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Characterisation of carbon paste electrodes for real-time amperometric monitoring of brain tissue oxygen

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ABSTRACT

Tissue O_2 can be monitored using a variety of electrochemical techniques and electrodes. *In vitro* and *in vivo* characterisation studies for O_2 reduction at carbon paste electrodes (CPEs) using constant potential amperometry (CPA) are presented. Cyclic voltammetry indicated that an applied potential of −650 mV is required for O_2 reduction at CPEs. High sensitivity (−1.49 ± 0.01 nA/μM), low detection limit (*ca.* 0.1 μM) and good linear response characteristics ($R^2 > 0.99$) were observed in calibration experiments performed at this potential. There was also no effect of pH, temperature, and ion changes, and no dependence upon flow/fluid convection (stirring). Several compounds (e.g. dopamine and its metabolites) present in brain extracellular fluid were tested at physiological concentrations and shown not to interfere with the CPA O_2 signal. *In vivo* experiments confirmed a sub-second response time observed *in vitro* and demonstrated long-term stability extending over twelve weeks, with minimal O_2 consumption (*ca.* 1 nmol/h). These results indicate that CPEs operating amperometrically at a constant potential of −650 mV (*vs.* SCE) can be used reliably to continuously monitor brain extracellular tissue O_2.

1. Introduction

Brain cells are critically dependent on a continuous supply of O_2 for their normal energy metabolism with the brain consuming approximately 20% of the total O_2 used by the body at any given time. The tissue concentration is determined by the balance between blood supply and local utilization. A decrease in supply, or a large increase in consumption without adequate compensation, can seriously compromise brain function due to the low reserve of dissolved O_2 present in the tissue; the distribution of concentrations reported ranges from 40 μM to 80 μM (Nair et al., 1987; McCreery et al., 1990; Kayama et al., 1991; Murr et al., 1994; Zauner et al., 1995) depending on the depth of measurement (Baumgärtl et al., 1989), and the heterogeneity of the tissue (Murr et al., 1994; Lübbers and Baumgärtl, 1997).

Oxygen levels in the brain can be measured in several ways: indirectly, using non-invasive near-infrared spectroscopy (Matsumoto et al., 1996; Rasmussen et al., 2007); globally, using fibre-optic catheters to monitor jugular venous O_2 saturation (SjO_2) (Coplin et al., 1998; Gopinath et al., 1999; Howard et al., 1999); and locally, using Clark-type electrode technology to directly monitor O_2 partial pressure (pO_2) (Clark et al., 1958; Thompson et al., 2003; Piilgaard and Lauritzen, 2009). The latter operates by measuring the electrochemical reduction of O_2 and can be used in freely moving animals. It also offers significant spatial (~10 μm) and temporal (millisecond) advantages compared to other techniques.

Since the pioneering 'brain polarography' research carried out by Clark and colleagues (Clark et al., 1953; Thompson et al., 2003) over 50 years ago a wide variety of electrodes (sensors) have been used. These can essentially be divided into two main groups: noble metal electrodes, such as Pt (Clark et al., 1958; Travis and Clark, 1965; Thompson et al., 2003; Offenhauser et al., 2005) and Au (Cooper, 1963; Holmström et al., 1998; El-Deab and Ohsaka, 2003); and carbon-based electrodes, such as glassy carbon (Clark and Clark, 1964), carbon fibre (Zimmerman and Wightman, 1991; Zimmerman et al., 1992; Venton et al., 2003), carbon epoxy (Bazzu et al., 2009), and carbon paste (CPE) (Lowry et al., 1996, 1997; Bolger and Lowry, 2005). While carbon electrodes tend to be more labour intensive in terms of their construction, they have the advantage that they are less prone to surface poisoning and as such do not require the use of protecting membranes which are a characteristic of metal-based O_2 electrodes. Of the three types used, glassy

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carbon can be restricted by its large size (>1 mm) (Jin et al., 2005), while with carbon fibre electrodes their small dimension (typically 10 μm) means that the concentration of O_2 observed can vary depending on the orientation of the electrode relative to the blood vessels and metabolically active sites, and on the depth of penetration into the tissue (Baumgärtl et al., 1989). The latter is particularly important when using cylinder electrodes. Since the dimension (typically 100–200 μm) (Justice, 1987) of carbon epoxy and CPEs is greater than the scale of a capillary zone (*ca.* 70 μm) (Silver, 1965), an average tissue O_2 level is detected, but this must be balanced against the increased tissue damaged caused by the larger electrode (Duff and O'Neill, 1994; Khan and Michael, 2003). While microdialysis probes of similar dimensions have been reported to alter dopamine levels (Khan and Michael, 2003) there is no evidence to suggest that this is the case for an electrochemically detected freely diffusing gaseous species such as O_2.

We have previously reported preliminary data demonstrating that CPEs can be used to cathodically monitor real-time changes in brain tissue O_2 during neuronal activation (physiological stimulation) in freely moving rodents using both differential pulse (Lowry et al., 1996; Bolger and Lowry, 2005) and constant potential (Lowry et al., 1997) amperometry (DPA and CPA). The increases observed with DPA were also found to correlate with increases in regional cerebral blood flow measured using the H_2 clearance technique (Lowry et al., 1997). While we have subsequently published detailed characterisation results for DPA O_2 monitoring at CPEs (Lowry et al., 1996) we now present the results of comprehensive characterisation studies for CPA, a technique which has several advantages compared to pulsed methods, including simple instrumentation and experimental design (i.e. it does not require optimized pulse sequences), and continuous real-time recording with high sensitivity and low background (baseline) noise. Such studies are important in establishing the properties confirming suitability for *in vivo* monitoring and include testing sensitivity, selectivity, consumption/depletion and stability (Phillips and Wightman, 2003).

