Analytical
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Cite this: *Anal. Methods*, 2012, **4**, 550

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PAPER

In vivo characterisation of a Nafion®-modified Pt electrode for real-time nitric oxide monitoring in brain extracellular fluid

Niall J. Finnerty,*[a] Saidhbhe L. O'Riordan,[a] Finbar O. Brown,†[a] Pier A. Serra,[a] Robert D. O'Neill[b] and John P. Lowry[a]

Received 21st December 2011, Accepted 21st December 2011
DOI: 10.1039/c2ay05924j

A Nafion®-modified Pt sensor developed for real-time neurochemical monitoring in freely-moving animals has now been characterised *in vivo* for the detection of nitric oxide (NO). Experiments were undertaken to test sensitivity, selectivity and stability. In control experiments, local administration of aCSF resulted in a decrease in signal (42 ± 12 pA, $n - 9$), while systemic administration of saline caused a transient increase in signal (22 ± 3 pA, $n - 9$) from baseline levels. Local MD perfusions of known concentrations of NO (1 mM) and L-arginine (720 μM) caused significant increases in current (4 ± 1 nA ($n - 7$) and 271 ± 37 pA ($n - 5$) respectively) while systemic administration of the non-selective NOS inhibitor L-NAME produced a significant decrease in current (91 ± 19 pA, $n - 4$) compared to baseline levels. Confirmation that the sensor remained selective was achieved by injection of ascorbic acid (2 g kg^{-1}, i.p). The oxidation current showed no variation over the selected time interval of 60 min, indicating no deterioration of the polymer membrane. Finally, after an initial decrease in sensitivity (*ca.* 50%) within the first 24 h of implantation the Nafion®-modified Pt sensor showed excellent stability under physiological conditions over extended periods (8 days) validating its use for continuous chronic *in vivo* recordings.

Introduction

Since its characterisation as the endothelium derived relaxing factor (EDRF) in the late 80's,[1,2] nitric oxide (NO) has been elucidated as having various other biomedical roles both intracellularly and extracellularly. Typical examples include a neurological function in synaptic plasticity, neurotransmission, learning and memory,[3–5] in addition to having a primary role in non-specific immunity,[6] penile erection[7,8] and platelet aggregation inhibition.[9] As NO is a gaseous free radical it is extremely reactive and has a high affinity for interaction with ferrous hemoproteins such as soluble guanylate cyclase[1] and hemoglobin,[10,11] while also reacting readily with molecular oxygen (O_2), peroxides and the superoxide anion (O_2^-).[12–14] The reaction between NO and O_2^- yields peroxynitrite ($ONOO^-$), a powerful oxidant through which it is hypothesised that NO carries out its neurotoxic effects[13,14] by oxidising lipids, proteins and DNA.[13,15]

[a]*Sensors Development Unit and Neurochemistry Research Unit, BioAnalytics Laboratory, Department of Chemistry, National University of Ireland Maynooth, Co. Kildare, Ireland. E-mail: niall.finnerty@nuim.ie; Fax: + 353-1-708 3815; Tel: + 353-1-708 6477*
[b]*UCD School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland*
† Pesticide Registration and Control Division, Department of Agriculture, Fisheries and Food, Backweston Laboratory Complex, Celbridge, Co. Kildare, Ireland. Department of Neuroscience, Medical School, University of Sassari, Viale S. Pietro 43/b, 07100 Sassari, Italy.

As a consequence of this neurotoxicity, NO has been implicated in a number of cardiovascular diseases such as hypertension and diabetes[14,16] and in brain disorders such as Alzheimer's disease[16–18] and schizophrenia.[19] Collectively this evidence suggests that the NO pathway may constitute an interesting target for novel pharmacological therapies in a wide range of disorders and possibly play a role in the pathophysiology of these disorders. Unfortunately, this hypothesis is a subject of contention, primarily due to the lack of direct evidence in support of this assumption.

