Fabrication of Enzyme Nanoparticles-based Nanosensor for Detection of Nitrate Content in Drinking Water

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Abstract

Aim: An advanced amperometric biosensor was designed based on immobilization of nitrate reductase nanoparticles (NaRNPs) on gold (Au) electrode. **Materials and Methods:** NaRNPs were prepared by chemical desolvation and glutaraldehyde cross-linking method to form nanoaggregate and characterized by transmission electron microscopy and ultraviolet–visible spectroscopy. NaRNPs/Au electrode was evaluated by scanning electron microscopy and Fourier-transform infrared spectroscopy. **Results and Discussion:** A linear relationship was observed between biosensor response, i.e., current milliampere and nitrate concentration in the range of 0.1 μM–200 μM showing lower detection limit of 0.14 mM with a response time of 2 s on 0.2 V–1.2 V at scan rate 50 mV/s under optimum pH 7.0 and temperature 35°C. **Conclusion:** A lower value of K_m (384.61 μM) with V_{max} (1219.512 μmol (min mg protein)-1), storage stability of 90 days, and low detection limit 0.14 μM nitrate content in drinking water samples was obtained.

Key words: Biosensor, enzyme nanoparticles, nitrate reductase, shelf life

INTRODUCTION

Nitrate is a widespread contaminant of soil surface and ground water[1] which is largely contributed by nitrogen fertilizers[2] present in human and animal waste.[3] The World Health Organization (WHO) guidelines[4] and US Environmental Protection Agency (EPA) set the maximum acceptable limit as 45 mg N/Lit to monitor nitrate level in drinking water US EPA.[5] Nitrate level above the maximum permissible level of 45 mg N/L, it may lead to several disorders and health issues. A form of infantile methemoglobinemia was reported Comly[6] due to the elevated level of nitrate in drinking water. Nitrate is reduced to nitrite after ingestion and causes fatal blood disorder named blue baby syndrome (MetHb) methemoglobinemia (cyanosis) having reduced oxygen transport in bloodstream.[7] Nitrate poisoning leads to bluish skin coloring around eyes, mouth, and nail beds[8] pancreatic and stomach cancer due to the formation of N-Nitroso compound in body. Pregnant women, infants, and persons with gastrointestinal disturbance are mainly susceptible to nitrate poisoning. Nitrate level in drinking water varies seasonally due to use of fertilizers;[9] thus, the need of water retesting is required in every

3–6 months. Nitrate cannot be detected without chemical analysis. A wide range of nitrate detection methods is: Indirect methods which include nitrite gas, followed by gasometry, ultraviolet (UV) visible spectroscopy,[10] and UV resonance Raman spectroscopy;[11] direct methods including liquid chromatography,[12] ion-selective electrode potentiometry[13] ion interaction liquid chromatography, colorimetry, and mass spectrophotometry,[14] differential pulse voltammetric method,[15] and polarography three-wavelength method.[16] However, all these methods are tedious, time-consuming needs sample preparation, not specific, and requiring expert handling. Among various kinds of biosensors, amperometric biosensors are most reliable as these are simple, sensitive, rapid, and economic to use.[17] However, lots of problems are reported in these biosensors such as loss of enzyme activity, narrow linear concentration range, less operational stability, long incubation time, and interference by various cations present in biological fluids. To overcome these problems,

immobilization of enzyme is done either covalently or directly onto nanoparticles/nanocomposite-coated electrode.[18] Enzyme nanoparticles exhibit unique structural and catalytic properties that enhance the conductivity and sensitivity of biosensor.[19] Cross-linked enzyme nanoaggregate could be easily immobilized on specific support with a high proportion of active enzyme and achieve a superior design with less time consuming and non-tedious procedure.[20] Therefore, it is expected that biosensor shows better sensitivity, detection limit, and response time using enzyme nanoparticles in detecting nitrate concentration in drinking water samples.

MATERIALS AND METHODS

Chemicals

Enzyme nitrate reductase source aspergillus from Sigma-Aldrich, N-ethyl-N-(3-dimethylaminopropyl)carbodiimide, N-hydroxysuccinimide, potassium nitrate (KNO_3), potassium dihydrogen phosphate and dipotassium hydrogen phosphate, N-(1-naphthyl)-ethylenediamine hydrochloride, nicotinamide adenine dinucleotide from HiMedia. Double-distilled water (DW), cold centrifuge, stirrer, (Zoology Department, M.D.U.) transmission electron microscopy (TEM) were carried out at AIIMS, New Delhi.

