Biosensors and Bioelectronics 24 (2009) 1574–1579

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

A novel nitrite biosensor based on conductometric electrode modified with cytochrome *c* nitrite reductase composite membrane

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ARTICLE INFO

Article history:
Received 23 May 2008
Received in revised form 5 August 2008
Accepted 6 August 2008
Available online 15 August 2008

Keywords:
Conductometric biosensor
Nitrite
Cytochrome *c* nitrite reductase (*cc*NiR)
Bovine serum albumin
Nafion®
Methyl viologen

ABSTRACT

A conductometric biosensor for nitrite detection was developed using cytochrome *c* nitrite reductase (*cc*NiR) extracted from *Desulfovibrio desulfuricans* ATCC 27774 cells immobilized on a planar interdigitated electrode by cross-linking with saturated glutaraldehyde (GA) vapour in the presence of bovine serum albumin, methyl viologen (MV), Nafion®, and glycerol. The configuration parameters for this biosensor, including the enzyme concentration, *cc*NiR/BSA ratio, MV concentration, and Nafion® concentration, were optimized. Various experimental parameters, such as sodium dithionite added, working buffer solution, and temperature, were investigated with regard to their effect on the conductance response of the biosensor to nitrite. Under the optimum conditions at room temperature (about 25 °C), the conductometric biosensor showed a fast response to nitrite (about 10 s) with a linear range of 0.2–120 μM, a sensitivity of 0.194 μS/μM [NO_2^-], and a detection limit of 0.05 μM. The biosensor also showed satisfactory reproducibility (relative standard deviation of 6%, $n = 5$). The apparent Michaelis–Menten constant ($K_{M,app}$) was 338 μM. When stored in potassium phosphate buffer (100 mM, pH 7.6) at 4 °C, the biosensor showed good stability over 1 month. No obvious interference from other ionic species familiar in natural waters was detected. The application experiments show that the biosensor is suitable for use in real water samples.

1. Introduction

Water containing a high concentration of nitrite can create serious problems, such as eutrophication and potential hazards to human health (Carpenter et al., 1998). Eutrophications in rivers, lakes, and costal waters have become one of the most prevalent environmental problems (Carpenter et al., 1998; Vitousek et al., 1997). On the other hand, with many potential hazards to human health, nitrite has been considered to be a main toxic agent (Almeida et al., 2001). Nitrite promotes the irreversible oxidization of hemoglobin to methemoglobin and reduces the blood capacity to transport oxygen (Amine and Palleschi, 2004). In addition, nitrite in the body can be converted into carcinogenic *N*-nitrosoamine compounds (Almeida et al., 2007). Consequently, there is a growing demand to detect nitrite in food, drinking water and environmental samples. The European Community, for instance, has established the maximum admissible level of nitrite in drinking water to 0.1 mg/L (Da Silva et al., 2004).

The present analytical technologies for nitrite detection mainly contain spectrophotometry (Griess reaction), ionic chromatography, polarography, capillary electrophoresis, and fluorescence spectrophotometric methods (Moorcroft et al., 2001). However, they all need centralized and sophisticated analytical systems, presenting delayed results in solving problems in emergency situations where the rapid diagnostic of diseases, food quality control or environmental pollution monitoring are issues of critical concern (Connolly and Paull, 2001; Monaghan et al., 1997; Stratford, 1999). As a consequence, a promising alternative for nitrite detection is the

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doi:10.1016/j.bios.2008.08.010

development of analytical probes based on the recognition properties of highly selective biological macromolecules, such as nitrite reductases (NiRs) (Almeida et al., 2001).

For the past decades, there has been a growing interest in the design of biosensors which intimately combine the recognition properties of biological macromolecules with the sensitivity of transducers. One of the most popular configurations of biosensors consists of enzyme immobilized within a polymer on an electrode. NiRs are natural candidates to build specific nitrite biosensors (Scharf et al., 1995; Wu et al., 1997). NiRs may be classified by their reaction products (NO or NH_4^+) or their prosthetic groups (siroheme, heme *c*, heme *cdl*, copper, etc.).

$$NO_2^- + 8H^+ + 6e^- \xrightarrow[\text{or heme } c\text{-NiR}]{\text{siroheme-NiR}} NH_4^+ + 2H_2O \quad (1)$$

$$NO_2^- + 2H^+ + e^- \xrightarrow[\text{or Cu-NiR}]{\text{heme } cdl\text{-NiR}} NO + H_2O \quad (2)$$

Cytochrome *c* nitrite reductase (*cc*NIR) is a multiheme enzyme that converts nitrite into ammonia on each active site (Reaction (1)) (Atkins et al., 2006). *cc*NiR exhibits several advantages as the biorecognition element of a nitrite biosensor: well-defined chemical reaction, high-specific activity, good stability and large yield (Almeida et al., 2007). The first example of a nitrite biosensor based on *cc*NiR was reported by Scharf et al. (1995). Strehlitz et al. (1996) reported a list of possible artificial redox mediators that shuttle electrons from *cc*NiR to the electrode, thus allowing the development of mediated biosensors. Several amperometric nitrite biosensors based on *cc*NiR and artificial redox mediators have been reported (Almeida et al., 2007; Chen et al., 2007; Da Silva et al., 2004). The development of amperometric nitrite biosensors, however, is faced with the major obstacles of the poor stability and of the difficulty in establishing an electrical communication with the immobilized reductase.