2. Materials and methods

2.1. Chemicals and solutions

The NaCl (SigmaUltra), NaH_2PO_4 (Sigma, A.C.S. reagent), NaOH (SigmaUltra), KCl (SigmaUltra), $CaCl_2$ (SigmaUltra), and $MgCl_2$ (SigmaUltra) were used as supplied (Sigma–Aldrich Ireland Ltd). Compounds used in the interference study were: L-ascorbic acid (AA; A.C.S. reagent, Sigma), dehydroascorbic acid (DHAA; Aldrich), uric acid (UA; sodium salt, Sigma), glutathione (oxidised disodium salt, Aldrich), dopamine (DA; hydrochloride, Sigma), 3,4-dihydroxyphenylacetic acid (DOPAC; Sigma), homovanilic acid (HVA; Fluka Biochemika), 5-hydroxytryptamine (5-HT; hydrochloride, Sigma), 5-hydroxyindole-3-acetic acid (5-HIAA; Fluka Biochemika), L-tryptophan (99%, Aldrich), L-cysteine (>98%, Sigma), L-tyrosine (99%, Aldrich). Stock standard solutions of all compounds were prepared from the supplied chemicals at the beginning of each experiment to avoid problems associated with gradual decomposition.

Unless otherwise stated *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). In pH studies the buffer pH was adjusted to between 6.5 and 8.0 using solutions of NaH_2PO_4 and NaOH. Experiments investigating the effects of ion changes (Ca^{2+} and Mg^{2+}) on O_2 sensitivity were performed in artificial cerebrospinal fluid (aCSF): 147 mM NaCl; 4 mM KCl; 1.2 mM $CaCl_2$; and 1 mM $MgCl_2$ (Deboer et al., 1990). All solutions were prepared using deoxygenated doubly distilled deionised water and stored at 4 °C when not in use.

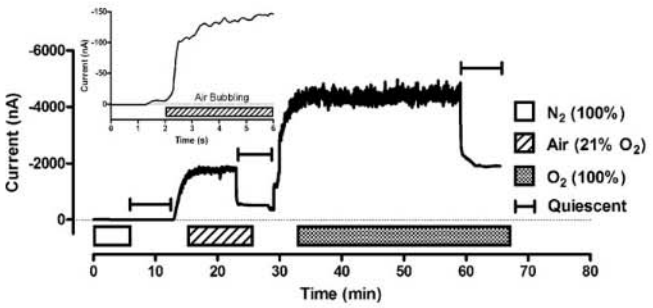

Fig. 1. Typical *in vitro* current–time response for an O_2 calibration (0–1200 μM; N_2, air and O_2-saturation) at a carbon paste electrode (CPE) carried out using constant potential amperometry (CPA) at −650 mV (*vs.* SCE) in PBS, pH 7.4. *Inset*: typical example of the effect of changing the O_2 concentration (bubbling air) on the CPE response characteristics for O_2 reduction. CPE background currents subtracted.

2.2. Working electrode preparation

Carbon paste was prepared by thoroughly mixing 0.71 g of graphite powder (1–2 μm, Aldrich) with 250 μL of silicone oil (high temperature, Aldrich) (O'Neill et al., 1982). CPEs (8 T, 200-μm bare diameter, 256-μm coated diameter) were either made in-house from Teflon-coated silver wire (Advent Research Materials, Suffolk, UK) as reported previously (Lowry et al., 1997) or supplied by Blue Box Sensors Ltd (Dublin, Ireland). When not in use all electrodes were stored in PBS at 4 °C.

2.3. Instrumentation and software

All electrochemical techniques (constant potential amperometry (CPA) and cyclic voltammetry) were carried out using a low-noise potentiostat (Biostat IV, ACM Instruments, Cumbria, UK). Data acquisition was performed with a notebook PC, a PowerLab interface system (ADInstruments Ltd., Oxford, UK) and LabChart for Windows software (ADInstruments Ltd.).

All data are presented as mean ± standard error (SEM), with n = number of electrodes. All analysis was performed using Microsoft Excel 2007 and the commercial packages Prism (version 5.01) and InStat (GraphPad Software Inc., CA, USA). The statistical significance of differences observed was calculated using Student's *t*-tests (two-tailed paired or unpaired observations where appropriate) or one-way ANOVA (Kruskal–Wallis test with Dunn's post test). Values of $P < 0.05$ were considered to indicate statistical significance.

2.4. Experiments in vitro

Experiments *in vitro* were performed in a standard three-electrode glass electrochemical cell containing 15 mL PBS at room temperature unless otherwise stated. A saturated calomel electrode (SCE) was used as the reference electrode, and a Pt wire served as the auxiliary electrode. CPEs were allowed settle under the influence of the applied CPA potential (−650 mV or 0 mV *vs.* SCE) until the non-faradaic current had reached a stable baseline level—typically 30 min.

To attain effective deaeration, the PBS solution was vigorously purged with O_2-free N_2 (BOC Ireland, average O_2 content 2 ppm, maximum O_2 content 5 ppm) for at least 30 min before recording began. In calibration experiments involving 0–1200 μM solution O_2 either N_2, atmospheric air (from a RENA air pump) or pure O_2 (compressed gas) was bubbled through the PBS for a similar period and the appropriate gaseous atmosphere then maintained over the cell solution during quiescent recording (see Fig. 1A). The concentrations of solution O_2 were taken as 0 μM (N_2-saturated), 240 μM

(air-saturated (Foster et al., 1993)) and 1200 μM (O_2-saturated (Bourdillon et al., 1982)) respectively.