The majority of methods currently used to monitor NO in biological systems tend to measure it indirectly. Typical examples include employment of NO-synthase inhibitors,[20,21] measurement of cGMP[22,23] or citrulline,[24] and the detection of NO-induced physiological effects such as vascular relaxation.[1,25] All of these are critically challenged by NO's unique chemical and physical properties, including its reactivity, rapid diffusion, and short half-life. Despite such problems, several analytical assays for measuring NO have been established. The most commonly used techniques are: UV-visible spectrophotometry of the diazotization product of the nitrite,[26] NO-hemoglobin or methemoglobin;[27,28] fluorometry of the fluorescent product of the nitrite;[29] detection of chemiluminescence by its reaction with ozone or luminol/H_2O_2;[30] and electron spin resonance spectrometry.[31,32] However, these techniques tend to be hindered by significant drawbacks including technical complexity, poor sensitivity, and

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interference such as detection of nitrite or nitrate derived from non-NO sources. As an alternative, the possibility of using microelectrochemical sensors for direct real-time NO measurement has recently gained considerable interest.[28,33–35] This is primarily because of their speed and sensitivity,[27,30,36] which are particularly important for NO as it is present at picomolar-to-micromolar concentrations[37] and has a half-life of 2–6 s *in vivo*.[38]

We have previously reported the *in vitro* development[39] and characterisation[40] of a Nafion®-modified Pt sensor designed for real-time monitoring of brain extracellular NO. Application of Nafion® (5% commercial solution) using a thermally annealing procedure involving 5 pre-coats, and 2 subsequent dip-bake layers results in a sensor (Nafion®(5/2)) which has a response time suitable of *in vivo* monitoring, linearity over the relevant concentration range for NO, freedom from protein and lipid fouling, and minimal interference from a variety of endogenous species, including ascorbic acid, dopamine and serotonin over physiologically relevant concentration ranges. However, before any sensor can be used reliably *in vivo* it is necessary to perform a detailed *in vivo* characterisation to ensure the properties obtained in the *in vitro* environment are maintained in the more complex and hostile biological milieu. In the present study we report sensitivity, selectivity and stability data for the Nafion®(5/2)-modified Pt sensor implanted in the striatum of awake freely-moving rats.

Materials and methods

Chemicals and solutions

All chemicals used throughout the experiments were purchased from Sigma Chemical Co. (Dublin, Ireland). A stock solution of artificial cerebrospinal fluid (aCSF) was made up in doubly distilled deionised water consisting of NaCl (0.15 M), KCl (0.004 M), $CaCl_2$ (0.0016 M) and $MgCl_2$ (0.021 M). A 0.9% solution of saline was prepared by dissolving 0.9 g NaCl in 100 mL doubly distilled water. In all cases, unless otherwise noted all systemic administration of L-arginine (300 mg kg^{-1}), L-N^{G}-Nitroarginine methyl ester hydrochloride (L-NAME (30 mg kg^{-1})), and sodium ascorbate (2 g kg^{-1}) were made up in a solution of 0.9% saline, while local perfusion of L-arginine (720 μM) was prepared in aCSF.

NO sensor preparation

Nafion® (5/2)-coated Pt disk electrodes were made from Teflon®-insulated platinum/iridium (Pt/Ir 90%/10%) wire (125 μM bare diameter 5T, Advent Research Materials, Suffolk, UK). The electrodes were approximately 4 cm in length and were prepared by carefully cutting 2 mm of Teflon® insulation from one end of the wire and soldering to this end a gold clip which provided rigidity and electrical contact. The other end of the wire acted as the active (disk) surface. The electrode was modified as previously described by Brown *et al.*[40]

NO stock synthesis

NO was synthesised in house using a very reproducible and inexpensive method which was described previously by Brown *et al.*[41] Due to NO being such a reactive gas it is important to ensure the collecting vial is free from oxygen contamination. The solution must be used within 3 h of synthesis.

Local and systemic administrations

All local administration was carried out in the striatum of Wistar rats by microdialysis (MD). A MD probe (Bioanalytical Systems, Inc, Indiana, USA) was implanted in close proximity to the NO sensor and perfused with aCSF. All systemic administrations were carried out by intraperitoneal injection (i.p.).

In vivo implantation and surgery protocol

Male Wistar rats (Biomedical Facility, University College Dublin, Ireland) weighing between 200 and 300 g were housed in a temperature-controlled (17–23 °C), humidity-controlled and light-controlled (12 h light, 12 h dark cycle) environment with access to food *ad libitum* prior to surgery. NO sensors and the combined sensor/MD probes were implanted following a previously described procedure[42] into the right/left striatum (coordinates with the skull levelled between bregma and lambda, were: A/P + 1.0, M/L $\pm$ 2.5 from bregma and D/V −5.0 from dura).[43] A reference and auxiliary electrode (8T Ag wires, 200 μm bare diameter) were placed in the cortex. The reference potential provided by the bare Ag wire in brain tissue is very similar to that of the saturated calomel electrode (SCE) used in the *in vitro* characterisation.[44] The electrodes and probe were fixed to the skull with dental screws and dental acrylate (Associated Dental Products, Swindon, UK). The rats were anesthetized with the volatile anaesthesia Isoflurane, placed in a Kopf stereotaxic instrument and kept on a heating pad to prevent hypothermia. A 1 mL kg^{-1} injection of the opioid analgesic buprenorphine is administered subcutaneously (s.c.) 30 min after the end of the surgery and the animal allowed to rest. The animal is monitored for the next few hours, before being transferred to a holding bowl where it remains for the duration of the experiment. The animal is allowed to recover for at least 24 h prior to connection to the potentiostat. The desired potential (+900 mV *vs.* Ag wire) is then applied to the NO sensor and the current is allowed to stabilise for approximately 24 h. Following this period of stabilisation, *in vivo* measurements were commenced. All experimental procedures were performed under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 and U.K. Animals (Scientific procedures) Act 1986).

