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Polypyrrole-based bilayer nitrate amperometric biosensor with an integrated permselective poly-*ortho*-phenylenediamine layer for exclusion of inorganic interferences

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ABSTRACT

A bilayer amperometric nitrate biosensor with an integrated permselective layer has been developed for exclusion of inorganic anion and cation interferences. The inner PPy(polypyrrole)–NaR–NADH layer of the biosensor is formed by galvanostatic polymerization of pyrrole (Py) in presence of nitrate reductase (NaR) and nicotinamide adenine dinucleotide (NADH), followed by formation of the outer permselective poly-*ortho*-phenylenediamine (P-*o*-PDA) layer by potentiodynamic polymerization of *ortho*-phenylenediamine (*o*-PDA). The exclusion efficiency (E_{eff}) of the outer layer in rejecting inorganic cation and anion interferences is evaluated by a new proposed relationship. 73–87% and 47–84% of anion and cation interferences, respectively, were efficiently rejected with the permselective layer. Further improvement in the exclusion efficiency for cations was accomplished by combining the use of the outer layer with the addition of 1 mM EDTA into the measurement solution. The addition of EDTA improved the E_{eff} achieved for cation rejection by 10–40% to give net E_{eff} of 89–94%. The inclusion of the outer layer also aided the retention of NaR and NADH in the inner PPy–NaR–NADH layer and, hence, enabled improved amperometric detection of nitrate, achieving a detection limit of 0.20 μM and a linear concentration range of 10–500 μM with a 3.4% rsd (n = 10).

1. Introduction

The use of permselective P-*o*-PDA layer as protective coatings for amperometric biosensors has attracted considerable interest because of its insulating nature (Malitesta et al., 1990), ease of formation at a neutral pH, and compatibility with enzymes and other biomolecules (Vidal et al., 1999). Several studies have reported the development of amperometric biosensors by immobilisation of enzymes into electropolymerised P-*o*-PDA layers (Centonze et al., 1994; Malitesta et al., 1990; O'Neill et al., 2008; Yuqing et al., 2004; McAteer and O'Neill, 1996; Mailley et al., 2000; Pan et al., 2005; Yao and Takashima, 1998). However, due to the self limiting, and insulating film growth of P-*o*-PDA, the amount of enzyme incorporated is usually lower than those achieved by entrapment in conducting polymer films (Mailley et al., 2000; Pan et al., 2005; Yao and Takashima, 1998).

The combination of PPy and P-*o*-PDA has also been considered for solving interference problems (Adeloju and Moline, 2001). However, most studies have focused mainly on the rejection of organic interferences with P-*o*-PDA layer (Palmisano et al., 1995; Vidal et al., 1999; Garjonyte and Malinauskas, 1999, 2000; Vidal et al., 2001; Moussy et al., 1993). But, for amperometric biosensing of nitrate, the presence of inorganic interferences poses a major challenge, never previously addressed by this approach (Willner et al., 1992; Quan et al., 2005). These inorganic interferences can suppress nitrate response to give negative quantification error. More significantly, a sequential electrochemical deposition of conducting PPy and non-conducting P-*o*-PDA has never been considered for fabrication of amperometric bilayer biosensors. Furthermore, no previous use of P-*o*-PDA layer or a composite of non-conducting PPy/P-*o*-PDA bilayer has ever been employed for fabrication of a nitrate biosensor.

This study investigates the integration of a permselective P-*o*-PDA layer with PPy–NaR–NADH film for rejection of inorganic interferences from a bilayer nitrate amperometric biosensor. Formation of the two integrated conducting and non-conducting layers will be accomplished by a two-step sequential electrochemical deposition. The first step will involve co-immobilization of NaR and NADH into PPy film during galvanostatic polymerization of pyrrole and the resulting single layer PPy–NaR–NADH biosensor will function as previously described (Sohail and Adeloju, 2008). The second step formation of an outer P-*o*-PDA layer will be accom-

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plished by potentiodynamic polymerization. The optimum use of the bilayer biosensor will be achieved by investigating the effect of various polymerization and measurement conditions. The exclusion efficiency of the bilayer for inorganic interference rejection will be compared with those of a single PPy–NaR–NADH layer. Also, the effect of inclusion of P-*o*-PDA outer layer on the operational stability of the bilayer biosensor will be investigated.

