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Nitrate quantification: recent insights into enzyme-based methods

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Abstract: Nitrate monitoring of environmental samples is essential to safeguard human and environmental health. Various non-enzymatic methods such as Griess reaction-based chemical method; Fourier transform infrared spectroscopy; chromatographic, electrochemical and optical sensors yield reproducible results but suffer from drawbacks such as use of hazardous chemicals, interference from coexistent anions, and bulky and expensive instrumentation and hence are not favored for routine analysis. On the other hand, nitrate reductase (NR)-based methods are simple, sensitive, specific, environment friendly, easy to carry out, and, therefore, suitable for routine analysis. NR in these methods is employed in both free (in commercially available kits) and immobilized form. In comparison to the native NR, immobilized NR shows better activity and stability accompanied by overall reduction in the cost of the method. The review gives a brief account of non-enzymatic nitrate quantification, whereas recent advances in enzyme-based determination have been explored in more detail.

Keywords: biosensors; immobilization; nitrate; nitrate reductase; quantification.

Introduction

Though nitrate is naturally present in the environment in moderate concentrations, excessive use of nitrogenous fertilizers to increase agricultural productivity has resulted in undesirable increase in its concentrations in the environment. When quantity of nitrogen added to the soil exceeds the amount that the plants can use, the excess nitrate leaches out from the root zone by water percolating through the soil profile and ultimately accumulates into the groundwater. Furthermore, surface discharge of untreated or poorly treated sewage, sewage irrigation, land filling of municipal solid waste, and animal excreta from dairy industry has also led to nitrate pollution of soil, ground and surface waters. High nitrate levels together with phosphate in water bodies have also been implicated in the frequent eutrophication of lakes and coastal waterways.

Owing to the possible adverse impacts on human health, most countries have imposed limits for nitrate in drinking water of 25–50 mg/l (0.4–0.8 mM). The World Health Organization and US Environmental Protection Agency (EPA) have defined a maximum permissible limit of 44 mg/l of nitrate in drinking water (Bendikov et al. 2005). Toxicity of nitrate to humans is associated with the ability of nitrate to oxidize hemoglobin to methemoglobin, which is unable to transport oxygen in the tissues; the condition is clinically known as “blue-baby” syndrome or met-hemoglobinemia, which may cause mortality by asphyxiation. Infants <6 months of age are at highest risk due to the presence of bacteria in their digestive systems that speed up the binding process. Nitrate is converted into nitrite endogenously which may act as precursor of endogenous nitrosamines and nitrosamides whose carcinogenic effects on the gastrointestinal apparatus as well as congenital malformation effects have been reported (American Public Health Association 1998). Furthermore, nitrate exposure beyond the permissible limits has also been reported to be associated with inflammatory diseases (Dykhuizen et al. 1996), cardiovascular conditions (Ayub et al. 2011), neurological conditions (Milstien et al. 1994) and recently hypotension (Jones et al. 2012, Kelly et al. 2013).

Considering the role of nitrates in environmental dynamics and their impact on human health, monitoring of nitrate concentration is extremely important worldwide to prevent exposure of populations to harmful levels. To address these concerns, determination of nitrate is of utmost significance. Chemical methods based on Griess reaction, Fourier transform infrared spectroscopy (FTIR), ion chromatography (IC), high performance liquid chromatography (HPLC) and several optical and

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electrochemical (enzymatic as well as non-enzymatic) sensors have so far been employed for nitrate analysis (Madasamy et al. 2013, Moo et al. 2016). These methods have also been incorporated into automated flow injected systems to increase throughput rate but are time consuming, show low reproducibility, use large volumes of toxic reagents and employ expensive and complicated instruments. Enzyme-based methods offer advantages like simplicity, rapidity and sensitivity. Moreover, these methods are user friendly and economical too if immobilized enzyme is employed.

Here, we have presented an account of recent advances in enzyme-based techniques for detection and determination of nitrates. An overview of non-enzyme-based techniques has also been presented in the beginning.

Non-enzymatic detection techniques

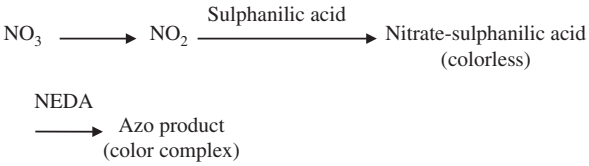
A variety of methods have been used to determine nitrate in soil, water, food and various other real-life samples. These methods can be divided into two broad categories: non-enzymatic and enzyme-based methods. A comparison of both the methods is given in Table 1.

Chemical methods

Chemical methods of nitrate detection using spectroscopy utilize Griess reaction, based on diazo coupling between the product of nitrite and sufanilic acid reaction and N-(1-naphthyl) ethylenediamine dihydrochloride (NEDA) to produce azo dyes which act as excellent chromophores absorbing light in the visible range, i.e. around 540 nm (Ivanov 2005). The overall reaction is as follows:

Table 1: Comparison of enzymatic and non-enzymatic techniques for nitrate determination.

Enzymatic	Non-enzymatic techniques
– Simple – Sensitive – Specific – Rapid – Ease of operation – Possibility of on-site analyte detection – Reusable and cost effective if immobilized enzyme is used	– Tedious analysis – Long sampling preparation – Time consuming – Use of extensive laboratory equipments – Requires skilled manpower



Chemical methods adapted for flow injection systems used cadmium or vanadium (III) chloride column (Wang et al. 2016) for reducing nitrate to nitrite. These analytical methods determined the sum of the nitrate (NO_3) and nitrite (NO_2) concentrations; to calculate the nitrate concentration, it was necessary to measure nitrite separately in the sample and subtract it from the combined measurement.

Fourier transform infrared spectroscopy

FTIR spectroscopy is based on the fundamental modes of vibration of the nitrate molecule upon absorption of infrared rays of the electromagnetic spectrum. Use of attenuated total reflection in conjunction with FTIR (FTIR-ATR) enables samples to be examined directly in the solid or liquid state without further preparation. In FTIR, spectra peak around 1400 cm^{-1} is the most promising location for predicting the presence of nitrate with minimal interference (Jahn et al. 2006). Kira et al. (2014) recently demonstrated that FTIR-ATR enables tracking of changes in the concentrations of the isotopic species of nitrate and ammonium and allows estimation of the gross reaction rates of N transformations in soil.

