Nitric oxide monitoring in brain extracellular fluid: characterisation of Nafion®-modified Pt electrodes *in vitro* and *in vivo*

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A Nafion®(5 pre-coats/2 dip-coats)-modified Pt sensor developed for real-time neurochemical monitoring has now been characterised *in vitro* for the sensitive and selective detection of nitric oxide (NO). A potentiodynamic profile at bare Pt established +0.9 V (*vs.* SCE) to be the most appropriate applied potential for NO oxidation. The latter was confirmed using oxyhaemoglobin and N_2, both of which reduced the NO signal to baseline levels. Results indicated enhanced NO sensitivity at the Nafion®(5/2) sensor (1.67 ± 0.08 nA μM^{-1}) compared to bare Pt (1.08 ± 0.20 nA μM^{-1}) and negligible interference from a wide range of endogenous electroactive interferents such as ascorbic acid, dopamine and its metabolites, NO_2^- and H_2O_2. The response time of 33.7 ± 2.7 s was found to improve (19.0 ± 3.4 s) when the number of Nafion® layers was reduced to 2/1 and an insulating outer layer of poly(*o*-phenylenediamine) added. When tested under physiological conditions of 37 °C the response time of the Nafion®(5/2) sensor improved to 14.00 ± 2.52 s. In addition, the NO response was not affected by physiological concentrations of O_2 despite the high reactivity of the two species for each other. The limit of detection (LOD) was estimated to be 5 nM while stability tests in lipid (phosphatidylethanolamine; PEA) and protein (bovine serum albumin; BSA) solutions (10%) found an initial *ca.* 38% drop in sensitivity in the first 24 h which remained constant thereafter. Preliminary *in vivo* experiments involving systemic administration of NO and L-arginine produced increases in the signals recorded at the Nafion®(5/2) sensor implanted in the striatum of freely-moving rats, thus supporting reliable *in vivo* recording of NO.

Introduction

Nitric oxide (NO) is an intracellular signalling molecule that performs a variety of roles throughout the body. It regulates vascular tone, acts as a neuronal signal in the gastrointestinal tract and central nervous system, and contributes to the pathology of several diseases including hypertension, Parkinson's disease and Alzheimer's disease.

The same properties that allow NO to carry out its diverse physiological and pathological processes also present problems concerning its qualitative and quantitative analysis in biological systems. Like all free radicals, NO is extremely reactive and has a high affinity for interaction with ferrous haemoproteins such as soluble guanylate cyclase[1] and haemoglobin,[2,3] while also reacting readily with O_2, peroxides and the superoxide anion (O_2^-).[4]

The majority of NO monitoring methods that are currently available measure NO indirectly and are hindered by significant drawbacks. The most commonly used methods found in the literature are electron paramagnetic resonance (EPR),[5,6] chemiluminescence,[7,8] UV-visible spectroscopy,[9–11] fluorescence[12,13] and electrochemistry.[14–18] Of these techniques, electrochemical methods are most advantageous because of their speed and sensitivity,[19–21] which are particularly important for NO as it is present at nanomolar concentrations and has a half-life of 2–6 s *in vivo*.[22] Thus, the possibility of using microelectrochemical sensors for real-time NO measurement has recently gained considerable interest.[14,23–27]

For a number of years we have been using Long-term *In Vivo* Electrochemistry (LIVE), *i.e.* the detection of substances using amperometric electrodes (sensors/biosensors) and electrochemical techniques *in vivo*, to study neurochemical phenomena in the living brain.[28,29] We have recently become interested in developing a new sensor for monitoring brain extracellular levels of NO. However, the mammalian brain is a hostile environment for implanted sensors as it contains electrode poisons (*e.g.* lipids and proteins) and a large number of possible interfering species present at relatively high concentrations (*e.g.* ascorbic acid (AA), uric acid (UA), and the catecholamines and their metabolites). As a first step in our development process various Nafion® coating procedures on Pt were examined in order to design a simple and reproducible coating method to maximise permselective characteristics.[30] Application of Nafion® (5% commercial solution) using a thermally annealing procedure involving 5 pre-coats, and 2 subsequent dip-bake layers resulted in elimination of interferent signals from AA (the principal endogenous electroactive interferent present in the brain), UA and dopamine. We have also recently established and characterised a consistent and reproducible method of the preparation of NO stock

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solutions in order to enable accurate calibration of our new NO sensor both prior to, and after, implantation.[31]

In this paper we present results of a detailed *in vitro* characterisation of the Nafion®(5/2)-modified microelectrochemical Pt sensor with respect to brain extracellular NO monitoring. We also present preliminary *in vivo* data from experiments designed to characterise the sensor's performance in its target biological environment.

Experimental

Reagents and solutions

The Nafion® (1100 EW, 5 wt% solution in a mixture of lower aliphatic alcohols and H_2O) was obtained from Aldrich Chemical Co., Dorset, UK. L-Ascorbic acid (AA; A.C.S. reagent), uric acid (UA; sodium salt), dopamine (DA; hydrochloride), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxytryptamine (5-HT; hydrochloride), 5-hydroxyindole-3-acetic acid (5-HIAA), glutathione (oxidised disodium salt), *o*-phenylenediamine (99+%), haemoglobin (bovine lyophilized powder), L-α-phosphatidylethanolamine (PEA; Type II-S, commercial grade), L-arginine and H_2O_2 (A.C.S. reagent, 30.4%) were obtained from Sigma-Aldrich Ireland Ltd. Bovine Serum Albumin (BSA; Fraction V) was obtained from Fluka Chemicals, Dorset, UK. The NaCl (SigmaUltra), NaH_2PO_4 (Sigma, A.C.S. reagent) and NaOH (SigmaUltra) were used as supplied.

NO stock solutions were prepared fresh using neutral Griess reagent following a previously described procedure.[31] Stock standard solutions of all other compounds were prepared at the beginning of each experiment to avoid problems associated with gradual decomposition. Solutions of oxyhaemoglobin (oxyHb; 0.1 mM) were prepared by dissolving 0.04 g of haemoglobin in 25 mL distilled deionised water and purging with O_2 gas. 10% solutions of BSA and PEA were prepared in distilled deionised water and chloroform respectively. *In vitro* experiments were carried out in phosphate buffer saline (PBS) solution, pH 7.4 (0.15 M NaCl, 0.04 M NaH_2PO_4 and 0.04 M NaOH), which was deaerated with O_2-free N_2 for 20 minutes prior to commencing electrochemical measurements. All solutions were prepared using deoxygenated doubly distilled deionised water unless otherwise stated, and stored at 4 °C between injections. In *in vivo* experiments, solutions of normal saline (1.0 mL/kg, 0.9% NaCl), NO (500–1200 μM), and L-arginine (300 mg/kg) were administered by intraperitoneal (i.p.) injection.

Working electrode preparation

Pt disk electrodes were made from Teflon-insulated platinum/iridium (Pt/Ir 90%/10%) wire (125 μm bare diameter, 175 μm coated diameter (5T), Advent Research Materials, Suffolk, UK). The electrodes were approximately 5 cm in length and were prepared by carefully cutting 5 mm of the Teflon insulation from one end of the wire. A gold electrical contact (Semat Technical, Herts, UK) was soldered to this end of the wire to enable connection with the instrumentation. The other end of the wire acted as the active (disk) surface of the electrode.

