinucleotide into polypyrrole films for potentiometric determination of nitrate.,Sens Actuat B 133(2008) 333-339.

[30] M. Sohail, B.Adeloju, Fabrication of Redox-Mediator supported potentiometric nitrate biosensor with nitrate reductase, Electroanalysis 12(2009) 1411-1418.

[31] A. Zatar, et.al., Spectrophotometric determination of nitrite and nitrate using phosphomolybdenum blue complex, Talanta 50 (1999) 819 – 826.