2. Materials and methods

2.1. Reagents

NaR (EC 1.6.6.1, 0.56 units mg^{-1}) derived from Aspergillus niger species, pyrrole, potassium nitrate, potassium chloride, β-NADH, barbitonic acid, and o-PDA were purchased from Sigma–Aldrich Co. Prior to use, pyrrole was vacuum distilled, stored in a nitrogen atmosphere to avoid air oxidation, covered with aluminium foil, and kept in a freezer to prevent UV degradation. All solutions were prepared with Milli-Q water (Millipore, North Ryde, NSW, Australia).

2.2. Instrumentation

A potentiostat/galvanostat was employed for fabrication of PPy–NaR–NADH biosensors, as well as for amperometric measurements. Formation of P-*o*-PDA layer by cyclic voltammetry (CV) was performed with a Voltalab 40 PGZ 301 voltammeter and recorded on a computer with VoltaMaster 4 version 2.00 (Radiometer Copenhagen).

2.3. Electrode preparation

Platinum working electrodes (area 0.024 cm^2) were polished with 0.05 μm alumina on a polishing pad, rinsed with Milli-Q water, acetone and again with Milli-Q water. The electrodes were sonicated for 10 min in Milli-Q water to remove any alumina particles and finally dried under vacuum. The working electrode was electrochemically cleaned in a 1 M H_2SO_4 solution by cycling the electrode potential from +200 mV to +1450 mV versus Ag/AgCl at a sweep rate of 75 mV s^{-1}, until a constant current–voltage response was obtained.

A three-electrode cell, comprising of Pt-disc, Pt-wire and Ag/AgCl/(3 M KCl) as working, auxiliary and reference electrodes, respectively, was used for electropolymerization and measurements. NaR was immobilized on the Pt-disc working electrode by galvanostatic polymerisation of pyrrole in a monomer solution which contained 0.4 M pyrrole, 1 U mL^{-1} NaR, 0.2 M KCl and 0.4 mM NADH (unless otherwise stated). The monomer solution was deoxygenated for 10 min prior to galvanostatic polymerization. Electropolymerization was accomplished by applying a current density of 0.7 mA cm^{-2} for 180 s. The resulting PPy–NaR–NADH electrode was rinsed with Milli-Q water and the outer layer was formed in an *o*-PDA monomer solution which contained 40 mM *o*-PDA, 0.5 M KCl and 0.05 M barbitone buffer by performing cyclic voltammetry between 0 mV and 600 mV at scan rates ranging from 10 mV s^{-1} to 150 mV s^{-1} (Malitesta et al., 1990; Losito et al., 2003).

2.4. Amperometric detection

A potential of −200 mV was applied to the bilayer biosensor in 0.1 M phosphate buffer solution and after the residual current has stabilised, standard nitrate solution was injected. Optimization of the biosensor response was accomplished by investigating the effects of film formation and measurement conditions.

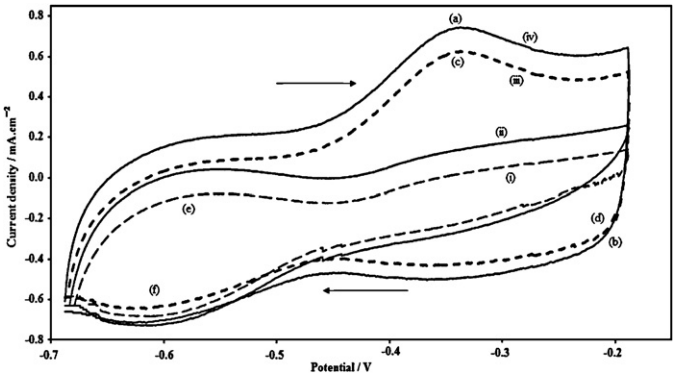

Fig. 1. Cyclic voltammograms obtained with PPy–NaR–NADH (curves i and iii) and PPy–NaR–NADH/P-*o*-PDA (curves ii and iv) electrodes in phosphate buffer (0.1 M, pH 7.3), containing 0.1 M KCl in absence (curves i and ii) and presence (curves iii and iv) of 500 μM of nitrate.

