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Amperometric nitrate biosensor based on Carbon nanotube/Polypyrrole/Nitrate reductase biofilm electrode

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ABSTRACT

This study describes the construction and characterization of an amperometric nitrate biosensor based on the Polypyrrole (PPy)/Carbon nanotubes (CNTs) film. Nitrate reductase (NR) was both entrapped into the growing PPy film and chemically immobilized via the carboxyl groups of CNTs to the CNT/PPy film electrode. The optimum amperometric response for nitrate was obtained in 0.1 M phosphate buffer solution (PBS), pH 7.5 including 0.1 M lithium chloride and 7 mM potassium ferricyanide with an applied potential of 0.13 V (vs. Ag/AgCl, 3 M NaCl). Sensitivity was found to be 300 nA/mM in a linear range of 0.44–1.45 mM with a regression coefficient of 0.97. The biosensor response showed a higher linear range in comparison to standard nitrate analysis methods which were tested in this study and NADH based nitrate biosensors. A minimum detectable concentration of 0.17 mM (S/N = 3) with a relative standard deviation (RSD) of 5.4% (n = 7) was obtained for the biosensor. Phenol and glucose inhibit the electrochemical reaction strictly at a concentration of 1 μg/L and 20 mg/L, respectively. The biosensor response retained 70% of its initial response over 10 day usage period when used everyday.

1. Introduction

Nitrate is a well-known pollutant to both environment and human health. Excess nitrate in environmental water systems causes algal blooms, a depletion of dissolved oxygen, and possible eutrophication. Nitrate in the human body, causes methemoglobinemia (so-called blue baby syndrome), and also may become a source of carcinogenic N-nitrosamines, some gastrointestinal cancers, and teratogenic effects [1–4]. These effects have increased the demand for sufficiently sensitive, accurate and uncomplicated analytical measurements for nitrate. Spectrophotometric methods for the determination of nitrate have been developed over the past several decades [5–9]. Ion exchange chromatography combined with spectrometric, conductometric or electrochemical detection has recently become popular for the detection of nitrate [10–13]. However, even though they have high sensitivity and good reproducibility, these methods require large and expensive instrument and extensive pre-treatment of the sample. Nitrate ion-selective electrodes (ISEs) and ion sensitive field-effect transistors (ISFETs), which provide fast response time, simplicity, low cost, and can be easily adapted to flowing streams and in situ measurements but suffer from poor stability and severe interference effect caused by other anions present in the sample [14]. Therefore, enzyme-based nitrate biosensors have been developed for the last two decades [4, 7, 9, 14–16] considering the advantage of the enzymes which are strongly substrate-selective. Nitrate reductase (NR) is used in the fabrication of nitrate biosensors. However, its multiredox center responsible to the biological conversion of nitrate to nitrite is generally not very active, and is deeply embedded in the protein structure, preventing thus a direct electron transfer with the electrode. Carbon nanotubes (CNTs) have emerged as new class nanomaterials that are receiving considerable interest owing to their ability to promote electron transfer reactions with enzymes showing low electroactivity. The high conductivity of this carbon material leading to level of $10^2\ \Omega^{-1}\cdot cm^{-1}$ improves electrochemical signal transduction, while its nano architecture imposes the electron contact between the redox centers, deeply inlaided in enzyme structure, and the smooth surface of the electrode. CNTs can donate and accept electrons in a wide range of potentials, and could therefore be used as mediators in biosensor systems [17]. In addition to this, enzymes can be chemically immobilized to composite films including CNTs which have functional amine and carboxyl groups [18].

The use of NR for fabrication of nitrate biosensors usually employs a cofactor such as NADH. The catalytic reduction of nitrate caused by NR in the presence of a cofactor shifts the electrode potential towards the NO_3^-/NO_2^- equilibrium potential, resulting in a sharp decrease in the electrode response which is proportional to the concentration of the substrate [19]. Unfortunately, there are two distinct disadvantages associated with the use of NADH for nitrate biosensors [20]: (a) low currents generated during electrochemical oxidation of NADH, and (b) byproducts are generated through dimerization and this can foul the electrode surface as the dimers (i.e. dimerization of NAD• radicals and other oxidation products) are readily adsorbed on the electrode surface. Consequently, this reduces

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the sensitivity, selectivity and stability of the biosensor. It has been found that a path for electron transfer between the redox centers of the enzyme and electrode was provided by using a redox mediator which is an effective way for overcoming or eliminating these problems for amperometric nitrate biosensors [21]. Another potential benefit of using a redox mediator instead of NADH is to acquire a low-cost biosensor. On an equal weight basis, NADH is considerably (up to 30 times) more expensive than redox mediators. Therefore, if an alternative redox mediator can be used, it will result in substantial reduction of the cost of fabricating a nitrate biosensor [16]. Several redox mediators as methyl violagen, azure A, safranin, neutral red, bromophenol etc. have been previously used for the amperometric nitrate biosensors [22–25].