On the other hand, conductometric biosensors have been reported increasingly (Muhammad-Tahir and Alocilja, 2003; Wang et al., 2006; Zhang et al., 2008). The principle of the detection is based on the fact that many biochemical reactions in solution produce changes in the electrical resistance (the reciprocal of conductance) (Wang et al., 2006). To our best knowledge, no previous work describing the immobilization of *cc*NiR composite membrane on an electrode resulting in a conductometric nitrite biosensor has been reported to date.

In this work, we present a conductometric nitrite biosensor based on the co-immobilization of *cc*NiR, bovine serum albumin, Nafion®, MV, and glycerol with saturated glutaraldehyde vapour on an interdigitated electrode. *cc*NiR is usually purified and stored in an oxidized form. However, this enzyme will only be active in a reduced state, which can be achieved with artificial electron donor such as MV in the reduced state ($MV^{\bullet+}$). MVs electrochemical behavior involves reduction of oxidated state (MV^{2+}). With sodium dithionite, MV^{2+} can be reduced to $MV^{\bullet+}$, which further reduces *cc*NiR to the active state (Brunetti et al., 2000; Ferreyra et al., 2000). In addition, since viologens are highly water soluble, any practical device containing the electron mediator should be based on immobilized viologens (Mao et al., 1999). The structure of MV^{2+} contains a hydrophobic part which is capable of hydrophobic–hydrophobic interaction with Nafion® and two cationic pyridinium groups that undergo ion exchange with the sulphonate sites of Nafion® polymer chains, according to the following reaction (Wang et al., 2006):

$$MV_{aq}^{2+} + 2(SO_3^-Na^+)_{film} \rightarrow [(SO_3^-)_2MV^{2+}]_{film} + 2Na_{aq}^+ \quad (3)$$

This interaction results in MV^{2+} accumulation in the membrane. In the presence of sodium dithionite as electron donor, the biocatalyzed reduction of NO_2^- to NH_4^+ is stimulated. The reaction is as follows:

$$NO_2^- + 6MV^{\cdot+} + 8H^+ \rightarrow NH_4^+ + 6MV^{2+} + 2H_2O \quad (4)$$

The subsequent local changes of conductance inside the enzymatic membrane are dependent on Reaction (4), and thus a

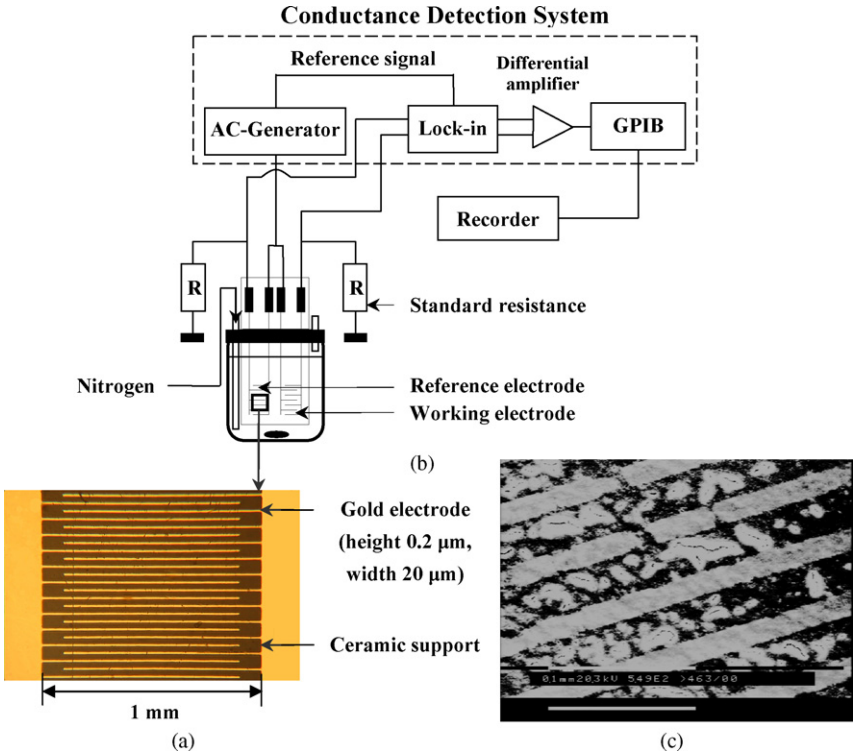

Fig. 1. (a) Optical microscopy view of the interdigitated electrode; (b) Schematic view of the conductance detection system; and (c) SEM image of the modified electrode.

conductometric nitrite biosensor is conceived. In light of conductance response to nitrite, the concentrations of the constituents in the enzymatic membrane will be optimized, and the characteristics of the developed biosensor will be investigated. At last, the performance of the biosensor will be evaluated by the detection of nitrite in some real water samples.