Instrumentation and software

Constant potential amperometry 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). All data presented had baselines normalized to zero to show the change in current (ΔI) and reported concentration changes are based on *in vitro* pre-calibration curves (average slope/sensitivity − 1.59 $\pm$ 0.04, n − 28 nA μM^{-1}). The significance of differences observed was

estimated using the Student's t-test for paired or unpaired observations where appropriate, or one way ANOVA followed by Bonferroni test. Two-tailed levels of significance were used with $P < 0.05$ considered to be significant. All data are presented as mean ± standard error (SEM), with n = number of sensors implanted in 5 animals (aCSF), 3 animals (saline), 5 animals (NO), 4 animals (L-arginine), 3 animals (L-NAME) and 3 animals (ascorbate). Stability investigations and post calibration sensitivities were carried out in 4 and 6 animals, respectively. The typical example data presented in Fig. 1–4 have been normalised and transformed to the average response obtained for the respective treatments. This removes both inter electrode and inter animal variability by ensuring that the presented current and concentration changes are representative of the data from all the animals used in each study. Scanning Electron Microscopy (S.E.M) images where carried out using a Hitachi S-3200 N to investigate the effect of implantation on the Nafion® polymer surface.

Experimental conditions

All experiments were carried out with the animal in its home bowl. Implanted electrodes were connected to the potentiostat through a six-pin Teflon socket and a flexible screened six core cable which was mounted through a swivel above the rats head (Semat Technical) at least 7–8 h prior to the start of the first experiment each day. This arrangement allowed free movement of the animal.

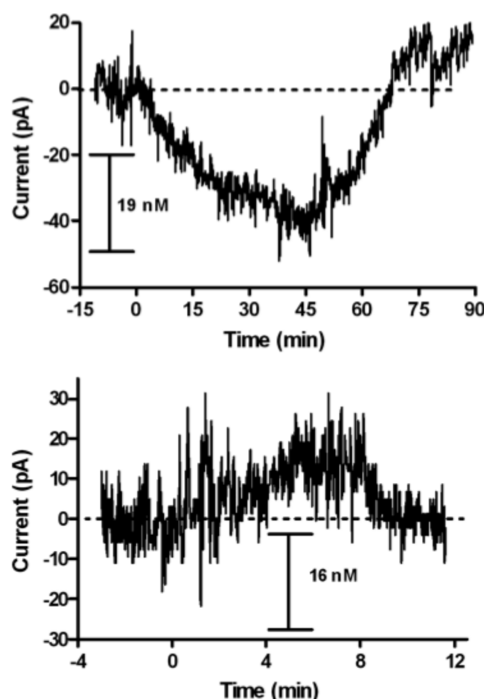

Fig. 1 Typical example of the effects of local perfusion (top) of aCSF ($2.0\ \mu L\ min^{-1}$, average pre-perfusion baseline = 981 ± 136 pA, n = 9) and (bottom) saline (1 mL i.p. injection, average pre-injection baseline = 828 ± 77 pA, n = 9), monitored in rat striatum with a Nafion®(5/2)-modified Pt sensor. Time zero indicates the start of 60 min perfusion (top) and point of injection (bottom).

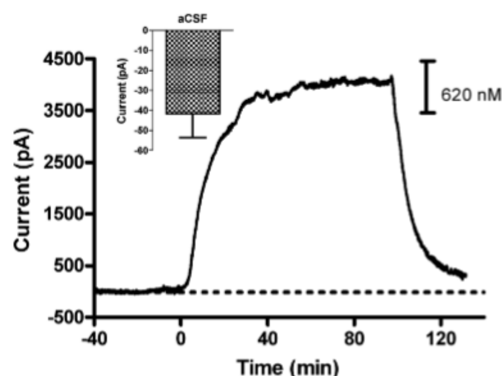

Fig. 2 Typical example of the effect of local perfusion of NO (1 mM, $2.0\ \mu L\ min^{-1}$, average pre-perfusion baseline = 726 ± 87 pA, n = 7), monitored in rat striatum with a Nafion®(5/2)-modified Pt sensor. Time zero indicates start of 60 min perfusion. *Inset* Average aCSF effect ($n = 9$).