In the present study, we fabricated an amperometric nitrate biosensor by the incorporation of carboxyl functionalized CNTs into the Polypyrrole (PPy) film at a glassy carbon electrode surface. Potassium ferricyanide [$K_3Fe(CN)_6$] was added into the electrochemical measurement medium and used as a redox mediator. $K_3Fe(CN)_6$, which has excellent redox properties [26], has not been used as a mediator for amperometric nitrate biosensors. The performance of Carbon nanotube/Polypyrrole/Nitrate reductase [CNT/PPy/NR] biofilm electrode was investigated by means of pyrrole concentration, mediator concentration, electropolymerization time of the composite film, pH, potential, supporting electrolyte concentration. The fabricated composite film electrode was then used for investigation of potential inhibition effect of some of the organic substances on the mediator or on the electrochemical reaction for nitrate analysis. Analytical nitrate measurement parameters such as detection limit, sensitivity, response time and linear range were calculated and compared with the results obtained from the spectrophotometric and photometric conventional nitrate analysis methods.

2. Materials and methods

2.1. Reagents

Nitrate reductase (E.C.1.7.1.2) from *Aspergillus niger* with an activity of ≥300 units/g solid (one unit will reduce 1.0 μmol of nitrate per min in the presence of β-NADPH at pH 7.5 at 25 °C performed by the supplier) and sodium dodecyl sulfate (SDS) were obtained from Sigma. Lithium chloride (as an electrolyte), potassium di-hydrogen phosphate, dipotassium hydrogen phosphate, sodium acetate and $K_3Fe(CN)_6$ were purchased from Merck. Pyrrole monomer (99%) and 1-cyclohexyl-3 (2-morpholinoethyl)carbodiimide metho-*p*-tolueno-sulfonate was obtained from Aldrich. Multiwalled CNTs (with a diameter of 100 nm, with a length of 50 μm) were supplied from Nanocs. Inc., Newyork, USA. Sodium nitrate was purchased from J. Baker. Stock solutions of various nitrates were daily prepared in 0.1 M PBS, pH 7.5.

2.2. Apparatus and electrochemical measurements

Electrochemical experiments were performed by using a CHI 840B Model electrochemical analyzer. A glassy carbon working electrode (3 mm diameter), a platinum wire counter electrode, a Ag/AgCl (3 M NaCl) reference electrode, and a conventional three-electrode electrochemical cell were obtained from CH Instruments. The electrochemical detection based on amperometric measurements was performed in an electrochemical cell (with a volume of 10 mL) containing a magnetically stirred (100 rpm) 0.1 M PBS, pH 7.5 included 0.1 M lithium chloride and 7 mM $K_3Fe(CN)_6$ under nitrogen purge to remove oxygen. Three-electrode system was immersed into the electrochemical cell, the working potential of 0.13 V was applied and current was allowed to reach a steady-state value. After reaching the steady-state current, increasing concentrations of nitrate solution were added to the electrochemical cell to produce current-time recordings of amperometric measurements. Calibration curves were constructed from the current-time recordings of nitrate additions.

2.3. Fabrication of CNT/PPy/NR biofilm electrode

The acid oxidation method was conducted to prepare water-soluble oxidized CNTs at a concentration of 0.03 mg/L according to the procedure given in Refs. [18, 27]. Additional carboxyl groups were formed on the walls of CNTs through the acid oxidation procedure [28]. No CNT precipitation was observed in oxidized CNT solution throughout the polymerization step. The electrode fabrication step was preceded by a cleaning phase of the electrode surface using gamma alumina powder then, rinsing with distilled water. CNT/PPy/NR composite film was deposited and coated onto the surface of the cleaned glassy carbon electrode by electropolymerization in the three electrode cell. The polymerization medium contained 10 mL of oxidized CNTs solution including 13 mM pyrrole, 0.6 mg/mL SDS, which was used as supporting electrolyte for the pyrrole polymerization, and 300 μL of 3.5 mg/mL nitrate reductase enzyme. Cyclic voltammogram of the CNT/PPy/NR biofilm was scanned between (−1.2) and (+1.2) V for 60 cycles with a scan rate of 100 mV/s. In this step, CNTs and enzyme were physically entrapped into the growing PPy film.