Chromatographic techniques

IC (Lopez-Moreno et al. 2016), HPLC coupled with mass spectrometry or UV spectroscopy, ultra performance liquid chromatography-mass spectrometry (Siddiqui et al. 2015) and reversed-phase liquid chromatography/electrospray ionization/mass spectrometry in the negative ion mode (Li et al. 2011) have so far been used for determination of nitrates. The US EPA's recommended method for the determination of inorganic anions in water samples uses IC with suppressed conductivity detection (EPA Method 300.0). Separation and determination of nitrate ions by IC is carried out in anion-exchange columns filled with a suitable exchanger and using a proper eluent (e.g. water solution of sodium carbonate and/or sodium hydrocarbonate) and most often conductometric or UV detection.

Electrochemical and optical sensors

Electrochemical sensors react with analyte and subsequently produce an electrical signal proportional to the analyte concentration. There are two main types of electrochemical sensors: potentiometric [ion-selective electrode (ISE) and ion-selective field effect transistor (ISFET)] and amperometric sensors have been reported for determination of nitrates. The ISEs are mainly membrane-based devices, consisting of ion-selective conducting materials, and ISFET incorporate the ion-sensing membrane directly on the gate area of a field effect transistor (FET). The multi-ISFET/flow injection analysis system allowed very low volumes of samples to be analyzed within 1.25 s.

To detect nitrate ions, a wide variety of electrode materials have been explored; among many of them, copper (Li et al. 2012, Silva et al. 2013, Stortini et al. 2015) has been found to be most efficient for electroreduction of nitrate. Still, other workers have reported diverse types of electrode materials such as Ag-doped zeolite-expanded graphite-epoxy composite (Manea et al. 2010), platinum (de Groot and Koper 2004), silver decorated carbon electrode (Guadagnini and Tonelli 2013), palladium (Mahmoudian et al. 2015), palladium-copper (Wang et al. 2006, Su et al. 2016), palladium tin (Fu et al. 2015), diamond microelectrode (Ward-Jones et al. 2005) and doped polypyrrole (PPy) nanowires (Aravamudhan and Bhansali 2008). A portable copper nanocluster-based microsensor chip with appreciable sensitivity was developed by Li et al. (2012) for nitrate determination in fresh waters.

In optical sensors, optical fibers deliver light from a light source to a sensing platform located at the fiber end. Modulation of light by the analyte is detected and analyzed by a light measurement device and correlated with the analyte concentration. The novel ultraviolet optical fiber sensor (Moo et al. 2016), etched fiber Bragg grating sensor (Lalasangi et al. 2011) and Lophine /polyvinylchloride polymeric matrix-coated optical fiber sensor (Camas-Anzueto et al. 2014) were used for measuring and detecting nitrates in water.

Non-enzymatic methods are the conventionally used methods for nitrate determination in a wide range of environmental and biological samples. Chemical methods are sensitive but are also time consuming, hazardous and interference susceptible. Other methods such as FTIR spectroscopy requires minimum treatment of sample (Du et al. 2009); IC is recommended for speciation analysis; i.e. simultaneous separation and determination of nitrate and nitrite ions (Mou et al. 1993) and sensors offer fast response, high throughput rates and broad linear response with increased selectivity. However, the expense, bulky instruments, interference from co-existing ions (Ito et al. 2005), extensive training and offsite analysis required by these methods impede their wide-scale use. Moreover, improvements in the sensitivity and reproducibility of these methods are often desired. Enzymatic methods overcome most of these limitations. Compared with the non-enzymatic methods, enzymatic methods available for nitrate determination are not only simple and ecofriendly but also substantially improve the sensitivity and specificity of the method.

Enzyme-based detection techniques

Enzyme-based determination of nitrates has relied on the catalytic activity of nitrate reductase (NR), wherein the enzyme is employed either in the soluble or immobilized form.

Nitrate reductases

NR are produced by a variety of animals, plants and microorganisms including fungi. NR is a complex multiredox center enzyme that catalyzes the reduction of nitrate to nitrite, with a pyridine nucleotide as the natural enzyme regenerator. The overall catalyzed reaction is as follows:

$$\text{Nitrate} + \text{NAD(P)H} + \text{H}^+ \longrightarrow \text{Nitrite} + \text{H}_2\text{O} + \text{NAD(P)}^+$$

The enzymes are usually homodimers or homotetramers of subunits whose molecular weight is approximately 95–100 kDa or 50 kDa, respectively. Each subunit contains flavin adenine dinucleotide (FAD), a b-type cytochrome and molybdenum-pterin group in a 1:1:1 stoichiometry. The sites for NAD(P)H and nitrate reduction are FAD and molybdenum domains, respectively, which also remain operative in the presence of synthetic electron donors (Campbell 2001). Electron flow between different subunits of NR is as below:

$$\text{NADH} \longrightarrow \text{FAD} \longrightarrow \text{Cyt } b_{557} \longrightarrow \text{Mo} \longrightarrow \text{NO}_3^- \longrightarrow \text{NO}_3^-$$

However, multiredox center of NR responsible for biological conversion of nitrate to nitrite is generally not very active and is deeply embedded in the protein structure. NR is also highly thermosensitive, which may be one of the reasons for its low *in vitro* stability (Kuo et al. 1982). Though NR has been purified and extensively studied from a number of bacterial, fungal and plant sources,

so far only limited sources have been exploited commercially for large-scale purification of NR, such as the Sigma Chemical Co., USA, sells purified NR only from *Arabidopsis thaliana*, *Escherichia coli* and *Aspergillus niger*. Out of these three sources, fungal NR has the highest catalytic activity (≥300.0 units/g), at least 60 times more than the other two sources. Though none of the authors have explicitly mentioned the specific reasons for choosing a particular NR in developing NR-based nitrate detection technologies, easy commercial availability and good catalytic activity might be one of the reasons for the extensive use of *A. niger* NR (Table 2). Additionally, factors such as the membrane-bound nature and maintenance of anaerobic conditions for majority of prokaryotes makes them a less preferred source for purification of NR. On the contrary, NR from eukaryotic sources is a cytoplasmic protein and, hence, is comparatively easier to purify (Morozkina and Zvyagilskaya 2007).

