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Mini Review: Nitrite Reductase and Biosensors Development

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ABSTRACT

Nitrite ions have emerged as one of the major problems in environmental and pollution issues nowadays. Food products are the major source of these ions to preserve color. However, there is a maximum permissible limit (MPL) for the use of nitrite in food. A lot of works in the literature have been carried out on nitrite quantification and most of them are using simple and quick analysis methods. Recently, the advent of biosensor has allowed the fabrication of sensors for the detection of nitrite. This paper specifically reviews the interest in the usage of the nitrite reductase enzyme as a biorecognition element for the application of nitrite biosensors.

INTRODUCTION

As one of the well-known inorganic pollutants, widespread presence of high level of nitrite ions (NO_2^-) has turned this ion into a toxic compound in the human body. Vermeer et al. [1] reported that NO_2^- was converted to the carcinogenic *N*-nitrosamines in stomach and reacts with hemoglobin in blood. When NO_2^- irreversible reacts with hemoglobin, methemoglobin is produced due to oxidation of divalent ferrous (Fe_2^+) ion to form trivalent ferric (Fe_3^+) ion [2, 3]. Methemoglobin caused oxygen binding blockage and interferes with oxygen transportation in the blood circulation. This disease is predominantly found in infants and is known as "blue baby syndromes" [4, 5, 6]. Chemical reactions of NO_2^- ion leads to nitric oxide (NO) metabolism which has critical roles in physiological processes and human disease pathophysiology. For example, NO is needed to regulate cardiovascular functions, infectious and degenerative disorders.

NO_2^- is a univalent radical compound and is known as sodium nitrite (NaNO_2) industrially. It is commonly found in soil and water due to its application as preservatives [7] and fertilising

agents [8]. Besides this, it is also used in the diazo dye manufacturing, photography, metal coating and rubber chemical industries [9]. Effects of continuous exposure to this compound is divided into three distinct fields: environmental risks, health hazards and physiological aspects. Environmentally, excess NO_2^- levels caused an unbalanced biogeochemical nitrogen cycle during denitrification/ nitrification as nitrate (NO_3^-) is commonly used as fertilizers [10]. Contamination of groundwater supplies also leads to excessive NO_3^- which converts into NO_2^- in the environment and these highly soluble ions are found in natural aquifers.

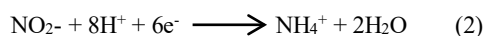
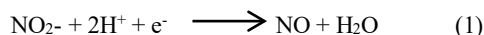
Biosensor is classified as one of the important analytical devices with the ability to record and transmits information of physiology, biological or chemical changes [11]. Thevenot [12] has stated that a biosensor is capable in providing quantitative or semi-quantitative analytical information with the help from biological element used, for examples DNA, enzyme and antibody.

Nitrite determination had been reported using different methods such as titrimetric [13], chromatographic [14], spectrophotometric [15, 16, 17] and electrochemical [18, 19]. The most common techniques were spectrophotometric using Griess

reagent where azo-dyes were produced by the reaction of sulfanilamide and *N*-(1 naphthyl) ethylenediamine with nitrite [20- 22]. These previous methods however require sample pre-treatment which is not an option if *in-situ* detection is to be done. Other than that, these methods are also neither subject to interferences nor are expensive and time consuming. Nitrite biosensor is one of the fast-growing technologies with applications in broad field such as agricultural, health care, environmental and food industry.

Why nitrite reductase enzyme?

The nitrite reductases (NiRs) enzyme are involved in nitrogen cycle and is consider as one of the key metabolic enzymes in plants. To date, NiRs acts in denitrification of nitrogen cycle processes and is classified to cytochrome *cd*₁ nitrite reductase with heme at work and copper nitrite reductase with copper as cofactor [23, 24]. Averill [25] and Wu et al. [26] had classified copper-containing nitrite reductase enzymes catalyzed both the one electron reduction of nitrite to NO as reaction 1 below, while cytochrome *cd*₁ nitrite reductase catalyzes a six-electron reduction of nitrite to ammonium (NH₄⁺) as shown in reaction 2. Both classes of NiRs have never been found together in the same microorganisms and are present based on their adaptability towards copper and iron [27].