Voltammetry techniques *in vivo*

All *in vivo* experiments utilised constant potential amperometry which involves the application of a constant potential. The resulting current is directly proportional to the concentration of the analyte at any given time. NO was detected by holding the implanted sensor at the oxidation potential of +900 mV *vs.* Ag wire which had been previously characterized as the optimum potential for NO detection.[40,45]

Results and discussion

Local perfusion of nitric oxide and L-arginine

In order to show that the sensor responds to changes in NO *in vivo* local perfusions of NO and its pre-cursor L-arginine were carried out through a MD probe situated in close proximity to the NO sensor. The vehicle for delivery was aCSF which was administered prior to all local drug perfusions. aCSF perfusion samples the concentration of the unbound fraction of endogenous substances in brain extracellular fluid.[46,47] Once perfusion

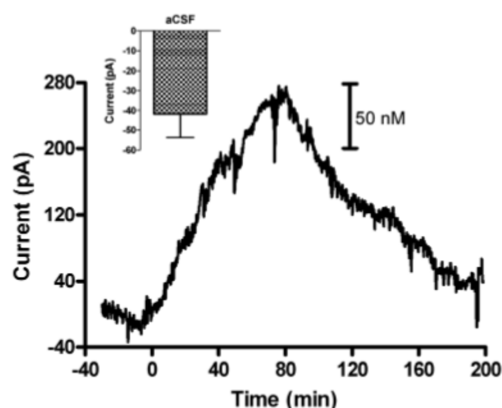

Fig. 3 Typical example of the effect of local perfusion of L-arginine (720 µM, $2.0\ \mu L\ min^{-1}$, average pre-perfusion baseline = 761 ± 111 pA, n = 5), monitored in rat striatum with a Nafion®(5/2)-modified Pt sensor. Time zero indicates start of 60 min perfusion. *Inset* Average aCSF effect ($n = 9$).

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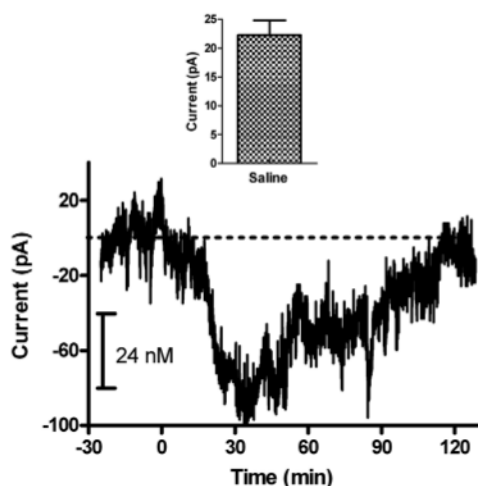

Fig. 4 Typical example of the effect of an i.p injection of L-NAME (30 mg kg^{-1}, average pre injection baseline = 957 ± 91 pA, n = 4), monitored in rat striatum with a Nafion®(5/2)-modified Pt sensor. Time zero indicates the point of injection. *Inset* Average effect of systemic saline administration (*n* = 9).

commences; compounds present in the tissue surrounding the MD probe including NO, diffuse down their concentration gradient across the MD membrane and into the dialysate. Continuous removal of this dialysate maintains the concentration gradient. A typical example of changes in the oxidation current following aCSF perfusion is shown in Fig. 1 (top). There was an immediate decrease in current which returned to baseline post administration. The signal (ΔI) displayed a maximum decrease of 42 ± 12 pA ($n = 9$) at 35 ± 4 min ($n = 9$) and had returned to baseline after 65 ± 7 min ($n = 9$). This current change was significantly different ($P < 0.01$) when compared to baseline and represented a 25 ± 7 nM ($n = 9$) decrease in concentration based on *in vitro* calibration curves. This decrease in current can be attributed to the diffusion of NO, in the tissue surrounding the sensor, down its concentration gradient and across the semi permeable MD membrane. An important issue to address is the potential for MD probes to damage tissue following implantation and the effect that this will have on the sensor. Khan and Michael have reported that stimulated neurochemical release in the tissue immediately adjacent to the MD probe is different than 1 mm away from the probe.[48] They attribute this to the traumatic brain injury caused by implantation of the micordialysis probe, which essentially eliminates the ability of nerve impulses to trigger dopamine release in the tissue adjacent to the probe. However, as we are not measuring stimulated release, the effect on our sensor is negligible. The area around the probe will become saturated with perfusate, causing an increase in NO concentration in the tissue surrounding the sensor. This technique is strictly a validation of the NO sensors ability to measure changes in NO concentration *in vivo*.