Methods using soluble enzyme

Commercially available colorimetric kits for nitrate determination such as from Sigma-Aldrich (Catalog no. 06239), Cayman chemical (Prod. no. 850-001-KI01) and Nitrate Elimination Company Inc (NECi; Product code: S-NTK-200 series) employ NR in soluble form. The kits have been variously adapted for determination of nitrates and nitrites in environmental samples, urine and plasma. For detection of low levels of nitrite a highly sensitive fluorometric version is also available from Cayman Chemical. These methods give a linear response from 10 to 100 μM.

Principle of nitrate determination

Detection of nitrate by NR is based on Griess assay, wherein in the first step nitrate is enzymatically reduced to nitrite in the presence of β-NADH as reducing agent. In the second step nitrite is treated with sulphanilamide to form a diazocompound, which reacts with NEDA to give an azo product (λ_{max} 540 nm). The redox reaction is represented in Scheme 1.

Merits and demerits: Primarily all the kit methods are reliable, accurate, sensitive, environmentally benign and a better alternative to the non-enzymatic methods but have certain limitations. Nitrate determination kit provided by NECi does not require any complex instrument, follows simple procedures which can be carried out by the end user and, most of all, offers onsite nitrate determination but does not guarantee lab-level precision as the nitrate content is determined by comparing the color with the nitrate standard and color chart. On the other hand, the methods of Sigma-Aldrich and Caymen chemical are highly precise but have an absolute requirement of plate reader and are essentially lab oriented. The methods are also slow as it takes about 2.5 h to prepare and read a 96-well plate. Above all, the biggest shortcoming that is holding back the popularity of these kits is their high cost since they employ NR in soluble form. The cost of one assay by the commercial kit method is around $7–8, which is quite high considering its use for routine purposes. Employing the enzyme in immobilized form may reduce the cost of the method. In addition, immobilization offers the advantages of easy separation of enzyme from the reaction mixture and a possible increase in the activity and stability of enzyme.

Methods using immobilized NR

Biosensors

Biosensors are based on the intimate contact between a biorecognition element that interacts with the analyte of interest and a transducer element that converts the biorecognition event into a measurable signal. Reduction of nitrate to nitrite may be followed either by detection of generated catalytic current (amperometry) or by measuring the change in working electrode potential (potentiometry) or by change in conductance of the channel (field-effect transistors) (Zayats et al. 2001).

Principle

Nitrate biosensors use immobilized NR as biological recognition element. The detection of nitrate with the immobilized NR is often achieved in the presence of NAD(P)H or other suitable co-factor/redox mediator by amperometry or potentiometry, optical and FET and cyclic voltammetry (Sohail and Adeloju 2016). The general flow of electrons for reduction of nitrate to nitrite is given in Scheme 2. Nirate is enzymatically reduced to nitrite and the oxidized form of NR is electrochemically reduced by an electron-transfer mediator.

NR immobilization

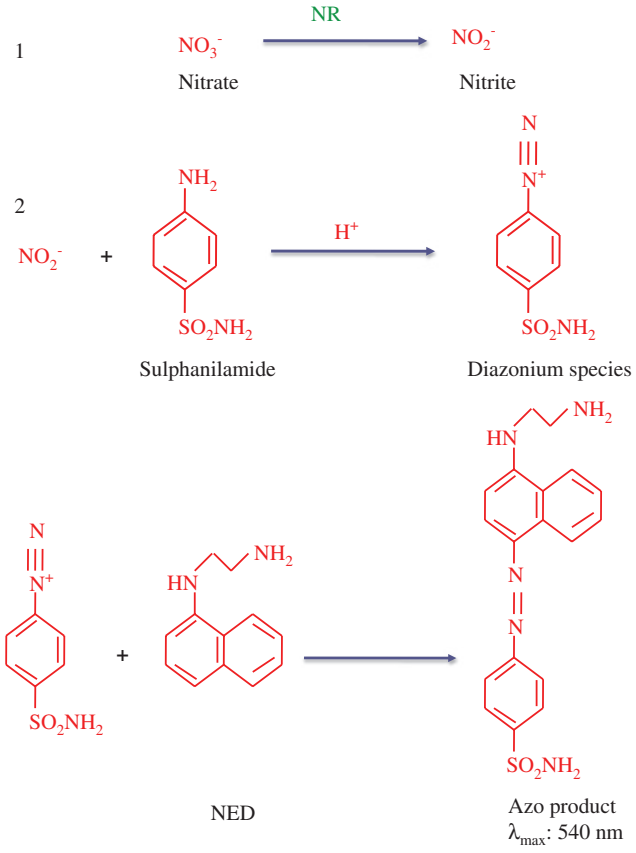
Three main factors that are critical for the proper performance of nitrate biosensor are the immobilization support, method of immobilization and synthetic electron donors. The materials that have been used for the immobilization of NR range from partially hydrophilic redox polymers such as nafion (Glazier et al. 1998), alkylpyrroleviologen (Ramsay

Table 2: Nitrate reductase immobilization: method, support and properties.