2.4. Enzyme immobilization procedure

The CNT/PPy/NR biofilm electrode was allowed to react for 3.5 h within the solution of 5 mg/mL 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-tolueno-sulfonate, prepared in 0.05 M, pH 4.8 acetate buffer, with a continuous stirring at 100 rpm. Then, the electrode was dipped into a solution of 3.5 mg/mL NR, dissolved in 0.1 M PBS, pH 7.5 for 18 h, and stored at +4 °C overnight. Enzyme was chemically immobilized via the carboxyl groups of CNTs to the CNT/PPy/NR biofilm.

2.5. Standard nitrate analysis

The standard UV spectrophotometric method was used to construct a nitrate calibration curve following Beer's law up to the nitrate concentration of 50 mg/L according to the procedure 4500-B in standard methods [29]. The direct photometric method based on the nitrate kits prepared by the supplier was used for measuring nitrate concentrations up to 60 mg/L. Standard nitrate solutions with concentration ranging between 1 and 60 mg/L were prepared in distilled water then added in nitrate kits. The kits were inserted in a photometer (Hach-Lange DR2800) to record absorbances at 315 nm. A nitrate calibration curve was obtained using the absorbances versus various concentrations of nitrate.

2.6. Optimization of experimental parameters

The performance of the biosensor was investigated by changing such parameters as pyrrole, $K_3Fe(CN)_6$ and supporting electrolyte (lithium chloride) concentration which both affect polymer film structure and measurement performance. CNT/PPy/NR biofilms were prepared on glassy carbon electrode with various pyrrole concentrations ranging between 4 and 16 mM, respectively. The prepared electrodes were tested for 1 mM nitrate additions in phosphate buffer containing 7 mM $K_3Fe(CN)_6$ at a working potential of 0.13 V.

In order to obtain a satisfactory performance of the biosensor, the CNT/PPy/NR biofilm electrode was also optimized with respect to the concentration of $K_3Fe(CN)_6$ in reaction medium. Since the catalytic reaction occurs between $K_3Fe(CN)_6$ (mediator) and NR, the amount of immobilized $K_3Fe(CN)_6$ must be sufficient to give a high amperometric response for the reaction. Otherwise, the fraction of mediated nitrate reduction limits the amperometric response instead of the nitrate concentration through the reaction. Because, the response of a calibrated nitrate biosensor must depend on the concentration of nitrate in the reaction medium. Amperometric responses of the

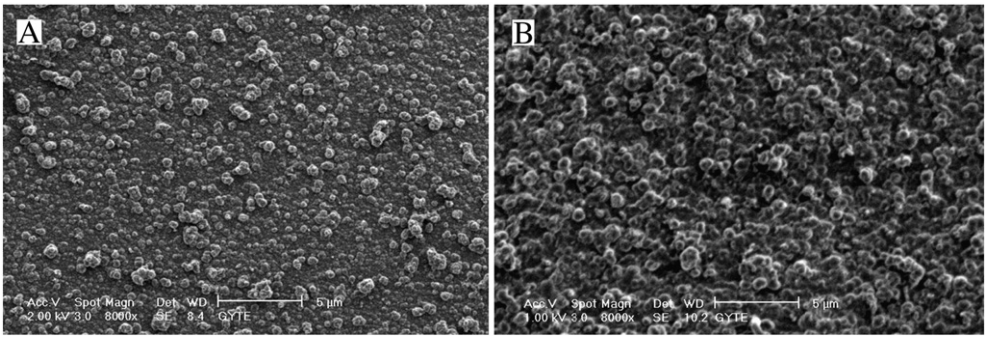


Fig. 1. SEM images of PPy (A), and CNT/PPy (B)film.

working electrode were tested by adding 1.4 mM nitrate into the reaction mediums at various $K_3Fe(CN)_6$ concentrations ranging between 1 and 9 mM at a working potential of 0.13 V.

Interference effect of some organic compounds such as glucose and phenol on biosensor response to 0.44–1.45 mM nitrate additions was investigated with an applied potential of 0.13 V (vs. Ag/AgCl). All measurements were performed in 0.1 M PBS, pH 7.5 containing 0.1 M LiCl and 7 mM $K_3Fe(CN)_6$ for various concentrations of phenol and glucose additions ranging between 1 and 500 μg/L and 1–50 mg/L, respectively.