3. Results and discussion

3.1. Electrochemical characterization of PPy–NaR–NADH and PPy–NaR–NADH/P-o-PDA layers

The CVs in Fig. 1 shows that in absence of nitrate (curves i and ii), one anodic (peak e) and one cathodic (peak f) peaks were observed at −0.55 and −0.60 V, respectively, for bilayer and single layer electrodes. These correspond to the PPy characteristic oxidation and reduction peaks and confirm that both bilayer and single layer electrodes still demonstrated the redox properties of conducting PPy films. As evident, the current magnitude obtained with the bilayer electrode (curve i) was lower than that of single layer electrode (curve ii). This indicates that the inclusion of outer layer did not adversely affect the electrochemical behaviour of the bilayer electrode. Two new peaks were observed for bilayer (curve iv, peaks a and b) and single layer electrodes (curve iii, peaks c and d) in presence of 500 μM nitrate. A considerable increase was observed for the cathodic wave (b and d) at between −0.2 V and −0.4 V, and the anodic peak (a and c) at −0.325 V. These increases are consistent with the involvement of a catalytic electrode process. NaR catalyses NADH reduction of nitrate to nitrite under these conditions by the following reaction (Campbell, 1999):

$$NO_3^- + NADH \rightarrow NO_2^- + NAD^+ + OH^- \qquad (\Delta G = -143 \ \text{kJ/mol}) \tag{1}$$

The large negative free energy indicates that the nitrate reduction is an irreversible reaction and is consistent with the CV results. The broad cathodic wave (b and d) which appeared between −0.2 V and −0.4 V suggests that the amperometric detection of nitrate with single layer and bilayer electrodes can be accomplished by applying a potential within this range. However, to achieve optimum amperometric detection of nitrate with the bilayer electrode, further optimization of the inner and outer layers is required.

3.2. Optimization of inner layer

Table 1 indicates that, besides NaR concentration, all other composition and conditions used for formation of an inner PPy–NaR–NADH layer were different to those used previously for potentiometric detection of nitrate (Sohail and Adeloju, 2008). The effect of NaR concentration on the amperometric response of nitrate was investigated between 0 and 2000 mU mL^{-1}. The current response increased with increasing NaR concentration, reaching an optimum sensitivity when 1000 mU mL^{-1} of NaR was added to the monomer solution. Further increase in NaR concentration

Table 1
Monomer composition and conditions used for formation of PPy–NaR–NADH film for potentiometric and amperometric detection of nitrate.

Composition/conditions	Potentiometric	Amperometric
Pyrrole concentration (M)	0.1	0.4
NaR concentration (U mL^{-1})	1	1
NADH (μM)	300	400
KCl (M)	0.3	0.2
Applied current density (mA cm^{-2})	0.5	0.7
Polymerization time (s)	240	180

resulted in monotonic decrease in nitrate response. Consequently, 1000 mU mL^{-1} of NaR was used for other investigations.

The other established film formation conditions for obtaining optimum nitrate amperometric response include 0.4 M pyrrole, 0.2 M KCl, applied current density (i_{app}) 0.7 mA cm^{-2}, 400 μM NADH and polymerisation time (t_{pol}) 120–180 s. It was noted that the reproducibility (2.5% rsd, n = 10) obtained with PPy–NaR–NADH film formed with a t_{pol} of 180 s was more superior than obtained with 120 s (6.2% rsd, n = 10). A t_{pol} of 180 s was therefore employed.

3.3. Optimization of outer layer

The irreversible oxidation of o-PDA occurred at 400–500 mV vs. Ag/AgCl, as shown in Fig. 2a, and the anodic peak current dropped rapidly from about 6 μA cm^{-2} to less than 2 μA cm^{-2} with successive scans due to the insulating nature of the P-*o*-PDA layer. Fig. 2b shows that, due to the self limiting (Vidal, 1999), insulating (Malitesta et al., 1990) and non-conducting nature of the film, the nitrate response decreased with increasing *o*-PDA concentration. The addition of 40–60 mM *o*-PDA gave more reproducible nitrate response, but with 22–25% reduction in sensitivity compared with the single layer electrode. An *o*-PDA concentration of 40 mM was therefore chosen for outer layer formation. 0.5 M KCl was also added as a dopant to initiate commencement of *o*-PDA polymerisation.

