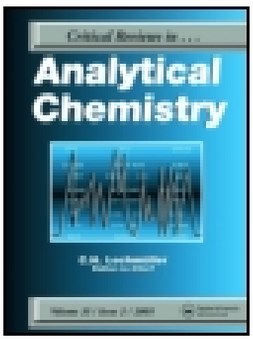

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Enzyme-Based Ultrasensitive Electrochemical Biosensors for Rapid Assessment of Nitrite Toxicity: Recent Advances and Perspectives

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ABSTRACT

In the present era of rapid international globalization and industrialization, intensive use of nitrite as a fertilizing agent in agriculture, preservative, dyeing agent, food additive and as corrosion inhibitor in industrial sectors is adversely effecting environment, natural habitats and human health. The issue of toxicity and carcinogenicity due to excessive ingestion of nitrites via the dietary intake has led to an imminent need for its efficient real-time monitoring in situ. Nitrite detection employing electrochemical biosensors has been gaining high credibility in the field of clinical research. Nitrite biosensors have emerged as an outstanding choice for portable point of care testing of nitrite quantification owing to the excellent properties, such as rapidity, miniaturization, ultra-low limits of detection, multiplexing and enhanced detection sensitivity. The article is enclosed with an interesting outlook on latest emerging trends in the development of nitrite biosensors utilizing nanomaterials, such as metal nanoparticles, carbon nanotubes, metal oxide nanoparticles, nanocomposites, polymers and biomaterials. The present review embarks on the highlights relevant to the nitrite quantification in real samples, then proceeds with a meticulous description of the most pertinent electrochemical nitrite biosensors, which have been proposed by adopting diverse materials and strategies of fabrication and finally end with the achievements and future outlook signifying the application of these nanoengineered biosensors for environmental surveillance and human safety.

KEYWORDS

Biosensor; electrochemical; enzyme; nitrite; nitrite reductase

Introduction

Growing concern worldwide about nitrite as an alarming environmental contaminant in natural or sewage water and soil, the last decades have witnessed an enormous interest in detection and quantification of nitrite (NO_2^-). Nitrite is an inorganic pollutant found extensively in fertilizers, industrial waste water, dyes, detergents and corrosion inhibitors.[1] It is often used as an additive and preservative to enhance the fragrance and taste of the food. In industries like textile, pharmaceutical industries, metal and petroleum, application of various products containing nitrite have been recognized.[2] The massive loading of N-fertilizers in agricultural fields, and the release of N-oxides into the atmosphere due to many domestic and industrial combustion processes is accountable for the contamination of soil, surface waters and groundwater supplies. The frequent eutrophication has been reported in the coastal waterways and lakes due to the presence of high levels of nitrite together with the phosphate in water bodies. The unbalance of global biogeochemical N-cycles adversely affects the environment as well as enhances the potential threat of human exposure to elevated amount of nitrite and the consequential health hazards especially in infants and children often known as the blue-baby syndrome.[3] Excessive ingestion of nitrite beyond normal human intake can lead to formation of carcinogenic nitrosamines by the interaction of nitrite with amines and methemoglobinemia by the irreversible oxidation of hemoglobin in blood.[4] Human exposure to nitrite comes via utilization of water from domestic wells for drinking and dietary intake of canned food like cheeses and meats, which are preserved by using nitrite salts. The significance of incessant evaluation of nitrite concentration in soil, food, biological fluids and water stems from its proven clinical and physiological impacts on human and environment safety. The issue of safety levels of nitrite have currently been under regulation with the promulgation of rules to limit their concentration in drinking water and packaged foodstuffs. In drinking water, the maximum contamination level (MCL) of nitrite ions as prescribed by the US Environmental Protection Agency (EPA) should be 1.0 mg NL^{-1} (71.4 μM).[5] According to the European Food Safety Authority (EFSA) recommendations, there should be strict control on the level of nitrite added to the various foodstuffs and not exceeding 150 mg kg^{-1} in case of meat products.[6] High nitrite concentration in human sera has currently been thought of as a serious physiological factor for clinical studies.

A hostile impact on ecological systems and human health has been observed as a result of excessive intake of nitrite. Nitric oxide plays a crucial task in cell signal transduction and (patho)physiological processes of numerous human diseases.

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Therefore, a lot of apprehension has been raised signifying the potential role of nitrite concentration in the environment as well as physiological fluids.[7] Up to now, several analytical assays have been reported for nitrite quantification, including chromatography,[8] capillary electrophoresis,[9] chemiluminescence,[10] spectrophotometry,[11] spectrofluorimetry and electrochemistry.[12–14] Among them, the electrochemical approach of nitrite detection is proven to be an environment friendly and powerful technique due to its outstanding performance such as fast procedure, selectivity, simplicity, high accuracy, low cost and no requirement of additional chemical loading. Biosensors are simple and economical to fabricate in miniature dimensions which bypass an elaborated laboratory protocol, making the task simple and quick to be executed onsite. Therefore, significant advances have been made to create innovative and enhanced apparatuses for nitrite analysis over the previous couple of years, prompting the growing field of enzyme-based nitrite biosensors.[15] Unfortunately, the electrochemical oxidation of nitrite at the most common electrodes suffers high redox potentials, decreasing the sensitivity of the electrodes that are easily poisoned by the electroactive interferences generated during the electrochemical process. In order to circumvent this problem, the surface of bare electrode is being modified with an appropriate catalyst to improve the sensitivity and lowering down the oxidation potential. Various nanomaterials-based modified electrodes, such as carbon materials and metal nanostructures have grabbed attention for the construction of enzymatic and non-enzymatic nitrite biosensors.

Enzymatic biosensors have been supported over different strategies since they are basic, sensitive, quick, simple to-utilize and exceedingly accurate. The chief enzyme involved in the determination of nitrite is nitrite reductase. The use of immobilized enzymes is preferred over the free enzyme for the fabrication of a biosensor to avoid high cost besides the cumbersome handling of the later. With the expanding information of nitrite function in physiology and related clinical implications, there is a significant boost in the awareness toward development of assays and tools for nitrite determination. There is currently exponential capability of nitrite diagnostic devices in clinical diagnosis, food industry as well as pollution control. From the most recent 20 years, the developing need of convenient apparatus for on location nitrite analysis prompts upheaval of various methodologies for improvement of efficient nitrite biosensors. The present review gives the worldwide point of view with respect to nitrite biosensors with versatile fabrication strategies and materials embraced.