3. Results and discussion

SEM image of pure PPy film without CNT, and CNT/PPy film prepared on the glassy carbon electrode were presented in Fig. 1A and B, respectively.

3.1. Electrochemical characterization of the CNT/PPy/NR biofilm

Fig. 2 shows the cyclic voltammograms (CV) of the CNT/PPy/NR biofilm electrode in pure 0.1 M PBS, pH 7.5 and in the same buffer containing 7 mM $K_3Fe(CN)_6$ at a potential scan between (−1.2) and (+1.2) V with a scan rate of 100 mV/s. The CV of the CNT/PPy/NR biofilm electrode in phosphate buffer including $K_3Fe(CN)_6$ showed the appearance of two peaks relative to $K_3Fe(CN)_6$ reagent at 0.13 and 0.4 V that corresponds to the redox process of $[Fe(CN)_6]^{-3}$/$[Fe(CN)_6]^{-4}$ couple. This result is similar with previously reported papers [30–32]. Electrochemical behavior of $K_3Fe(CN)_6$ involves reduction of $Fe(CN)_6^{-3}$ to $Fe(CN)_6^{-4}$ at a potential of 0.13 V. The reduction of NR to its native form is conducted by electron capture of the oxidized NR via the $Fe(CN)_6^{-4}$ form. The reduction current of $Fe(CN)_6^{-3}$ is proportional with nitrate concentration added in the reaction medium according to the following mechanism (Scheme 1).

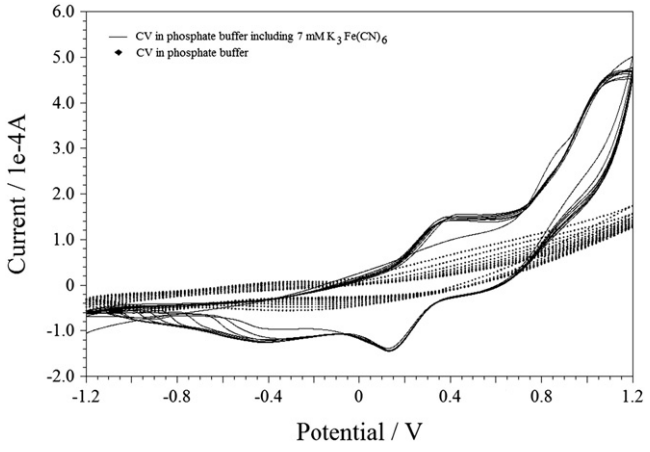


Fig. 2. Cyclic voltammograms of the CNT/PPy/NR biofilm electrode at a scan rate of 100 mV/s.

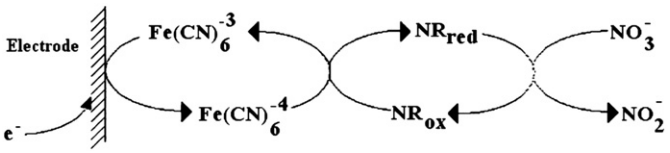
3.2. Optimization of monomer, mediator and supporting electrolyte concentration

Fig. 3A shows that amperometric nitrate response increased up to the pyrrole concentration of 13 mM. Due to the increased film thickness with excessive pyrrole concentration higher than 13 mM it resulted a decrease in amperometric nitrate response. Because, thick film layer behaved as diffusion barrier for electron transfer, and, hence, decreased amperometric nitrate response. Pyrrole concentration of 13 mM was chosen for further experiments since the maximum amperometric nitrate response was observed with the CNT/PPy/NR biofilm electrode.

In the experiment of the mediator optimization mentioned in Section 2.6., paragraph 2, results showed that the response was increased up to the mediator concentration of 7 mM, and no change in nitrate response was observed higher than 7 mM (Fig. 3B). The result indicated that a mediator concentration of 7 mM was best for our purpose, taking into consideration both maximum response and biosensor economy.

Since enzyme activity is strongly affected by pH, the effect of pH in phosphate buffer on amperometric nitrate response of the biosensor was also examined. The optimal pH for the CNT/PPy/NR biofilm electrode was determined in the pH range of 5.5–9.5. The results obtained by the addition of nitrate at a concentration of 1 mM in 0.1 M PBS at various pH levels showed that the maximum amperometric response was observed at pH 7.5 (*not shown*). The optimized pH level of 7.5 was then used in subsequent experiments. This result is strongly correlated with those obtained using NR-based biosensors [33–35].