We have previously reported preliminary *in vivo* characterisation data for the Nafion®(5/2) sensor. Systemic administration of stock NO (1 mM) resulted in a significant increase in the current (35 ± 5 pA, $n = 5$, $P < 0.01$) above baseline at 23 ± 7 min post-injection.[40] A typical example of the effects of local NO administration on the signal is displayed in Fig. 2. There was an immediate increase in oxidation current following perfusion and a subsequent return to baseline post administration. The signal (ΔI) increased maximally by 4 ± 1 nA ($n = 7$) and had returned to baseline by 122 ± 22 min ($n = 7$). This resulted in a significant change from baseline ($P < 0.01$) corresponding to a concentration change of *ca.* 2.0 ± 0.4 μM (n = 7). Local perfusion of exogenous NO caused an increase in endogenous levels that reached a maximum after 33 ± 5 min. The delayed response is attributed to the perfusion rate (2 μL min^{-1}) of the reterodialysis technique and the time taken for the perfusate to diffuse out and saturate the tissue surrounding the sensor. This will not happen immediately. Stock perfusions of NO ensured local delivery of the analyte to the tissue where the sensor is located, a process known as retrodialysis.[49] The perfusate contains a higher concentration of NO in relation to the surrounding tissue resulting in the gaseous molecule diffusing across the dialysis membrane and into the brain extracellular fluid. The NO sensor implanted in close proximity to the dialysis membrane records an increase in response to this saturation of target analyte. The recorded current reaches a maximum level remaining elevated until cessation of perfusion, after which the signal returns to a pre-administration baseline. The discrepancy between the initial NO concentration in the perfusate and the measured concentration can be explained by the high reactivity of NO in physiological conditions. Local perfusion of exogenous NO is not expected to result in 100% detection at the sensor surface due to a number of factors. As NO is a gaseous free radical it is extremely reactive and has an extremely short half-life *in vivo*. Previous reports have calculated the half-life of NO at physiological concentrations (1 μM to 10 nM) to range from seconds to minutes.[38,50] However, the half-life is inversely proportional to the NO concentration, suggesting that at higher concentrations (1 mM) the half-life is greatly reduced due to its high tendency to react with molecular oxygen and reactive oxygen species (ROS) such as O_2^-, hydrogen peroxide (H_2O_2) and the hydroxyl anion (OH^-). The short life time of NO in physiological conditions, especially when present at such high concentrations, would cause this decrease in measured concentration. Another factor may be the deterioration of the stock solution of NO perfused over time. Earlier work carried out reported an in house method of synthesising NO.[41] The NO stock solution showed excellent stability (*ca.* 6% variation) over the first 30 min following synthesis, however, a dramatic deterioration of the NO concentration occurred over the next 90 min (*ca.* 47%). It is possible that a decrease in the stock concentration had occurred prior to perfusion in addition to the physiological factors. Previous work from various groups have reported using a similar characterisation protocol.[7,33,51,52] Escrig *et al.*[7] delivered commercial NO gas to the penis corpus cavernosa while Friedemann *et al.*[33] reported an increase in observed current at a Nafion®/o-phenylenediamine modified carbon fiber electrode implanted in anaesthetized animals following a 25 nL perfusion of 2 mM stock NO solution from a single-barrel micropipette with a return to baseline a few minutes after administration. Park *et al.*[51] have also reported the use of a similar type sensor in anaesthetized animals while more recently Heinzen and Pollack[52] have reported validation data in freely moving animals using commercial NO sensors.