Nafion® modification of the active surface was performed following a previously reported pre-cast procedure. Briefly, pre-casting involves placing an under layer of concentrated Nafion® onto the electrode surface. This is achieved by placing a droplet of Nafion® onto a watch glass. This droplet is then allowed to air dry at room temperature for 5 minutes. After drying, further individual drops (5) are placed on top of the initial droplet using the same procedures. This produces a localised concentrated layer of Nafion® on the watch glass. A further drop of Nafion® is then placed on top of this concentrated pre-coated Nafion® layer. The active surface of the electrode is dipped into this concentrated layer and then immediately removed and allowed to air dry at room temperature for 2 minutes. The final fresh Nafion® droplet is required to adhere the concentrated Nafion® layer to the electrode. This electrode is then placed into an oven and baked for 5 minutes at 210 °C. After baking, the electrode is then further coated with a second Nafion® layer by repeating the dip-bake procedure, resulting in what we term a 5 pre-coat and 2 dip-coat (Nafion®(5/2)) sensor.

For some experiments the number of coatings was reduced to produce a Nafion®(2/1) sensor which was then coated with an outer layer of poly(*o*-phenylenediamine) (Nafion®(2/1)-PPD) following a previously described electropolymerisation procedure.[32–34] When not in use all electrodes where stored dry at room temperature.

Characterisation of Nafion®-modified Pt electrodes

All calibrations were performed in a standard three-electrode glass electrochemical cell which was constructed in-house. A saturated calomel electrode (SCE) was used as the reference electrode and a large Pt wire served as the auxiliary electrode. To facilitate mixing, solutions were bubbled with N_2 for *ca.* 5 s following the addition of each aliquot unless otherwise stated. The current was then measured under quiescent conditions with an N_2 atmosphere maintained over the solution.

Concentrations of NO injected into the electrochemical cell were not mixed using N_2 gas due to the possibility of being displaced from the PBS electrolyte. Mixing was facilitated instead by use of a magnetic stirrer for approximately 5 s. For NO sensors calibrated in PBS containing 50 μM O_2, a second pre-calibrated O_2 electrode (bare Pt) was used to continuously monitor the solution concentration of O_2 by measuring the O_2 reduction current at −550 mV *vs.* SCE. The measured current was maintained at that for 50 μM O_2 by accurately controlling a gaseous mixture of air (RENA 102 air pump) and N_2 (British Oxygen Co. (BOC)) entering the electrochemical cell. Once the reduction current was stable and representative of 50 μM O_2 the NO calibration was commenced. All calibrations were performed at room temperature unless otherwise stated.

Instrumentation and software

Scanning electron microscopy was performed on a JEOL JSM-5410 SEM (EDAX, UK) operating with a 15 kV accelerating voltage. Constant potential amperometry (CPA) was performed in all electrochemical experiments using a low-noise potentiostat (Biostat II, Electrochemical and Medical Systems, Newbury, UK). Data acquisition was carried out with a Gateway GP6-350 computer, a Powerlab/400 interface system (ADInstruments Ltd., Oxford, UK) and Chart for Windows (v 4.0.1) software (ADInstruments Ltd.).

All analysis was performed using Microsoft Excel and Prism (GraphPad Software Inc., CA, USA). The significance of differences observed was estimated using the Student's *t*-test for paired or unpaired observations where appropriate. Two-tailed levels of significance were used and $P < 0.05$ was considered to be significant. All data are presented as mean ± S.E.M.

Surgical procedures

Male Wistar rats weighing 200–300 g were anesthetised, following published guidelines,[35] with a mixture of Hypnorm (fentanyl citrate/fluanisone, Janssen Pharmaceuticals Ltd., Oxford, UK), Hypnovel (midazolam, Roche Products Ltd., Herts, UK), and sterile water, mixed 1 : 1 : 2 and injected i.p. at a volume of 3.3 mL/kg, as described previously.[36,37] Once surgical anaesthesia was established, animals were placed in a stereotaxic frame and the electrodes implanted following a previously described procedure.[36] Pt/Nafion®(5/2)-modified electrodes, for monitoring NO, were implanted bilaterally in the right and left striatum: coordinates with the skull levelled between bregma and lambda, were: A/P + 1.0 from bregma, M/L ± 2.5, and D/V −5.0 from dura.[38] A reference electrode (8T Ag wire, 200 μm bare diameter; Advent Research Materials) was placed in the cortex and an auxiliary electrode (8T Ag wire) attached to one of the support screws (see below). The reference potential provided by the Ag wire in brain tissue is very similar to that of the SCE.[39] The electrodes were fixed to the skull with dental screws and dental acrylate (Associated Dental Products Ltd., Swindon, UK). Surgery typically lasted 40 min and anesthesia was reversed by an i.p. injection of naloxone (0.1 mg/kg, Sigma Chemical Co.). Post-operative analgesia was provided in the form of a single injection (0.1 mg/kg, subcutaneous) of Vetergesic (Buprenorphine hydrochloride, Reckitt and Colman Pharmaceuticals, Hull, UK) given immediately following the surgery. Animals were allowed to recuperate for 24 h after surgery and were assessed for good health according to published guidelines[40] immediately after recovery from anesthesia and at the beginning of each day. This work was carried out under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 – Amendment of Cruelty to Animals Act 1876).

Experimental conditions *in vivo*

Rats were housed in large plastic bowls (diameter *ca.* 50 cm), in a windowless room under a 12 h light, 12 h dark cycle, lights coming on at 8 am, with free access to water. Food was available *ad libitum*. All experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat after the 24 h recuperation period, through a six-pin Teflon socket and a flexible screened six core cable which was mounted through a swivel above the rat's head (Semat Technical Ltd.). This arrangement allowed free movement of the animal which remained continuously connected to the instrumentation. After application of the applied potential each animal was given a further 24 h before experiments were begun in order to ensure that the background currents for the electrodes were completely stabilised. A low-pass digital filter (50 Hz cut-off) was used to eliminate mains AC noise and all data were recorded at 10 Hz.

Results and discussion

Potentiodynamic profile of NO

Previous reports indicate that NO can be oxidised or reduced at bare Pt disk electrodes.[41–44] However, NO reduction requires an applied potential of *ca.* −900 mV[17] and is thus not an ideal detection method at electrodes designed for *in vivo* applications. This is primarily because other low molecular weight highly permeable species, such as tissue O_2, can be reduced at similar reduction potentials (*e.g.* −550 mV *vs.* SCE).[21,45] Oxidation of NO is thus the preferred method of detection as the faradaic NO signal is not contaminated by O_2 at positive potentials[23] even though the gaseous O_2 molecule can permeate most interferent-rejecting membranes. NO oxidation has previously been reported to occur between +600 mV and +900 mV at bare Pt electrodes.[23,46–48]