In calibrations involving 0–240 μM O_2 known volumes (+200, +204, +208, +212, and +216 μL) of a saturated (100%) O_2 solution were added to 10 mL of N_2-purged PBS. Mixing in these, and interference experiments, was achieved by placing the electrochemical cell on a magnetic stirrer (IKA MST Mini Magnetic stirrer, Lennox Laboratory Supplies Ltd, Dublin, Ireland) and agitating (*ca.* 10 Hz) the cell solution using a stirring bar (20 mm × 5 mm diameter) for *ca.* 5 sec following each injection aliquot. In studies investigating the effects of fluid convection stirring was carried out 1 Hz under an air-saturated atmosphere. Calibrations at 37 °C were performed by placing the cell on a temperature regulated magnetic stirrer/hot plate (IKA MST Basic C, Lennox Laboratory Supplies Ltd, Dublin, Ireland). The solution temperature was controlled using a TC 1 temperature controller (IKA) which was placed in the solution as near as possible to the sensor. Unless otherwise stated a N_2 atmosphere was maintained over all solutions throughout recording. Post *in vivo* calibrations were carried out in the 0–1200 μM O_2 range.

2.5. Surgery

Male Sprague–Dawley or Wistar rats weighing 200–350 g were anesthetised and implanted with CPEs (Day 0), following protocols similar to those previously described (Lowry and Fillenz, 2001; Bolger and Lowry, 2005). The level of anesthesia was checked regularly (pedal withdrawal reflex). Typical 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 (striatum); A/P −3.6 from bregma, M/L ±2.2, and D/V −3.2 (hippocampus); A/P +2.7 from bregma, M/L ±1.2, and D/V −3.8 (medial prefrontal cortex); A/P +1.9 from bregma, M/L ±0.8, and D/V −6.9 (nucleus accumbens). A reference electrode (8 T Ag wire, 200-μm bare diameter; Advent Research Materials) was placed in the cortex and an auxiliary electrode (8 T 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 (O'Neill, 1993). Unless otherwise stated animals were allowed recover from surgery with the electrodes fixed to the skull using dental screws and dental acrylate. All animals were assessed for good health according to published guidelines (Morton and Griffiths, 1985) immediately after recovery from anesthesia and at the beginning of each day. All procedures were performed under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 and UK Animals (Scientific Procedures) Act 1986).

2.6. Experiments in vivo

Rats were housed 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 (MS363, Plastics One, Roanoke, VA, USA), and a flexible screened six core cable (363-363 6TCM, Plastics One) which was mounted through a swivel (SL6C, Plastics One) above the rat's head. This arrangement allowed free movement of the animal which remained continuously connected to the instrumentation.

Mild hypoxia and hyperoxia were produced by the administration of N_2/air and O_2/air mixtures; plastic tubing, connected to the respective gas cylinder (BOC), was held *ca.* 2–3 cm from the animal's snout for either 30 (N_2/air) or 60 s (O_2/air) periods. A flow rate of *ca.* 150 mL/min was used. This procedure resulted in the inhalation of an air/gas mixture.

3. Results and discussion

3.1. Oxygen reduction

Electrochemical reduction of O_2 at carbon electrodes is a two-electron processes producing H_2O_2:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$$

$$H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O$$

Since the direct reduction (Martel and Kuhn, 2000) (and oxidation (Taylor and Humffray, 1975; Zimmerman and Wightman, 1991)) of H_2O_2 is severely inhibited at carbon electrode surfaces the rate-limiting step is the initial one-electron step followed by protonation of the superoxide ion and further reduction (Taylor and Humffray, 1975). We have previously determined the position of O_2 reduction on the voltage axis of CPEs using cyclic voltammetry (Lowry et al., 1996). Characterisation voltammograms recorded at 100 mV/s in N_2 (background) and air-saturated (21% O_2) PBS solutions indicate that a potential of −650 mV (*vs.* SCE) is appropriate for CPA O_2 detection; this potential is in the mass-transport limited region after the peak potential for O_2 reduction (*ca.* −500 mV). In order to determine the O_2 sensitivity of CPEs operating at −650 mV calibrations were performed over a 0–1200 μM O_2 range (N_2, air and O_2-saturated PBS). It takes approximately 5 min for the buffer solution to reach the new level of gaseous saturation (Fig. 1). The decrease in current during the quiescent periods is a result of the removal of forced convection due to the bubbling associated with the introduction of the atmospheric air (air pump) and pure O_2 (compressed gas). The response was linear ($R^2 = 0.999$, $n = 8$) with a slope of -1.49 ± 0.01 nA/μM, $n = 8$. There is an instantaneous change in the current upon increasing the O_2 concentration via bubbling with the response time <1 s (*inset* Fig. 1). This is comparable to the O_2 response characteristics reported for Clark-type noble metal electrodes (Piilgaard and Lauritzen, 2009), but with the added advantage of long-term *in vivo* stability (see Section 3.6). Calibrations performed over a range of low, physiologically relevant, concentrations (0–125 μM O_2) produced a similar sensitivity (-1.09 ± 0.03 nA/μM, $R^2 = 0.998$, $n = 4$) and subsecond response time (Fig. 2A and B). The calculated limit of detection (3 × SD of the background noise level) of 0.09 μM is smaller than that found for DPA[1] (8 μM) reflecting the low background currents that distinguish CPA from DPA (Lowry et al., 1997; Bolger and Lowry, 2005). When the same calibrations were repeated at 0 mV (*vs.* SCE) there was no change in the background signal in response to increasing concentrations of O_2 (Fig. 2C).