Basic principle of an electrochemical detection technique

In the electrochemical biosensing technique, electrical properties are measured for extracting the information from biological systems and a bioelectrochemical component act as the main transducing element. The electrochemical detection techniques predominantly utilize enzymes as the recognition element. The specific confining capacities and biocatalytic activity of enzymes imply their potential role in biosensors. Typically, an electrochemical biosensor operates by reacting with the analyte of concern to generate an electrical signal proportional to the amount of analyte. Electrochemical sensor usually consists of a working electrode (sensing or redox electrode), a reference electrode and a counter or auxiliary electrode separated by an electrolyte. The reference electrode is regularly made up of Ag/AgCl and stayed away from however much as could be expected from the reaction site to sustain a known and constant potential. The counter or auxiliary electrode sets up a connection to the electrolytic solution so that a current can be applied to the working electrode, which serves as the transduction element in the biochemical reaction. These electrodes should be chemically inert as well as conductive. For that reason, carbon (e.g., graphite), gold, platinum and silicon compounds are generally employed, reliant upon the analyte of concern. The reaction under investigation in (bio-)electrochemical techniques would either generate a measurable potential or charge accumulation (*potentiometric sensors*), a measurable electric current due to the reduction or oxidation of electroactive species (amperometric sensors), quantifiable change in the conductive properties of a medium (conductometric sensors) between electrodes or a measurable impedance including both resistance and reactance (impedimetric sensors). The basic principle of an electrochemical biosensor has been depicted in Figure 1.

Enzymes involved in the electrochemical determination of nitrite

Over the last 15 years, significant advances have been made in nitrites detection by employing exceptionally specific, dynamic and stable nitrite reducing enzymes isolated from different bacterial sources. In line with the selectivity of the biorecognition event, non-specific proteins and nitrite reductases are the two groups that are able to perform nitrite quantification and utilized in the fabrication of nitrite biosensors.

Nitrite detection using non-specific proteins

The development of some electrochemical biosensors based on the employment of non-specific proteins such as hemoglobin, myoglobin, HRP/catalase, cytochrome c and superoxide dismutase (SOD) have also been reported in the literature.

Hemoglobin and myoglobin are hemeproteins whose physiological significance is mainly identified with their capacity to bind with molecular oxygen. Myoglobin is a monomeric hemeprotein mainly found in muscle tissue where it serves as an intracellular storage site for oxygen.[16] Hemoglobin is an iron-containing metalloprotein found in erythrocytes of numerous vertebrates where it combines with oxygen and carry it to the cells.[17] Bovine blood Hb and horse heart Mb have been reported

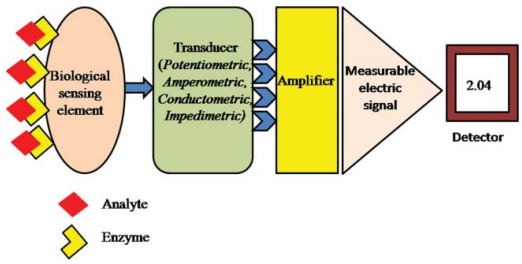

Figure 1. Basic principle of an electrochemical biosensor.

to be used as model proteins in the fabrication of electrochemical nitrite biosensors. Various nitrite biosensors based on Mb and Hb developed in last few years has been shown in Table 1[18–24] along with the comparison of their analytical parameters.

In 2003, Titov and Petrenko reported that the interaction of nitrite-catalase is a significant aspect of nitrite toxicity. Nitrite behaves as a competitive inhibitor and inhibits the activity of catalase even when present in micro-molar concentration, without altering the essence of the enzymatic procedure.[25] UV–Visible spectroscopy confirmed the inhibition of catalase activity by the presence of nitrite. Since hydrogen peroxide is an essential feature for eliciting a number of significant lethal effects of nitrite, so the latter enhances its toxicity by inhibiting the enzyme-catalase. Inactivated catalase shows low affinity for nitrite (substrate) called enzyme inhibition and the degree to which enzyme is inhibited is directly proportional to the amount of nitrite present in the sample. Recently this year, a novel mediator free biosensor based on the direct electrochemistry of horseradish peroxidase (HRP) has been fabricated. The enzyme was immobilized on Co_3O_4 nanosheets along with the reduced graphene oxide composite modified glassy carbon electrode (GCE). The prepared nitrite biosensor displayed an excellent performance with the low detection limit of 0.21 μM and a wide linear range of 1–5400 μM. Some HRP/Catalase-based nitrite biosensors have been shown in Table 1.[26–29]

The employment of enzyme-SOD has also been reported for the fabrication of nitrite biosensor. In 2010, Rajesh et al. developed a novel sensitive biosensor by immobilizing Cu, ZnSOD (SOD1) on the carbon nanotube (CNT)/polypyrrole (PPy) nanocomposite modified Pt electrode for the simultaneous determination of nitrite (NO_2^-). The biosensor exhibited a linear range of 0.5–2000 μM and the detection limit was found to be 0.5 ± 0.025 μM for NO_2^-. The proposed biosensor showed good reproducibility and high sensitivity with stability period over 30 days.[30] In 2014, Madasamy et al. constructed a novel bienzymatic nitrite biosensor by coimmobilizing copper, zinc superoxide dismutase (SOD 1) and nitrate reductase on CNT/PPy nanocomposite modified platinum electrode for the instantaneous determination of nitrite and nitrate. The bienzyme electrode (SOD1-NaR-CNT-PPy-Pt) showed excellent sensitivity and fast response. The electrocatalytic activity of SOD was linear from 100 nM to 1 mM and a detection limit of 50 nM toward NO_2^-.[31]