Copper-containing nitrite reductase (Cu NiR) is a homotrimer structures enzyme containing both type-1 and type-2 copper sites with subunits molecular mass of between 30-40 kDa [25]. Each type is classified based on its spectroscopic character of four blue type I copper sites (Cys, His, His, and Met ligands) within subunits and three mononuclear type II copper sites (three His ligands) at the subunits interface [28-30]. The enzyme is commonly isolated from *Achromobacter cycloclastes*, *Alcaligenes faecalis*, *Pseudomonas* sp. and *Rhodobacter sphaeroides* and exists in both gram-positive [31, 32] and gram-negative bacteria [33]. Type-1 copper site is responsible for accepting electrons from an electron donor and transfer it to type-2 copper site to build an active site of three His ligands within it. The reaction is catalyzes bidirectionally [34]. Previously, Asterir *et al.* [35] and Silveira & Almeida [36] use this Cu-NiR enzyme in nitrite biosensor development.

Cytochrome *cd*₁ nitrite reductase (ccNiR) is also known as ferredoxin: nitrite oxidoreductase with the ability to catalyze the six-electron reduction of nitrite to ammonium ions [37]. Ammonia was produced through assimilatory or dissimilatory processes and NO is through the dissimilatory pathway. Nitrite reduction is measured when nitric oxide is released in iron of *d1* heme, and released as reaction products [38]. The same paper also reported that nitrite binding to iron involved proton transfer to one of the oxygen in nitrite forming water as a product. The most common source for ccNiR are plants. However, Blackmore *et al.* [39] has purified the enzyme from the bacterium *Wolinella succinogenes* which is found in cow's rumen. The purified enzyme is reported with a high yield with activity detected in high and low *M_r* fractions. It is classified in this group due to its spectral characteristics showing *c*-type haem groups.

Immobilisation methods of enzyme

Enzyme immobilisation was developed to overcome problems to reuse enzyme and became one of the important process involved in biosensors development. Immobilisation of either enzymes or whole cells is done by confinement of the elements on matrix [40, 41]. Immobilization of enzymes improved the biological element's stability, half-life and allowed it to work on a larger environment [42]. The most common chemical method used includes covalent binding and cross-linking (Fig. 3.1). Covalent binding appears when enzymes functional groups like imidazole, indolyl, phenolic hydroxyl, etc. form stable bonds with enzymes or microorganisms [41, 43]. It can also be illustrated as biological element's wall such as amine, carboxylic or sulphhydryl against the transducer used, for examples amine, carboxylic, epoxy and tosyl [44]. However, problems might occur when whole cells are used due to direct exposure towards harmful chemical and harsh reaction environment, thus affecting their viability [44, 45]. This method has the lowest cell loading achievement reported.

Cross-linking is also classified as one of the chemical methods for enzyme immobilisation which is achieved by intermolecular cross-linking of protein towards either protein molecules or matrix's functional group. D'Souza [45] reported that cross-linking techniques suitable in immobilisation of active intracellular enzymes found in non-viable cell during microbial biosensor development. This irreversible method is able to decrease costs but still increased time specific and volumetric activity of biocatalysts [46]. Besides easier to be recycled and of controllable size, this method comes with a high price of several disadvantages. The main disadvantages of cross-linking binding can be seen when aggregate or crystal sizes were increase and affecting its diffusions. Sheldon [47] also stated that the cross-linking enzyme have low activity retention, poor reproducibility and low of mechanical stability besides hard to handle.

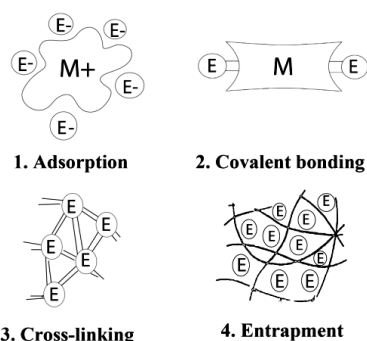


Fig. 1. All four main immobilization methods often used in enzyme (E) fabrication on a matrix/ supports (M).

Fig. 1 shows the four main methods in enzyme immobilization. This review will focus on adsorption and entrapment methods only. Adsorption is the simplest immobilization method for organic material, but has weak bonding. This method does not involve any covalent bond formations, making it much different from previous two methods reported. Physical adsorption involved of microbial suspension onto electrode or matrix and rinsed with buffer. Immobilization

occur if adsorptive interaction such as polar, ionic or hydrogen bonding and hydrophobic interactions. These interactions are considered as weak forces but with efficient binding process [46]. Entrapment is also classified as physical immobilization method with entrapped of particles or cells in support matrix using dialysis or filter membrane. Studies on this method aim for particle arrangement control. The main disadvantages of this physical method are that they offer lower sensitivity and detection limit of the developed biosensors [48].