Oxygen electrode signals are generally tested for their dependence on flow which is usually achieved *in vitro* by examining the signal sensitivity to fluid convection (Schneiderman and Goldstick, 1978; Gotoh et al., 1961). The O_2 reduction current at CPEs was thus monitored in stirred and unstirred solutions with convection produced using a magnetic stirrer (Sharan et al., 2008). While a small signal increase in the air-saturated (240 μM O_2) quiescent response was observed with stirring this was not significant (-607 ± 70 nA (quiescent), -628 ± 73 (1 Hz); $P = 0.83$, $n = 8$) and represents an increase in current (3.8 ± 2.4%) similar to that observed at Pt microelectrodes (*ca.* 2–3%) (Sharan et al., 2008). While this data is more relevant for *in vitro* use in brain slice or cell culture experiments

[1] For DPA O_2 reduction, two equally sized cathodic pulses are applied, the first from a resting potential at −150 mV to −350 mV that corresponds to the foot of the reduction wave for O_2 at treated (tissue modified) CPEs, and the second from −350 mV to −550 mV that corresponds to the peak of the reduction wave. The difference in the current (ΔI) sampled during these respective pulse pairs is calculated, and changes in ΔI used as a measure of changes in O_2.

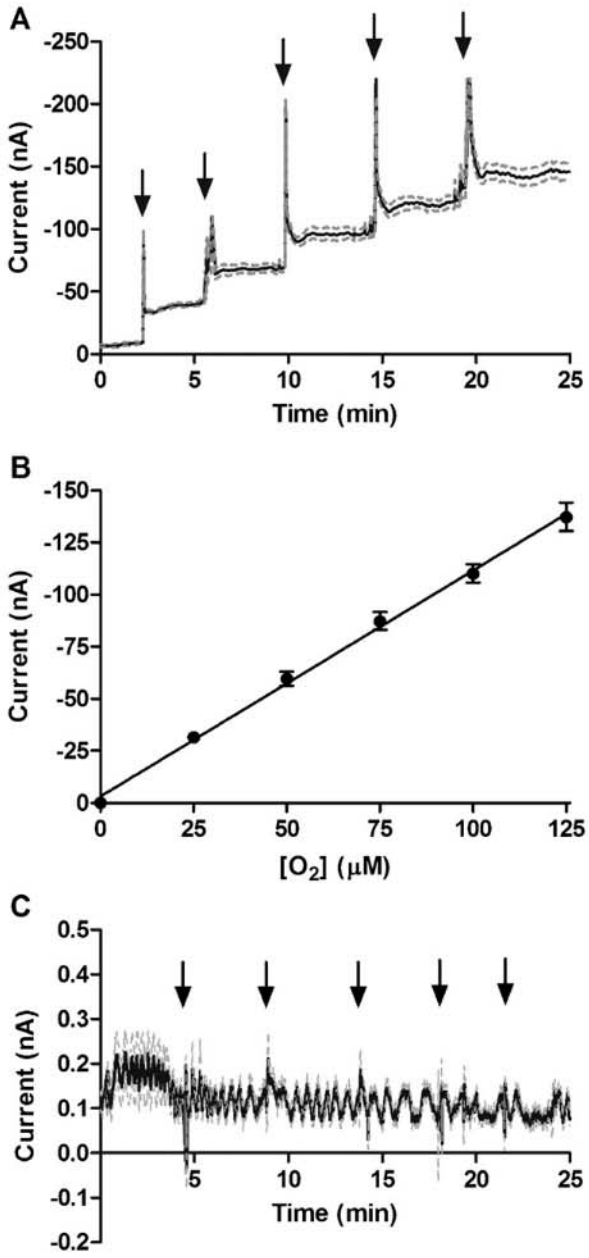

Fig. 2. (A) Current–time responses for CPE O_2 reduction ($n = 4$) at physiologically relevant concentrations carried out in PBS, pH 7.4, at −650 mV *vs.* SCE. (B) Amperometric calibration plot for the quiescent steady-state data shown in (A). (C) Current–time responses for CPE O_2 reduction ($n = 4$) at 0 mV (*vs.* SCE). Arrows indicate injections (+200, +204, +208, +212, and +216 μL) of a saturated O_2 solution (1.2 mM) yielding concentrations of 25, 50, 75, 100 and 125 μM O_2. The hashed (gray) lines in (A) and (C) represent the SEM.

we would, in reality, not expect to see even such small changes *in vivo* as it has been reported that O_2 electrodes in brain tissue do not show a dependence upon flow (Cooper, 1963). While convection results in a reduced diffusion layer thickness at the sensor surface (Bard and Faulkner, 1980a; Amatore et al., 2000), and hence an increase in current, Cooper found that the "self stirring" action of blood movement through the tissue causes this diffusion layer effect to be negligible *in vivo* (Cooper, 1963). This was explained by the fact that the O_2 concentration varies in accordance with the acceleration and not the velocity of the blood.