Synthesis of NaR nanoparticles (NaRNPs) and characterization

Nitrate reductase NPs were made by desolvation and glutaraldehyde cross-linking method[21] and dispersed in 0.1M phosphate buffer, sonicated, and stored at 4°C. The aggregated NaRNPs were characterized by TEM at (AIIMS) New Delhi and Fourier-transform infrared (FTIR) at the Department of Zoology, M.D.U, Rohtak.

Construction of NaRNPs/gold (Au) modified electrode

The Au electrode was ultrasonically in conc. H_2SO_4 and H_2O_2 solution (3:1 ratio) for 30 min and then cleaned by alumina slurry to remove dust particle from surface. Further, the Au electrode was dipped into 1 μl/mL NaRNPs solution for 24 h at 4°C. NaRNPs were chemically immobilized on Au electrode. The NaRNPs/Au electrode was washed with DW to discard unbound protein, further dried to use as a working electrode, and stored at 4°C. An amperometric NaRNPs/Au biosensor was formed by means of three electrochemical cell systems, containing NaRNPs coated Au electrode serving as a working electrode (Ag/AgCl) as reference, and platinum (pt) wire as a secondary electrode. The electrode system was dipped in a reaction mixture having 25 mL of 0.1 M phosphate buffer (pH 7.0) having 1 mL nitrate solution, connected through Autolab potentiostat/galvanostat. The reaction involved the reduction of nitrate and production

of nitrite on applying potential among working and counter current electrode. The released electrons are transferred to working electrode relay; signal and response are measured in terms of milliampere (mA) by applying a potential range. The potential range was fixed between 0.2V and 1.2V.

Construction of immobilized NaRNPs/Au electrode biosensor

A NaRNPs based sensor was constructed by three electrochemical cell system was dipped in a reaction mixture having 25 mL of 0.1 M phosphate buffer, pH 7.0, and 1 mL of 10 mM nitrate solution. There were two peaks that appeared on cyclic voltammogram corresponds to redox reaction cycle, in which the upper one represents the oxidation peak at 0.96 V while lower one as reduction peak 0.48 V at 50 mV/s scan rate and response measured in mA was observed [Figure 1].

Optimization study of NaRNPs/Au electrode

The current from cyclic voltammetry is considered as input variables, while the current produced due to nitrate concentration in a given sample is measured as output variables. To check optimum working conditions of NaRNPs/Au electrode, the pH was varied from a range of 6.0 to 9.0 pH at an interval of 0.5 by assaying with 25 mL (0.1M) potassium phosphate buffer containing 1 mL of (10 mM) nitrate. To determine incubation temperature, NaRNPs/Au electrode was incubating in reaction mixture incubating at a temperature range from 20°C to 50°C with 5°C interval. For reaction time, the incubation of electrode was performed from 2 to 12 s, with interval of 2 s and for optimum substrate concentration, different nitrate concentrations varying from 0.1 μM to 800 μM were assayed in the reaction mixture at pH 7.0 and 35°C temperature.

Determination of nitrate in drinking water samples

Forty samples (4 × 10 = 40) of fresh drinking water of four different sources were collected from 10 different areas located nearby Bahadurgarh city. The tap water, river/pond water, submersible/well water, and supply water 1.0 mL of the sample from each were collected in a test tube and tested for nitrate. The samples were kept at room temperature when not used.

Evaluation of the NaR/NPs/Au electrode fabricated biosensor

A linear association was studied between current (mA) and nitrate concentration ranging from 0.1 to 20.0 μM in 0.1M PB, pH 7.0 for NaRNPs/Au electrode. The detection limit was determined at a signal to noise ratio of 3 (S/N = 3). The mean analytical revival of exogenously mixed nitrate at 0.5 mM and 1.0 mM concentration was determined for
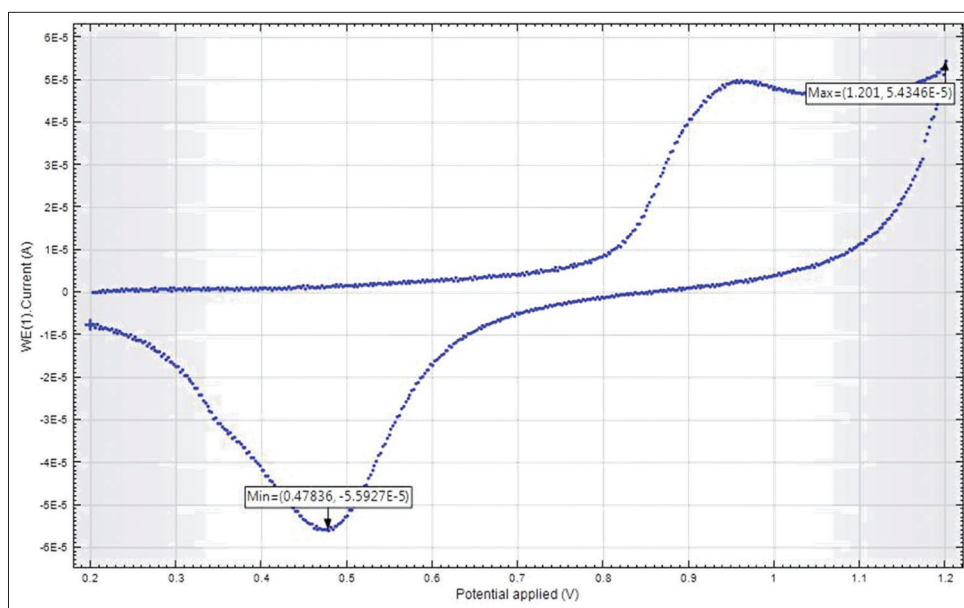
Figure 1: Cyclic voltammogram of nitrate reductase nanoparticles/gold electrode and response measurement