The amino acid L-arginine is the precursor for NO production in the body. Its subsequent reaction with molecular O_2 results in the formation of NO and L-citrulline with the latter regenerated back to arginine resulting in NO metabolism fitting into the normal nitrogen metabolism of the body.[6] Previous work has demonstrated that systemic L-arginine significantly increased the NO sensor's signal (71 ± 14 pA, $n - 6$, $P < 0.01$) after 22 ± 6 mins compared to pre-injection baseline levels.[40] Here we investigated the effect of 60 min local perfusions. Fig. 3 illustrates a typical example of the effects of local L-arginine (720 μM) delivery to the striatum. There is a significant increase in signal reaching a maximum after 36 ± 7 min ($n - 5$) and producing an overall current increase of 271 ± 37 pA ($n - 5$). A similar phenomenon is observed with exogenous NO perfusion; however, the slightly increased duration (36 ± 7 min) recorded with L-arginine can be attributed to the biosynthesis of NO. Upon saturation of the tissue with the precursor a downstream increase in NO concentration at the sensor surface occurs. This confirms that the NO sensor is responding to increased production of endogenous NO. A return to pre-administration levels occurred after 90 ± 30 min ($n - 5$). This current increase corresponds to a concentration change of *ca.* 162 ± 22 nM ($n - 5$) and is significantly different compared to baseline recordings ($p < 0.01$). These results validate previous findings from other groups who reported an increase in NO current accompanying L-arginine perfusion, using an amperometric NO sensor implanted in the hippocampus of rats.[52,53] Other reports confirm the antioxidant effects of L-arginine in the early and late stages of ischemia with deficiencies of the amino acid in brain neurons along with inadequate levels of NO formation within them leading to increases in superoxide formation.[54] Our finding supports the role of L-arginine in the biosynthesis of NO in the body; however, contradictory reports have been published discussing the possible role of L-arginine in one mechanism of NO inhibition. Castellano *et al.* detailed how glutamate-stimulated NO production was markedly increased by 32 nM L-arginine infusion, however, 128 nM infusion had no significant effect on glutamate-evoked NO production as the NO levels were significantly lower than the corresponding value obtained with 32 nM L-arginine. This lead to their hypothesis that L-arginine despite being a precursor for NO synthesis may be involved in a mechanism of NO inhibition.[55]

Systemic L-NAME injection

Since the NOS inhibitor L-NAME, and primary interferent ascorbate, were administered by i.p. injection we first examined the effect of i.p. injection of normal saline (NaCl 0.9%). A typical example of changes in signal is shown in Fig. 1 (bottom). There was an immediate increase in the recorded current following injection. The signal (ΔI) increased maximally by 22 ± 3 pA ($n -$ 9) at 4 ± 1 min ($n - 9$) and had returned to baseline by 13 ± 4 min ($n - 9$). This increase represented a significant change from baseline ($p < 0.0001$, $n - 9$) corresponding to an increase in concentration of *ca.* 13 ± 2 nM ($n - 9$). Similar initial and brief injection effects have also been observed for tissue O_2[56] and regional cerebral blood flow (rCBF)[57] during the injection of saline, with a comparable return to baseline levels. The stress of the i.p. injection stimulates neuronal activation,[58] increasing rCBF and thus O_2, with the supply of the latter exceeding utilisation. The observed increase may be attributed to this increase in blood flow following vasodilation brought about by the EDRF.[1,2]

Next it was imperative to characterise the sensor against a known inhibitor of NO production. L-NAME is a non-selective nitric oxide synthase (NOS) inhibitor which acts by competing with L-arginine for its binding site on the NOS enzyme.[59] A typical example of the effects of a 30 mg kg^{-1} i.p injection is illustrated in Fig. 4. The signal (ΔI) decreased maximally by $91 \pm$ 19 pA ($n - 4$) at 52 ± 16 min ($n - 4$) and had returned to baseline by *ca.* 180 min. This current change represented a significant decrease ($P < 0.05$) from baseline, corresponding to a concentration change of *ca.* 55 ± 12 nM ($n - 4$). Previous reports detailing L-NAME have shown that the NOS inhibitor attenuates or completely blocks the effects of phencyclidine hydrochloride (PCP), an NMDA receptor antagonist that is assumed to carry out its actions through an NO-mediated mechanism.[3,4,60,61] A number of behavioural studies incorporating a series of different paradigms have confirmed this, for example, prepulse inhibition, which is the reduction in startle amplitude to a startling stimulus when this stimulus is immediately preceded by a weaker pre-stimulus.[20,62] Another is latent inhibition, which is a referral to a phenomenon whereby pre-exposure to a stimulus weakens the subsequent association of that stimulus with a reinforcer in classical conditioning.[63,64] We have recently reported direct evidence that L-NAME inhibits NO production following systemic administration of PCP[21] which confirms all hypothesis of the previous behavioural investigations. Other paradigms include the elevated plus-maze which is based on exploratory behaviour of rats. NO is believed to play a role in learning and memory and L-NAME is shown to induce a learning deficit in this avoidance learning test.[65] Since nitric oxide has been characterized as the EDRF, it is very closely associated with CBF and cerebrovasodilation. A number of existing reports have reported that L-NAME induced a reduction in levels of CBF that were increased following a period of hyperbaric oxygen exposure by a nitric oxide mediated mechanism.[25,66,67]