In order to determine the most appropriate oxidation potential for NO at our Pt disk microelectrodes we carried out a potentiodynamic profile for NO from 0 to +900 mV. A complete calibration ($n = 4$) for NO was performed over a relevant physiological concentration range (0–1 μM, see *inset* Fig. 1) at 0, 200, 400, 600, 700, 800 and 900 mV at bare Pt. A plot of the slope *vs.* applied potential is shown in Fig. 1. NO oxidation commenced at *ca.* 400 mV and increased with increasing applied potential reaching a plateau after *ca.* 800 mV: 0.13 ± 0.02 nA μM^{-1} (400 mV); 0.49 ± 0.06 nA μM^{-1} (600 mV); 1.22 ± 0.13 nA μM^{-1} (700 mV); 2.04 ± 0.05 nA μM^{-1} (800 mV); 2.32 ± 0.29 nA μM^{-1} (900 mV). All calibrations were linear (mean $r^2 = 0.98 \pm 0.01$, $n = 5$) and although there was no significant difference in the sensitivities observed at +800 and +900 mV ($P = 0.91$) we decided, because of the short half-life of NO and that fact that most interferents would oxidise below +800 mV, that it would be preferable to operate the sensor at the higher applied potential given the likely advantage of a faster response time.[21]

Verification of NO signal

NO is a very difficult gaseous analyte to work with in an experimental setup, primarily because of its high reactivity with O_2.[48–50] NO can react with O_2 to give the oxidation product NO_2^- and then undergo even further oxidation to produce NO_3^-.[46,51] We thus decided to confirm the authenticity of the

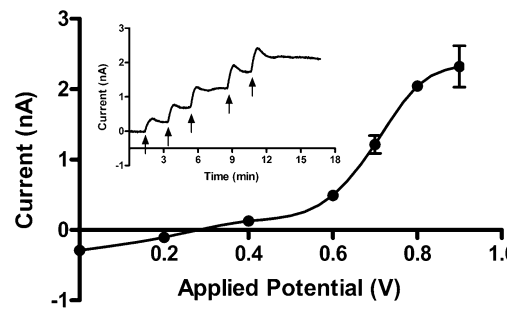

Fig. 1 The current–potential profile for 1 μM NO at bare Pt disk electrodes at 0, +200, +400, +600, +700, +800 and +900 mV *vs.* SCE ($n = 4$). *Inset*: Typical steady state currents obtained for a 0–1 μM NO calibration at +900 mV *vs.* SCE; arrows indicate injections of 0.2, 0.4, 0.6, 0.8 and 1 μM NO.

in vitro NO signal recorded in calibrations at our Pt microelectrodes. This was achieved using oxyhaemoglobin (OxyHb) and N_2. OxyHb is an efficient NO scavenger[21,27,46] – NO reacts rapidly with oxyHb to from nitrate and methaemoglobin,[2] while N_2 displacement of NO in solution has previously been reported by other research groups.[46,47]

Addition of N_2 at the end of a full NO calibration resulted in a rapid decrease in the 1 μM signal to background levels (see Fig. 2A) while injection of 6 μM OxyHb to a solution containing 200 nM NO had a similar effect with the faradaic current also rapidly decreasing to background levels (see Fig. 2B). These results confirm that our analyte signal is due to NO oxidation.

Interference studies

As already outlined, the mammalian brain is a hostile environment for implanted sensors as it contains electrode poisons (*e.g.* lipids and proteins) and a large number of possible interfering species present at relatively high concentrations (*e.g.* ascorbic acid (AA), uric acid (UA), and the catecholamines (*e.g.* dopamine, DA) and their metabolites). The success or failure of a sensor designed for *in vivo* neurochemical applications is generally decided by its ability to eliminate interferent signals while maintaining sufficient sensitivity for the target analyte. One of the most common methods of achieving this desired characteristic is the use of permselective membranes[52–58] which are generally dip-coated[46,59–62] or electropolymerised onto the electrode surface.[14,32,54,55,58,63–68]

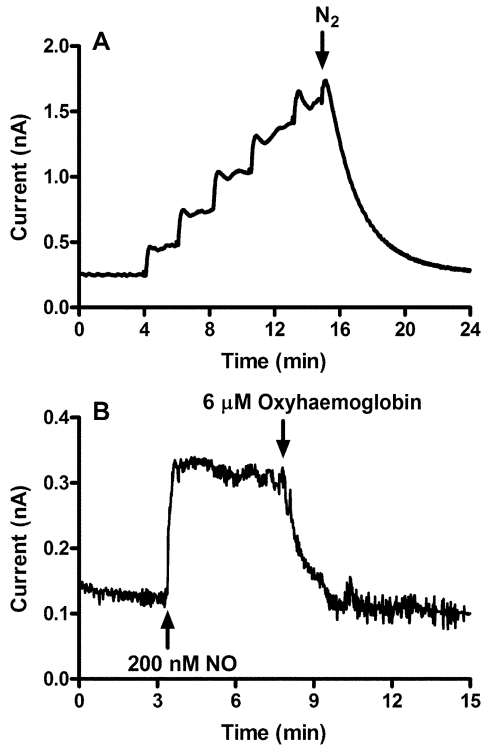

Fig. 2 (A) An example of the effect of bubbling N_2 into an electrochemical cell containing 1 μM NO on the response of a Nafion®-modified Pt electrode at +900 mV *vs.* SCE. Increases in current represent injections of 0.2, 0.4, 0.6, 0.8 and 1.0 μM NO. (B) Typical response to injection of 200 nM NO followed by the addition of 6 μM OxyHb.

However, because NO is a gaseous molecule present a low concentrations *in vivo* (tens of nanomoles to low micromoles)[49] conventional membranes and application procedures rarely produce the desired sensitivity and selectivity. One polymer with the potential to successfully address this is Nafion®. Electrodes modified with Nafion® have traditionally been used for the detection of species such as DA[67,69,70] due to their ability to preconcentrate the cationic catecholamine and minimise interference from the various endogenous anionic species (*e.g.* AA) present in the extracellular fluid (ECF).[16,26,27,71–73] Such permselective Nafion® coatings have been applied to electrode surfaces using a variety of techniques including dip-coating,[74] spin-coating[75] electrostatic spray[76] and by Langmuir–Blodgett techniques,[77,78] producing a variety of characteristics in terms of sensitivity, selectivity and response time. However, it is widely accepted that Nafion® does not adhere to metal surfaces well and that the process of thermal annealing of a cast Nafion® membrane produces a more adherent film.[14,79–84] More importantly, it has been shown that thermal annealing also produces a more selective membrane.[14,85] However, the scientific literature appears undecided on the most efficient method of Nafion® application regarding the number of applications, the temperature at which the application is applied, and the time for which the application is allowed to dry before the addition of further applications.[4,59,61,63,74,86–94]

We have recently addressed this issue by examining various Nafion® coating procedures on Pt in order to design a simple and reproducible coating method to maximise permselective characteristics.[30] Application of Nafion® (5% commercial solution) using a thermally annealing procedure involving 5 pre-coats and 2 subsequent dip-bake layers resulted in elimination of interferent signals from AA (0–1000 μM), the principal endogenous

