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In vitro characterisation of ortho phenylenediamine and Nafion®-modified Pt electrodes for measuring brain nitric oxide

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ABSTRACT

Incorporation of Nafion® pre-coat layers (Nafion®(1/2) and Nafion®(2/1)), and poly-*o*-phenylenediamine (PPD) on platinum disk electrodes (127 μm) for the electrochemical detection of nitric oxide (NO) is discussed. The extensive *in vitro* development and characterisation of Nafion®(1/2) PPD and Nafion®(2/1) PPD modified sensors demonstrated excellent sensitivity towards NO (1070 ± 19 pA μM^{-1}, $n = 38$ and 990 ± 14 pA μM^{-1}, $n = 38$) respectively which were both significantly different from bare Pt electrodes (352 ± 25 pA μM^{-1}, $n = 14$). There was negligible interference, <1% of the overall signal, from the various electroactive interferents investigated at their respective physiological concentrations. The Nafion®(1/2) PPD sensor demonstrated a response time of 18 ± 2 s, $n = 38$ at 25 °C which decreased to 14 ± 1 s, $n = 6$ at 37 °C with no significant increase in sensitivity observed (1287 ± 48 pA μM^{-1}, $n = 6$). Similarly the Nafion®(2/1) PPD sensor demonstrated a response time of 16 ± 2 s, $n = 38$ at 25 °C which decreased to 10 ± 1 s, $n = 6$ at 37 °C with a non significant decrease in sensitivity observed (791 ± 41 pA μM^{-1}, $n = 6$). The limit of detection was 8 ± 1 nM, $n = 12$ for the Nafion®(1/2) PPD modified sensor and 7 ± 1 nM, $n = 12$ for the Nafion®(2/1) PPD modified sensor. Sensocompatibility studies undertaken in proteins (BSA), lipids (PEA) and brain tissue for 28 days resulted in an AA current which is *ca.* 1% of the bare Pt response (37 ± 1 nA mM^{-1}, $n = 14$). Shelf-life studies for both the Nafion®(1/2) PPD and Nafion®(2/1) PPD modified sensors demonstrated negligible differences after 56 days storage at 4 °C.

1. Introduction

The physiological role of nitric oxide (NO) as the endothelium derived relaxing factor (EDRF), involved in the process of vasodilation and regulation of blood pressure was discovered in the late 1980s [1,2]. Since this discovery, many researchers have attempted to understand and unravel the various roles of NO in physiology. NO is derived from three main NO synthase enzymes (NOS) consisting of inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) [3–5]. The source of NOS in the body determines the specific type of NO produced. The global physiological function of NO has been extensively studied in processes such as the immune response [6], anti-microbial activity [7] and penile erection [8]. Of the three isoforms of NOS, nNOS is present in the most abundance in the brain. Neuronal NO has been implicated in a variety of neurological processes, specifically sleep and appetite regulation [9,10] synaptic plasticity, neurotransmission and learning and memory [11–13]. Hence the detection of NO in the central nervous system (CNS) provides an insight into a variety of vital neurological functions.

Owing to its importance and relevance in biological processes, it is necessary to develop fast and sensitive detection methods of measuring NO in biological samples and organisms [14]. Electrochemical techniques provide the appropriate temporal and spatial resolution necessary to enable long-term *in vivo* NO recordings. However, detecting the free radical NO, is a very challenging task for electrochemical sensors for a variety of reasons. It has an extremely short half-life *in vivo* (typically < 10 s) due to its reaction with a vast number of diverse molecules [15]. The concentration of NO *in vivo* has been established as *ca.* 0.1 μM [16] and trying to detect such a small and reactive molecule in the presence of a myriad of endogenous interferents (ascorbic acid (AA), dopamine (DA), nitrite (NO_2^- etc.) can prove particularly challenging. Notwithstanding this, a number of studies have reported the electrochemical detection of NO release from neuronal environments [17–21]. The first direct oxidation of NO on a platinum (Pt) electrode was reported by Shibuki and Okada in 1991 using a miniature Clark-type electrode [22]. Over subsequent years, there have been numerous different reports of electrochemical sensors utilising carbon, glassy carbon, Pt or gold electrodes covered by different

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types of membranes [23–28]. More recently Xu et al. have extensively reviewed the chemical modification of electrode surfaces with biomolecules, organic molecules and protection membranes that have expanded the application of NO sensing electrodes [14].