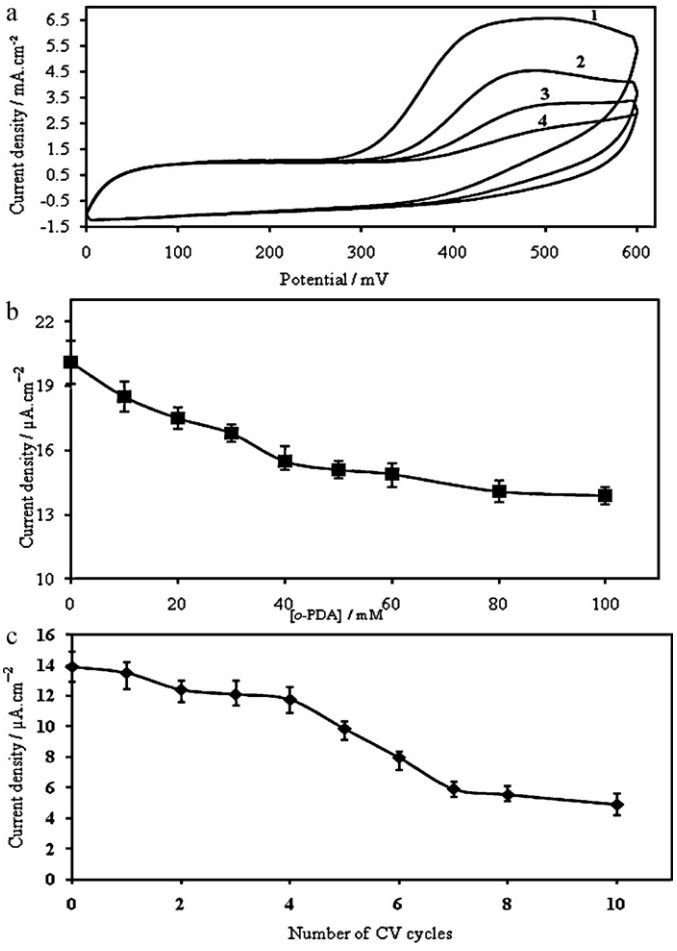

Fig. 2. Influence of (a) successive cyclic voltammetric scans, (b) *o*-PDA concentration, and (c) number of CV cycles used for P-*o*-PDA film growth on nitrate amperometric response of PPy–NaR–NADH/P-*o*-PDA biosensor. Film formation conditions are as given in Section 2.4; additional conditions for inner layer: scan rate = 100 mV s^{-1}, number of cycles = 4. Nitrate concentration measured was 500 μM (n = 3).

Fig. 2c shows that formation of the outer layer with up to four CV cycles is sufficient to ensure attainment of sensitive nitrate response. The rapid decrease in sensitivity observed when more than four cycles is employed was due to increased thickness and insulating nature of the outer layer. Typically, the use of 6–10 CV cycles resulted in 60–93% reduction in nitrate response. Four CV cycles was therefore employed at a scan rate of 100 mV s^{-1}.

3.4. Optimization of measurement conditions

The choice of applied potential, pH and buffer concentration is critical for reliable amperometric measurement of nitrate with the bilayer biosensor. Fig. 3a shows that the nitrate response increased with increasing applied potential from −0.375 V to −0.225 V. Beyond this range, the application of more positive potential inhibits the catalytic reduction of nitrate to nitrite in presence of NaR and NADH (Tkáč, 2000). This is consistent with previous observation that NaR catalysed reduction of nitrate could not take place at potentials more positive than −180 mV (Kirstein et al., 1999; Ferreyra and Solís, 2004). The chosen potential of −225 mV gave lower background current and resulted in more sensitive nitrate response (Miao, 2000).

The nitrate response also increased with increasing pH to an optimum at 7.3. Beyond pH 7.3, the response decreased gradually due to reduced enzyme activity with up to 66% loss in sensitivity at pH 8.5. Similar observations were made in previous studies (Sohail and Adeloju, 2008; Ferreyra and Solís, 2004). All subsequent amperometric measurements were therefore performed with a solution pH of 7.3.

Buffer concentrations less than 0.05 M gave low nitrate response due to low buffering capacity and enzymatic activity (Dzyadevych et al., 2004). In contrast, the response increased considerably in 0.05 M phosphate buffer, but was less reproducible (5.7% rsd, n = 10). The use of 0.1 M phosphate buffer solution gave better reproducibility (2.5% rsd, n = 10) with slightly lower sensitivity. Buffer concentrations >0.1 M led to gradual decrease of the response due to the presence of excessive ions (Vorotyntsev et al., 1998; Takashima, 2004). Consequently, 0.1 M phosphate buffer (pH 7.3) solution was used for all measurements.