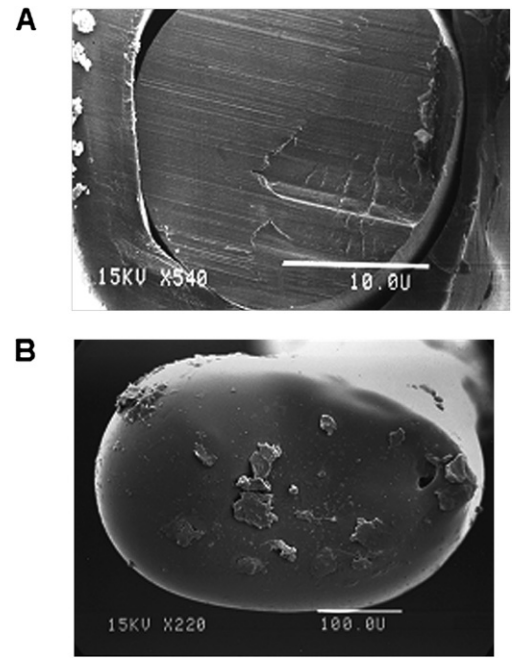

Fig. 3 (A) Surface SEM of bare Pt disk electrode (10 μm dimensional bar). (B) SEM of Pt-Nafion®(5/2)-modified electrode (100 μm dimensional bar).

electroactive interferent present in the brain, UA (0–60 μM) and DA (0–100 μM). Fig. 3 shows a typical SEM of this Nafion®(5/2)-modified electrode (×220 magnification) compared to bare Pt (×540 magnification). We have now characterised this sensor in terms of its sensitivity for NO (see Fig. 4) and its permselective properties with respect to a wide range of potential endogenous interferents.[95] The results of this characterisation are presented in Table 1 and are compared to the average responses observed at bare Pt. All interferents tested yielded a significant decrease in response upon application of the Nafion® membrane with the majority producing no response in calibrations performed over physiologically relevant concentration ranges, similar to that previously reported for AA.[30] A typical example for NO_2^- is shown in Fig. 5. NO sensitivity on the other hand, was increased (see Fig. 4; 1.67 ± 0.08 nA μM^{-1}, $r^2 = 0.99$, $n = 14$) compared to bare Pt (1.08 ± 0.20 nA μM^{-1}, $r^2 = 0.99$, $n = 28$, $P = 0.05$) with a calculated limit of detection (LOD) (3 × SD of the background noise level) of 5 nM. In addition, NO sensitivity was not affected by the presence of physiological concentrations of AA (500 μM) and DA (0.05 μM) in the electrochemical cell during calibrations: AA, 1.75 ± 0.058 nA μM^{-1}, $n = 4$, $P = 0.8552$; DA, 1.79 ± 0.37 nA μM^{-1}, $n = 6$, $P = 0.8541$. The hydrophobic nature of NO permits it to pass freely across cell boundaries and hydrophobic membranes. Nafion® consists of a hydrophobic fluorocarbon backbone and hydrophilic sulfonic groups. The latter facilitate the interference rejection (*i.e.* anion repulsion) while hydrophobic interactions, such as those previously observed for other neutral molecules such as ferrocene[96] and methylmercury chloride,[97] facilitate the permeation of the neutral NO through the Nafion® film.

Although the majority of NO sensors that have been modified with a selective membrane (*e.g.* cellulose acetate) have shown decreased sensitivity towards NO compared to the unmodified electrode[46,47,71,98] a similar increase in sensitivity to that reported here has previously been observed by Diab and Schuhmann,[66] also at a Nafion®-modified electrode, although no reason was given. A possible explanation is that adsorbed oxidation products decrease the sensitivity at electrodes not modified with Nafion®. Pariente *et al.*[47] have reported that the mechanism of NO oxidation at Pt surfaces involves the presence of adsorbed intermediates according to the reaction:

$$NO_{(a)} + H_2O \rightleftharpoons HNO_{2(a)} + H^+ + e^-$$

where (a) is used to represent an adsorbed species. However, HNO_2 may not be formed when NO is oxidised at Nafion®-coated Pt because the negatively charged Nafion® stabilises the reaction product NO^+, thus preventing a complicated series of reactions that could lead to NO_2^- and NO_3^- formation.[14,26,47,62,99–102]

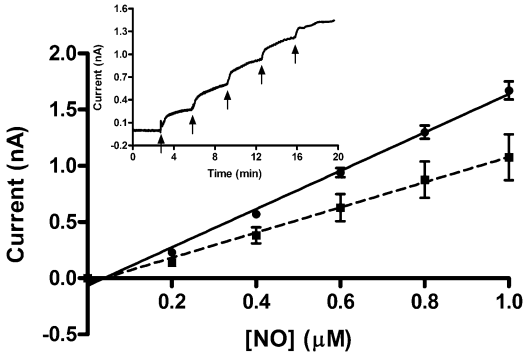

Fig. 4 Amperometric calibration plots for NO at bare Pt (dashed line) and Nafion®(5/2)-modified Pt (solid line) electrodes carried out in PBS, pH 7.4, at +900 mV *vs.* SCE. *Inset*: Typical steady state currents obtained for a 0–1.0 μM NO calibration using a Nafion®(5/2)-modified Pt electrode; arrows indicate injections of 0.2, 0.4, 0.6, 0.8 and 1 μM NO.

Response time

Although Nafion® can provide a sensor with selectivity against electroactive interferents, it is commonly known that it reduces the temporal response for target analytes.[103] The relatively short lifetime of NO in tissue (10–60 s) requires a sensor to respond rapidly in order to continuously monitor changing concentrations of NO *in vivo*.[20] The response time achieved with the Nafion®(5/2)-modified Pt electrodes was 33.7 ± 2.7 s ($n = 14$, see Fig. 4). This is slow compared to bare Pt where the response time was 6.8 ± 1.1 s ($n = 28$, see Fig. 1).

In order to improve the response time, while at the same time trying to retain the selectivity and sensitivity required to detect the low concentrations of NO in brain ECF, we decided to investigate the effects of reducing the thickness of the Nafion® coating in combination with incorporating poly(*o*-phenylenediamine) (PPD). PPD has previously been used in sensor design as an efficient permselective barrier against electroactive species such as AA and DA without adversely affecting the response time.[14,71,104] It has also been used in the development of a Nafion®-modified carbon fibre-based NO sensor with a reported response time at 50% of 1 s.[105] Reducing the number of Nafion® layers to 2 pre-coats/1 coat annealed at a temperature of 210 °C, and adding an insulating outer layer of PPD resulted in an improved response time of 19.0 ± 3.4 s ($n = 8$) compared to the standard 5 pre-coats/2 coats sensor (33.7 ± 2.7 s, $n = 14$, $P = 0.022$). In addition, there was no significant effect on NO sensitivity: Nafion®(2/1)-PPD, 1.38 ± 0.21 nA μM^{-1} ($n = 8$); Nafion®(5/2), 1.67 ± 0.08 nA μM^{-1} ($n = 14$, $P = 0.1475$). There was also no effect on the interference rejection characteristics with respect to the AA (Nafion®(2/1)-PPD: 0.069 ± 0.069 nA mM^{-1}, $n = 3$; Nafion®(5/2): −0.110 ± 0.080 nA mM^{-1}, $n = 3$,‡ $P = 0.1661$) and the NO_2^- response (Nafion®(2/1)-PPD: 0.29 ± 0.17 nA mM^{-1}, $n = 3$; Nafion®(5/2): 0.028 ± 0.016 nA mM^{-1}, $n = 5$, $P = 0.1814$).