Interference studies

It has been previously demonstrated *in vitro* that the Nafion®(5/2)-modified Pt NO sensor displayed excellent rejection of a wide range of electroactive interferents (*e.g.* ascorbic acid, dopamine, DOPAC, NO_2^-, serotonin) found endogenously in brain extracellular fluid.[40] The most abundantly present interferent is ascorbic acid with a reported ECF concentration of *ca.* 500 μM.[68] However, it was important to investigate the effect of systemic administrations of ascorbate on the Nafion®-modified Pt sensor current *in vivo*. A typical example of the effects of a 2 g kg^{-1} i.p injection is illustrated in Fig. 5. The current was monitored over a 60 min period as previous investigations have reported this time frame allows for a maximum response to occur.[69] It is hypothesised that any effect following ascorbate injection will have occurred within this time frame. Fig. 5 (inset) illustrates this effect from a carbon paste electrode (CPE) implanted in the striatum. It is clear from the inset that a maximum response measured by the CPE has occurred within the first hour. It is apparent from the Nafion®-modified Pt sensor data that there is no significant change ($p > 0.05$) in oxidation current

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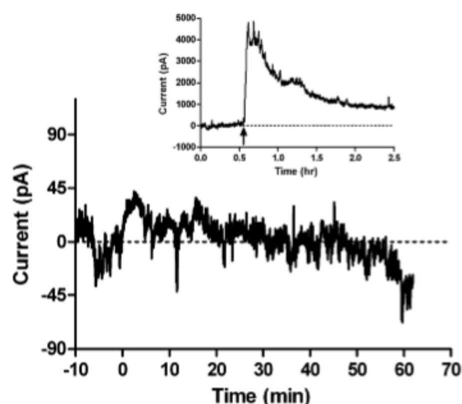

Fig. 5 Typical example of the effect of an i.p injection of sodium ascorbate (2 g kg^{-1}, average pre-injection baseline = 883 ± 86 pA, n = 3) on NO current monitored in rat striatum with a Nafion®(5/2)-modified Pt sensor. Time zero indicates point of injection. *Inset*: Typical example of the effect of an i.p. injection of sodium ascorbate on the response of a carbon paste electrode implanted in rat striatum.

($\Delta I = -8 \pm 7$ pA, n = 3) over the 60 min post ascorbate injection. The slight decrease in current can be attributed to baseline drift recorded over the 60 mins. A similar trend was reported during *in vitro* investigations.[39] Collectively these results support the assumption that the Nafion® membrane has not degraded when placed in the *in vivo* environment and that the sensor exhibits similar selectivity characteristics to those recorded *in vitro*.[39,40] Indeed S.E.M performed post implantation confirms an intact Nafion® polymer on the electrode surface (see Fig. 6).

Stability investigations

Direct contact of electrochemical sensors with biological samples *in vivo* can result in a decrease in sensitivity as a result of fouling by lipids and proteins.[70] Nafion® has previously been reported to enhance stability by protecting against surface fouling in brain extracellular fluid,[33,51,71,72] corpora cavernosa[7,8] and aorta.[73] The mean baseline current recorded with the Nafion®(5/2)-modified sensor (see Fig. 7) implanted in the striatum of freely moving animals showed no significant variation ($p > 0.05$, two-way ANOVA: $n = 4$) over a successive 8 day period from day 1 (*ca.* 24 h following recovery from surgery) to day 8: 742 ± 71 pA

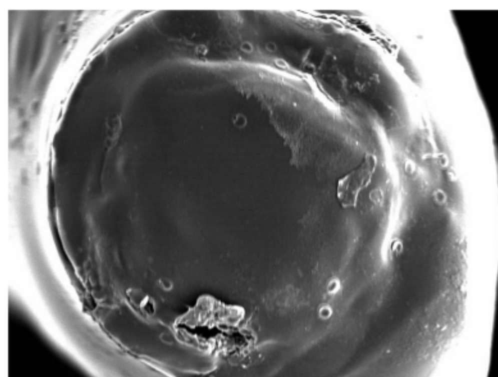

Fig. 6 Surface S.E.M of a Pt-Nafion® (5/2)-modified electrode after 14 days implantation in the brain of a freely moving rat.