the present NaRNPs/Au electrode fabricated biosensor. For reproducibility and reliability of biosensor, the nitrate content of the sample in one run (within batch) and (between batch) was determined for 1 week.

Storage stability and reusability of NaRNPs/Au electrode

For stability, the working electrode was studied for 90 days in 0.1 M phosphate buffer, pH 7.0 at 35°C on a weekly basis. The electrode was dried and stored at 4°C while not used.

RESULTS AND DISCUSSION

Preparation of NaRNPs

NaRNPs were synthesized by desolvation with ethanol and subsequent glutaraldehyde cross-linking method by drop-wise ethanol addition. The synthesized enzyme nanoparticles showed more activity as compared to the free enzyme which is due to the formation of aggregates. Aggregate formation results into increased surface area which further enhances the attachment over the given surface and hence minimizing the loss of activity of the enzyme. The enzyme nanoaggregates have higher shelf life and stability and held by non-covalent interactions. Enzyme nanoparticles of nitrate reductase have not been reported until now [Figure 2].

Characterization of NaRNPs

TEM studies

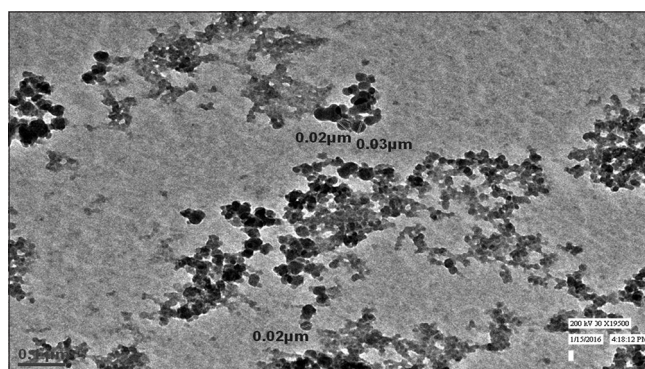The TEM images and electron diffraction patterns of NaR nanoparticles reveal the sphere-shaped particles

Figure 2: Transmission electron microscopy image of synthesized nitrate reductase nanoparticles
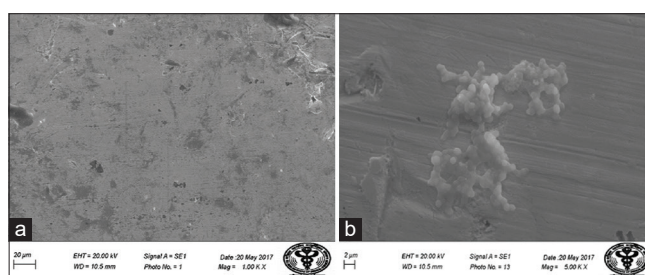

Figure 3: Scanning electron microscopy micrographs of gold electrode (a) bare (b) with immobilized nitrate reductase nanoparticles

with an average 20–30 nm size with the crystalline structure. It indicates that the synthesized NaR nanoparticles are not single crystal but aggregates of several crystals [Figure 3].

Scanning electron microscopy (SEM)

The SEM micrograph of bare Au electrode revealed a homogenous, consistent surface while NaRNPs

immobilized Au electrode presented globular structure morphology. The appearance of cluster aggregates on the surface of electrode confirms the successful immobilization of enzyme nanoaggregate on the surface of electrode [Figure 4].