3.5. Exclusion of inorganic interferences with permselective layer

3.5.1. Anion interferences

Nitrate determination in water samples are often affected by inorganic interferences. Fig. 4a shows that the nitrate response obtained with single layer biosensor was considerably more suppressed than with the bilayer biosensor when100–500 μM each of Cl^-, CH_3COO^-, SO_4^{2-}, CO_3^{2-} and OH^- was present. Traditionally, the interference rejection rejection by P-*o*-PDA layer is assessed by determining the permselectivity (S_p%) as follows (Rothwell et al., 2009; Kirwan et al., 2007):

$$S_p\% = \frac{P(\text{int})\%}{P(\text{anal})\%} \times 100\% \quad (2)$$

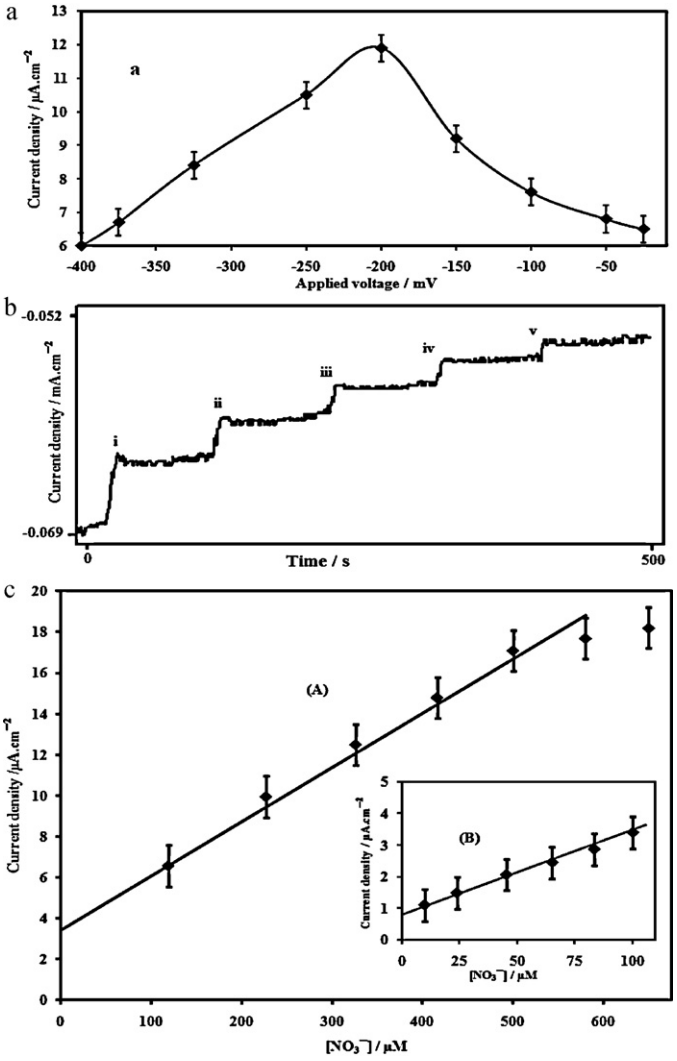

Fig. 3. (a) Effect of applied potential on nitrate amperometric response, (b) typical amperometric responses and (c) calibration plots obtained with bilayer biosensor. Nitrate concentration measured was 500 μM (n = 3). Nitrate additions: (i) 120, (ii) 230, (iii) 330, (iv) 420, and (v) 500 μM; 0.1 M phosphate buffer (pH 7.3); rsd = ±2%; n = 6.