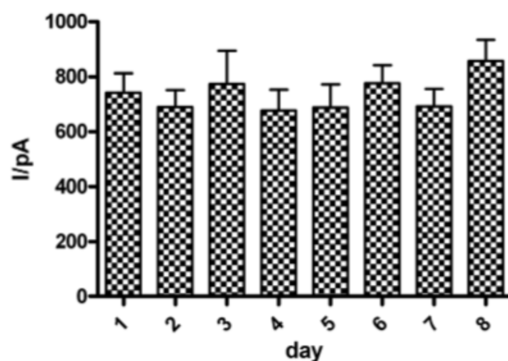

Fig. 7 Average (± SEM) baseline *in vivo* data for Pt-Nafion® (5/2)-modified sensors implanted in four animals over a successive 8 day period.

(day 1); 690 ± 62 pA (day 2); 774 ± 120 pA (day 3); 678 ± 75 pA (day 4); 689 ± 84 pA (day 5); 777 ± 65 pA (day 6); 692 ± 64 pA (day 7); 856 ± 78 pA (day 8). No difference in sensitivity after 8 days recording supports the assumption that the sensor remains stable for extended periods *in vivo* confirming its suitability for chronic recording. We also compared the sensitivity of the sensor pre and post *in vivo* to evaluate the overall effect of implantation on the performance of the sensor. The pre-implantation sensitivity determined from *in vitro* calibration data was 1.3 ± 0.1 nA μM^{-1} ($n = 7$). The post-implantation sensitivity determined following euthanasia was 0.6 ± 0.2 nA μM^{-1} ($n = 7$). This corresponds to a 54 ± 11% decrease in sensitivity ($P < 0.01$) over implantation periods ranging from 11 to 23 days and is in line with other reports where decreases of between 20 and 50% have been observed following initial exposure of sensors to brain tissue.[74,75] It is also similar to the 38% decrease previously reported for this sensor following *in vitro* exposure to lipid (L-α-phosphatidylethanolamine) and protein (bovine serum albumin) solutions.[40] The latter occurred within the first 24 h of exposure with no significant effect of further exposure thereafter. This is supported by the *in vivo* baseline stability data reported above.

Conclusions

A novel Pt-based electrochemical sensor modified with cured Nafion® (5 pre-coats/2 dip-coats) previously characterised for use *in vitro*[40] has now been characterised *in vivo* for the sensitive and selective detection of NO in brain extracellular fluid. Previously reported preliminary *in vivo* results confirmed NO changes following systemic administrations of stock NO and L-arginine.[40] A detailed characterisation has been carried out in the striatum of Wistar rats (see Table 1) and significant NO changes were recorded against baseline following local administrations of stock NO and L-arginine. Systemic administration of L-NAME produced a significant decrease in NO current compared to baseline levels, corroborating previously reported systemic investigations. Ascorbate selectivity studies confirmed minimal deterioration of the Nafion® modified surface. Finally, the stability of the sensor was analyzed over 8 days confirming no significant change in baseline following an initial drop in sensitivity which occurs within the first 24 h after implantation.

Table 1 Summary of *in vivo* characterisation data for Nafion® (5/2)-modified sensors

Administration	ΔI (pA, unless stated)	Max Response Time (min)	Return to baseline (min)	Conc. (nM, unless stated)
aCSF (local)	−42 ± 12 (*n* – 9)	35 ± 4 (*n* – 9)	65 ± 7 (*n* – 9)	25 ± 7 (*n* – 9)
Saline (systemic)	22 ± 3 (*n* – 9)	4 ± 1 (*n* – 9)	13 ± 4 (*n* – 9)	13 ± 2 (*n* – 9)
NO (local)	4 ± 1 nA (*n* – 7)	33 ± 5 (*n* – 7)	122 ± 22 (*n* – 7)	2.0 ± 0.4 μM (n – 7)
L-arginine (local)	271 ± 37 (*n* – 5)	36 ± 7 (*n* – 5)	90 ± 30 (*n* – 5)	162 ± 22 (*n* – 5)
L-NAME (systemic)	−91 ± 19 (*n* – 4)	52 ± 16 (*n* – 4)	180	55 ± 12 (*n* – 4)
Ascorbate (systemic)	−8 ± 7 (n – 3)	n/a	n/a	n/a

Acknowledgements

We gratefully acknowledge financial support from the Analytical Chemistry Trust Fund, Royal Society of Chemistry (RSC) Analytical PhD Studentship 03/F/018, Enterprise Ireland (BR/1999/159), and Science Foundation Ireland (03/IN3/B376).

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