Physiological conditions *in vitro*

Before performing experiments *in vivo* we tested the effects of two important physiological conditions which could dramatically affect the sensor response *in vivo*, namely temperature and concentration of tissue O_2. Calibrations for the Nafion®(5/2) sensor carried out at 37 °C (1.53 ± 0.28 nA μM^{-1}, $n = 5$) displayed a similar sensitivity ($P = 0.5002$) to those carried out at

‡ A negative sensitivity is reported as there was no response to AA additions and the background current drifted slightly below the previously recorded baseline value over the time scale of the calibration.[30]

Table 1 Comparison of sensitivities of bare Pt and Nafion®(5/2)-modified Pt electrodes to NO (CPA +900 mV *vs.* SCE) and potential endogenous electroactive interferents at reported physiological concentrations.[95] Number of electrodes in parenthesis

Analyte	Bare Pt sensitivity	Nafion®-modified sensitivity[a]
NO	1.08 ± 0.20/1.0 μM (28)	1.67 ± 0.08 nA/1.0 μM (14)
AA	26.3 ± 2.0 nA/500 μM (12)	0 nA/500 μM (3)
UA	1.53 ± 0.05 nA/50 μM (4)	0 nA/50 μM (3)
5-HT	0 nA/0.01 μM (4)	0 nA/0.01 μM (4)
DOPAC	0.94 ± 0.17 nA/20 μM (4)	0 nA/20 μM (4)
5-HIAA	0.99 ± 0.10 nA/50 μM (4)	0 nA/50 μM (4)
DA	0.009 ± 0.001 nA/0.05 μM (4)	0 nA/0.05 μM (4)
Glutathione	0.009 ± 0.001 nA/50 μM (4)	0 nA/50 μM (4)
HVA	0.10 ± 0.01 nA/10 μM (4)	0 nA/10 μM (4)
NO_2^-	0.56 ± 0.11 nA/100 μM (3)	0.003 ± 0.002 nA/100 μM (5)
H_2O_2	0.0043 ± 0.0007 nA/0.1 μM (3)	0.0012 ± 0.0002 nA/0.1 μM (3)

[a] 0 nA is reported in cases where there was no response to analyte addition. For example, 0.05 μM DA produced no change in the response of the Nafion®(5/2)-modified Pt electrodes: 0.043 ± 0.009 nA, $n = 4$ (background); 0.039 ± 0.008 nA, $n = 4$, $P = 0.8234$ (0.05 μM).

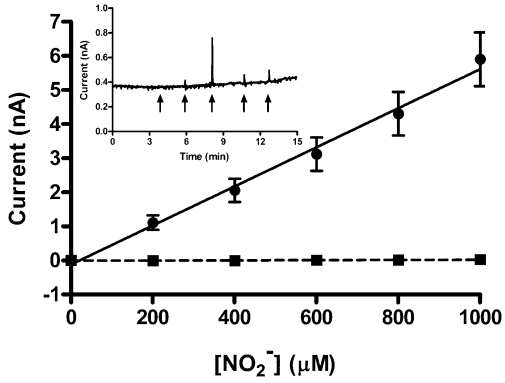

Fig. 5 Amperometric calibration plots for NO_2^- at bare Pt (solid line) and Nafion®(5/2)-modified Pt (dashed line) electrodes carried out in PBS, pH 7.4, at +900 mV *vs.* SCE. *Inset*: Typical steady state currents obtained for a 0–1000 μM NO_2^- calibration using a Nafion®(5/2)-modified Pt electrode; arrows indicate injections of 200, 400, 600, 800 and 1000 μM NO_2^-.

room temperature (25 °C: 1.67 ± 0.08 nA μM^{-1}, $n = 14$). This is in contrast to other reports where a doubling in NO sensitivity was found on increasing the calibration temperature over the same range.[21] We did, though, observe an increase in background current (25 °C: 0.24 ± 0.03 nA, $n = 14$; 37 °C: 3.10 ± 0.38 nA, $n = 5$) which has also been observed by other research groups.[106] However, the most encouraging characteristic displayed while calibrating the sensors at 37 °C was the significant decrease ($P = 0.0029$) in electrode response time to 14.00 ± 2.52 s ($n = 5$), compared to 33.67 ± 3.71 s ($n = 14$) at 25 °C. Results presented in the remaining sections of this manuscript are thus for the characterisation of the Nafion®(5/2) sensor only.

Since NO has a high tendency to react with molecular oxygen, it was decided to characterise the NO response in the presence of physiological concentrations of O_2 (*ca.* 50 μM[86]). Understanding the effects of tissue O_2 on the NO response is important if we are to use *in vitro* calibration data to estimate *in vivo* concentrations. No significant difference ($P = 0.0882$) was observed in the NO sensitivity under N_2-saturated (*ca.* 0 μM O_2,[95] 1.67 ± 0.08 nA mM^{-1}, $n = 14$) and 50 μM O_2 conditions (1.34 ± 0.19 nA μM^{-1}, $n = 4$) indicating that physiological concentrations of O_2 have negligible effect on the NO response characteristics of the sensor.

Stability

We have previously reported on the shelf-life of the Nafion®(5/2) sensor where 14 days of storage at room temperature had no significant effect on its AA interference characteristics.[31] However, electrochemical detection of NO *in vivo* is not only made difficult by the presence of electroactive interferents but also by the presence of electrode poisons such as lipids and proteins. While unmodified Pt electrodes have previously been shown to be susceptible to electrode poisoning[107] it has been reported that Nafion® enhances stability by protecting the electrode surface against fouling.[49,51,73] In fact, Mercado and Moussy have shown that annealed Nafion® is more biocompatible than room temperature cast Nafion®.[108]

In order to test the biocompatibility of our Nafion®(5/2) sensor we investigated the effect of its exposure to lipid (L-α-phosphatidylethanolamine, PEA) and protein (bovine serum albumin, BSA) solutions[109,110] on the NO sensitivity (1.67 ± 0.08 nA μM^{-1}, $n = 14$). Storage for 24 h in 10% BSA resulted in a significant decrease in sensitivity (1.19 ± 0.24 nA μM^{-1}, $n = 3$, $r^2 = 0.998$, $P = 0.0346$). However, on increasing the exposure time to 72 h no further decrease was observed: 0.87 ± 0.05 nA μM^{-1}, $n = 4$, $r^2 = 0.971$, $P = 0.2068$. Exposure to 10% PEA resulted in a similar decrease in sensitivity after 24 h (0.97 ± 0.12 nA μM^{-1}, $n = 4$, $r^2 = 0.992$, $P = 0.0008$), with an additional 48 h exposure having no further effect (1.12 ± 0.01 nA μM^{-1}, $n = 2$, $r^2 = 0.954$, $P = 0.4676$). Pooling the data for 24 h and 72 h for each type of treatment resulted in a 38.32% decrease in both cases. This is in line with other reports where decreases of between 20 and 50% have been observed following initial exposure of sensors to brain tissue.[111,112] These results highlight the need to adjust pre-implantation calibration data to allow for lipid and protein fouling or preferably to use post-*in vivo* data when estimating concentration changes.