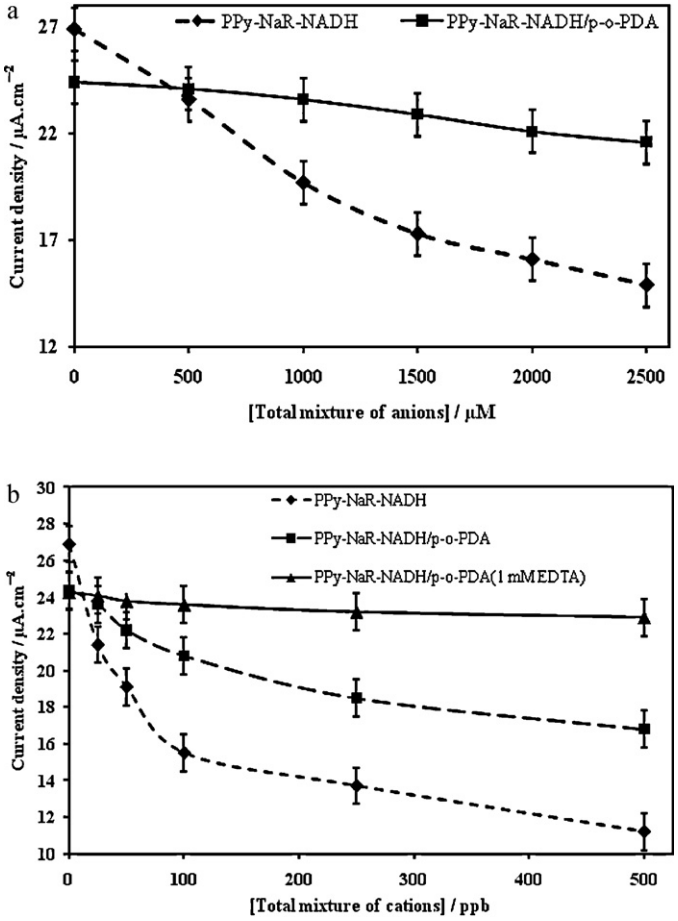

Fig. 4. Comparison of nitrate amperometric responses of optimized single layer and bilayer biosensors in presence of: (a) mixture of Cl^-, CH_3COO^-, SO_4^{2-}, CO_3^{2-} and OH^-, and (b) mixture of Fe^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Ca^{2+} with and without 1 mM EDTA.

where P(int)% is permeability of interferent and P(anal)% is permeability of analyte. This relationship applies for single layer where both interferents and analytes are electroactive. However, when P-*o*-PDA layer is employed in a bilayer arrangement where the interferent is not electroactive and its effect is mainly suppressive, this relationship cannot be employed. Instead, the efficiency of the outer layer inclusion in reducing the suppressive effect of interferents is more relevant. The suppression of nitrate response caused by a given interferent concentration at single layer (SL) and bilayer (BL) biosensors can be defined as:

$$S_{SL} = 1 - \frac{i_{(INT)SL}}{i_{SL}} \quad (3)$$

and

$$S_{BL} = 1 - \frac{i_{(INT)BL}}{i_{BL}} \quad (4)$$

where $i_{(INT)}$ is current response in presence of an interferent; i_{SL} or i_{BL} is current response in absence of interferent with single or bilayer biosensor. As S_{SL} represents the maximum suppression on the single layer biosensor for a given interferent concentration, the difference between this value and S_{BL} gives an indication of interferent exclusion achieved with the bilayer biosensor. Consequently, the exclusion efficiency accomplished with outer layer inclusion can be calculated from these values as follows:

$$E_{eff} = \frac{S_{SL} - S_{BL}}{S_{SL}} \times 100\% \quad (5)$$

Thus, the E_{eff} value provides a measure of the ability of the outer layer to exclude inorganic interferences. The calculated exclusion efficiencies given in Table 2 indicate that the achieved E_{eff} with outer layer inclusion for a total anion concentration of 500–2500 μM ranged from 73% to 88%. As expected, the achieved E_{eff} decreased with increasing anion concentration. Nevertheless, the achievement of over 70% exclusion of interference caused by up to 2500 μM anions demonstrates high degree of permselectivity with the outer layer. Evidently, the effectiveness of the outer layer in reducing interferences from anions is best for total anion concentration <1000 μM. The total anion concentration in most unpolluted natural waters is less than this value. For example, calculated total anion concentrations, based on measured sulphate, chloride and hydroxide (derived from pH) concentrations (Water Encyclopedia, 2009) were 558 μM in rainwater (California), 39 μM in rainwater (North Carolina), 405 μM in Rhine river, 15.3 μM in stream (Washington cascades), 289.5 μM in creek SW Oregon in wet season, 943 μM in creek SW Oregon in dry season, 635 μM in groundwater from volcanic rocks (New Mexico) and 24.5 μM in groundwater from a spring (Sierra Nevada mountains). Acetate and carbonate concentrations were not measured for these natural

Table 2
Suppression and exclusion efficiency of PPy–NaR–NADH and PPy–NaR–NADH/P-*o*-PDA nitrate biosensors in presence of anion and cation interferences.