The presence of a supporting electrolyte (LiCl) was critical for electrical current of a working electrode. In the absence of a supporting electrolyte, much higher working potential is required to observe redox mechanism on the working electrode surface. Such a high potential can lead to the denaturation of the enzyme. Evidently, the nitrate reduction was very difficult to achieve in the absence of the supporting electrolyte. Fig. 3C presents the increasing addition of LiCl up to 0.2 M into the nitrate reaction medium. Nitrate at a concentration of 0.5 mM was added into the 0.1 M PBS, pH 7.5 including various concentrations of LiCl, and amperometric responses were observed at 0.13 V. The increasing addition of LiCl up to 0.1 M to the reaction medium resulted in the increasing of nitrate response. Further increase in the LiCl



Scheme 1. The electron transfer mechanism of NR in the presence of the mediator [$K_3Fe(CN)_6$].

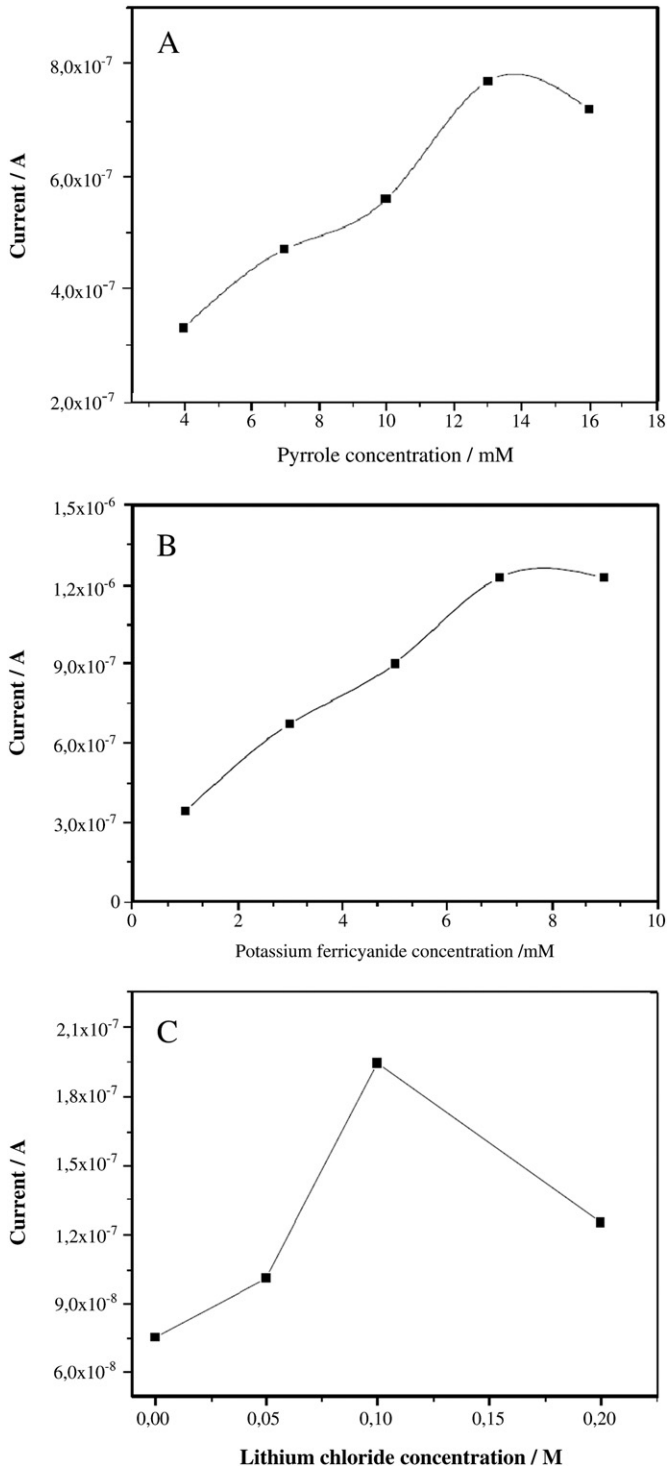


Fig. 3. Effect of pyrrole concentration on response of 1 mM nitrate addition (A), effect of $K_3Fe(CN)_6$ concentration on response of 1.4 mM nitrate addition (B), effect of LiCl concentration on response of 0.5 mM nitrate addition (C) in 0.1 M PBS, pH 7.5. Applied potential 0.13 V vs. Ag/AgCl, 3 M NaCl.

concentration beyond this level resulted with a considerable decrease of the nitrate response. Thus, 0.1 M was used as an optimized supporting electrolyte concentration.