2. Experimental

2.1. Reagents

Cytochrome *c* nitrite reductase (ccNiR, 1.0 mg/mL, 150 U/mg) was extracted from the sulfate-reducing bacteria *Desulfovibrio desulfuricans* (*Dd*) ATCC 27774 in Faculdade de Ciênciase Tecnologia, Universidade Nova de Lisboa, Portugal and stored in potassium phosphate buffer (PBS, 100 mM, pH 7.6) at −20 °C. Bovine serum albumin (BSA), glutaraldehyde (GA), methyl viologen (MV), Nafion®, glycerol, and sodium dithionite were purchased from Sigma–Aldrich Chemie GmbH. Potassium nitrite and sodium bicarbonate were purchased from Merck. All chemicals were of analytical grade. Millipore Milli-Q nanopure water (resistivity 18.2 MΩ cm) was used throughout for the preparation of the solutions.

2.2. Apparatus

The measurement setup is shown in Fig. 1. The interdigitated electrodes (Fig. 1(a)) were fabricated at the Institute of Semiconductors Physics, Kiev, Ukraine. A pair of Au (150-nm-thick) interdigitated electrodes was made by lift-off photolithography on a ceramic support (10 mm × 30 mm). A 50-nm-thick intermediate Cr layer was used to improve the adhesion of Au to the support. The pads of the sensor chip were covered manually with epoxy resin for their insulation. Both the digit width and the interdigital distance were 20 μm, and their length was about 1 mm. As a result, the sensitive area of each electrode was about 1.0 mm^2. A low-frequency wave-form generator (Schlumberger, type 4431) was used to generate a sinusoidal wave with a frequency of 100 kHz and a peak-to-peak amplitude of 10 mV around a fixed potential of 0 V to each sensor, forming a miniaturized conductance cell (Fig. 1(b)).

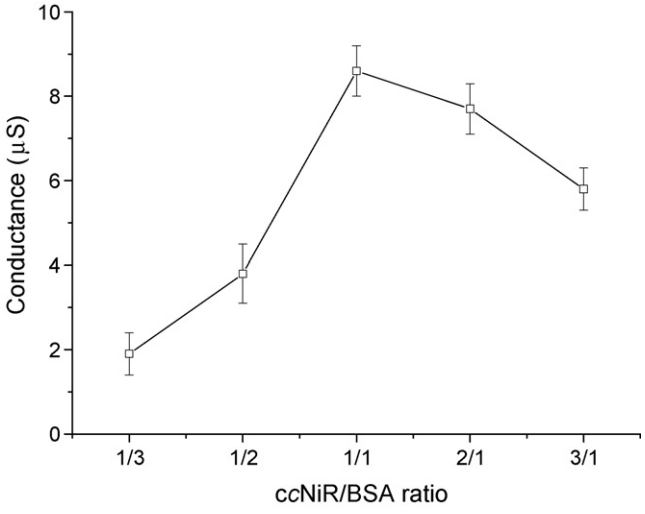

Fig. 2. Dependence of the conductometric nitrite biosensor performance on ccNiR/BSA ratio in the enzymatic membrane. Measurements were performed with 50 μM nitrite in PBS (5.0 mM, pH 7.6) at room temperature.

2.3. Biosensor preparation

The enzymatic membrane was prepared on the transducer surface by the cross-linking of the enzymatic mixture in saturated GA vapour (Dzyadevych et al., 1994). The enzyme was deposited together with BSA, MV, Nafion®, and glycerol in order to obtain a stable and active enzymatic membrane. A mixture of ccNiR and BSA (ccNiR/BSA ratio equal to 1:1), 10% (w/v) MV, 3% (v/v) Nafion®, and 10% (v/v) glycerol in PBS (100 mM, pH 7.6) was deposited on the sensitive area of the working sensor using a microlitre syringe, while another mixture of BSA (two times weight of the enzyme), 10% (w/v) MV, 3% (v/v) Nafion®, and 10% (v/v) glycerol in PBS (100 mM, pH 7.6) was similarly deposited on the other sensor, which was configured to be the reference sensor. The sensor chips were placed in saturated GA vapour for 40 min followed by drying in air for 10 min at room temperature (about 25 °C). The prepared biosensors (Fig. 1(c)) were then stored in PBS (100 mM, pH 7.6) at 4 °C.