Total interferent concentration (μM)	PPy–NaR–NADH (S_{SL})	PPy–NaR–NADH/P-*o*-PDA (S_{BL})	E_{eff} (%)	ΔE_{eff} (%)
Anions				
500	0.126	0.016	87.3	
1000	0.270	0.033	87.8	
1500	0.359	0.066	81.6	
2000	0.404	0.099	75.5	
2500	0.441	0.119	73.0	
Cations				
25	0.204	0.033	83.8	
50	0.289	0.086	70.2	
100	0.426	0.143	66.4	
250	0.493	0.238	51.7	
500	0.593	0.311	47.6	
Cations (with 1 mM EDTA)				
25	–	0.012	94.1	10.3
50	–	0.020	93.0	22.8
100	–	0.033	92.3	25.9
250	–	0.049	90.1	38.4
500	–	0.066	88.9	41.3

waters, but their concentrations would be less than 50 μM in most cases. Thus, it can be concluded that the high E_{eff} obtained with bilayer biosensor reflects the intrinsic properties of outer layer in achieving effective permselective exclusion of anionic interference from the inner PPy–NaR–NADH biosensor and such effectiveness can be achieved for most unpolluted natural waters.

3.5.2. Cation interferences

Cations can even have more significant effect on the amperometric detection of nitrate with single layer and bilayer biosensors. As shown in Fig. 4b, the presence of 100 ppb of each of Fe^{3+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, and Ca^{2+} resulted in considerably less suppression of nitrate response of the bilayer biosensor than with the single layer biosensor. However, the levels of suppression caused by common cations were in both cases very high. By applying Eqs. (3)–(5), the E_{eff} achieved for cation rejection with the permselective layer was evaluated.

The calculated E_{eff} values given in Table 2 indicate that the cation rejection achieved with the permselective layer ranged from 47% to 84% when 25–500 ppb of total cations was present. Again, as expected, the E_{eff} decreased with increasing total cation concentration. However, the decline in the E_{eff} below 70% when >50 ppb of total cations was present indicate that the permselective layer was not as effective as for anion rejection. The low E_{eff} obtained when total cation concentration ≥50 ppb was present indicate that the outer layer alone was not sufficiently effective when high metal ion concentration is present.

The best and quickest option for overcoming the problem associated with the presence of high metal ion concentration is by addition of a complexing agent into the measurement solution. The data in Table 2 reveal that a considerable improvement in the E_{eff} was obtained with the bilayer biosensor when 1 mM EDTA was added into the measurement solution. The combined use of the outer layer and complexing agent gave an E_{eff} value of 94% in the presence of a total cation concentration of 25 ppb. Even in the presence of 20-fold increase in total cation concentration (500 ppb), it still achieved an E_{eff} value of 89%. Evidently, the improved cation rejection reflect both the intrinsic properties of the outer layer in excluding cation interference, as well as the ability of EDTA to complex metal ions with minimal effect on nitrate response. The change in the exclusion efficiency, ΔE_{eff}, data in Table 2 indicate that the addition of EDTA alone contributed 10–40% of the achieved exclusion when a total cation concentration of 25–500 ppb was present.

3.6. Analytical performance

The detection limit (3σ) achieved with the bilayer biosensor for nitrate is 0.20 μM compared with 0.55 μM for single layer biosensor. This detection limit is also 75 times lower than the 15 μM achieved with potentiometric detection of nitrate with single layer biosensor (Sohail and Adeloju, 2008). The detection limit obtained for the amperometric detection of nitrate with bilayer biosensor is also lower than the 0.4 μM achieved with viologen-based nitrate amperometric biosensor (Cosnier et al., 1994).

Although the sensitivity of the bilayer biosensor (34.2 nA μM^{-1} cm^{-2}) was slightly lower than that obtained with the single layer biosensor (38.8 nA μM^{-1} cm^{-2}) due to the presence of the outer layer, it was much better than those obtained with other NaR-based nitrate amperometric biosensors (Cosnier et al., 1994, 1997; Takayama et al., 1996; Ramsay and Wolpert, 1999; Da Silva et al., 2004; Quan et al., 2005). Also, the linear range of 10–500 μM (R^2 = 0.978) achieved with the bilayer biosensor was slightly better than 25–500 μM (R^2 = 0.945) obtained with the single layer biosensor and far superior than those obtained with other NaR-based nitrate amperometric biosensors (Cosnier et al., 1994, 1997; Takayama et al., 1996; Ramsay and Wolpert, 1999; Da Silva et al., 2004; Quan et al., 2005). Also, the reproducibility of the bilayer biosensor (2.7% rsd, n = 10) was more superior than that of the single layer biosensor (5.1% rsd, n = 10). However, the average response times of both single (18–22 s) and bilayer (19–22 s) biosensors were similar and confirmed that the inclusion of the outer layer did not affect nitrate detection with inner layer.