In 2008, Geng et al. reported the first Cytochrome c based oxidative nitrite biosensor and illustrated the probable mechanism of biocatalytic oxidative property of nitrite as shown in the reactions given below:

$$Fe^{2+}\text{–Cyt c} \rightarrow Fe^{3+}\text{–Cyt c} + e- \quad (1)$$

$$Fe^{3+}\text{–Cyt c} \rightarrow [Fe^{4+}\text{–Cyt c}]\bullet + 2e- \quad (2)$$

$$[Fe^{4+}\text{–Cyt c}]\bullet + NO_2^- + H_2O \rightarrow Fe^{3+}\text{–Cyt c} + NO_3^- + 2H^+ \quad (3)$$

First, ferrous Cyt c was oxidized to ferric form at low potential, and then the ferric Cyt c was further oxidized into $[Fe^{4+}$-Cyt c$]^\bullet$ at higher potential. $[Fe^{4+}$-Cyt c$]^\bullet$ is a highly reactive Cyt c π-cation, which could oxidize NO_2^- into NO_3^-

Table 1. Various nitrite biosensors based on non-specific proteins.

Protein	Transducer	Sensor preparation	Linearity range	Detection limit	Sensitivity	Reference
Mb	Amperometric	GC + zirconium phosphate nanosheet films +Mb	3–800 mM	700 μM	N.D.	[18]
Mb	Amperometric	GC + clay-chitosan-gold nanoparticle nanohybrid + Mb between matrix layers	2.49–63.3 mM	500 μM	N.D.	[19]
Mb	Voltammetric	GC+ multi-walled carbon nanotubes/ionic liquid + Nafion/Mb between matrix layers	0.2–11.0 mM	N.D.	26.9 μA mM^{-1}	[20]
Hb	Amperometric	GC + hexagonal mesoporous silica + PVA + Hb	0.2–3.8 μM	0.61 μM	1.79 μA μM^{-1}	[21]
Hb	Amperometric	GC + Hb electrospin followed by cross-linking with glutaraldehyde	N.D–4.5 mM	0.47 μM	N.D.	[22]
Hb	Amperometric	One-dimensional gold nanoparticles + Hb	0.4–14.8 μM	6.5 × 10^{-8} M	N.D.	[23]
Hb	Amperometric	Gold colloids modified carbon paste electrode + Hb	9.0 × 10^{-7}–3.0 × 10^{-4} M	1.0 × 10^{-7} M	N.D.	[24]
HRP	Voltammetric	GC + casting of gemini surfactant C_{12}-C_{12}-C_{12} + PVA+ HRP	0.03–12 mM	N.D.	−0.0302 μA mM^{-1}	[26]
HRP	Amperometric	Layered double hydroxides + HRP + catalase	N.D.	4 μM	102 μA M^{-1} cm^{-2}	[27]
HRP	Conductimetric	An interdigitated electrode + HRP + catalase	0.3 μM to 446 μM	0.03 μM	N.D.	[28]
HRP	Amperometric	Porous Co_3O_4 hexagonal nanosheets (PCHNSs) + reduced graphene oxide + HRP + glassy carbon electrode	1–5400 μM	0.21 μM	N.D.	[29]
SOD	Amperometric	Carbon nanotubes + polypyrrol (PPy) matrix + Pt electrode + Cu, ZnSOD	0.5–2000 μM	0.5 μM	N.D.	[30]
SOD	Amperometric	Carbon nanotubes + polypyrrol (PPy) + Pt electrode+ SOD1	100 nM to 1 mM	50 nM	N.D.	[31]
Cyt c	Amperometric	Cyt c + multi-walled carbon nanotubes-poly (amidoamine) (PAMAM)-chitosan nanocomposite + glass carbon electrode (GCE).	0.1–29 μM	0.01 μM	N.D.	[33]
Cyt c	Amperometric	Conducting polymer poly-(3-methylthiophene) (P3MT) + MWCNT + GCE + Cyt c/l-Cys	10–100 μM	0.5 μM	N.D.	[34]
Cyt c	Amperometric	PANI-g-ND + Au film + Cyt c	0.5 μM to 3 mM	0.16 μM	88.2 μA/mM	[35]
Cyt c	Amperometric	Pt nanoparticles loaded ITO (PtNPs/ITO) electrode + Cyt c	5–800 μM	0.4 μM	N.D.	[36]
Cyt c	Amperometric	Titanium nitride (TiN) nanoparticles + MWCNTs + Cyt c	1–2000 μM	0.0014 μM.	121.5 μA μM^{-1} cm^{-2}	[37]
Cyt c	Amperometric	Cyt c + TiO_2(G) NWs@PANI(SH)-Au MCNC + 3DNE	10 μM to 720 mM	0.05 μM	9.2 μA/mM	[38]

N.D. – Not determined.

in the solution.[32] In 2010, Eguilaz et al. fabricated a Cyt **c**/L-Cys/P3MT/MWCNT/GCE biosensor for the determination of nitrite. This cytochrome c (Cyt c) based biosensor was prepared by immobilizing the enzyme onto L-cysteine (L-Cys) self-assembled monolayer (SAM) modified GCE together with the conducting polymer poly-(3-methylthiophene) (P3MT) and multi-walled carbon nanotubes (MWCNT). The biosensor showed excellent sensitivity, linearity range from 10 to 100 μM and a detection limit of 0.5 μM.[34] In the same year, Gopalan et al. developed a nitrite biosensor based on immobilization of the cytochrome c (Cyt c) on PANI-g-ND/gold nanoparticles (GNPs) composite film. The polyaniline chains were firmly attached onto the nanodiamond (PANI-g-ND) via electrochemical polymerization. The biosensor showed sensitivity of 88.2 μA/mM and a wide linear range from 0.5 μM to 3 mM. The low detection limit of 0.16 μM was observed.[35] In 2012, Liang et al. developed an amperometric nitrite biosensor by immobilizing Cyt c on a facile Pt-implanted indium tin oxide (PtNPs/ITO) electrode. The modified electrode along with cytochrome c exhibited a linear concentration range of 5–800μM and detection limit of 0.4 μM toward nitrite determination.[36] In 2016, Haldorai et al. fabricated a cytochrome c based amperometric nitrite biosensor using direct electrochemistry. In the proposed biosensor, the enzyme was immobilized onto the titanium nitride (TiN) nanoparticles decorated MWCNTs nanocomposite modified GCE. Electrocatalytic activity of immobilized cytochrome c displayed the linear current response ranging from 1 μM to 2000 μM toward nitrite concentration with a low detection limit of 0.0014 μM. The biosensor showed excellent sensitivity of 121.5 μA μM^{-1} cm^{-2} and long-term stability. The validity and successful application for nitrite detection in sea water and tap water samples was confirmed.[37] In the same year, an electrochemical nitrite biosensor based on cytochrome c was designed and fabricated by Shanmugasundaram et al. The biosensor was designated as Cyt c/TiO_2(G) NWs@PANI(SH)-Au MCNC/3DNE in which a GCE was modified into three-dimensional nanoarchitectured electrode (3 DNE) by immobilizing Cyt c onto the multi-component nanocomposite (MCNC) film consisting of graphene embedded with titanium dioxide nanowires (TiO2 (G) NWs), GNPs (Au) and thiol-functionalized polyaniline (PANI(SH). The fabricated electrode showed a detection limit of 0.05 μM and a wide linear range of 10μM-720mM along with high stability.[38]