3.3. Amperometric response of the biosensor for nitrate detection

Under optimized conditions, nitrate measurement was carried out according to the procedure described in Section 2.2 at a working potential of 0.13 V (vs. Ag/AgCl). Fig. 4A illustrates the typical amperometric response (current-time recording) of the biosensor after the addition of successive aliquots of nitrate solutions (0.25–1.55 mM)

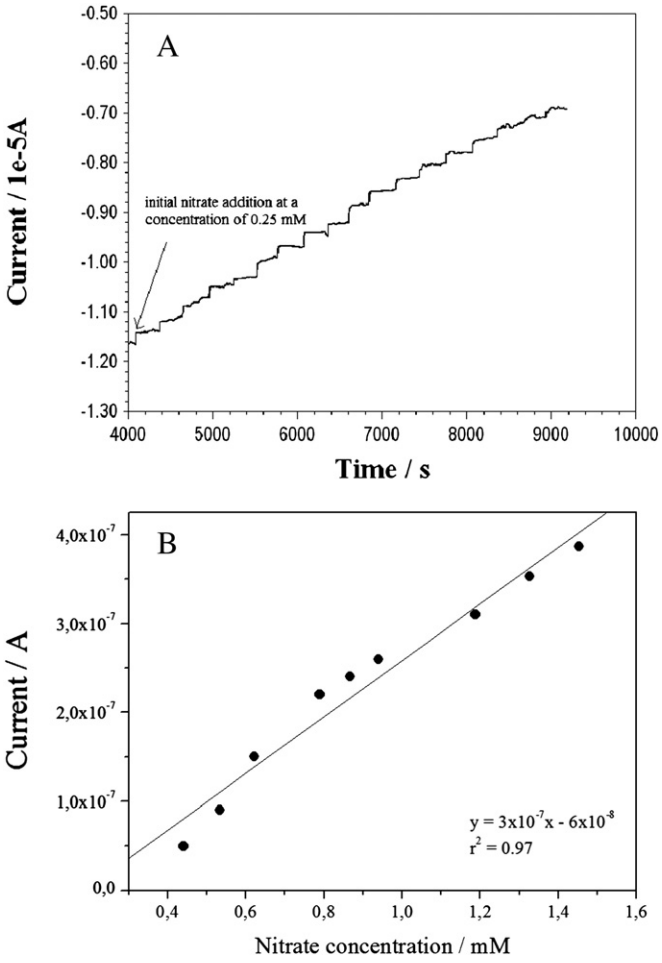


Fig. 4. Current-time recording of the biosensor to increasing nitrate concentrations ranged between 0.25–0.3–0.35–0.4–0.45–0.5–0.6–0.7–0.8–0.85–0.95–1–1.05–1.15–1.25–1.35–1.45–1.55 mM. Applied potential: 0.13 V vs. Ag/AgCl, 3 M NaCl (A). Nitrate calibration curve obtained from current-time recording in linear range of 0.45–1.45 mM nitrate (B).

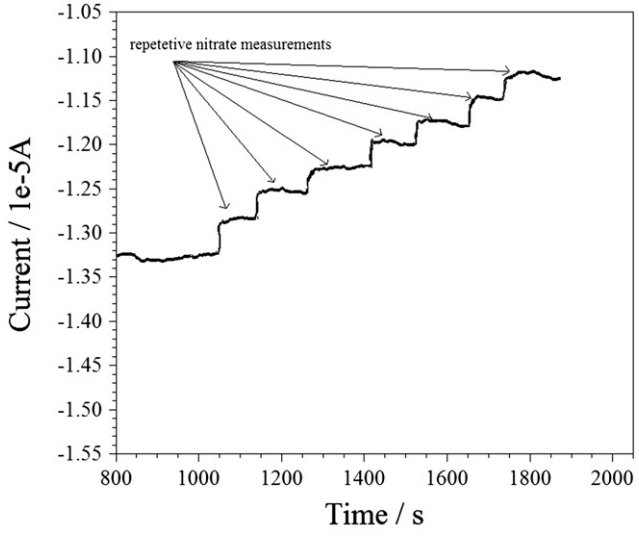


Fig. 5. Operational stability of CNT/PPy/NR biofilm electrode to successive addition of 1 mM nitrate. Applied potential: 0.13 V vs. Ag/AgCl, 3 M NaCl.