Calculation of the approximate Michaelis–Menten constants $[K_M]^{app}$ revealed that the inclusion of P-*o*-PDA layer resulted in a slight decrease in the $[K_M]^{app}$ values from 0.23 mM to 0.20 mM, indicating a greater affinity towards the substrate. It could therefore be inferred that the specificity of the bilayer biosensor was improved with the inclusion of the outer layer (Liu, 2005). However, the K_M of 0.20 mM obtained with the bilayer biosensor is higher than 0.13 mM obtained with free NaR–NADH (Savidov et al., 1997) due to entrapment of lower concentrations in the PPy film, but is much lower than 0.62 mM achieved for free NaR–NAD(P)H (Savidov et al., 1997), 0.28 mM for genetically modified NaR–NarGHI and 1.3 mM for NaR–NapAB free NaR species (Stewart et al., 2002). This observation shows that NaR immobilized with NADH in the bilayer biosensor still has high affinity for nitrate.

Fig. 3b shows that the magnitude of nitrate response increased with increasing nitrate concentration. Typical calibration curves for (a) higher and (b) lower nitrate concentrations are given in

Fig. 3c. These curves indicated that the bilayer biosensor gave a good linear concentration range from 10 μM to 500 μM. A different slope and R^2 values were observed for lower nitrate concentrations (10–100 μM) which is consistent with previous observations (Geise et al., 1991).

3.7. Operational stability

The re-use of the same single layer biosensor after 24 h resulted in about 50% (7.6% rsd, $n = 10$) reduction in nitrate response due to a change in porosity of the film during storage in 0.1 M phosphate and associated losses of NaR and NADH from the film. In contrast, the re-use of the bilayer biosensor only gave 20% (6.1% rsd, $n = 10$) reduction of the response. This observation supports earlier view that the inclusion of the P-*o*-PDA layer aids the retention of NaR and NADH in bilayer biosensor.

Further reuse of the same single layer biosensor after 48 h gave 57% (8.1% rsd, $n = 10$) reduction of the response, while reuse of the bilayer biosensor after the same period gave only 25% (6.3% rsd, $n = 10$) reduction. Evidently, much improved operational stability was obtained with bilayer biosensor than with single layer counterpart, as well as those reported previously (Ferreyra and Solís, 2004; Kirwan et al., 2007). Also, with repeated use and storage in between use in 0.1 M phosphate, the bilayer biosensor could be used for more than 30 days with no more than 30% reduction in sensitivity. When coupled with quantification by standard additions method, such small change in sensitivity has no effect on the analytical reliability of the bilayer biosensor.

4. Conclusions

The integration of the permselective P-*o*-PDA outer layer with PPy–NaR–NADH inner layer resulted in a considerable improvement of the analytical performance and operational stability of the nitrate bilayer biosensor. The bilayer arrangement resulted in much improved detection limit, linear concentration range and sensitivity for nitrate detection. The detection limit of 0.2 μM achieved in this study was several orders of magnitude better than achieve for potentiometric detection of nitrate (Sohail and Adeloju, 2008, 2009) and more than twice better than with a single layer biosensor. The detection limit was also better than those reported for other NaR-based nitrate amperometric biosensors. The working linear concentration range of 10–500 μM achieved with the bilayer biosensor is also better than that of a single layer biosensor and other previously reported NaR-based nitrate amperometric biosensors. The integrated permselective P-*o*-PDA layer was effective in achieving exclusion efficiency of 73–88% for anions and 47–84% for cations. The E_{eff} achieved for cations with the P-*o*-PDA layer was improved substantially to 89–94% with the addition of 1 mM EDTA into the measurement solution. However, further improvement in sensitivity is still required to enable the use of the bilayer biosensor for amperometric detection of nitrate in pristine natural waters. Further work is being undertaken in our laboratory to achieve this goal.

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