Common sensing techniques

Biosensor development has grown enormously day by day, with different sensor type or systems representing its sensing techniques. The types of biosensors developed differ on the different type of transducers used.

i) Amperometric biosensor

Amperometric biosensor is one of the common electrochemical transducers used and most widespread biosensors developed. In amperometric biosensors, enzyme-catalyzed electro-oxidation or current produced, allowed the reaction of electroreduction measured [49, 50]. Fundamentally, when a potential applied between two electrodes (working electrode and reference electrode), current produced is carried between electrodes. A biocatalyst separated the electrode compartment, for example glucose oxidase, which is permeable towards oxygen. Enzyme electrodes are often used in amperometric biosensor as reported by Wang [51]. The sensor sensitivity towards analytes had encouraged researchers to develop more sensors based on this method [52, 53] and parts of it is summarized in **Table 1**.

Table 1. Amperometric nitrite biosensor.

Protein	Sensor preparation	Detection limit	Ref.
Hb	GC/mesoporous silica / PVA / protein	0.61 μ M	[54]
Hb	GC/ colloidal silver nanoparticles / protein/ titania sol-gel	34 μ M	[55]
Hb	GC/ CdS nanospheres/Nafion/ protein	0.08 μ M	[56]
Hb	1D Au nanoparticles electrodeposition/ alumina template	6.5 \times 10 ⁻³ M	[57]
Mb	GC/ hexagonal mesoporous silica / PVA / protein	0.8 μ M	[58]
Mb	graphite/nanoporous ZnO electrodeposition/protein dip coating	4 μ M	[59]
Mb	MWCNT/ cysteamine/ Nafion modified gold electrode	0.01 μ M	[60]
ccNiR	GC/ [ZnCr-AQS] LHD /enzyme/ glutaraldehyde	4 nM	[61]
ccNiR	pyrolytic graphite/ EETMS sol/ enzyme	120 nM	[62]
ccNiR	graphite and mediator composite/ enzyme/ poly(carbamoylsulfonate) hydrogel membrane	1 mM	[63]
ccNiR	Gold casting/ Nafion/ Cu-LDH / enzyme	2 \times 10 ⁻⁷ M	[64]
cd ₂ NiR	graphite/enzyme entrapment /mediator	N.D	[65]
cd ₂ NiR	graphite/enzyme entrapment / mediator	10 mM	[63]
cuNiR	GC/ poly(vinyl alcohol) / mediator / enzyme/ poly(allylamine hydrochloride)/ hydrophilic polyurethane	1.5 mM	[66]
cuNiR	gold/enzyme entrapped /mediator	0.22 mM*	[67]

Note: Hb for Haemoglobin; Mb for Myoglobin

ii) Potentiometric biosensor

Potentiometric biosensor is also classified as one of the electrochemical biosensors developed extensively nowadays. Potentiometric differ than amperometric biosensor due to its

electrical changes either based on measured voltage between electrodes (potentiometric) or changed in measured current at fixed voltage (amperometric) [68]. This biosensor is divided into two types based on ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET) [69, 70]. Ion-selective electrodes were used to detect changes in biosensor charge. Those electrodes changed the biological reaction present in the sensor into an electrical signal, for example pH meter. The collected electrical potential was measured without interference and at very high impedance allowing zero current effectively flowing [71]. **Table 2** shows some of the examples of developed nitrite potentiometric sensors.

Table 2. Potentiometric nitrite biosensor.

Protein	Sensor preparation	Detection limit	Ref.
SirohemeNiR	enzyme/ BSA /glutaraldehyde	- (Linear range:0.1–50 mM)	[72]
Hydroxylamine oxidase (HAO)	HAO / ZrO ₂ NPs/CPE electrodes	- (Linear range: 3 - 117 μ M)	[73]
-	Sol-gel /Chloro (5, 10, 15, 20 Tetraphenylporphyrinato) Cobalt (III)	- (Selectivity between 10 ^{-5.4} and 10 ^{-1.0} M)	[74]
-	PVC /Cobalt(III) 5,10,15-tris(4-tert-butylphenyl) corrole	5 μ M	[75]

CONCLUSION

Biosensors have become important tools in technology especially in the field of ion detection for environmental quality; in medicine and industry. The various formats of biosensor development especially on nitrite ions detection has revolutionized the detection of this ion and it is expected that further improvement of biosensor technology would allow an even more sensitive detection of this ion.

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