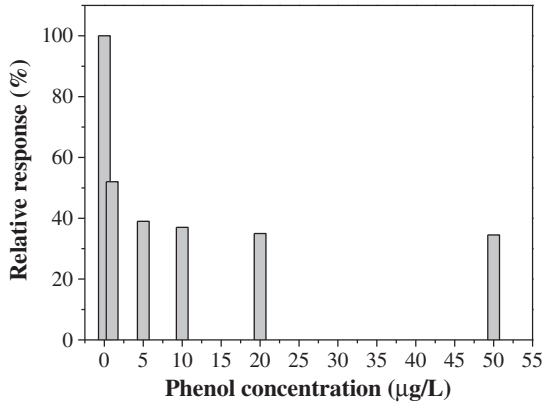


Fig. 6. Interference effect of phenol concentration on biosensor response in presence of 1 mM nitrate. Applied potential: 0.13 V vs. Ag/AgCl, 3 M NaCl.

under continuous stirring. Response time of the biosensor for nitrate additions was observed to be 20 s which indicated that a fast electron transfer was achieved by using $K_3Fe(CN)_6$. The response time is faster than recently reported time of 30 s [3], 15–60 s [36], and 40 s [37]. Nitrate calibration curve was constructed from the current-time recording and presented in Fig. 4B.

Analytical parameters were calculated from the obtained calibration plot. Detection limit (LOD) was found to be 0.17 mM with a signal-to-noise ratio of 3 according to the $3s_b/m$ criteria in Ref. [18]. The biosensor was responsive to nitrate within a linear concentration range of 0.44–1.45 mM ($r^2 = 0.97$). The detection of nitrate with the immobilized NR was achieved in the presence of NADH or other suitable cofactor by amperometry in previous studies [22, 23, 35, 37–39]. However, most of these biosensors have a narrow linear concentration range and the upper concentration limit was less than 1 mM. Linear range upper concentration up to 0.25, 0.16 and 0.5 mM was reported in the previously published nitrate biosensor studies operated with mediator of methylviologen, polyviologen and azure A, respectively [9, 15, 40]. In our study, the biosensor sensitivity obtained from the slope of the nitrate calibration curve was 300 nA/mM. Silvia et al. reported a sensitivity value of 94.7 nA/mM by using a new hydrophilic poly(pyrrole-viologen) film and viologen was used as the redox mediator of the sensor [15]. The analytical parameters of previously published nitrate biosensors were presented in Table 1. Since NADH is the original cofactor of NR, the affinity of the enzyme to NADH is higher than an ordinary redox mediator. Therefore, reaching to higher sensitivity values for the nitrate biosensors prepared with NADH is not a surprise. However, as we mentioned in the Introduction section,

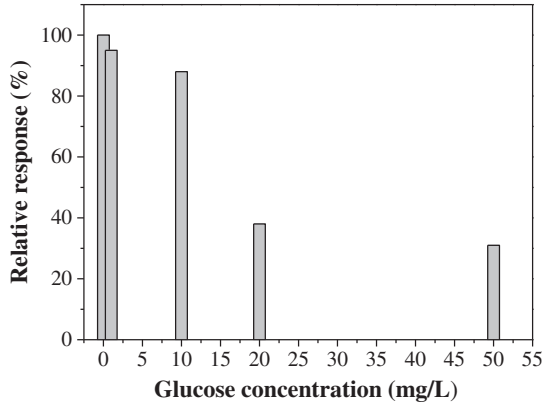


Fig. 7. Interference effect of glucose concentration on biosensor response in presence of 1 mM nitrate. Applied potential: 0.13 V vs. Ag/AgCl, 3 M NaCl.

Table 1
Analytical performance characteristics of some of the previously published nitrate biosensors.