Confirmation that CPEs operating at −650 mV respond rapidly to changes in brain tissue O_2 *in vivo* was obtained by administering N_2 and O_2 gas (Fig. 3A): 1-min of an O_2/air mixture resulted in an increase of 50.1 ± 5.9 nA ($n = 4$) in the signal from baseline while 30-s of N_2/air produced a decrease of 16.2 ± 4.3 nA ($n = 4$). Changes in both cases were immediate. On cessation of inhalation of N_2/air the signals quickly returned to baseline levels indicating a rapid return to normoxic conditions. For O_2/air the return was slower and the signals had not reached baseline during the period of recording. When the same experiments were repeated at an applied potential of 0 mV (Fig. 3B) no change in signal was observed, supporting the *in vitro* calibration results obtained at 0 mV, and confirming that an applied potential of −650 mV is required for O_2 reduction at CPEs. Such data is in agreement with previously reported O_2 characterisation data from *in vivo* experiments performed using DPA in freely moving animals (Lowry et al., 1996, 1997; Bolger and Lowry, 2005): periods of mild hypoxia and hyperoxia produced rapid (subsecond) decreases and increases in the CPE signal; vasodilators such as the carbonic anhydrase inhibitor acetazolamide (Diamox) increased the signal; while neuronal activation (tail pinch and stimulated grooming) produced similar increases in both regional cerebral blood flow (rCBF) and O_2 indicating that CPE O_2 currents provide an index of increases in rCBF when such increases exceed O_2 utilization. The two techniques were found to have very similar sensitivities post implantation (Lowry et al., 1998), and in preliminary *in vivo* experiments involving tail pinch (Lowry et al., 1997) and insulin administration (Lowry et al., 1998) both methods produced similar response characteristics. The advantage of CPA over DPA is that it has much lower background (capacitance) currents *in vivo* (−49 nA *vs.* −748 nA) (Lowry et al., 1998), but is limited to the detection of a single analyte (see Section 2.4), whereas DPA, on the other hand, can be used to simultaneously measure several species (e.g. O_2 and ascorbic acid) (Lowry et al., 1996).

3.2. Oxygen consumption

As already outlined, *in vivo* voltammetry involves the detection of substances in the extracellular fluid (ECF) using electrochemistry with implanted microelectrodes (e.g. amperometric sensors and biosensors) (Lowry and O'Neill, 2005); by implanting the electrode in a specific brain region, applying a suitable potential profile and recording the resulting Faradaic current, changes in the concentration can be monitored in real-time. However, such concentrations have the potential to be altered by the voltammetric measurement itself through depletion in the vicinity of the electrode. While this is certainly the case for brain microdialysis (Sam and Justice, 1996; Borland et al., 2005), such effects tend to be small for voltammetry and can be minimised for example with appropriate selection of sampling intervals and other parameters (Ewing et al., 1981).

The consumption of O_2 *in vivo* by the CPE polarised at −650 mV was thus determined using the following rate equation (Bard and Faulkner, 1980b): $v = i/nFA$, where v is the rate of the electrode reaction, i is the baseline current, n is the number of electrons per molecule of O_2 reduced, F is the Faraday constant, and A is the area of the sensor. Taking −60 nA (nC/s) as a typical baseline current, 2 for the value of n (O_2 reduction at a carbon surface), 9.65×10^4 C/mol for the Faraday constant, and 3.14×10^{-4} cm^2 for the area of the sensor, yields a value of 0.001 μM O_2 reduced/cm^2/s. This corresponds to a consumption rate of *ca.* 1.13 nmol/h which is comparable to the calculated value (*ca.* 0.92 nmol/h) for noble metal electrodes of similar dimension. Assuming that the CPE samples from a fluid pool of 50 μm radius at the electrode tip (Rice and Nicholson, 1995) and a reported tissue sampling area of 15 mm^2 (Ma and Wu, 2008) this corresponds to a localized utilization (depletion) rate of *ca.* 12.5 nmol/g/min which is small relative to reported values 3.9 μmol/g/min (Madsen et al., 1998) for the cerebral metabolic rate of O_2 consumption ($CMRO_2$).