FTIR spectra of NaRNPs/Au electrode

FTIR spectroscopy is used to identify the groups in the biomolecules. FTIR analysis of NaRNPs was performed in 500–4000 range. It was further evidenced from an intense OH stretching band of 1° and 2° amines in the range of 3434 cm^{-1}. The representative spectra of nitrate reductase nanoaggregate showed small absorption peaks located at about 2914, 1425 (1400 cm^{-1} predicts the existence of nitrate), 1018 cm^{-1}, and 560 cm^{-1}, as well as some intense peaks, were observed such as 3434, 1636, and 1044 cm^{-1}. The crest of 1636 cm^{-1} indicates C-O stretching vibration as well as the deformation of O-H bonds, which corresponds to the presence of organic compounds.[22] The band area around 2200–2400 cm^{-1} would indicate the possible presence of a triple bond between N-O, depicting the presence of enzyme on the surface of the nanoparticles [Figure 5].

Effect of substrate concentration on fabricated NaRNPs/Au electrode-based biosensor

When the activity of NaRNPs based Au electrode was obtained by assaying at different nitrate concentrations from 10 μM to 1000 μM, it was observed that the current density was highly dependent on substrate concentration (1 mL of 10 mM KNO$_3$). The catalytic current response of NaRNPs based electrode initially increases at applied voltage, up to enzyme saturation point at which the rate of reaction is at maximum and further increase in substrate concentration causes no effect because all active sites of enzyme get engaged. Thus, the optimum nitrate concentration was reported as 200 μM. Initially, there is a linear relationship between nitrate concentration and response of sensor; after that, it became almost constant. However, the fabricated enzyme NPs based biosensor showed a quite lower value of K$_m$ 384.61 μM (0.38 nM) and good V$_{max}$ value of 1219.512 μmol (min mg protein)$^{-1}$ calculated from the Lineweaver-Burk plot that was formed between reciprocal of nitrate concentration (I/[S]) versus enzyme activity (1/V) using Michaelis-Menten equation presenting better results than 0.809 nM,[23] 0.58 nM,[24] 1.81 nM, and 1.66 nM (2016)[25] earlier reported biosensors.
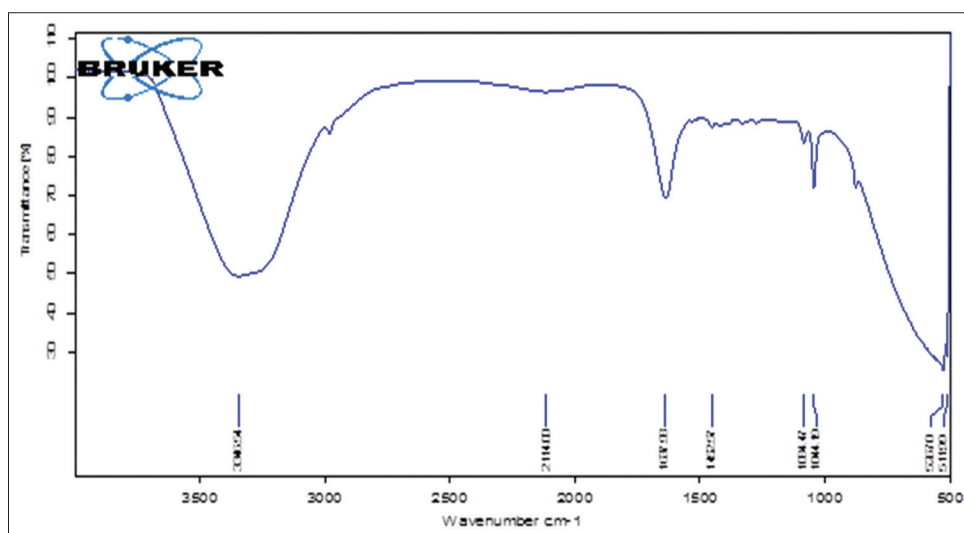

Figure 4: Fourier-transform infrared spectra of nitrate reductase nanoparticles/gold electrode
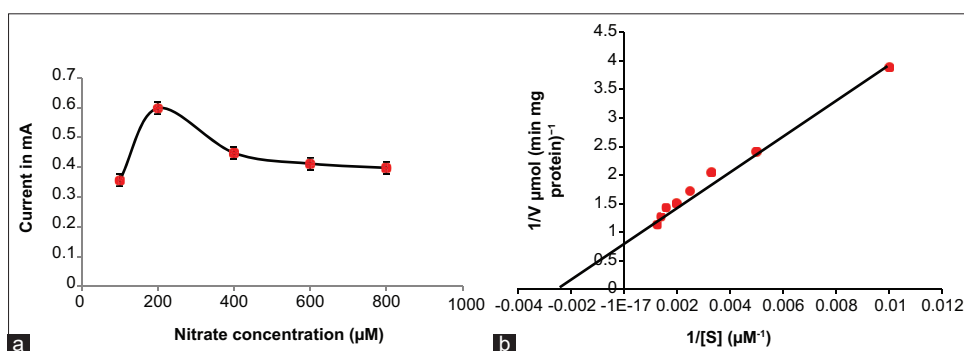