S. no.	Source of NR	Method of immobilization	Immobilization matrix	Electrical wiring	Enzyme regenerator	Amount of NR immobilized	pH	*K*m (mM)	References
1	*Aspergillus niger*	Entrapment by electropolymerization	Amphiphilic pyrrole viologen	Amphiphilic pyrrole viologen	Pyrrole viologen	0.165 mg	7.5	≈0.75	Cosnier et al. (1994)
2	*Escherichia coli*	Adsorption on electrode surface followed by entrapment via electropolymerization	Viologen Ppy films	Pyrrole viologen	MV	0.12 mg	7.5	0.15	Cosnier et al. (1997)
3	*E. coli*	Adsorption	MV/Nafion	MV	MV	3.0 μM	7.0	≈0.25	Glazier et al. (1998)
4	*Pseudomonas stutzeri*	Adsorption	Graphite electrode surface	MV	Phenothiazine (azure A, thionin), triphenylmethane, sulfonphthaleine and viologen	–	8.0	0.51	Kirstein et al. (1999)
5	*A. niger*	Entrapment	Laponite clay gel	Pyrrole viologen	Water soluble PV	–	7.5	0.21	Da Silva et al. (2004)
6	*A. niger*	Adsorption on electrode surface followed by entrapment via electropolymerization	Pyrrole viologen	Pyrrole viologen	Pyrrole viologen	–	7.5	–	Da Silva et al. (2004)
7	*A. niger*	Adsorption	Glassy carbon electrode (GCE) surface	MV	Phenosafranin	8.75×10^{-10} U	7.5	0.809	Ferreyra and Solis (2004)
8	*Yeast*	Entrapment	[Poly(vinyl alcohol)]	MV		–	7.0	~0.58	Quan et al. (2005)
9	*A. niger*	Adsorption on electrode surface followed by glutaraldehyde cross linking	Gold interdigitated electrode surface	Nafion, MV	Sodium dithionite/MV	–	7.5	–	Xuejiang et al. (2006)
10	*A. niger*	Entrapment followed by glutaraldehyde crosslinking	Laponite clay gel	MV	MV	0.37 μg	7.5	0.007	Cosnier et al. (2008)
11	*Yeast*	Co-entrapment with β-NADH by electropolymerization	Ppy film	Ppy	β-NADH	–	7.0–7.3		Sohail and Adeloju (2008)
12	*A. niger*	Co-entrapment with redox mediators by electropolymerization	Ppy	MV	Thionin acetate Safranin and azure A	–	7.0–7.3		Adeloju and Sohail (2011)
13	*E. coli*	Adsorption on electrode surface followed by glutaraldehyde cross-linking in the presence of redox mediators and BSA	Screen printed electrode (SPE) surface	MV	Azure A and MV	0.05 mg/μl	6.8		Albanese et al. (2010)
14	*A. niger*	Chemical bonding via carboxyl groups of CNTs	Ppy/CNTs film	Ppy/CNTs film	Potassium ferricyanide	–	7.5	–	Can et al. (2012)
15	*A. niger*	Covalent immobilization	Self assembled monolayer (SAM) of cysteine on GNPs/PPy film	GNPs	β-NAD(P)H	–	7.0	–	Madasamy et al. (2013)

Table 2 (continued)

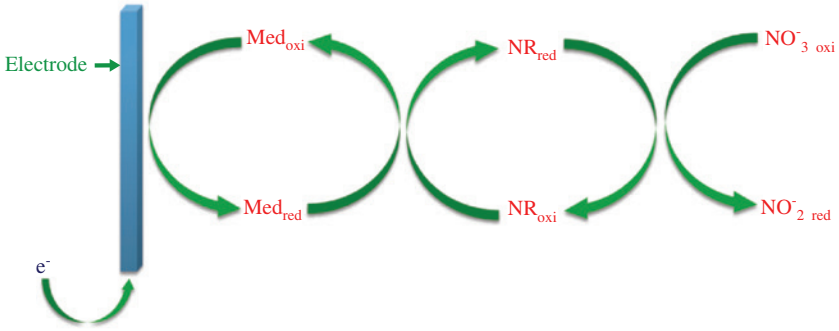
S. no.	Source of NR	Method of immobilization	Immobilization matrix	Electrical wiring	Enzyme regenerator	Amount of NR immobilized	pH	*Km* (mM)	References
16	*A. niger*	Adsorption on PPy/CNTs film followed by glutaraldehyde crosslinking	PPy/CNTs	CNTs	β-NAD(P)H	–	7.0	–	Madasamy et al. (2014)
17	*A. niger*	Covalent binding	Epoxy/GNPs	GNPs	NADH	35.40±0.01 μg/cm²	7.5	1.66	Sachdeva and Hooda (2014)
18	*A. niger*	Covalent binding	Epoxy/SNPs	SNPs	NADH	37.6±0.01 μg/cm²	7.5	1.81	Sachdeva and Hooda (2015)
19	*A. niger*	Covalent binding	Epoxy/ZnONPs	ZnONOs	NADH	33.20±0.01 μg/cm²	7.0	1.66	Sachdeva and Hooda (2016)
20	*A. niger*	Covalent binding	Epoxy/Fe_3O_4NPs	Fe_3O_4NPs	NADH	35.8±0.01 μg/cm²	7.5	1.54	Sachdeva and Hooda (2016)
21	-	Entrapment by electropolymerization	PEDOT nanowires	MV	–	–	–	63 mM	Gokhale et al. (2015)



Scheme 1: Two-step enzymatic colorimetric assay for nitrate. In the first step nitrate is converted to nitrite utilizing nitrate reductase. In the second step sulfanilamide and *N*-(1-naphthyl) ethylenediamine (NED) are added which convert nitrite to a deep pink-purple azo compound, which absorbs maximally at 540 nm.

and Wolpert 1999), polyviologen (Reipa et al. 1999, Ferreyra et al. 2003), viologen-acrylamide (Willner et al. 1990) and azure A (Kirstein et al. 1999, Sohail and Adeloju 2009, Adeloju and Sohail 2011) to hydrophilic matrices such as laponite clay gel (Da Silva et al. 2004, Cosnier et al. 2008), sol-gel matrix and carboxylated carbon nanotubes (CNTs) (Madasamy et al. 2014). NR has either been directly adsorbed on the electrode surface or entrapped inside the electropolymerized redox polymer for development of nitrate biosensors. However, these sensors suffered from poor NR activity either due to denaturation or leaching of enzyme from the support. In order to improve the binding and stability, NR has been crosslinked to other protein and redox mediators in the immobilization medium. Covalent attachment of NR to carboxyl terminated CNTs has also been reported.

Redox mediators are crucial for the success of nitrate biosensors as they establish electrical connectivity between the deep-seated enzyme active centers and electrode (Strehlitz et al. 1994). In fact, in many studies, redox



Scheme 2: Schematic representation of flow of electrons in nitrate biosensor. Redox mediators (Med) are often employed to improve electron flow between electrode and the deep-seated reaction centers of nitrate reductase (NR).

mediators themselves were used as enzyme supports, thus enabling both immobilization and electrical conduction (Albanese et al. 2010, Adeloju and Sohail 2011). Table 2 lists the major immobilization supports, the method of immobilization and some properties of the immobilized enzyme systems used to develop nitrate biosensors.