2.4. Nitrite measurement

Except especial note, all nitrite measurements were carried out in a closed glass cell at room temperature. 5 mL of magnetic-stirred PBS (5.0 mM, pH 6.5) was put into the cell and purged using a nitrogen flux for at least 15 min before use. To avoid any oxygen interference, the closed cell was maintained under nitrogen during the entire experiments. Injections of nitrite solutions were performed with a syringe. The sensor chip was immersed in the measuring cell and nitrite concentrations were adjusted by adding defined volumes of stock nitrite solution (1 mM). After the stabilisation of the output signal, freshly prepared sodium dithionite (200 mM) was added in the vessel, which initiated the series of reducing reactions. Since the working electrode and the reference electrode were integrated on the same ceramic support, the differential output signal between the working sensor and the reference sensor was logged by SR 510 lock-in amplifier (Stanford Research System) and the conductance response (C) was calculated by (Eq. (5)).

$$C = (C_n - C_0) \quad (5)$$

where C_n is the conductance value obtained in the presence of nitrite and C_0 is the conductance value obtained in the absence of nitrite.

The steady-state conductance response of the biosensor was plotted as a function of the nitrite concentration.

3. Results and discussion

3.1. Optimization of the enzymatic membrane

Without enzyme nor mediator in the composite membrane, no conductance signal was observed, implying that no direct reduction of NO_2^- occurred. These phenomena indicate that the conductometric biosensor detection process is nitrite-dependent and enzyme-catalyzed. BSA can disperse enzyme to keep an appropriate distance between enzyme molecules, so as to obtain the maximum enzymatic activity (Zhang et al., 2008). 10% (v/v) of glycerol has been used in various conductometric biosensors to maintain the humidity in the membrane and protect the enzymatic activity (Anh et al., 2004; Dzyadevych et al., 2001; Wang et al., 2006; Zhang et al., 2008), which was also used in this study.

In view of both energy and time consumed to concentrate ccNiR solution and the strong signal at the concentration of 10 mg/mL, this enzyme concentration was fixed in all the following experiments. The ratio of ccNiR/BSA in the enzymatic membrane was

varied to investigate its effect on the performance of the conductometric nitrite biosensor (Fig. 2). It can be seen that the maximum of the enzyme activity was achieved when ccNiR/BSA ratio equaled 1:1. For a lower ccNiR/BSA ratio, more protein may lead to an increase of the diffusional resistance for the substrate to arrive at the electrode surface and then to a decrease in the biosensor response (Giovannoni et al., 1997). On the other side, for a higher ratio, the aggregation of the enzyme molecules may make them become too tightly bonded, leading to most of the active sites in the enzyme being sealed (Zhang et al., 2008). As a result, the ratio of ccNiR/BSA was settled to 1:1 and was used in the following experiments.

The dependence of the biosensor performance on the mediator concentration in the enzymatic membrane was investigated. The applied MV concentration of 10% (w/v) was sufficient to saturate the working electrode surface with mediator molecules. A decrease in MV concentration was not enough compared to the quantity of ccNiR molecules, whereas an increase of the mediator concentration did not lead to any further increase in the sensor response. Therefore, MV concentration of 10% (w/v) in the enzymatic membrane was fixed and used in the following experiments.

The effect of the Nafion® concentration in the enzymatic membrane on the biosensor response to nitrite was investigated. The maximum conductance response of the biosensor to nitrite occurred at the concentration of 3% (v/v) of Nafion® in the enzymatic membrane. A lower Nafion® concentration means that less MV could be reserved by Nafion® in the enzymatic membrane. On the other hand, in view of the electrostatic repulsion of the sulphonate groups, Nafion® ionomers have been commonly used in the biosensor field as a membrane barrier, preventing interferences from anionic species, such as ascorbate, urate and nitrite itself (Manowitz et al., 1995; Matsumoto et al., 2001; Pan and Arnold, 1996; Park et al., 1998; Xu et al., 2002). A higher Nafion® concentration may then repulse NO_2^- to lay aboard the enzymatic membrane and block the reduction reaction. The good results at 3% Nafion® concentration may be an outcome of neutralization of the negative charges by the cationic viologens, which makes the enzymatic membrane slightly permeable to NO_2^- (Almeida et al., 2007). Therefore, Nafion® concentration of 3% (v/v) in the enzymatic membrane was fixed and used in the following experiments.