Figure 5: (a) Calibration curve showing peak current versus nitrate concentration (scan rate = 50 mV/s) of nitrate reductase nanoparticles/gold biosensor (b) L-B plot for determination of K$_m$ and V$_{max}$

Evaluation of biosensor

A linear association between current (mA) produced and nitrate concentration ranging from 0.1 to 200 μM in 0.1M phosphate buffer pH 7.0 for NaRNPs immobilized Au electrode was observed which was better linear range than earlier reported biosensor 15–250 μM, 20–250 μM,[26] and 300 μM.[27] The detection limit was measured as 0.14 μM at S/N = 3 that is comparable and better than earlier biosensors.[28] The mean analytical revival of nitrate mixed exogenously at 0.5 mM concentration is 78.24 ± 0.12% and at 1.0 mM concentration 78.24 ± 0.04% representing the satisfied precision and reproducibility of current nanosensor. To confirm the reproducibility and consistency of this biosensor, nitrate was added in the sample in one run within batch and between batch for 1 week, and results were noticed. It was observed that nitrate content was according to each other within and between batches. The coefficient of variation measured within and between batches was 2.25% and 2.74%, which reveals the high reproducibility of the NaRNPs/Au biosensor [Table 1]. To measure accuracy, total nitrate concentration in 10 drinking water samples was measured by standard colorimetric method and compared with values obtained by NaRNPs/Au biosensor. The value of nitrate content revealed by present biosensor (y) signifying a good correlation with the standard colorimetric method (x) showing correlation having regression as (R2) = 0.9988, which reveals the high accuracy of present biosensor method.

Amperometric determination of total nitrate concentration

From all the 40 samples of fresh drinking water (taken from four different sources, tap water, river/pond water, submersible/well water, and supply water) collected from 10 different locality, nearby Bahadurgarh city maximum nitrate concentration (0.443 μM) was observed in hand pump water sample while minimum concentration (0.148 μM) was observed in the supply water. However, in other water sources, a quite comparative value of nitrate (average 0.320–0.372 μM) was obtained, as shown in Table 2.

Storage stability and reusability storage stability of NaRNPs/Au biosensor

To check the stability, enzyme nanoparticles immobilized Au electrode was studied for 90 days by conducting the weekly assay. The working electrode was dried and stored at 4°C when not used. The main limitation for amperometric detection for nitrate by NaRNPs/Au electrode is its poor reproducibility when used repeatedly. The response decreases by 50% during its testing on a weekly basis on 40 days when store in 0.1M phosphate buffer at pH 7.0, temperature 35°C.

Table 1: Evaluation of the present NaRNPs/Au electrode fabricated biosensor

Parameters studied by NaRNPs/Au electrode	NPs/Au electrode
Linearity (mM)	4.5
Minimum detection limit (μM)	0.14
% Recoveries of added nitrate (Mean±SD, n=7)	
0.5 mM	±0.12
1.0 mM	79.82±0.04
Coefficient of variation% (n=7)	
I. Within batch	2.25%
II. Between batch	2.74%
Coefficient of determination, R2 (n=20)	0.9988

NaRNPs/Au: Nitrate reductase nanoparticles/gold

Table 2: Nitrate content of various water samples by colorimetric method and NaRNPs/Au biosensor

Source of water samples	By colorimetric method	By present biosensor
Hand pump water (μM)	3.425±0.281	3.417±0.311
River water (μM)	3.435±0.138	3.415±0.168
Submersible water (μM)	3.545±0.793	3.523±0.995
Supply water (μM)	3.692±1.008	3.667±0.169

NaRNPs/Au: Nitrate reductase nanoparticles/gold

CONCLUSION

Immobilized NaRNPs based nanosensor showed a linear graph with substrate concentration from 0.1 μM–200 μM with optimum pH 7.0, temperature 35°C with good shelf life, and storage stability of 90 days. It could be used again and again without leaching action with a high proportion of enzyme activity. The nanosensor showed a response time of 2 s with the lower value of K_m 384.61 μM and good value of V_{max} of 1219.512 μmol (min mg protein)-1 along with low detection limit of 0.14 μM. In the present study, the synthesized NaRNPs based nanosensor is used in nitrates detection in drinking water samples and showed superiority as compared to earlier reported biosensors in terms of linear range, sensitivity, and response time.

ACKNOWLEDGMENTS

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