Immobilization methods

Adsorption followed by entrapment

Cosnier et al. (1994) for the first time reported the immobilization of enzymes based on electropolymerization of pyrrole amphiphilic monomer-enzyme mixture previously adsorbed on an electrode surface. Later on, Cosnier et al. (1997) functionalized the pyrrole monomers by viologen redox groups in order to achieve better electrical wiring between the immobilized NR and electrode surface. Use of conducting polymers was imperative for better electrical communication between NR and electrode, but partial hydrophobic character of this matrix was highly nonconducive for NR immobilization. As a result, immobilized NR was highly unstable and inactive.

As an improved approach Da Silva et al. (2004) and Cosnier et al. (2008) first entrapped NR in highly porous and hydrophilic laponite clay gel, which was then used as template for electropolymerization of pyrrole-viologen derivative. The process resulted in both efficient immobilization and electrical wiring of NR. These polymers also reinforced the mechanical stability of the clay and improved the stability of NR. However, redox potential of methyl viologen (MV) is very low (-0.446 volts) (Michaelis and Hill 1933) and despite the fact that MV has been widely used as an enzyme mediator in NR-based biosensors, Ferreyra et al. (2000) presented strong evidence that it might react with products of the enzymatic reaction. Hence, Ferreyra and Solis (2004) used phenosafranin as enzyme regenerator, whose redox potential is 0.238 V less negative than MV and does not chemically react with the enzymatic product. In the developed biosensor, NR was directly adsorbed on the surface of glassy carbon electrode rather than being entrapped inside the electropolymerized mediator, and the modified surface was covered with a cellulose dialysis membrane. Electron mediator was externally used in the reaction mixture.

Xuejiang et al. (2006) developed the first conductometric sensor based on coimmobilization of NR and MV in the films at an interdigitated thin film electrode surface. During immobilization NR was also crosslinked with bovine serum albumin in saturated glutaraldehyde vapor to prevent leaching of enzyme.

Sohail and Adeloju (2008) constructed the first potentiometric nitrate biosensor utilizing the original electron mediator, i.e. β-NADH. For this purpose, NR along with β-NADH was immobilized galvanostatically into conducting PPy film. Use of immobilized β-NADH substantially enhanced the performance of the sensor in comparison to when it was present in soluble form in the solution. As an improvement, the authors replaced β-NADH with azure A and were able to lower down the minimum detection limit to 0.5 μM (about 20 times lower than that achieved using β-NADH) (Adeloju and Sohail 2011). Further, the method avoided oxygen interference as the electrode operated at a lower potential (-0.25 V) and required less amount of enzyme. Albanese et al. (2010) also used azure A and developed amperometric NR biosensors by coadsorption of NR with azure A, MV and BSA on screen-printed electrode using glutaraldehyde as cross linking agent.

Covalent bonding

Recently, with the invent of nanotechnology, gold nanoparticles (GNPs) and CNTs have been extensively used for biosensor development due to their unique chemical and

electrical properties. Madasamy et al. (2013) covalently immobilized NR in self-assembled monolayer (SAM) of cysteine on GNPs/PPy modified platinum electrode. Can et al. (2012) and Madasamy et al. (2014) employed CNTs for developing nitrate biosensor to improve electrochemical signal transduction between the enzyme redox centers and electrode surface. A mixture of carboxyl terminated CNTs, PPy and NR was electropolymerized on glassy carbon electrode. As a result, enzyme was both entrapped into the growing PPy film and covalently linked to carboxyl groups of CNTs via its amino groups. Potassium ferricyanide was used as redox mediator and added externally to the electrochemical measurement medium by Can et al. (2012).

Analytical performance

Table 3 represents a comparison of analytical performance of some of the recently developed nitrate biosensors. Madasamy et al. (2014) developed the sensors with least detection limit of 0.2 μM. Kirstein et al. (1999) presented the results of their experiments with various dyes coadsorbed with NR onto graphite electrode. Redox mediators belonging to the phenothiazine (azure A, thionin) and to the triphenylmethane dye group performed best. Azure A gave the highest current density under saturating nitrate concentration with nitrate detection limit of 4.8 μM. Detection limit was substantially reduced to 0.5 μM by employing hydrophilic laponite clay gel and pyrrole-viologen in the biosensing system (Da Silva et al. 2004).

Conductometric nitrate biosensor designed using NR/MV/nafion films showed linear calibration in the range of 20 and 25 μM with detection limits of 5.0 μM (Xuejiang et al. 2006). A minimum detectable concentration of 15 μM and a linear concentration range of 100–5000 μM with substantial enhancement in nitrate response were achieved with the nitrate biosensor having NADH co-entrapped with NR in PPy films rather than being free in the solution (Sohail and Adeloju 2008). Improved sensitivity, detectable concentration, linear concentration range and response time for nitrate was achieved by replacing NADH with redox mediators such as thionin acetate, safranin, and azure A. Use of these redox mediators also improved the Nernstian behavior of the electrode process beyond the capability of the PPy-NR-NADH biosensor. A minimum detectable concentration of 0.50 μM and a linear concentration range of 20–500 μM was achieved with azure A (Adeloju and Sohail 2011).

The broadest linear range from 100 to 10,000 μM and minimum sensitivity for nitrate (0.12 mA/M^{-1}) was achieved by the sensor designed by Albanese et al. (2010). This biosensor showed the detection limit of 0.1 mM and storage stability of 36 days for the analysis of nitrate ions in water and food samples.

Analytical parameters of nitrate biosensors were tried to improve using nanomaterials. The nanobiosensor employing NR covalently linked to SAM of cysteine on GNPs/PPy surface showed a wide linear range of response over the concentration of nitrate from 1 μM to 1 mM, with detection limit of 0.5 μM (Madasamy et al. 2013). Sensitivity of the biosensor employing CNTs was found to be 0.3 mAM^{-1} cm^{-2} in a linear range of 440–145 μM, and a minimum detectable concentration of 170 μM was obtained (Can et al. 2012). Another biosensing system developed by Madasamy et al. (2014) using NR/CNTs/PPy nanocomposite modified platinum electrode exhibited linear response from 500 nM to 10 mM with a detection limit of 200 nM. Recently, poly(3,4-ethylenedioxythiophene) (PEDOT)/NR was electropolymerized under similar conditions (1.1 V, 300 s) to yield two different structures viz., nanowires and 2D flat films. Nanowires were grown in the pores of polycarbonate (PC) membrane, and the film was deposited on a gold-coated electrode. The flat 2D PEDOT/enzyme sensor displayed improved sensitivity, and detection limit (34.4 ppm) was several orders of magnitude higher than the one displayed by the nanoarray sensor (0.16 ppm) (Table 3).