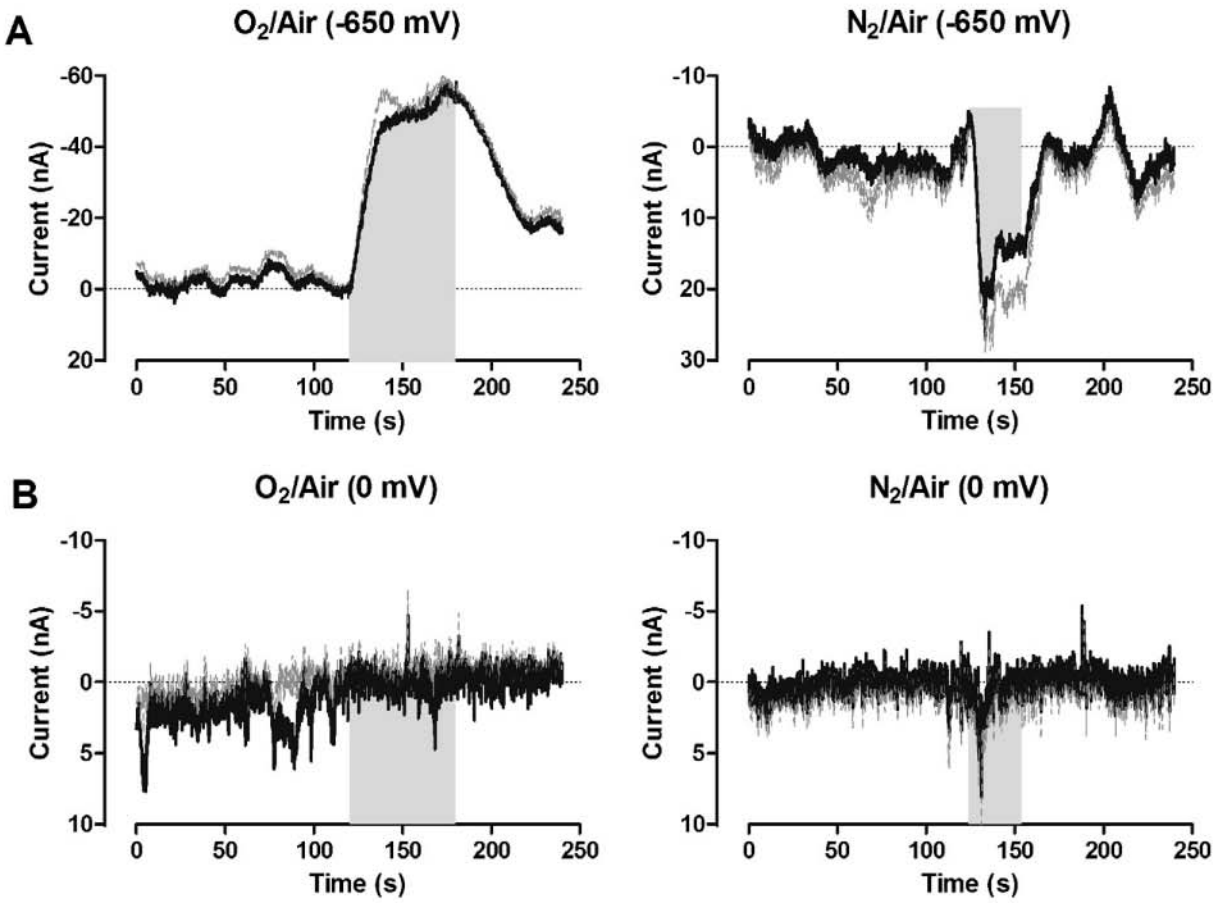

Fig. 3. Typical raw data (current–time) examples of the effects of mild hyperoxia (100% O_2/Air) and mild hypoxia (100% N_2/Air) on brain tissue O_2 levels monitored using CPEs ($n = 4$) at −650 mV (A) and 0 mV (B). Data from bilaterally implanted CPEs in the medial prefrontal cortex and hippocampus of an anaesthetised rat. Filled gray areas represent periods of gas administration. Hashed gray lines (shown above the data only for clarity) represent the SEM.

While electrodes with smaller dimensions will have smaller consumption rates (e.g. 4×10^{-4}–5×10^{-3} nmol/h for 10-μm Pt; Revsbech, 1989; Piilgaard and Lauritzen, 2009) the concentrations monitored can vary depending on proximity to blood vessels and metabolically active sites.

3.3. Effect of temperature and pH

Classical membrane covered O_2 sensors tend to have significant temperature dependence; the signal typically increasing by 1–6% for a rise of 1 °C (Hitchman, 1978; Jeroschewski and zur Linden, 1997). This is primarily due to the effects of temperature on the diffusion coefficient and the solubility of the gas in the membrane with temperature. As our *in vitro* experiments are routinely carried out at room temperature (*ca.* 22.5 ± 0.2 °C), we thus examined the effect of increasing temperature on the CPE O_2 sensitivity. Calibrations ($n = 4$) in the range 0–240 μM O_2 performed at the physiological temperature of 37 °C (−1.93 ± 0.39 nA/μM) were not significantly different ($P = 0.95$) from those at room temperature (−1.90 ± 0.28 nA/μM). This is likely the result of the absence of an outer membrane on the CPE.

Changes in pH may occur during physiological experiments *in vivo* (Zimmerman and Wightman, 1991), and these could also affect the cathodic reduction of O_2 which involves proton transfer. Previous reports for carbon-based electrodes found that for pH 12–14 the reduction of O_2 appears to be independent of pH, but as pH decreases the reduction becomes pH dependent (Taylor and Humffray, 1975; Yang and McCreery, 2000). To test for the pH sensitivity the buffer pH was changed from the standard physiological 7.4 to 6.5 and 8.0. No change was observed in the O_2 sensitivity: −1.83 ± 0.14 nA/μM (7.4, $n = 8$); −1.64 ± 0.17 nA/μM (6.5, $n = 4$, $P = 0.43$); −1.91 ± 0.48 nA/μM (8.0, $n = 4$, $P = 0.84$). Similar findings have previously been reported for O_2 reduction at carbon fibre electrodes (CFEs) using fast cyclic voltammetry (FCV) (Zimmerman and Wightman, 1991) and at multi-walled carbon nanotube modified glassy carbon electrodes using rotating disk electrode voltammetry (Kruusenberg et al., 2009).

3.4. Effect of ion changes

The media-dependence of redox reactions for electroactive species such as dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) have previously been investigated by several groups. Rice and co-workers found that the FCV sensitivities to DA and 5-HT at CFEs were 2–3-fold higher in non-physiological phosphate or HEPES-buffered saline compared to artificial cerebrospinal fluid (aCSF) which more accurately reflects the ionic composition of the brain (Kume-Kick and Rice, 1998; Chen and Rice, 1999). Interestingly, the reverse was observed for the sensitivities of the acid metabolites (DOPAC, 5-HIAA). Crespi reported shifts in oxidation peaks and higher sensitivities using differential pulse voltammetry for aCSF that contained Ca^{2+} but not Mg^{2+}, than in PBS that contained Mg^{2+} but not Ca^{2+} (Crespi, 1996). Such findings indicate that electrode surface interactions differ for anions and cations and are consistent with adsorption or repulsion effects at the modified (Nafion® or electrically pre-treated) CFE surface.