Nitrite detection using nitrite reductases

Nitrite reducing enzymes (NiRs) play a valuable role as a bio-recognition element in analytical devices for nitrite bio-sensing. There are two broad categories of nitrite reductases – nitric oxide forming nitrite reductases and ammonia forming nitrite reductases.

Nitrite reductases (nitric oxide forming)

Two different types of NiRs proficiently catalyze the one electron reduction reaction of nitrite to nitric oxide (Equation 4). They are called cytochrome cd_1 nitrite reductases (cd_1NiRs) and copper-containing nitrite reductases (CuNiRs).

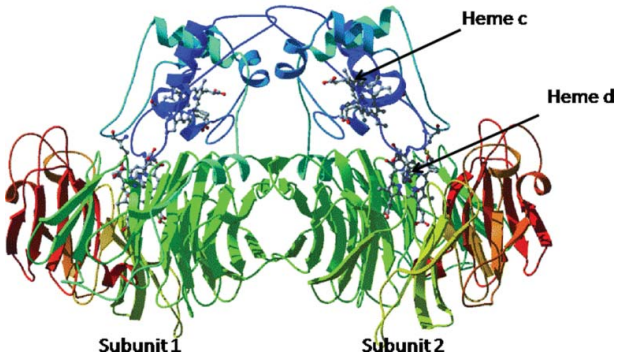

Figure 2. Three-dimensional structure of Pseudomonas cytochrome cd_1 nitrite reductase (dimeric).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (4)$$

cd_1NiRs or *Pseudomonas* cytochrome oxidases are homodimeric (2 × 60 kDa), soluble enzymes, which contains two c-type hemes (electron acceptor site) and two d type hemes (substrate-binding site) with two polypeptide chains as shown in Figure 2.[39] The reduced d hemes bind nitrite and convert it to nitric oxide. CuNiRs are trimeric enzymes consisted of three identical subunits (3 × 37 kDa), each having two copper ions. Type-1 Cu is involved in electron transfer and type-2 Cu constitutes the catalytic center as shown in Figure 3.[40] The cysteine bound to the type 1 copper center is situated directly next to a histidine residue, which is ligated to type 2 Cu center and responsible for binding and reducing nitrite. There is significant role of this Cys-His bridge in assisting the transfer of electrons from type 1 center to type 2 with rapidity.

Nitrite reductases (ammonia forming)

Two different types of NiRs catalyze the six electron reduction of nitrite to ammonia (NH_4^+) as shown in equation given below:

$$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O \quad (5)$$

The dissimilatory cytochrome c nitrite reductases (ccNiR) isolated from sulfate or sulfur reducing bacteria are multi-heme

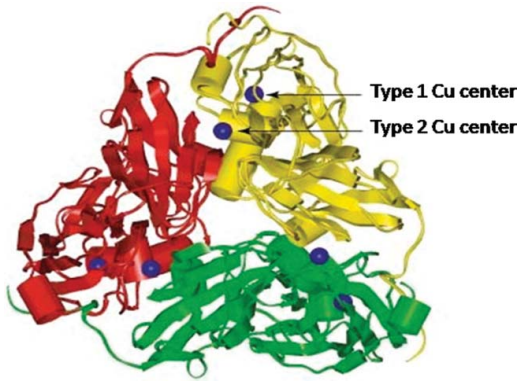

Figure 3. X-ray crystal structure of copper containing nitrite reductase (trimeric).

enzymes, which play a significant role in respiratory processes. They are normally consisting of subunits NrfA and NrfH in $NrfA_4NrfH_2$ stoichiometry and isolated from the periplasmic membrane as high molecular mass multimers as depicted in Figure 4.[41] NrfH is the small tetrahemic polypeptide, which is hydrobhobic in nature and involved physiologically in donating electron of NrfA. The catalytic active subunit-NrfA contains five hemes for every monomer in a firm packing, so that high rates of electron transfer can be accomplished. Another category of NH_4^+ forming NiRs belong to the ferredoxin-dependent nitrite reductases. They are usually isolated from photosynthetic organisms like cyanobacteria, plants and algae. These assimilative enzymes are characterized by the presence of a unique active site which is composed of a siroheme coupled to an iron–sulfur cluster [4Fe–4S] through a bridging sulfur atom from a cysteine residue as shown in Figure 5.[42] The siroheme group is the binding site for nitrite.