Interference from oxygen

Portability, easy handling, ability to carry out fast analysis without sample pretreatment, simple procedure and low cost are some of the prerequisites for successful, point-of-use application of any analytical method. Several attempts to use nitrate biosensors for on-site nitrate sensing have been made, but success has so far been limited due to interference by oxygen (Plumeré 2013). Purging argon or degassing the working solution prior to analysis is often the most commonly employed technique, but apart from this several oxygen scavenging mechanisms such as the use of chemical and enzymatic scavengers and changing the working potential of electrode have been attempted. Although use of sodium sulfite, a popular chemical scavenger to remove dissolved oxygen, was satisfactory, the method was applicable only to closed large volume system (Quan et al. 2005). On the other hand, using glucose oxidase-catalase as oxygen scavenging components, low volume samples (200 μl) could be analyzed in an open system, but it was difficult to maintain near-optimal pH conditions because of the continuous production of gluconic acid from glucose oxidase catalyzed oxidation of glucose. This problem was circumvented by using

Table 3: Nitrate quantification using NR: comparison of analytical parameters.

S. no.	Working electrode	Electrode coating	Biosensor type	Applied potential/ current density	Detection limit	Linear range (μM)	Sensitivity	Response time (s)	References
1	GCE	PPy/viologen/NR	Amperometric	-0.7 V	0.4 μM	<35	14.0 mAM^{-1} cm^{-1}	40	Cosnier et al. (1994)
2	GCD	PPy/viologen/NR	Amperometric	-0.7 V	5.0 μM	<300	13.8 mAM^{-1} cm^{-2}	40	Cosnier et al. (1997)
3	Nafion-coated GCE	Nafion/MV/NR/dialysis membrane	Amperometric	-0.8 V	3.0 μM	3.0–17.9	9.0 mAM^{-1} cm^{-1}	<60	Glazier et al. (1998)
4	Graphite	*Paracoccus denitrificans*/ dialysis membrane	Amperometric		1.0 μM	1.0–50.0	–	<30	Takayama (1998)
5	Graphite	Azure A/NR	Amperometric	-300 and -400 mV	4.8 μM	<100	530 mAM^{-1} cm^{-1}	12	Kirstein et al. (1999)
6	–	Direct bacterial conversion	Amperometric		3.6 μM	4.0–86.0	–	30	Larsen et al. (2000)
7	GCE	Laponite clay/pyrrole viologen/NR		-0.7 V	0.5 μM	0.5–160	94.7 mAM^{-1} cm^{-2}	–	Da Silva et al. (2004)
8	GCD	Cellulose dialyis membrane/NR	Amperometric	-0.750 V	3.0 μM	–	60 mAM^{-1} cm^{-2}	60	Ferreyra et al. (2004)
9	Gold interdigitated thin-film	Nafion/MV/BSA/NR	Conductometric	-	5.0 μM	20–250	–	15	Xuejiang et al. (2006)
10	Platinum	PPy/β-NADH/NR	Potentiometric	0.5 mA cm^{-2}	15.0 μM	100–5000	–	–	Sohail and Adeloju (2008)
11	Platinum	PPy/azure A/NR	Potentiometric	–	10.0 μM	50–5000	-65 mV/decade	2–4	Sohail and Adeloju (2009)
12	Platinum	Ppy/AzA/NR	Amperometric	-0.25 V	0.50 μM	20–500	4.08 mAM^{-1} cm^{-2}	18–20	Adeloju and Sohail (2011)
13	Screen printed	Azure A/MV/BSA/NR			100.0 μM	100–10000	0.11833 mAM^{-1} cm^{-2}		Albanese et al. (2010)
14	GCE	PVA/MV/NR	Amperometric	-0.85 V	4.1 μM	15–300	7.3 nA/μM	–	Quan et al. (2005)
15	Screen printed carbon paste	PVA/MV/NR	Amperometric	0.90 V	5.5 μM	15–250	5.5 nA/μ M	–	Quan et al. (2005)
16	GCE	PPy/CNTs film/NR	Amperometric	0.13 V	170.0 μM	440–1450	0.3 mAM^{-1} cm^{-2}	20	Can et al. (2012)
17	Platinum	NR/SAM/GNPs/PPy	Amperometric	-0.76 and -0.62 V	0.50 μM	1–1000	84.5 nA μM^{-1}	–	Madasamy et al. (2013)
18	Platinum	CNTs/Ppy/SOD/NR	Amperometric	+0.76 V	0.20 μM	0.50 to 10,000	84.5±1.56 mAM^{-1} cm^{-1}	–	Madasamy et al. (2014)
19	Gold coated PC membrane	PEDOT/NR nanowires	Amperometric	+1.1 V	0.16 ppm	200 ppb–1100 ppb	92.0 μAmM^{-1}	2–5	Gokhale et al. (2015)
20	Gold coated PC membrane	PEDOT/NR 2D flat film	Amperometric	+1.1 V	34.4 ppm		10 μAmM^{-1}	2–5	Gokhale et al. (2015)
21	GCD	NR	Amperometric	-400 mV	0.00076 μM	10–400	14 nA/l M)	–	Kalimuthu et al. (2015)
22	Platinum	Salicylate hydroxylase	Amperometric	+0.42 V	5.6 μM	10–1000	–	2	Cui et al. (2006)

BSA, Bovine serum albumin; SOD1, zinc superoxide dismutase; GCE, glassy carbon electrode; GCD, glassy carbon disk; PVA, polyvinyl alcohol.

pyranose 2-oxidase, which oxidizes glucose to ketone, a pH neutral product (Plumeré et al. 2012). A bienzymatic nitrate biosensor employing soluble NR in conjunction with platinum electrode immobilized salicylate hydroxylase resolved oxygen interference by carrying out decarboxylation of salicylic acid to catechol in the presence of oxygen and NADH. As a result, nitrate determination was fully insensitive to oxygen but suffered from the drawback of using the expensive NADH molecule at high concentrations (Cui et al. 2006).