In vivo studies

While the *in vitro* characterisation results indicate that the Nafion®(5/2) sensor has the necessary properties in terms of

selectivity, sensitivity and stability for *in vivo* neurochemical monitoring of NO it is important to perform an *in vivo* characterisation of the sensor to ensure the properties obtained in the *in vitro* environment are maintained in the more complex biological *milieu*. We thus performed some preliminary experiments in freely-moving animals involving systemic administration of NO and the NO precursor L-arginine.

Systemic administration of stock NO resulted in a maximum increase in signal of 35 ± 5 pA ($n = 5$, $P = 0.0020$) above baseline at 23 ± 7 min post-injection, whereas L-arginine produced a maximum signal increase of 71 ± 14 pA ($n = 6$, $P = 0.0043$) at 22 ± 6 min. Both signals remained elevated for *ca.* 10 ± 2 min and 25 ± 15 min respectively before returning to baseline levels: 917 ± 14 pA (pre-injection) *vs.* 913 ± 71 pA (post-injection) at 64 ± 15 min ($P = 0.1936$). *In vitro* calibration data suggest that these increases correspond to maximum concentration changes of 22 nM and 45 nM respectively. Examples of both NO and L-arginine injections are shown in Fig. 6 and Fig. 7, respectively, and are compared with the effect of normal saline injection: a small maximum increase in signal occurred after 3.0 ± 0.7 min before returning to baseline levels within 5.8 ± 0.9 min ($n = 4$). A similar short-lived injection stress-related response has previously been reported for other sensors and attributed to an increase in cerebral blood flow.[113] When compared with the maximum increases associated with NO and L-arginine, significant differences were found in both cases (NO, $P = 0.0043$; L-arginine, $P = 0.0091$) indicating that the change in signal associated with systemic administration of both compounds is not due to the stress linked with injection.

These preliminary *in vivo* results suggest that the Nafion®(5/2) sensor responds to changes in NO in the striatum of freely-moving rats. In the case of L-arginine the data provide the first direct evidence of an NO increase associated with systemic administration of the precursor, something which has been indirectly assumed in previous reports where direct real-time NO measurement was not possible.[113,114]

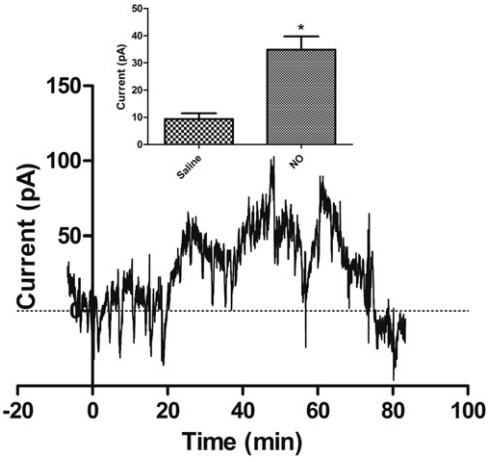

Fig. 6 An example of the effect of an intraperitoneal injection of stock NO solution (1 mM) on the response (background subtracted) of a Nafion®(5/2)-modified Pt electrode implanted in the striatum of a freely-moving rat. Time 0 is the point of injection. *Inset*: Comparison of the effect of saline *vs.* NO stock injection ($n = 5$, * $P = 0.0033$).

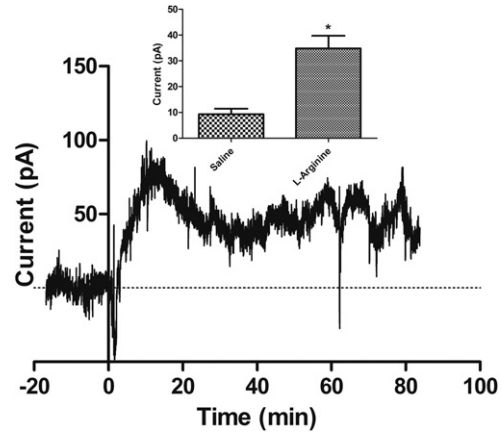

Fig. 7 An example of the effect of an intraperitoneal injection of the NO precursor L-arginine (300 mg/kg) on the response (background subtracted) of a Nafion®(5/2)-modified Pt electrode implanted in the striatum of a freely-moving Wistar rat. Time 0 is the point of injection. *Inset*: Comparison of the effect of saline *vs.* L-arginine injection ($n = 6$, * $P = 0.0091$).

Conclusions

A novel Pt-based electrochemical sensor modified with cured Nafion® (5 pre-coats/2 dip-coats) and previously developed for *in situ* real-time neurochemical monitoring[30] has now been characterised *in vitro* for the sensitive and selective detection of NO in brain ECF at +0.9 V (*vs.* SCE). Results indicate enhanced NO sensitivity compared to bare Pt, negligible interference from potential endogenous interferents, and good operational characteristics in terms of response time and stability, all suggesting high potential for reliable *in vivo* recording of NO. Preliminary *in vivo* experiments involving systemic administration of NO and L-arginine in freely-moving rats support the latter. Future experiments will involve a full *in vivo* characterisation of the Nafion®(5/2) sensor in terms of sensitivity, selectivity and stability to ensure that the properties presented in this report are maintained in the target biological environment of the brain.

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References

1 L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 9265.
2 Z. Huang, J. G. Louderback, M. Goyal, F. Azizi, S. B. King and D. B. Kim-Shapiro, *Biochim. Biophys. Acta, Gen. Subj.*, 2001, **1568**, 252.
3 H. Kosaka, *Biochim. Biophys. Acta, Bioenerg.*, 1999, **1411**, 370.
4 S. Archer, *FASEB J.*, 1993, **7**, 349.
5 R. Cammack, J. K. Shergill, V. Ananda Inalsingh and M. N. Hughes, *Spectrochim. Acta, Part A*, 1998, **54**, 2393.
6 D. M. Hall, G. R. Buettner and C. V. Gisolfi, *Microchem. J.*, 1997, **56**, 165.