The different biosensors fabricated for nitrite detection by using nitrite reductase has been mentioned in Table 2.[43–52] In the literature, it has been reported that many insoluble supports were employed for the immobilization of enzyme to overcome the challenges faced in the fabrication of efficient working electrodes. In 2010, Silveira et al. worked on the construction of an efficient non-mediated electrochemical biosensor for the determination of nitrite in complex samples. In the proposed device, cytochrome c nitrite reductase (c**c**NiR) from bacteria *Desulfovibrio desulfuricans* was incorporated with a porous silica glass and immobilized onto the pyrolytic graphite (PG) surfaces. The working electrode exhibited a wide linear range of 0.25–50 μM and the minimum detectable limit of 120 nM. The method was highly accurate for the nitrite analysis in freshwaters with sensitivity of mA M^{-1} cm^{-2}.[49] In 2011, Serra and coworkers developed a novel enzymatic biosensor based on the cooperative use of two enzymes for the quantification of nitrite. The

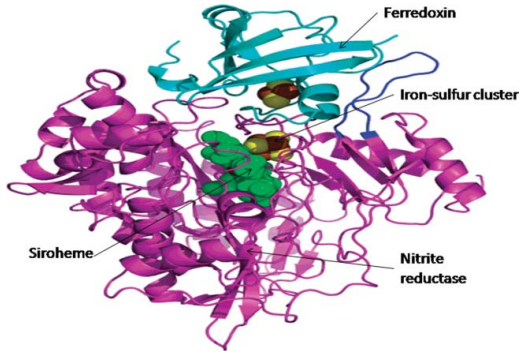

Figure 5. Structure of ferredoxin-dependent nitrite reductase isolated from spinach.

co-immobilization of cytochrome-cd(1) (cyt-cd(1)) nitrite reductase as biological recognition element and cytochrome-c (552) (cyt-c(552)) as electron mediator was done using a photopolymerizable polyvinyl alcohol (PVA) derivative onto the surface of carbon paste screen printed electrodes (CPSPEs). The amperometric measurements were assessed as follows: linear concentration range from 10 to 200 μM; quantification and detection limits of 24 and 7 μM, respectively.[50] In 2015, Monteiro et al. developed an effective disposable biosensor for point of care quantification of nitrite using various samples, such as plasma, milk, urine and water. Nitrite reductase (ccNiR) and carbon ink composite homogenized in organic solvents were coated on CPSPEs. A wide linear response range from 0.7 to 370 μM and sensitivity of 0.55 A $M^{-1}cm^{-2}$ was observed toward nitrite reduction.[51] In 2017, Santharaman et al. reported a cytochrome c reductase (CcR) based highly sensitive portable electrochemical analyzer for point-of-care sensing of nitrite. The enzyme was immobilized onto the biofunctionalized SAM decorated with GNPs in PPy nanocomposite modified screen printed carbon electrode (SPCE). Based on

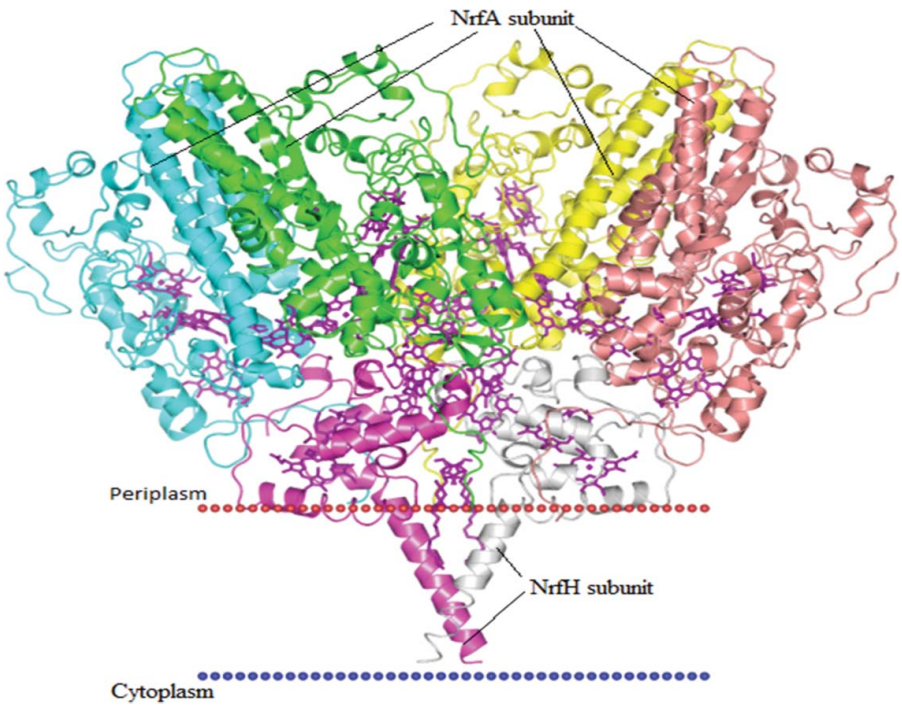

Figure 4. Three-dimensional structure of multi-heme *c* nitrite reductase ($NrfA_4NrfH_2$ complex) isolated from *Disulfovibrio vulgaris*.

Table 2. Various nitrite biosensors based on nitrite reductases.

Protein	Transducer	Sensor preparation	Linearity range	Detection limit	Sensitivity	Reference
NiR	Amperometric	1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) + NiR + Au electrode	0–1 mg/l	0.01 mg/l	N.D.	[43]
ccNiR	Amperometric	poly(pyrrole–viologen) matrix + ccNiR + glassy carbon electrode	5.4–43.4 μM	5.4 μM	1721 mAM^{-1} cm^{-2}	[44]
NiR	Amperometric	NiR + methyl viologen (MV) + glassy carbon electrode	1.5–260 μM	1.5 μM	11.8 nA/μM	[45]
ccNiR	Amperometric	ccNiR + Nafion ionomeric matrix + methyl viologen	75–800 μM	60 μM	445 mA M^{-1}cm^{-2}	[46]
ccNiR	Amperometric	ccNiR + [ZnCr-AQS] + glassy carbon electrode	0.015–2.35 μM	4 nM	1.8 A M^{-1} cm^{-2}	[47]
ccNiR	Conductometric	ccNiR + glutaraldehyde + Bovine serum albumin + methyl viologen + Nafion + glycerol + planar interdigitated electrode	0.2–120 μM	0.05 μM	0.194 μS/μM	[48]
ccNiR	Amperometric	ccNiR + sol-gel + pyrolytic graphite electrode	0.25–50 μM	120 nM	430 ± 8 mA M^{-1} cm^{-2}	[49]
cd1 NiR	Amperometric	Cyt cd1 + Cyt c552 + polyvinyl alcohol (PVA) + carbon paste screen printed electrode (CPSPEs)	10–200 μM	7 μM	2.49 ± 0.08 mA M^{-1} cm^{-2}	[50]
ccNiR	Amperometric	ccNiR + carbon ink composite + carbon paste screen printed electrode (SPE)	0.7–370 μM	N.D.	0.55 A M^{-1} cm^{-2}	[51]
ccNiR	Amperometric	ccNiR + self-assembled monolayer + AuNPs + polypyrrole (PPy) nanocomposite + screen printed carbon electrode	0.1–1600 μM	60 nM	0.172 μA μM^{-1} cm^{-2}.	[52]