The use of the polymeric ion exchanger Nafion®, which is a perfluorinated sulfonic acid ionomer, to provide a barrier for a variety of anionic interfering species, has been successful in the detection of NO. In fact, Nafion® is the most widely used permeable membrane in the selective detection of NO. Our research group has demonstrated a highly sensitive, selective and stable NO sensor for the purpose of *in vivo* monitoring [29–31] utilising a Nafion® modified electrochemical sensor. This sensor possesses the required operational characteristics i.e., response time and detection limit to enable the detection of NO *in vivo*. Furthermore the application of this sensor in the physiological environment for the continuous real-time detection of NO in the brain has been extensively characterised [32,33]. It is a well-established phenomenon that the mass transport properties of Nafion® membranes are determined by the thickness of the polymer film [14,34]. Previously groups have investigated the simultaneous combination of Nafion® and the polymer film *o*-phenylendiamine (*o*-PD) on carbon fibre electrodes in the hope of improving sensor performance [23,35]. *o*-PD may be electropolymerised onto the surface of a Pt electrode to form an insulating poly-*o*-phenylenediamine (PPD) polymer which can act as a permselective membrane that permits access of small molecules such as NO to the Pt surface and prevents the access of larger interfering molecules. The use of PPD in electrochemical sensor design has previously been demonstrated by Lowry et al. amongst others [36–40].

It is anticipated that incorporation of PPD into our previously characterised Nafion® pre-coat electrode design will allow for improvement in response time and sensocompatibility under physiological conditions. The work described within focuses on the *in vitro* development and characterisation of novel Nafion® PPD modified Pt sensors suitable for the electrochemical detection of NO *in vivo*.

2. Materials and methods

2.1. Reagents and solutions

The Nafion® (5 wt.% solution in a mixture of lower aliphatic alcohols and H_2O) was obtained from Aldrich Chemical Co., Dorset, UK. The L-ascorbic acid (AA; A.C.S. reagent), uric acid (UA; sodium salt), dopamine (DA; hydrochloride), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanilic acid (HVA), 5-hydroxytryptamine (5-HT; hydrochloride), 5-hydroxyindole-3-acetic acid (5-HIAA), glutathione (oxidised disodium salt), *o*-phenylenediamine (*o*-PD, 99 + %), L-α-phosphatidylethanolamine (PEA, Type II-S, commercial grade), 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 [29]. Stock standard solutions of all other compounds were prepared at the beginning of each experiment to avoid problems associated with gradual decomposition. 10% solutions of BSA and PEA were prepared in distilled deionised water. *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 min 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.

2.2. Sensor manufacture

Bare Platinum (Pt) disk electrodes were made from Teflon-insulated Platinum/Iridium (Pt/Ir 90%/10%) wire (127 μm bare diameter, 203 μm coated diameter (5T), Science Products GmbH, Hofheim, Germany) as previously described. Briefly, 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 (Bilaney, 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®(5/2) refers to the previously described 5 pre-coat, 2 applications sensor which has been extensively characterised *in vitro* [30,31] and *in vivo* [32,33]. The active surface is coated with a pre-coat Nafion® membrane which allows excellent sensitivity and selectivity towards NO.

Nafion®(1/2) PPD refers to a novel electrode design described as 1 pre-coat, 2 applications of Nafion®, annealed after each application at 210 °C, followed by polymerisation in *o*-PD for 30 min. The pre-coat application method involves placing a fixed volume (5 μL) of Nafion® on a clock glass using a syringe which is allowed to air dry at room temperature for 5 min. A fresh application of Nafion® is placed onto the concentrated pre-coat of Nafion. The active surface of the electrode is dipped into this concentrated layer and then immediately removed and let air dry at room temperature for 2 min. The electrodes are then placed in the oven and annealed for 5 min at 210 °C. After the annealing process has been completed, the electrode is coated again using the same procedure i.e., placing another application of Nafion® onto the concentrated layer and dipped into this concentrated layer and then immediately removed and let air dry at room temperature for 2 min. The modified electrode is then annealed again at 210 °C for 5 min. The electropolymerisation of *o*-PD onto the Nafion® modified Pt disk electrode, was carried out by applying a constant potential of +700 mV vs. SCE in a 300 mM solution made up in N_2 saturated PBS. The electrodes were polymerised for 30 min and the electrochemical cell was kept under a N_2 atmosphere as the monomer is readily oxidised in air.