3.2. Influence of measurement parameters

Since the oxidated viologens (MV^{2+}) need to be converted to the reduced state ($MV^{\bullet+}$) first in this study with sodium dithionite, the addition of sodium dithionite was investigated. The concentration of sodium dithionite was varied between 0 and 5.0 mM. In the absence of sodium dithionite, no response was observed, while for concentrations higher than 1.0 mM of sodium dithionite, the response became constant, and this addition (1 mM) was used for all the following experiments.

As the enzyme activity is strongly affected by the solution pH, the effect of pH on the biosensor response was examined with 50 μM nitrite in 5.0 mM PBS. The maximum response of the biosensor based on the composite membrane occurred at pH 6.5. This result strongly corresponds to the optimal working pH for ccNiR obtained by Chen et al. (2007). pH 7.6 is the best value for storing ccNiR and maintaining its activity, while Reaction (4) indicates that the nitrite reduction requires consuming a lot of H^+. As a consequence, the compromise pH for the above two factors was achieved at pH 6.5, assuring a good response of the conductometric biosensor to nitrite. We used the buffer solution of pH 6.5 throughout the following experiments to obtain the maximum sensitivity.

The influence of the concentration of the buffer solution on the biosensor response was also investigated. The best sensitivity

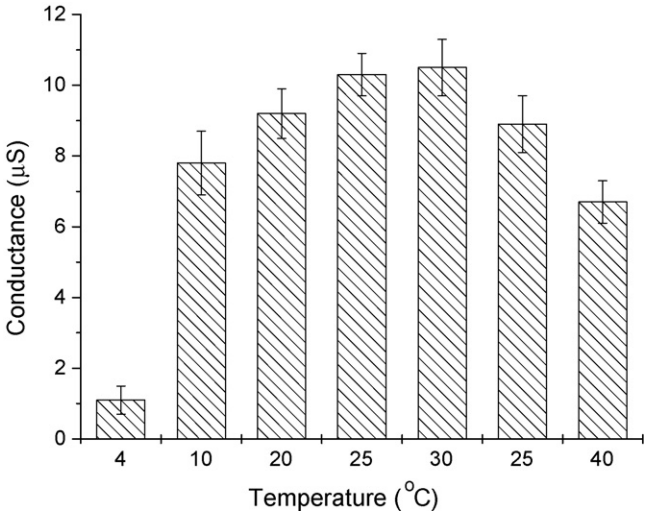

Fig. 3. Effect of temperature on the conductance response of the nitrite biosensor. Measurements were performed with 50 μM nitrite in PBS (5.0 mM, pH 6.5).

was obtained when the PBS concentration was 5.0 mM, which was also used in all the following experiments. Such dependence of the response on the buffer concentration is due to a kind of "carrier-mediated" transport of protons out of the enzymatic membrane in the presence of mobile buffer species (Soldatkin et al., 1994). It means that when the buffer species are present in the solution, protons can associate with them and this is "an additional channel" for their diffusion out of enzymatic membrane (the first one being the diffusion of protons as free ions). A decrease in the ionic strength would result in a weak buffer function, while an increase in the ionic strength would result in an increase in the overall conductivity of the solution and a decrease in the sensitivity (Dzyadevych et al., 2001).

Fig. 3 shows the effect of temperature on the sensitivity of the same biosensor. Like most of enzymes, the activity of ccNiR is related to temperature. The biosensor worked well in the temperature range of 20–35 °C; the best response was achieved at 30 °C. But once the temperature over 40 °C, the activity of ccNiR sharply

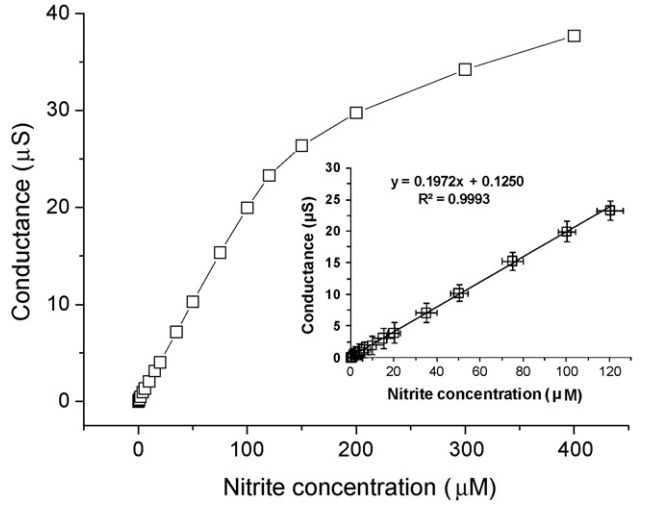

Fig. 4. Calibration curve of the optimized conductometric nitrite biosensor. Measurements were performed in PBS (5.0 mM, pH 6.5) at room temperature. *Note*: The vertical and horizontal bars refer to the relative standard deviations between measurements done with the same biosensor and between measurements done with different biosensors prepared in the same conditions, respectively.

decreased. These results are also consistent with the study of Chen et al. (2007).