Mediator	Linear range (mM)	Response time (s)	Sensitivity (nA/mM)	Reference
$K_3Fe(CN)_6$	0.44–1.45	20	300	This study
Methylviologen	0.02–0.25	15	–	[9]
Polyviologen	0.005–0.16		94.7	[15]
Azure A	0.02–0.5	18–20	40.8	[39]
No mediator	0.015–0.3	40	7300	[36]

byproducts generated through dimerization (i.e. dimerization of NAD• radicals and other oxidation products) can foul the electrode surface resulting to low dynamic measurement range of the NADH-based biosensors. Fortunately, in this study the use of $K_3Fe(CN)_6$ overcame this drawback, and enlarged the dynamic range of nitrate measurement up to the concentration of 1.45 mM. Furthermore, the presence of CNTs in the composite film most probably enhanced the electroactivity of the enzyme by promoting the electron transfer among deeply embedded active center of the enzyme and its mediator and substrate. It was previously reported that CNTs acted as an electron transferring bridge or a wire for the desired electrochemical reaction [18, 27].The sensitivity of a biosensor also depends on the electrode material, enzyme immobilization method and the magnitude of the applied potential [41–43].

Operational stability and long-term stability are considered as some of the key factors of a biosensor performance. The operational stability of the biosensor was evaluated by means of 7 repetitive measurements of nitrate at a concentration of 1 mM (Fig. 5). The relative standard deviation (RSD) was found to be 5.4%. The long-term stability was studied over a 10 day period by monitoring amperometric response of the biosensor to the addition of nitrate at a concentration of 1 mM with everyday usage. The biosensor response retained 70% of its initial response after the usage in this time period. The working electrode was stored in 0.1 M PBS, pH 7.5 at +4 °C when not in use.

The analytical parameters of the biosensor were compared with those obtained for the standard nitrate analyses. Standard nitrate analyses were performed as described in Section 2.5. The analytical parameters were calculated for both UV spectrophotometric and direct photometric methods in the same way with the biosensor parameters. The biosensor response showed the widest linear range with a regression coefficient of 0.97 with respect to the UV spectrophotometric method (0.16–0.8 mM) and the direct photometric method (0.016–0.96 mM). The biosensor was also tested by the nitrate recovery experiment, which showed a satisfactory result, with a recovery value of 97% in comparison to the UV spectrophotometric method (96%) and the direct photometric method (90%). Recovery values were calculated according to the formula given as *(measured nitrate concentration / actual nitrate concentration)* × 100.

3.4. Interference of organic substances on biosensor response

Fig. 6 shows the relative response of the biosensor to the nitrate addition at a concentration of 1 mM in presence of various concentrations of phenol (1–50 μg/L). When the reaction medium consisted of 1 μg/L phenol, the response of the biosensor decreased to its half value in comparison to the reaction medium without phenol. The relative response was about 40% of its initial response in the presence of 50 μg/L phenol. Hence, the biosensor response retained stable when phenol was added over the concentration of 50 μg/L. $K_3Fe(CN)_6$ is known as a strong oxidizer of phenol, and used in spectrophotometric phenol assay for this purpose. The decrease of the biosensor response might be a result of the interaction between $K_3Fe(CN)_6$ and phenol. Another reason of response decrease might be inhibition effect of

phenol on enzyme activity [44]. Glucose was also investigated for its interference effect (Fig. 7). The response of the biosensor decreased sharply (38%) in presence of glucose concentration of 20 mg/L in comparison to the reaction medium without glucose. The decrease of the response is probably due to the relatively low electrical conductivity of the reaction medium consisting glucose. In brief, although the enzyme is strongly selective to its substrate which is nitrate ion, enzyme based electrochemical nitrate biosensors have still some other interference risks depending on the content of the reaction medium. It should be noted that the redox mediators are highly reactive substances, and the necessary conditions for the sufficient electroactivity should be conducted.

4. Conclusion

In this study, the CNT/PPy/NR biofilm electrode was fabricated for amperometric nitrate measurement. For the first time, $K_3Fe(CN)_6$ was used to be a mediator instead of NADH for an amperometric nitrate biosensor. Analytical parameters of sensitivity, linear range, detection limit etc. were investigated under the optimized reaction condition. Detection limit, sensitivity and linear range were found to be 0.17 mM, 300 nA/mM and 0.44–1.45 mM, respectively. Response time of the biosensor was observed to be 20 s. The relative standard deviation (RSD) was found to be 5.4%. The biosensor response retained 70% of its initial response after the usage in this time period. The biosensor response showed the widest linear range with a regression coefficient of 0.97 with respect to the UV spectrophotometric method (0.16–0.8 mM) and the direct photometric method (0.016–0.96 mM). The biosensor had a recovery value of 97% in comparison to the UV spectrophotometric method (96%) and the direct photometric method (90%).

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