Alternate approach to avoid oxygen interference was detecting nitrates at more positive potentials. Though reversible reduction of nitrate to nitrite at pH 7.0 occurs at 0.431 V versus standard hydrogen electrode (SHE), still most of the nitrate biosensors work at potentials, negative enough for reduction of oxygen at cathode. The problem is aggravated by employing electron mediators such as MV, which has excessively negative redox potential of -0.44 V vs. SHE, and hence either contributes significantly to the overpotential or react directly with oxygen (Yoon and Kochi 1988). Several other mediators such as phenothiazine dyes, azure A, thionin, and patent blue that work at comparatively less negative redox potentials (-0.16 V) abated oxygen interference to some extent (Kirstein et al. 1999). Working potentials more positive than the oxygen reduction may also be achieved via direct electron transfer between the electrode and the enzyme (Plumeré 2013). However, this direct bioelectrochemistry did not go well with NR since it is a complex enzyme where the electron acceptor centers are deep seated. To overcome the complexity issue, a smaller and less complex NR, consisting of molybdenum-molybdopterin binding site and nitrate-reducing active site was engineered by Barbier et al. (2004). The engineered NR fragment lacked the FAD domain and was able to reduce nitrate at less negative potentials. A similar truncated NR from *Arabidopsis thaliana* was purified from *Escherichia coli* expression system and used for developing a highly selective and sensitive amperometric biosensor with glassy carbon electrode and artificial electron transfer partner anthraquinone-2-sulfonate (AQ), but oxygen interference could not be avoided (Kalimuthu et al. 2015). Real on-site application of oxygen-insensitive nitrate biosensors is still to be demonstrated.

Merits

These analytical devices are gaining momentum over classical analysis techniques due to their high selectivity and sensitivity, low instrumentation cost, ease of use and rapidity of the assay. They can also be employed for analysis of turbid samples (Glazier et al. 1998).

Demerits

Practical utility of NR-based biosensors has been limited because of the inherent properties of NR that hinder direct electron transfer between an enzyme and an electrode. Two major constraints are as follows: (i) a large distance between the electrode surface and the redox active site of the enzyme which is normally inside the globular protein and (ii) the inadequate orientation of donor to acceptor sites depending on the method of the immobilization of the enzyme at the electrode. As a consequence, the biosensors displayed weak sensitivities for nitrate. Besides this, very low stability and activity of immobilized NR often due to partially hydrophobic nature of the immobilization matrix, poor conjugation yield (Willner et al. 1992), slow leakage of NR and redox mediators into the bulk solution and oxygen sensitivity were responsible for instability and irreproducibility in the biosensor response. Further, entrapment of NR by electropolymerization requires the presence of polymer and enzyme in large quantities, and the amount of enzyme immobilized cannot actually be controlled.

Direct colorimetric dip strip method

In order to improve the activity and stabilize the three-dimensional structure of NR, it has been immobilized onto polyethylene supported epoxy/metal or metal oxide nanoparticles conjugates and used as a dip strip sensor for determination of nitrates in soil and water samples.

Principle

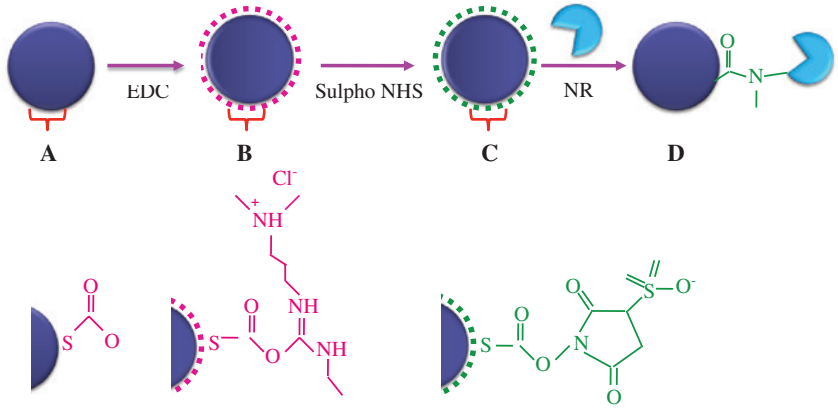
The dip strip method is based on colorimetric detection of nitrates. The strip was fabricated by immobilizing NR separately on to epoxy affixed and surface modified GNPs, silver nanoparticles (SNPs), zinc oxide (ZnONPs) and iron oxide nanoparticles (Fe_3O_4NPs) (Sachdeva and Hooda 2014, 2015, 2016). Metal or metal oxide nanoparticles were first capped with mercaptoundecanoic acid (MUA), and then MUA group was terminated with N-hydroxysuccinimide (NHS) based on carbodiimide hydrochloride (EDC)/NHS coupling reaction (Scheme 3). The modified nanoparticles were adhered onto polyethylene supported epoxy layer and used for immobilizing NR. Thus formed strip was dipped in the sample, and nitrate was reduced to nitrite using immobilized NR in the presence of β-NADH as reducing agent. Further, nitrite was treated with sulphanilamide, which was coupled with NED to form a diazocompound that gave nitrite color complex (Scheme 4).