Consequently, the optimal conditions for nitrite measurement are as follows: 1-mM sodium dithionite as the electron donor to initiate the serial reactions, PBS (5 mM, pH 6.5) as the working buffer solution, 30 °C as the working temperature.

3.3. Calibration curve

As can be seen from Fig. 3, the conductance response obtained at room temperature (about 25 °C) is very close to that obtained at 30 °C. To simplify the experiment operations, the calibration cure was plotted under the optimal conditions except for the temperature which was room temperature (Fig. 4). From this figure, the linear regression equation is: $C\,(\mu S) = 0.1250 + 0.1972\,[NO_2^-]\,(\mu M)$, $R^2 = 0.9993$. The optimized biosensor exhibited a fast response to nitrite (about 10 s), a linear range of 0.2–120 μM nitrite, a sensitivity of 0.194 μS/μM $[NO_2^-]$, and a low detection limit of 0.05 μM. The same sensitivity was obtained for five different biosensors prepared in the same conditions with a relative standard deviation of around 6%.

At high nitrite concentrations, the conductance response deviated from linearity and approached a constant value reflecting the maximum rate of the enzymatic reaction under saturating nitrite conditions. As most enzymatic reactions can be modelled with Michaelis–Menten equation (Eq. (6)), the data of the whole curve were plotted using Lineweaver–Burk method (Eq. (7)).

$$C = \frac{C_{max} \times [NO_2^-]}{K_{M,app} + [NO_2^-]} \quad (6)$$

$$\frac{1}{C} = \frac{K_{M,app}}{C_{max}} \times \frac{1}{[NO_2^-]} + \frac{1}{C_{max}} \quad (7)$$

The Lineweaver–Burk plot showed that $K_{M,app}$ and C_{max} were 338 μM and 80 μS, respectively. Apparent Michaelis–Menten constant $K_{M,app}$ can denote the affinity of enzymes to substrate. Higher the constant value is, worse the affinity of the enzymatic membrane in the biosensor to nitrite. The reported $K_{M,app}$ value of the amperometric nitrite biosensor based on ccNiR immobilized in the Nafion® matrix loaded with MV is 1.27 mM (Almeida et al., 2007). Compared with the amperometric nitrite biosensor, the conductometric nitrite biosensor showed better affinity to nitrite, which may be resulted from both the addition of BSA and the immobilization method used for the enzymatic membrane.

3.4. Biosensor stability

The long-term operational and storage stability is a key biosensor performance parameter. An exponential time decay of the enzyme activity caused by the thermal or chemical inactivation of the enzyme has been reported (McDonald and Coddington, 1974; Reinhard et al., 1986). The conductometric nitrite biosensor was stored in PBS (100 mM, pH 7.6) at 4 °C. In order to evaluate the long-term stability of the biosensor, the conductance at a fixed nitrite concentration of 50 μM was checked periodically with the same sensor (figure not shown). The conductance response of the sensor was found to be fairly stable during the first week, and then to drop gradually with time. After about 3 weeks, it still maintained over 50% of the initial response. Other experiments showed that the conductometric biosensor could be maintained at about 75% of the initial response for about 1 month when the checking was reduced to less than five times within that period. The reduced sensor response may then be partly related to both the lifetime of ccNiR and the progressive depletion of MV (Almeida et al., 2007).

3.5. Interference

Both ammonia and nitrate are often appearing simultaneously with nitrite in the same samples. Hence, the potential interference of ammonia and nitrate on the biosensor response was examined, as well as that of some other ions familiar in natural waters. The interference level was calculated in percentage by comparing the response of the biosensor to each of the potential interfering species with the one recorded in the presence of an equal amount of nitrite. The conductance responses produced by the ions from NH_4Cl, KNO_3, $CaCl_2$, K_2SO_4, K_2SO_3, Na_2CO_3, and $NaHCO_3$ were measured at the same concentration with nitrite (50 μM). All the ions caused no or negligible interference (<5% when compared to nitrite).