the electrochemical and catalytic properties, the planned biosensor was investigated for the detection of nitrite. It showed a high sensitivity of 0.172 μA μM^{-1} cm^{-2} and linear range from 0.1 to 1600 μm. LOD was found to be 60 nM.[52]

Methods of enzyme immobilization

The accomplishment of a biosensing system is not merely an issue of finding a suitable enzyme but also attaching it firmly onto a transducing surface without denatuartion or leakage. In order to make a viable biosensor, an appropriate immobilizing method should be chosen as it plays the significant role in determining the activity of the enzyme and its characteristics in a particular reaction. Various factors are accountable for the selection of immobilization method, for instance the physicochemical properties of the analyte, the type of transducer used, the nature of the biological element and the optimum conditions in which the biosensor is to operate, and it is essential for the biological element to supersede all these considerations to exhibit maximum activity in its immobilized microenvironment.[53] Biosensors are generally designed with high enzyme loading for the assurance of adequate biocatalyst activities, and a suitable environment is provided to the enzymes to maintain their structural stability. There are various kinds of physical and chemical interactions between the enzyme and the electrode material. Several strategies of enzyme immobilization that have been used for the construction of biosensors are depicted in Figure 6.

Physical adsorption

It is one of the simplest methods of reversible enzyme immobilization. In this method, enzyme is physically adsorbed on the surface of an inert support through hydrobhobic interactions and salt linkages. The support or carrier used may be organic (DEAE-cellulose, starch, DEAE-sephadex, carboxymethyl cellulose) or inorganic (e.g., calcium phosphate gel, alumina, glass, silica gel). The use of eco-friendly supports like microcrystalline cellulose, coconut fibers, kaolin has also been reported in the literature.[54–56] The support is either bathed into a solution of enzyme or the enzyme is dried on the surface of electrode for physical adsorption.[57,58] Only weak bonds (low energy bonds) such as hydrogen bonds, Van der Waal forces, ionic interactions[59] are involved in stabilizing the enzyme to the support or carrier. When the enzymatic activity has decayed, the reversibly immobilized enzymes can be removed from the support by controlling physicochemical parameters.[60]

Advantages: No reagents are required; no pore diffusion limitation, cheap, retain high enzyme activity,[61] relatively chemical free enzyme binding,[62] less disruptive to enzyme.

Disadvantages: Quite liable to changes of pH, ionic strength of buffer and temperature,[63] less efficient, desorption of enzymes from the support.[64]

Covalent bonding

It is the most widely used technique of irreversible enzyme immobilization. The covalent bond between the chemical groups of enzyme and the chemical groups of support forms a stable complex. Covalent association of enzymes to supports

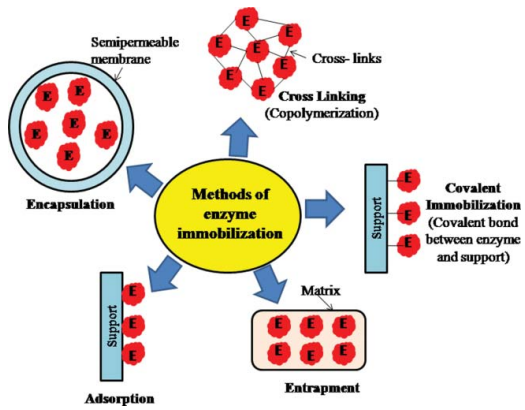

Figure 6. Different methods of enzyme immobilization.

involves binding through the side chains of cysteine (thiol group), lysine (-amino group), glutamic and aspartic acids (carboxylic group),[65] hydroxyl group, imidazole group, methylthiol group, guanidyl group, phenolic group and imino group. The size and shape of the carrier material, composition of the carrier material, nature of the coupling method and explicit conditions during coupling are responsible for the activity of covalent bonded enzyme.[66] Generally mild conditions such as low ionic strength, low temperature and pH in the physiological range are required for the occurrence of covalent bonding.[67] Researchers have reported enhancement in thermal stability and half-life of enzyme via covalent coupling with various supports like chitosan, mesoporous silica, etc.[68]

Advantages: Simple method, firm linkage of enzyme to the support and reusability,[69] no leakage problem,[65] availability of various supports with different functional groups.

Disadvantages: Loss of native conformation of enzyme due to chemical modifications.

Entrapment

In entrapment, enzymes are physically entrapped inside a polymer or a gel matrix that allows the substrate and products to pass through but retains the enzyme.[70,71] It is an irreversible method of enzyme immobilization. There may be covalent or non-covalent bonding involved in stabilizing the enzyme to the matrix. Polymers such as starch, collagen, polyacrylamide gel, gelatin, cellulose acetate, silicone rubber, calcium alginate, polyurethane, carrageenan and PVA with styrylpyridium group are utilized as matrices for entrapping of enzymes.[72] Mesoporous silica has been used successfully for entrapment owing to its high adsorption capacity, high surface area, tunable pore size and uniform pore distribution.[68] Efficient encapsulation has been achieved by nanostructured supports like pristine materials and electrospun nanofibers.[73]

Advantages: Minimum loss of enzyme activity, fast method of immobilization, cheap, easy to practice at small scale, improved mechanical stability.[74]

Disadvantages: Enzyme leakage,[75] chances of microbial contamination, pore diffusion limitation, less successful in industrial processes.