7 M. K. Weih, S. Weikert, D. Freyer and U. Dirnagl, *Brain Res. Protoc.*, 1998, **2**, 175.
8 N. P. Evmiridis and D. Yao, *Anal. Chim. Acta*, 2000, **410**, 167.
9 S. Mesároš, S. Grunfeld, A. Mesárošová, D. Bustin and T. Malinski, *Anal. Chim. Acta*, 1997, **339**, 265.
10 R. W. Nims, J. F. Darbyshire, J. E. Saavedra, D. Christodoulou, I. Hanbauer, G. W. Cox, M. B. Grisham, F. Laval, J. A. Cook, M. C. Krishna and D. A. Wink, *Methods*, 1995, **7**, 48.
11 L. A. Ridnour, J. E. Sim, M. A. Hayward, D. A. Wink, S. M. Martin, G. R. Buettner and D. R. Spitz, *Anal. Biochem.*, 2000, **281**, 223.
12 S. A. Hilderbrand, M. H. Lim and S. J. Lippard, in *Topics in Fluorescence Spectroscopy - Advanced Concepts in Fluorescence Sensing, Vol. 9, Part A: Small Molecule Sensing*, eds. C. D. Geddes and J. R. Lakowicz, Springer, New York, 2005, pp. 163–188.
13 X. Ye, S. S. Rubakhin and J. V. Sweedler, *Analyst*, 2008, **133**, 423–433.
14 M. N. Friedemann, S. W. Robinson and G. A. Gerhardt, *Anal. Chem.*, 1996, **68**, 2621.
15 F. Bedioui and S. Trevin, *Biosens. Bioelectron.*, 1998, **13**, 227.
16 S. Burlet and R. Cespuglio, *Neurosci. Lett.*, 1997, **226**, 131.
17 D. Christodoulou, S. Kudo, J. A. Cook, M. C. Krishna, A. Miles, M. B. Grisham, R. Murugesan, P. C. Ford and D. A. Wink, in Methods in Enzymology. Nitric Oxide, Part A: Sources and Detection of NO; NO Synthase, ed. L. Packer, vol. 268, 1996, Academic Press, San Diego, CA, p. 69.
18 J. Jin, T. Miwa, L. Mao, H. Tu and L. Jiu, *Talanta*, 1999, **48**, 1005.
19 Y. Hirata, H. Hayakawa, E. Suzuki, K. Kimura, K. Kikuchi, T. Nagano, M. Hirobe and M. Omata, *Circulation*, 1995, **91**, 1229.
20 F. L. Kiechle and T. Malinski, *Am. J. Clin. Pathol.*, 1993, **100**, 567.
21 K. Shibuki, *Neurosci. Res.*, 1990, **9**, 69.
22 T. Malinski, in *Encyclopedia of Electrochemistry: Bioelectrochemistry*, eds. A. J. Bard, M. Stratmann and G. S. Wilson. Wiley-VCH, Weinheim, 2002, vol. 9, pp. 229–256.
23 F. Bedioui, S. Trevin and J. Devynck, *Electroanalysis*, 1996, **8**, 1085.
24 F. Bedioui, S. Trevin, J. Devynck, F. Lantoine, A. Brunet and M. A. Devynck, *Biosens. Bioelectron.*, 1997, **12**, 205.
25 E. Casero, M. Darder, F. Pariente and E. Lorenzo, *Anal. Chim. Acta*, 2000, **403**, 1.
26 T. Malinski and Z. Taha, *Nature*, 1992, **358**, 676.
27 X. J. Zhang, J. Lin, L. Cardoso, M. Broderick and V. Darley-Usmar, *Electroanalysis*, 2002, **14**, 697.
28 R. D. O'Neill and J. P. Lowry, in *Encyclopedia of Analytical Chemistry*, ed. R. A. Meyers, John Wiley & Sons Ltd., Chichester, pp 676–2000.
29 R. D. O'Neill, J. P. Lowry and M. Mas, *Crit. Rev. Neurobiol.*, 1998, **12**, 69.
30 F. O. Brown and J. P. Lowry, *Analyst*, 2003, **128**, 700.
31 F. O. Brown, N. J. Finnerty, F. B. Bolger, J. Millar and J. P. Lowry, *Anal. Bioanal. Chem.*, 2005, **381**, 964.
32 J. P. Lowry and R. D. O'Neill, *Electroanalysis*, 1994, **6**, 369.
33 J. P. Lowry, K. McAteer, S. S. El Attash, A. Duff and R. D. O'Neill, *Anal. Chem.*, 1994, **66**, 1754.
34 J. P. Lowry, M. Miele, R. D. O'Neill, M. G. Boutelle and M. Fillenz, *J. Neurosci. Methods*, 1998, **79**, 65.
35 S. Wolfensohn and M. Lloyd, *Handbook of Laboratory Animal Management and Welfare*, Oxford University Press, Oxford, 1994.
36 J. P. Lowry and M. Fillenz, *J. Physiol.*, 1997, **498**, 497.
37 A. E. Fray, R. J. Forsyth, M. G. Boutelle and M. Fillenz, *J. Physiol.*, 1996, **496**, 49.
38 G. Paxinos and C. Watson, *The Rat Brain in Stereotaxic Coordinates*, Academic Press, Sydney, 1986.
39 R. D. O'Neill, *Analyst*, 1993, **118**, 433.
40 D. B. Morton and P. H. M. Griffiths, *Vet. Rec.*, 1985, **116**, 431.
41 S. L. Vilakazi and T. Nyokong, *Electrochim. Acta*, 2000, **46**, 453.
42 K. C. Pan, C. S. Chuang, S. H. Cheng and Y. O. Su, *J. Electroanal. Chem.*, 2001, **501**, 160.
43 C. H. Fan, J. T. Pang, P. P. Shen, G. X. Li and D. X. Zhu, *Anal. Sci.*, 2002, **18**, 129.
44 F. Lisdat and F. W. Scheller, *Anal. Lett.*, 2000, **33**, 1.
45 J. P. Lowry, M. G. Boutelle and M. Fillenz, *J. Neurosci. Methods*, 1997, **71**, 177.
46 Y. Kitamura, T. Uzawa, K. Oka, Y. Komai, H. Ogawa, N. Takizawa, H. Kobayashi and K. Tanishita, *Anal. Chem.*, 2000, **72**, 2957.
47 F. Pariente, J. L. Alonso and H. D. Abruna, *J. Electroanal. Chem.*, 1994, **379**, 191.
48 A. Cserey and M. Gratzl, *Anal. Chem.*, 2001, **73**, 3965.
49 X. J. Zhang, Y. Kislyak, H. Lin, A. Dickson, L. Cardosa, M. Broderick and H. Fein, *Electrochem. Commun.*, 2002, **4**, 11.
50 A. Meulemans, *Neurosci. Lett.*, 2002, **321**, 115.
51 M. Yasuzawa and A. Kunugi, *Electrochem. Commun.*, 1999, **1**, 459.
52 L. Agüí, A. González-Cortés, P. Yáñez-Sedeño and J. M. Pingarrón, *Anal. Chim. Acta*, 1999, **401**, 145.
53 S. L. Alvarez-Crespo, M. J. Lobo-Castanon, A. J. Miranda-Ordieres and P. Tunon-Blanco, *Biosens. Bioelectron.*, 1997, **12**, 739.
54 L. T. Cai and H. Y. Chen, *Sens. Actuators, B*, 1999, **55**, 14.
55 L. A. Coury Jr., E. W. Huber, E. M. Birch and W. R. Heineman, *J. Electrochem. Soc.*, 1989, **136**, 1044.
56 Z. H. Fan and D. J. Harrison, *Anal. Chem.*, 1992, **64**, 1304.
57 M. G. Garguillo and A. C. Michael, *Anal. Chim. Acta*, 1995, **307**, 291.
58 R. Garjonyte and A. Malinauskas, *Sens. Actuators, B*, 2000, **63**, 122.
59 M. Huang and P. W. Faguy, *J. Electroanal. Chem.*, 1996, **406**, 219.
60 J. Katrlík and P. Zálešáková, *Bioelectrochemistry*, 2002, **56**, 73.
61 Y. M. Maksimov, B. I. Podlovchenko and T. L. Azarchenko, *Electrochim. Acta*, 1998, **43**, 1053.
62 M. Pontie, H. Lecture and F. Bedioui, *Sens. Actuators, B*, 1999, **56**, 1.
63 E. K. W. Lai, P. D. Beattie, F. P. Orfino, E. Simon and S. Holdcroft, *Electrochim. Acta*, 1999, **44**, 2559.
64 J. C. Vidal, S. Mendez and J. R. Castillo, *Anal. Chim. Acta*, 1999, **385**, 203.
65 J. C. Vidal, E. Garcia and J. R. Castillo, *Biosens. Bioelectron.*, 1998, **13**, 371.
66 N. Diab and W. Schuhmann, *Electrochim. Acta*, 2001, **47**, 265.
67 W. W. Kubiak and J. Wang, *Anal. Chim. Acta*, 1996, **329**, 181.
68 S. Myler, S. Eaton and S. P. J. Higson, *Anal. Chim. Acta*, 1997, **357**, 55.
69 W. A. Cass, G. A. Gerhardt, R. D. Mayfield, P. Curella and N. R. Zahniser, *J. Neurochem.*, 1992, **59**, 259.
70 M. E. Rice and C. Nicholson, *Anal. Chem.*, 1989, **61**, 1805.
71 J. K. Park, P. H. Tran, J. K. T. Chao, R. Ghodadra, R. Rangarajan and N. V. Thakor, *Biosens. Bioelectron.*, 1998, **13**, 1187.
72 J. P. Rivot, A. Sousa, J. Montagne-Clavel and J. M. Besson, *Brain Res.*, 1999, **821**, 101.
73 F. H. Wu, G. C. Zhao and X. W. Wei, *Electrochem. Commun.*, 2002, **4**, 690.
74 J. P. Lowry and R. D. O'Neill, *J. Electroanal. Chem.*, 1992, **334**, 183.
75 T.-H. Lu and I.-W. Sun, *Talanta*, 2000, **53**, 443.
76 B. Hoyer, N. Jensen and L. P. Busch, *Electroanalysis*, 2001, **13**, 843.
77 P. Bertoncello, M. K. Ram, A. Notargiacomo, P. Ugo and C. Nicolini, *Phys. Chem. Chem. Phys.*, 2002, **4**, 4036.
78 P. Ugo, P. Bertoncello and F. Vezza, *Electrochim. Acta*, 2004, **49**, 3785.
79 R. B. Moore III and C. R. Martin, *Anal. Chem.*, 1986, **58**, 2569.
80 L. D. Whiteley and C. R. Martin, *J. Phys. Chem.*, 1989, **93**, 4650.
81 B. Hoyer and N. Jensen, *Talanta*, 1994, **41**, 449–453.
82 J.-H. Ye and P. S. Fedkiw, *Electrochim. Acta*, 1995, **40**, 291.
83 J.-H. Ye and P. S. Fedkiw, *Electrochim. Acta*, 1996, **41**, 221.
84 B. Ilic, P. Neuzil, T. Stanczyk, D. Czaplewski and G. J. Maclay, *Electrochem. Solid-State Lett.*, 1999, **2**, 86.
85 D. M. Zhou, H. X. Ju and H. Y. Chen, *Sens. Actuators, B*, 1997, **40**, 89.
86 H. Y. Liu and J. Q. Deng, *Anal. Chim. Acta*, 1995, **300**, 65.
87 H. Matsuyama, K. Matsui, Y. Kitamura, T. Maki and M. Teramoto, *Sep. Purif. Technol.*, 1999, **17**, 235.
88 A. A. Mikhaylova, O. A. Khazova and V. S. Bagotzky, *J. Electroanal. Chem.*, 2000, **480**, 225.
89 J. Langmaier, A. Trojanek, J. Weber and Z. Samec, *J. Electroanal. Chem.*, 1999, **469**, 11.
90 D. Chu, *Electrochim. Acta*, 1998, **43**, 3711.
91 J. S. Jiang, D. B. Greenberg and J. R. Fried, *J. Membr. Sci.*, 1997, **132**, 273.
92 L. X. Sun, F. Xu and T. Okada, *Talanta*, 1998, **47**, 1165.
93 M. Pontié, C. Gobin, T. Pauporté, F. Bedioui and J. Devynck, *Anal. Chim. Acta*, 2000, **411**, 175.
94 W. Y. Tu, W. J. Liu, C. S. Cha and B. L. Wu, *Electrochim. Acta*, 1998, **43**, 3731.