3.6. Comparison

It is interesting to compare the rather simple and cheap conductometric nitrite biosensor with recently reported amperometric nitrite biosensors using the same nitrite reductase ccNiR from *Dd* ATCC 27774 cells (Table 1). The linear range of the

Table 1
Comparison of the performance parameters between the conductometric nitrite biosensor and some amperometric nitrite biosensors reported in the literature

Type	Enzyme	Linearity (μM)	Detection limit (μM)	Response time (s)	Stability	Interference	Ref.
Amperometric	ccNiR	5.4–43.4	5.4	–	Full response for 4 days, and then dropped by 53%, 72% and 92% for 5, 6, and 7 days, respectively.	No obvious interference from nitrate and sulfite	Da Silva et al. (2004)
Amperometric	ccNiR	75–800	60	–	The response decreased progressively along 1 week, and ceased after 8 days	Neglected interference from nitrate and sulfite, but noteworthy interference from hydroxylamine (49%)	Almeida et al. (2007)
Amperometric	ccNiR	0.015–2.35	0.004	5	Retaining 60% of its initial activity after 32 days	No or negligible interference from chloride, nitrate, sulfate, bicarbonate and sulfite	Chen et al. (2007)
Conductometric	ccNiR	0.2–120	0.05	10	Retaining about 75% of the initial response after 1 month	No or negligible interference from ammonia, nitrate, chloride, sulphate, sulphite, carbonate and bicarbonate	This study

Table 2
Analytical results of the conductometric nitrite biosensor for four real water samples in France ($n = 5$)

Water sample	Initial concentration (μM)	Added concentration (μM)	Obtained concentration (μM)	Recovery (%)
Chaudanne River	0.7 ± 0.2	1.0	1.8 ± 0.3	106
Saône River	1.5 ± 0.2	1.0	2.6 ± 0.3	105
Givors Entrée	0.7 ± 0.3	1.0	1.9 ± 0.3	109
Givors Intermédiaire	2.8 ± 0.3	3.0	6.0 ± 0.4	107

poly(pyrrole–viologen)-nitrite reductase biosensor developed by Da Silva et al. (2004) was 5.4–43.4 μM at 30 °C. For the amperometric nitrite biosensor using the nitrite reductase/Nafion®/methyl viologen system developed by Almeida et al. (2007), it showed a linear range between 75 and 800 μM, and the lowest detected nitrite concentration was 60 μM. Both biosensors, however, showed no response to nitrite after only 7 days. Chen et al. (2007) reported an amperometric nitrite biosensor based on the electrical wiring of nitrite reductase with [ZnCr–AQS] LDH, which exhibited a linear range from 0.015 μM to 2.35 μM nitrite with a low detection limit of 4 nM. This nitrite biosensor retained 60% response over 32 days. In comparison, the conductometric nitrite biosensor, aside from expanding the linear range of the nitrite detection, also shows an instant response, a satisfactory reproducibility, a good stability of operation and storage.

3.7. Application

The optimized conductometric nitrite biosensor was used to analyze nitrite in four real water samples from four locations in France using the method of standard addition, following the procedure described in Section 2.4. Before any measurement, the samples were filtered through a PTFE membrane (0.45 μm), and purged by nitrogen flux for at least 15 min. The addition reclaim method was adopted to confirm the reliability of the biosensor. The results are listed in Table 2. As shown in this table, the recoveries (105–109%) for the analysis of nitrite in the samples are satisfactory. These results indicate that the developed conductometric nitrite biosensor can be successfully applied to the analysis of nitrite at the usual concentrations in water samples. Further practical use of this biosensor in determination of the nitrite concentration for on-site monitoring (river, lake, wastewater, etc.) is being investigated.

4. Conclusions

In the present paper, an original conductometric biosensor for the nitrite detection in water solution has been developed. The enzymatic mixture mainly consisting of ccNiR, BSA, MV, Nafion®, and glycerol was immobilized on an interdigitated electrode by cross-linking with saturated GA vapour. The proposed conductometric biosensor is relatively reliable and inexpensive. It has a linear range of 0.2–120 μM with a detection limit of 0.05 μM. The apparent Michaelis–Menten constant $K_{M,app}$ is 338 μM. The conductometric biosensor exhibited a satisfactory reproducibility as well as a long-term storage and operational stability. No obvious interference from other ionic species familiar in natural waters was detected. The application experiments show that the biosensor is suitable to be used for real water samples.

Acknowledgements

This work thanks the Programme de Bourses d'Excellence Eiffel (Ministère des Affaires Etrangères et Européennes, France), China Scholarship Council, the National Natural Science Foundation of China (20707014), ARCUS program (France), the Specialized Research Fund for the Doctoral Program of Higher Education of China (No. 20050247016) and the Program for New Century Excellent Talents in University of China (NCET-05-0387).