Cross-linking (copolymerization)

Cross-linking is another irreversible method of enzyme immobilization. In cross-linking, enzymes are directly linked by creating cross-links between them via polyfunctional reagents. The absence of matrix or solid support is the characteristic of this method.[75] Enzyme acts as its own carrier and does not require a support to prevent enzyme loss into the substrate solution as well as eliminating the drawbacks associated with carriers.[76,77] Glutaraldehyde and diazonium salts are the most widely used cross-linking reagents as they are cost-effective and easily available in large quantities.[78] Recently, cross-linking of enzymes to nanodiametric supports and electrospun nanofibres has shown better residual activity in the field of biocatalyst immobilization due to increased surface area and porosity.[79]

Advantages: Little desorption, simple and economic method.

Disadvantages: Risk of denaturation of enzyme.

Encapsulation

It is a type of entrapment. In encapsulation, the enzyme is enclosed within a semi permeable membrane forming a spherical particle called as microcapsule. The nature of membrane may be polymeric, non-ionic or lipoidal. This immobilization method depends upon the difference in size of enzyme and substrate or product molecules in correlation with pore size of the membrane. The small sized molecules such as substrate/product diffuse in and out through the membrane, whereas larger size enzyme molecules retain free floating inside the capsule. Additionally, more than one enzyme can be trapped inside the membrane to develop multi-enzyme system.[80]

Advantages: Large quantity of enzymes can be immobilized, minimal enzyme leakage, simple and cheap method.

Disadvantages: Pore size limitation.

Role of mediators in ameliorating progress of nitrite biosensors

In direct electron transfer, the proper orientation of the enzyme on the surface of the electrode and an adequately short separation between the redox active site and the electrode interface is the precondition for attaining a proficient electron exchange response. ccNiR from *D. desulfuricans* is the only NiR that has been reported to be employed in DET biosensors.[81] In 2010, Silveira and co-workers had utilized PG electrodes as transducing material, which offered a good interface for DET with ccNiR.[82] Soon after, a highly efficient non-mediated nitrite biosensor was developed by Silveira et al.[49] They deposited a ccNiR film on the surface of single-wall carbonnanotubes (SWCNTs) multi-layered on PG electrodes. One of the major hindrances in DET is the confining nature of the polypeptide chains that wrap around the redox center in numerous enzymes. If cofactors are secured by the apoprotein, it is difficult to accomplish electrochemical communication with electrodes. To facilitate the rate of electron transfer between the enzyme and the electrode, a common approach – mediated electron transfer (MET) is followed in which artificial electron donors are used as mediators. These redox mediators are small electroactive molecules that can easily interact with the protein's redox centers and consequently exchange electrons between electrodes and the biocatalyst. Direct electron transfer (DET) and MET have been shown in Figure 7. In enzymatic electrochemical biosensors, an efficient mediator must be capable of diffusing between the active site of enzyme and surface of electrode with high speed. It must be non-toxic and effectively soluble in both reduced and oxidized form. In literature, various research articles have been described pertaining to the ability of NiR to accept electrons from mediators to improve the progress of amperometric nitrite detection. In this perspective, a broad range of synthetic electron carriers or mediators such as 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate),[43] poly(pyrrole–viologen),[44] methyl viologen,[45,46,48] [ZnCr-AQS],[47] PPy,[52] either in solution or immobilized, were tested prospecting the advancement of mediated nitrite biosensing devices.

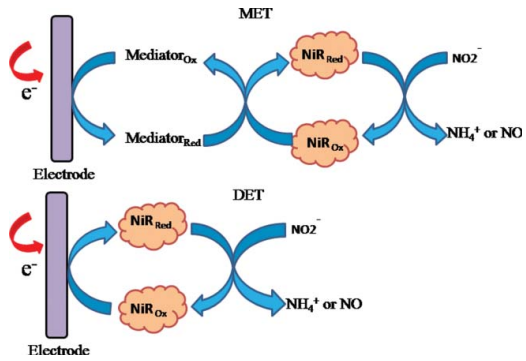

Figure 7. Role of a mediator in electron transfer.

Role of nanoparticles in scientific research related to fabrication of nitrite biosensors

Recent progresses in nano-technological approaches intend innovative applications in bioanalytical devices employed for the detection of various toxins concerning environmental and food safety. The unique properties of nanomaterials such as high surface to volume ratio, strong adsorption capability, distinctive size tunable electrocatalytic activity and enhanced electron-transfer kinetics offer excellent prospects for designing highly efficacious new generation biosensor devices. The integration of nanomaterials for the advancement of biosensing devices has been exploited to improve their sensitivity, electronic conductivity, stability, extreme rapidity devices for point of care nitrite analysis. Various strategies have been exhaustively scrutinized for recuperating the performance of nitrite biosensors with multiplexed capabilities. The use of various kinds of nanomaterials such as metal nanomaterials, up-conversion nanoparticles, carbon nanomaterials, magnetic nanoparticles, nanocomposites and quantum dots for the construction of nitrite biosensors has been reported in the literature. Some of the nitrite biosensors based on nanomaterials have been listed in Table 3[83–95] and the detection limits and linear range of nitrite concentration of different biosensors have been compared. In 2012, Zhang et al. constructed a simple gold plate electrode decorated with a film of gadolinium-doped titanium dioxide (Gd/TiO_2) nanoparticles for the detection of trace nitrite in cured foods. The nanoparticles modified electrode showed excellent sensitivity toward nitrite concentrations with the linear range of 8.0×10^{-7} to 4.0×10^{-4} mol l^{-1} and a detection limit of 5.0×10^{-7} mol l^{-4}.[88] In 2016, an electrochemical biosensor based on CuS–MWCNT nanocomposites was fabricated by Zhang et al. CuS–MWCNT nanocomposites modified glass carbon electrode displayed good reproducibility, anti-interference and remarkable catalytic performance for sensitive determination of nitrite. The biosensor could detect nitrite with the sensitivity of 131.2 μA mM^{-1} cm^{-2} and linear concentration range of 1.0 μM to 8.1 mM.[89] In the same year, Wang et al. also developed a straw cellulose/molybdenum sulfide (TOSC-MoS_2) nanocomposite-based non-enzymatic nitrite biosensor. The TOSC-MoS_2 modified GCE exhibited a wide linear nitrite concentration range of 6.0–3140 and 3140–4200 μM with a detection limit of 2.0 μM.[91]

In 2017, a large number of research papers have been published till now on utilization of different nanoparticles for fabrication of nitrite biosensors. Sheng et al. worked on non-enzymatic electrochemical biosensor based on Pt/$Ni(OH)_2$/MWCNTs nanocomposites.[92] Losada et al. prepared GNPs on ferrocenyl-dendrimer film modified electrodes electrochemically and used them for electrocatalytic oxidation of nitrite.[93]

Table 3. Various nitrite biosensors based on nanoparticles.