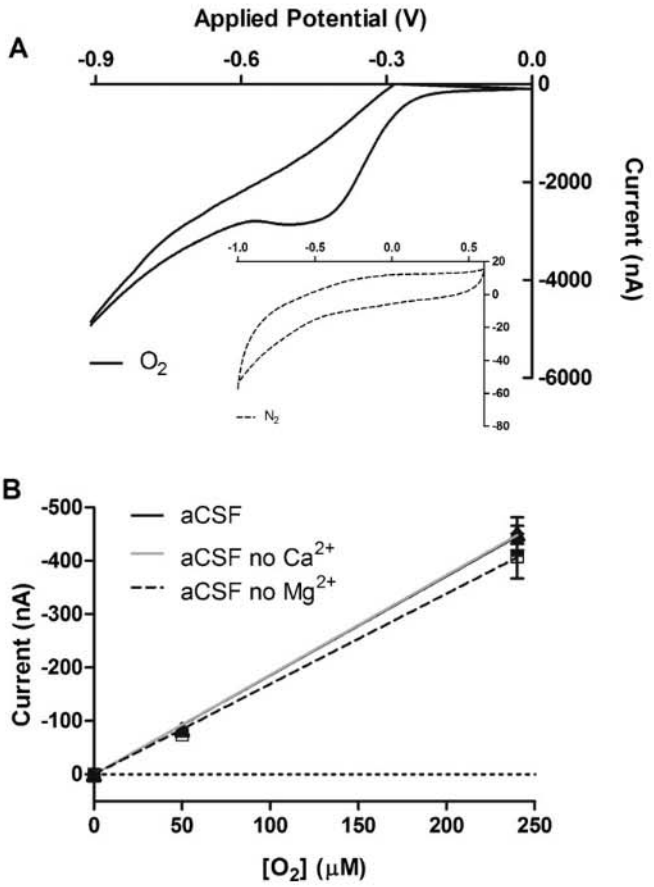

Fig. 4. Typical cyclic voltammograms recorded *in vitro* at 100 mV/s with a CPE in O_2 (A) and N_2 (A, *inset*) saturated artificial cerebrospinal fluid (aCSF). (B) Amperometric calibration plots for O_2 (0–240 μM) at CPEs ($n = 4$) carried out using constant potential amperometry at −650 mV (*vs.* SCE) in aCSF with and without either Ca^{2+} or Mg^{2+} ions.

As it has also been reported that the extracellular Ca^{2+} concentration can fall under conditions of both electrical stimulations (e.g. *ca.* 80 μM for 10 Hz at 10 s) (Kume-Kick and Rice, 1998) and intense tissue depolarisation (e.g. >1 mM for anoxic depolarisation or spreading depression) (Nicholson and Rice, 1988), we decided to test the effect of ion changes (Ca^{2+} and Mg^{2+}) on the CPE O_2 response in aCSF. No compensation was made for the decrease in osmolality associated with removal of the ions as this change has previously been reported to be small (Chen and Rice, 1999). We first confirmed the position for O_2 reduction on the voltage axis. Fig. 4A shows typical cyclic voltammograms recorded at 100 mV/s in N_2 (background) and O_2-saturated aCSF. As with PBS, −650 mV (*vs.* SCE) is in the mass-transport limited region after the peak potential for O_2 reduction, and all experiments in aCSF were thus performed at this potential. O_2 sensitivities for calibrations performed in the range 0–240 μM O_2 (Fig. 4B, $n = 16$) in the presence and absence of ions were similar (aCSF, -1.62 ± 0.11 nA/μM, $n = 16$; aCSF (no Ca^{2+}), -1.68 ± 0.14 nA/μM, $n = 16$, $P = 0.73$; aCSF (no Mg^{2+}), -1.50 ± 0.11 nA/μM, $n = 16$, $P = 0.43$) indicating that typical (small) ion changes in physiological media will not affect the CPE O_2 reduction signal. In fact, there was no significant difference between PBS (-1.68 ± 0.09 nA/μM, $n = 16$) and aCSF (-1.62 ± 0.11 nA/μM, $n = 16$, $P = 0.67$).

3.5. Selectivity

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 varying concentrations ranging from low nM (e.g. catecholamines and their metabolites) to high μM (e.g. ascorbic acid, AA). 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 its target analyte (Phillips and Wightman, 2003). The selectivity of CPEs for O_2 reduction using CPA, relative to a variety of potential interferents present in the brain (Lowry et al., 1996), was thus characterized *in vitro*. The compounds tested included the neurotransmitters DA and 5-HT, their metabolites DOPAC, homovanillic acid (HVA) and 5-HIAA and other electroactive species such as L-tyrosine, L-cysteine, L-tryptophan, L-glutathione, dehydroascorbic acid (DHAA) and the purine metabolite uric acid (UA). While possible interferents for oxidised analytes (e.g. DA) have been well documented (Phillips and Wightman, 2003; Lowry and O'Neill, 2005) this is not the case for species such as O_2 which are detected by electrochemical reduction. The above list is thus not exhaustive but represents a range of standard interferents. It excludes other gaseous species (e.g. NO) which have standard potentials more positive than O_2 as their effect on the current response has been reported to be negligible due to their low (nM) concentrations (Falck, 1997).

Table 1
In vitro CPA (−650 mV *vs.* SCE) response of carbon paste electrodes ($n = 4$) for a variety of potential interferents at physiologically relevant concentrations.[a] Data expressed as a percentage of the O_2 current at the reported extracellular level of *ca.* 50 μM (Zimmerman and Wightman, 1991).

Compound[b]	*I* (nA)	O_2 (%)
O_2 (50 μM)	−52.03 ± 2.43	100
AA	−0.12 ± 0.01	0.06 ± 0.03
HVA	−0.31 ± 0.01	0.06 ± 0.01
L-Glutathione	−0.22 ± 0.02	0.41 ± 0.02
L-Cysteine	−0.13 ± 0.02	0.25 ± 0.02
Uric acid	−0.13 ± 0.02	0.25 ± 0.02
5-HT	−0.14 ± 0.02	0.27 ± 0.03
L-Tryptophan	−0.08 ± 0.02	0.16 ± 0.03
DHAA	−0.09 ± 0.03	0.16 ± 0.05
L-Tyrosine	−0.29 ± 0.05	0.55 ± 0.08
DA	−0.20 ± 0.07	0.37 ± 0.11
DOPAC	−0.04 ± 0.05	0.08 ± 0.10
5-HIAA	−0.03 ± 0.06	0.06 ± 0.12

[a] 100 μM interferent or brain extracellular fluid concentration if known (O'Neill, 1994; Yang et al., 1994; Brand et al., 1993): AA, 500 μM; HVA, 10 μM; L-glutathione, 50 μM; L-cysteine, 50 μM; uric acid, 50 μM; 5-HT, 0.01 μM; dopamine, 0.05 μM; DOPAC, 20 μM; 5-HIAA, 50 μM.
[b] AA, ascorbic acid; HVA, homovanillic acid; 5-HT, 5-hydroxytryptamine; DHAA, dehydroascorbic acid; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid.