95 J. P. Lowry, M. G. Boutelle, R. D. O'Neill and M. Fillenz, *Analyst*, 1996, **121**, 761.
96 I. Rubinstein, *J. Electroanal. Chem.*, 1984, **176**, 359.
97 L. M. Moretto, P. Ugo, R. Lacasse, G. Y. Champagne and J. Chevalet, *J. Electroanal. Chem.*, 1999, **467**, 193.
98 A. G. Wu and Y. G. Liu, *Brain Res.*, 1999, **850**, 249.
99 A. Kitajima, T. Teranishi and M. Miyake, *Electrochemistry*, 2001, **69**, 16.
100 A. Kitajima, M. Miyake, T. Kobayashi, H. Koyama, O. Ikeda, K. Kijima, T. Komura, A. Uno and A. Yamatodani, *Electrochemistry*, 1999, **67**, 784.
101 Y. Z. Xian, W. L. Sun, J. A. Xue, M. Luo and L. T. Jin, *Anal. Chim. Acta*, 1999, **381**, 191.
102 T. Malinski and L. Czuchajowski, in *Methods in Nitric Oxide Research*, eds. M. Feelisch and J. S. Stamler, John Wiley and Sons Ltd., New York, 1996, pp. 320–339.
103 D. J. Michael and R. M. Wightman, *J. Pharm. Biomed. Anal.*, 1999, **19**, 33.
104 S. V. Sasso, R. J. Pierce, R. Walla and A. M. Yacynych, *Anal. Chem.*, 1990, **62**, 1111.
105 N. R. Ferreira, A. Ledo, J. G. Frade, G. A. Gerhardt, J. Laranjinha and R. M. Barbosa, *Anal. Chim. Acta*, 2005, **535**, 1.
106 A. J. Gow, S. R. Thom, C. Brass and H. Ischiropoulos, *Microchem. J.*, 1997, **56**, 146.
107 P. Pantano and W. G. Kuhr, *Electroanalysis*, 1995, **7**, 405.
108 R. C. Mercado and F. Moussy, *Biosens. Bioelectron.*, 1998, **13**, 133.
109 D. E. Ormonde and R. D. O'Neill, *J. Electroanal. Chem.*, 1990, **279**, 109.
110 A. Nelson and N. Auffret, *J. Electroanal. Chem.*, 1988, **248**, 167.
111 M. G. Garguilo and A. C. Michael, *Anal. Chem.*, 1994, **66**, 2621.
112 Y. Hu, K. M. Mitchell, F. N. Albahadily, E. K. Michaelis and G. S. Wilson, *Brain Res.*, 1994, **659**, 117.
113 J. P. Lowry and M. Fillenz, *Bioelectrochemistry*, 2001, **54**, 39.
114 I. C. DaCunha, R. F. Jose, L. Orlandi Pereira, J. A. Pimenta, I. A. Oliveira de Souza, R. Reiser, H. Moreno Jr., J. Marino Neto, M. A. Paschoalini and M. S. Faria, *Physiol. Behav.*, 2005, **84**, 351.