Immobilization of NR

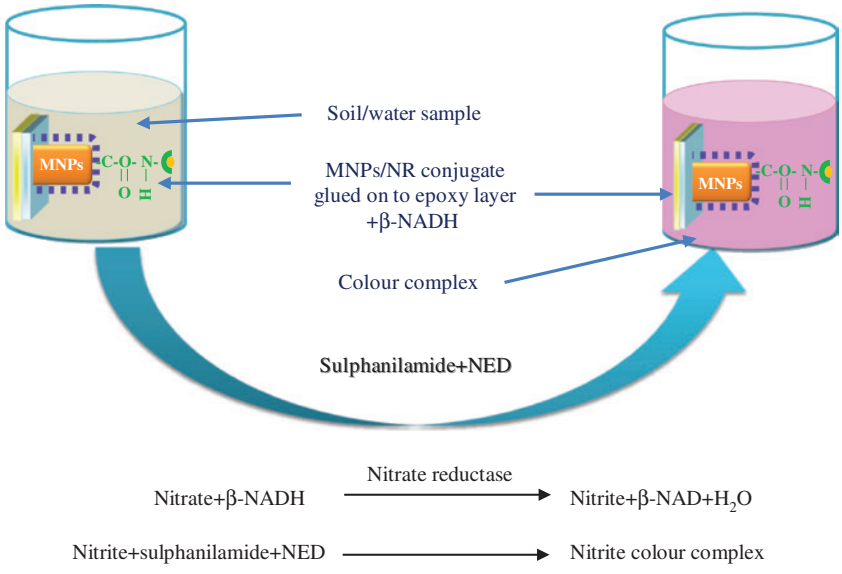
Epoxy adhered GNPs, SNPs, ZnONPs and Fe_3O_4NPs have so far been successfully employed as supports for immobilization of NR. All of these nanoparticles are extensively used for attaching biomolecules of interest as they are biocompatible, have high surface area to volume ratio and impose minimal diffusional limitations. Additionally, they preserved the structure and activity of NR since the enzyme was immobilized covalently. In NHS terminated nanoparticles, the side chain amino groups on NR surfaces displaced the terminal NHS groups and formed amide bonds with the carboxyl groups on the nanoparticles. NR in particular retained 85–93.90% of specific activity on these metal and metal oxide nanoparticles, which was pretty good considering the multisubunit and complex structure of NR. An increase in affinity for nitrate as evident by decreased *K*m values was also reported for immobilized NR. Table 2 enlists the *K*m values for NR immobilized onto different supports.

Analytical performance

All the four epoxy/nanoparticles/NR strips prepared individually for gold, silver, iron oxide and zinc oxide nanoparticles yielded valid and duplicable results for nitrate determination. The minimum detection limit by strip method was 0.05 mM, and linearity between nitrate concentration and A_{540} was obtained from 0.1 to 11.0 mM with



Scheme 3: Modification of metal and metal oxide nanoparticles for attaching nitrate reductase (NR). Citrate stabilized (A), N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) modified (B), N-hyhroxysuccinimide (NHS) terminated (C) and NR bound nanoparticles (D).



Scheme 4: Nitrate determination by colorimetric dip strip method using MNPs/NR/epoxy conjugates. MNPs represent metal (gold and silver) and metal oxide (iron and zinc) nanoparticles.

SNPs and Fe_3O_4NPs (Sachdeva and Hooda 2015) and up to 10.0 mM using GNPs and ZnONPs (Sachdeva and Hooda 2014). The strip method also corelated well with the chemical method.

Merits

Nitrate determination using nanoparticle conjugated NR is a good alternative to other chemical and electrochemical methods. The method is simple, reproducible, reliable and does not require any sophisticated instrument or a trained person and hence may be adopted for routine measurement of nitrates. Use of nanoparticles resolved the dual issues of electrical conduction and stability of NR to a certain extent (Sachdeva and Hooda 2015). An account of the stability of immobilized NR along with reproducibility of nitrate determination and applicability of the method in natural or spiked samples is given in Table 4.

Demerits

The technique is comparatively slower than the biosensor, but speed may be enhanced using automated systems. The technique can only be used for clear samples, which are not opaque.

Summary and conclusion

Nitrate determination has great relevance in environmental monitoring and sustainable agriculture. An attempt has been made to summarize various methods used for nitrate determination. Every method offers certain advantages and disadvantages, but at large enzymatic methods are considered superior and more practical over non-enzymatic methods. Compared with non-enzyme-based methods, they are very selective, sensitive, rapid and environment friendly. Immobilized NR-based methods if used for developing nanobiosensors or discrete nanoparticle conjugated enzymatic strips, in all likelihood, may become a commercially successful technology.

Future perspectives

In vitro stability, poor activity and electrical conductivity of NR have been consistent issues with all the enzyme-based methods for nitrate determination. In the area of nitrate biosensors, research has so far been centered around effective communication between NR and electrode, largely ignoring the issues of NR activity and stability. Hence, most of the nitrate biosensors perform poorly. Improvement in NR stability using different immobilization supports is essential for development of cheap and commercially viable nitrate sensors. Use of nanomaterials in this regard has great potential since they are biocompatible as well as have good electrical conductivity. In addition, use of functionalized polymers for the covalent binding of NR should also be explored. Problems related to portability and requirement of large sample volumes may be overcome by using modified screen-printed, microtrench and nano-gap electrodes. Furthermore, microtrench and nano-gap electrodes are also oxygen insensitive and should rather be explored for developing low-cost nitrate biosensors. Developing

Table 4: Storage stability, reproducibility and application of various immobilized NR-based methods.

S. no.	Stability (activity retained)	Reproducibility (% RSD)	Application	References
1	50% after 14 days	1.01–3.12, n=6	Groundwater	Sachdeva and Hooda (2014)
2	50% after 18 days	0.55–1.63, n=6	Agricultural soil	Sachdeva and Hooda (2015)
3	50% after 1–2 days	–	–	Kirstein et al. (1999)
4	50% upon reuse	1.90, n=6	Spiked water samples	Sohail and Adeloju (2008)
5	20% after 5 days	–	Spiked water samples	Da Silva et al. (2004)
6	70% >14 days	6.0–8.0, n=10	Spiked and natural water samples	Xuejiang et al. (2006)
7	89.6% after 30 days	1.5–2.9, n=5	Human plasma, whole blood, saliva samples	Madasamy et al. (2014)
8	50% after 36 days	2.98	Drinking water	Albanese et al. (2010)
9	70% after 10 days	5.4, n=7	Spiked water samples	Can et al. (2012)
10	35% after 3 days	–	Spiked water samples	Ferreyra and Solis (2004)
11	100% after 10 h	3.5, n=6	Spiked water samples	Adeloju and Sohail (2011)
12	100% after 1 month	–	Beetroot juice and human breast cancer cells	Madasamy et al. (2013)
13	0.0% within 2 days	–	Fertilizer and drinking water samples	Glazier et al. (1998)

electrode materials having working potentials for oxygen reduction more negative than that required for nitrate reduction may also resolve the issues of oxygen interference and pave the way for developing oxygen-insensitive nitrate biosensors suitable for field applications.

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