The results are summarized in Table 1. Although slight positive changes (<0.5 nA) were observed for most compounds following injection the signal tended to return towards baseline within the recording period (5 min) before the next injection. Also, such changes are negligible compared to the O_2 sensitivity at physiological concentrations, and will thus have no effect on the CPE CPA response for O_2, resulting in interference-free signals *in vivo*. Similar selectivity characteristics were found for O_2 reduction at CPEs using DPA (Lowry et al., 1996).

3.6. Stability

It is well accepted that noble metal (i.e. Clark-type) electrodes are susceptible to electrode poisoning in physiological media and generally require a protective membrane, which in some cases, depending on the membrane, improves specificity (Clark, 1959; Pantano and Kuhr, 1995; Papkovsky, 2004; Hynes et al., 2006). CPEs in contrast are less prone to surface poisoning and are very stable, even after weeks of continuous recording in the brain (Fillenz and O'Neill, 1986). As such, they do not require the use of pro-

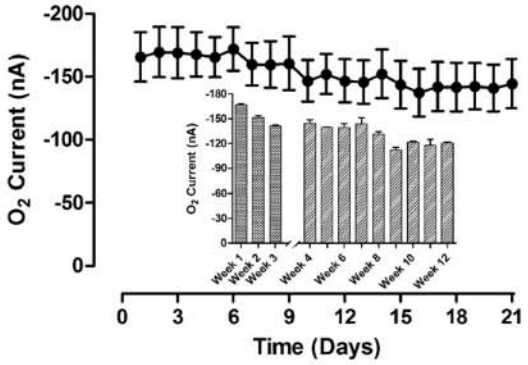

Fig. 5. Average (±SEM) baseline *in vivo* data (pooled from 12 animals—striatum, hippocampus, prefrontal cortex and nucleus accumbens) for CPEs ($n = 17–26$) recorded using CPA at −650 mV over 21 days. *Inset*: average weekly baseline data recorded over the 3 week period and extended to 3 months using data from 3 animals ($n = 12$). All data taken from a six-hour period covering morning/afternoon (10 am–4 pm).

tecting membranes. The basis of this stability was investigated by Kane and O'Neill (1998) who examined the effects of lipid and protein media on the electrooxidation of AA at both CPEs and CFEs. CFEs were extensively poisoned whereas CPEs showed no evidence of fouling. This novel resistance to poisoning appears to be due to the presence of the pasting silicone oil. Examination of baseline *in vivo* data for CPEs implanted in freely moving animals suggests that this stability also extends to the reduction of O_2 (Fig. 5): although there was a gradual decrease over the first three weeks (Week 1, -167.0 ± 1.5 nA; Week 2, -151.9 ± 2.3 nA; Week 3, -141.7 ± 0.9 nA), no significant variation was observed over the 21-day period ($P = 0.89$, one-way ANOVA; $n = 17–26$, 12 animals). Using data from three animals (four implanted sensors each) recorded out to 84 days indicates a stable baseline signal over twelve weeks (*inset* Fig. 5). It is important, however, to point out that during a 24 h period the signal can exhibit numerous naturally occurring deviations from baseline levels. These changes can be rapid, occurring over periods ranging from seconds to minutes, or more prolonged, lasting one or more hours. The former tends to be associated with physiological phenomena such as naturally occurring grooming and feeding, while the latter occurs mainly with periods of intense activity typically observed during the dark cycle (unpublished observations). Both are reflective of the fact the measured real-time [O_2] is the dynamic balance between supply and utilisation.

It is noteworthy that such changes in signal (nA) can be converted to units of pressure (mmHg), often used to represent pO_2 (Offenhauser et al., 2005; Lecoq et al., 2009; Piilgaard and Lauritzen, 2009), using post *in vivo* calibration data and literature reported concentration and pressure data associated with the air-saturated [O_2] at 37 °C (Forstner and Gnaiger, 2010): typical increases of 22 nA observed for grooming, and 26 nA for feeding/drinking correspond to *ca.* 25 μM O_2 and 29 μM O_2 respectively, or 18 mmHg and 24 mmHg.

4. Conclusions

CPEs operating amperometrically at a constant potential of −650 mV (*vs.* SCE) can be used reliably to monitor tissue O_2 in brain extracellular fluid, and offer several advantages compared to pulsed amperometric methods including simple experimental design, continuous real-time recording and low background noise. *In vitro* and *in vivo* characterisation studies indicate a low limit of detection, high sensitivity and interference free signals at physiological concentrations, rapid response time, no effect of pH, temperature, and ion changes, no dependence upon flow (stirring), and long-term stability *in vivo* extending over weeks, with minimal consumption of O_2. The significance of these results is highlighted by the recent finding that the tissue [O_2] monitored using CPA at a carbon-based O_2 sensor can serve as an index of changes in the magnitude of the blood oxygenation level dependent (BOLD) functional magnetic resonance imaging (fMRI) response (Lowry et al., 2010). The amperometric O_2 signal thus provides a reliable awake animal surrogate of human fMRI experimentation, and is thus an effective translational tool which can better enable the comparison of pre-clinical and clinical research.

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