References

Almeida, M.G., Silveira, C.M., Moura, J.J.G., 2007. Biosens. Bioelectron. 22 (11), 2485–2492.
Almeida, M.G., Tavares, P., Lampreia, J., Moura, J.J.G., Moura, I., 2001. J. Inorg. Biochem. 86 (1), 121–1121.
Amine, A., Palleschi, G., 2004. Anal. Lett. 37 (1), 1–19.
Anh, T.M., Dzyadevych, S.V., Van, M.C., Jaffrezic-Renault, N., Duc, C.N., Chovelon, J.M., 2004. Talanta 63 (2), 365–370.
Atkins, P., Overton, T., Rourke, J., Weller, M., Armstrong, F., 2006. Biological Inorganic Chemistry, 4th ed. W.H. Freeman & Company, New York.
Brunetti, B., Ugo, P., Moretto, L.M., Martin, C.R., 2000. J. Electroanal. Chem. 491 (1–2), 166–174.
Carpenter, S.R., Caraco, N.F., Correll, D.L., Howarth, R.W., Sharpley, A.N., Smith, V.H., 1998. Ecol. Appl. 8 (3), 559–568.
Chen, H., Mousty, C., Cosnier, S., Silveira, C.M., Moura, J.J.G., Almeida, M.G., 2007. Electrochem. Commun. 9 (9), 2240–2245.
Connolly, D., Paull, B., 2001. Anal. Chim. Acta 441 (1), 53–62.
Da Silva, S., Cosnier, S., Almeida, M.G., Moura, J.J.G., 2004. Electrochem. Commun. 6 (4), 404–408.
Dzyadevych, S.V., Arkhypova, V.N., Korpan, Y.I., Elskaya, A.V., Soldatkin, A.P., Jaffrezic-Renault, N., Martelet, C., 2001. Anal. Chim. Acta 445 (1), 47–55.
Dzyadevych, S.V., Soldatkin, A.P., Shulga, A.A., Strikha, V.I., Elskaya, A.V., 1994. J. Anal. Chem. 49 (8), 789–792.
Ferreyra, N.F., Dassie, S.A., Solis, V.M., 2000. J. Electroanal. Chem. 486 (2), 126–132.
Giovannoni, G., Land, J.M., Keir, G., Thompson, E.J., Heales, S.J.R., 1997. Ann. Clin. Biochem. 34, 193–198.
Manowitz, P., Stoecker, P.W., Yacynych, A.M., 1995. Biosens. Bioelectron. 10 (3–4), 359–370.
Mao, L.Q., Jin, J.Y., Song, L.N., Yamamoto, K., Jin, L.T., 1999. Electroanalysis 11 (7), 499–504.
Matsumoto, T., Ohashi, A., Ito, N., Fujiwara, H., 2001. Biosens. Bioelectron. 16 (4–5), 271–276.
McDonald, D.W., Coddington, A., 1974. Eur. J. Biochem. 46 (1), 169–178.
Monaghan, J.M., Cook, K., Gara, D., Crowther, D., 1997. J. Chromatogr. A 770 (1–2), 143–149.
Moorcroft, M.J., Davis, J., Compton, R.G., 2001. Talanta 54 (5), 785–803.
Muhammad-Tahir, Z., Alocilja, E.C., 2003. IEEE Sens. J. 3 (4), 345–351.
Pan, S.T., Arnold, M.A., 1996. Talanta 43 (7), 1157–1162.
Park, J.K., Tran, P.H., Chao, J.K.T., Ghodadra, R., Rangarajan, R., Thakor, N.V., 1998. Biosens. Bioelectron. 13 (11), 1187–1195.
Reinhard, R., Florian, S., Frieder, S., 1986. Trends Biochem. Sci. 11 (5), 216–220.
Scharf, M., Moreno, C., Costa, C., Vandijk, C., Payne, W.J., Legall, J., Moura, I., Moura, J.J.G., 1995. Biochem. Biophys. Res. Commun. 209 (3), 1018–1025.
Soldatkin, A.P., Elskaya, A.V., Shulga, A.A., Jdanova, A.S., Dzyadevych, S.V., Jaffrezic-Renault, N., Martelet, C., Clechet, P., 1994. Anal. Chim. Acta 288 (3), 197–203.
Stratford, M.R.L., 1999. Methods Enzymol. 301, 259–269.
Strehlitz, B., Grundig, B., Schumacher, W., Kroneck, P.M.H., Vorlop, K.D., Kotte, H., 1996. Anal. Chem. 68 (5), 807–816.
Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Ecol. Appl. 7 (3), 14.
Wang, X.J., Dzyadevych, S.V., Chovelon, J.M., Jaffrezic-Renault, N., Chen, L., Xia, S.Q., Zhao, J.F., 2006. Electrochem. Commun. 8 (2), 201–205.
Wu, Q., Storrier, G.D., Pariente, F., Wang, Y., Shapleigh, J.P., Abruna, H.D., 1997. Anal. Chem. 69 (23), 4856–4863.
Xu, J.J., Yu, Z.H., Chen, H.Y., 2002. Anal. Chim. Acta 463 (2), 239–247.
Zhang, Z.Q., Jaffrezic-Renault, N., Bessueille, F., Leonard, D., Xia, S.Q., Wang, X.J., Chen, L., Zhao, J.F., 2008. Anal. Chim. Acta 615, 73–79.