Transducer	Sensor preparation	Linearity range	Detection limit	Sensitivity	Reference
Amperometric	Pt nanocluster + polypyrrole (PPy) nanowires + glassy carbon electrode	5.0×10^{-7}–1.0×10^{-3} M	1.5×10^{-7} M	0.566 μA μM^{-1}	[83]
Amperometric	Gold nanoparticles + poly(3-methylthiophene) (P3MT) + glassy carbon electrode	10–1000 μM	2.3 μM	N.D.	[84]
Amperometric	Cu nanoparticles/carbon nanotube/chitosan (Cu_{nano}/CNTs/CS) film + glass carbon electrode	1.0×10^{-7} M–2.5×10^{-3} M	2.4×10^{-8} M	−48.92 μA mM^{-1}	[85]
Amperometric	Hollow hematite nano-polyhedrons (Fe-HNPs)	0.009–3 mM	N.D.	19.83 μA mM^{-1}	[86]
Voltammetric	Cyclodextrin (CD)+ cyclodextrin prepolymer (pre-CD)+ graphene sheets (GS) + MWCNTs	5 μM to 6.75 mM	1.65 μM	N.D.	[87]
Amperometric	gadolinium-doped titanium dioxide (Gd/TiO^2) + gold plate electrode (GPE)	8.0×10^{-7}–4.0×10^{-4}) mol l^{-1}	5.0×10^{-7} mol l^{-1}	N.D.	[88]
Amperometric	copper sulfide nanoparticles (CuS) + multi-walled carbon nanotubes (MWCNTs)+ glass carbon electrode	1.0 μM to 8.1 mM	0.33 μM	131.2 μA mM^{-1} cm^{-2}	[89]
Amperometric	poly(3,4-ethylenedioxythiophene) (PEDOT)+ gold nanoparticles (AuNPs)+ glass carbon electrode	0.2–1400 μM	60 nM	N.D.	[90]
Amperometric	TEMPO oxidized straw cellulose/molybdenum sulfide (TOSC-MoS2) nanocomposite + glass carbon electrode	6.0–3140 and 3140–4200 μM	2.0 μM	N.D.	[91]
Amperometric	Pt nanoparticles + $Ni(OH)_2$/multi-walled carbon nanotubes + glass carbon electrode	N.D.	0.13 mM	145 μA mM^{-1} cm^{-2}	[92]
Amperometric	AuNPs/ferrocenyl-dendrimer/Pt or GC electrodes	10 μM to 5 mM	2.0×10^{-7} M	44.8 $\mu A \mu M^{-1}$	[93]
Amperometric	MnO_2 decorated graphene oxide (MnO_2/GO) nanocomposite + screen printed electrode	0.1 μM to 1 μM and 1 μM to 1000 μM	0.09 μM	1.25 $\mu A \mu M^{-1}$ cm^{-2} and 0.005 $\mu A \mu M^{-1}$ cm^{-2}	[94]
Amperometric	Au nanoparticles + 3D flower-like structure graphene+ glassy carbon electrode (GCE) (Au/f-GE/GCE)	0.125–20375.98 μM,	0.01 μM	N.D.	[95]

Jaiswal et al. fabricated a screen-printed sensor utilizing bulk-modified MnO_2 decorated graphene oxide (MnO2/GO-SPE) nanocomposite for the on-site monitoring of nitrite.[94] Zou et al. developed a highly sensitive nitrite biosensor based on GNPs decorated flower-like graphene.[95]

Conclusion and future directions

Elevated concentration of nitrite in the drinking water and packaged food is noxious to human health, especially infants. Thus, it is obligatory to limit the continuous rise of nitrite levels in the surrounding environment as well as insuring the safety standards of public water systems and food products. The diagnostic diligence has been challenged to develop newer approaches that display accuracy, sensitivity, selectivity, rapidity and cost-effectiveness for nitrite quantification to overcome the shortcomings of centralized conventional methods. Immense potentiality of enzymes- and nanomaterials-based biosensors as an analytical tool for nitrite analysis has boosted scientific enthusiasm toward evolution of miniaturized, optimized and field portable sensor technologies for environment monitoring, water and food safety standards. The synergistic outcome of nanotechnology and bioelectronics has exposed new prospects for developing disposable nitrite biosensors with excellent performance. It is anticipated that the nanomaterials such as nanorods, nanowires, nanoparticles, nanohybrids, CNTs and nanocomposites will emerge as powerful elements of future bioelectronic devices as they helps in improving the sensitivity of a biosensor, which is an ever-lasting goal in research field. The employment of enzymes such as HRP/Catalase, cytochrome c, SOD and nitrite reductase in fabricating biosensors has proved momentous as they modify surface of working electrodes to circumvent the non-specific adsorption and enhancing selectivity. The employment of enzyme-based biosensors has dominated over the other analytical devices as a valuable technique for quantitative analysis of nitrite due to their brilliant specificity as well as efficient catalytic properties. Next step of development should include achievement of multiplexing in this field so that a large number of assays can be performed simultaneously, which is significant for saving assay time. The prime challenge is to develop an ideal highly automated, miniaturized, sleek and economic wearable biosensor that can be commercialized for the point of care testing of nitrite. In near future, the user-friendly, inexpensive and disposable biosensing devices will undoubtedly draw attention in diagnostic field of health area for commercial successes.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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