Nafion®(2/1) PPD refers to a novel electrode design described as 2 pre-coats, 1 application of Nafion®, annealed at 210 °C followed by 1 dip into 5% Nafion® solution, annealed at 210 °C and finally polymerisation in *o*-PD for 30 min. The pre-coat application was performed as described above, however, a second pre-coat layer was allowed to air dry at room temperature on the clock glass. Following a single application of these 2 pre-coats, the electrode surface was dipped in 5% Nafion® solution and placed in the oven and annealed for 5 min at 210 °C. The modified electrode was then electropolymerised in N_2 saturated *o*-PD for 30 min.

2.3. Characterisation of NO sensors

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. All calibrations were performed at +900 mV vs. SCE. To facilitate mixing, solutions were agitated using a magnetic stirrer for approximately 5 s following the addition of each aliquot. The current was then measured under quiescent conditions with a N_2 atmosphere maintained over the solution.

2.4. Sensocompatibility investigations

To elucidate the effect of the brains response to the implanted sensor, sensocompatibility studies were investigated in various brain constituents to mimic conditions encountered *in vivo*.

The Nafion®(1/2) PPD and Nafion®(2/1) PPD modified sensors were calibrated with AA after manufacture and stored immediately at 4 °C in either BSA (10%), PEA (10%) or homogenised brain tissue (BT). After 28 days, the sensors were removed from these storage conditions, rinsed with deionised water and recalibrated with AA. No calibrations were performed at any stage during the 28 days. The calibration was performed with AA as it was deemed a more appropriate analyte to determine the effect of longer exposure times on the integrity of the Nafion® PPD layer. A compromised Nafion® PPD layer will result in a much larger AA change as opposed to a smaller NO current change and thus highlight the sensocompatibility in a more accurate way.

2.5. Instrumentation and software

Constant potential amperometry (CPA) was performed as previously described. Briefly, all electrochemical experiments used a low-noise four channel biostat (ACM instruments; Cumbria, UK). Data acquisition was carried out with a logiQ M760TG notebook with an Intel dual core processor, an eight channel powerlab® 8/30 from (ADInstruments Ltd.; Oxford, UK) and LabChart for Windows (v 6.1) software (ADInstruments Ltd., Oxford, UK).

3. Results and discussions

3.1. Effect of decreasing Nafion® layers on sensitivity and response time

Previously we have illustrated the electrochemical detection of NO at bare Pt and Nafion®(5/2) sensors [30]. An increase in NO current was reported following modification of the bare Pt surface with 5 pre-coats, 2 applications of Nafion®. During this body of work, a linear ($R^2 = 0.99$) sensitivity value of 1104 ± 46 pA μM^{-1}, $n = 17$ was observed for the Nafion®(5/2) sensor over a concentration range of 0–1 μM NO which was significantly different ($P < 0.0001$) than that obtained for bare Pt electrodes (352 ± 25 pA μM^{-1}, $n = 14$, $R^2 = 0.99$).

Fig. 1(top) details a linear ($R^2 = 0.99$) current concentration profile for the Nafion®(1/2) PPD sensor which reduced the number of Nafion® layers to 1 pre-coat, 2 applications. This had no significant effect ($P > 0.05$) on NO sensitivity (1070 ± 19 pA μM^{-1}, $n = 38$) when compared against the Nafion®(5/2) sensor, however, a significant increase ($P < 0.0001$) against bare Pt was recorded. In addition the Nafion®(2/1) PPD sensor, which too reduced the pre-coat layers, yielded a comparable linear ($R^2 = 0.99$) sensitivity of 990 ± 14 pA μM^{-1}, $n = 38$ (see Fig. 1(bottom)) which demonstrated no significant difference from the Nafion®(5/2) sensor ($P > 0.05$) and Nafion®(1/2) PPD sensor ($P > 0.05$). Once again a significant increase ($P < 0.0001$) was observed over bare Pt. The limit of detection (LOD), defined as the analyte concentration producing a signal equal to three times the standard deviation of the baseline current was 8 ± 1 nM, $n = 12$ for the Nafion®(1/2) PPD modified sensor and 7 ± 1 nM, $n = 12$ for the Nafion®(2/1) PPD modified sensor. These are in close alignment with the 5 nM which has previously been characterised for the Nafion®(5/2) sensor. Fig. 2 compares the sensitivities of the various electrode types and clearly identifies an improvement in all Nafion® modified electrode sensitivities over the bare Pt electrode sensitivities. Modification of the electrode surface with the perfluorinated ionomer has resulted in significantly increased detection of NO at the sensor surface. We attribute this improved response to the negatively charged sulfonate groups on the Nafion® layers which stabilise the nitrosonium ion (NO^+) formed upon oxidation of the analyte at the electrode surface. This prevents the adsorption of said species on the Pt surface which may inhibit NO detection as has been previously reported

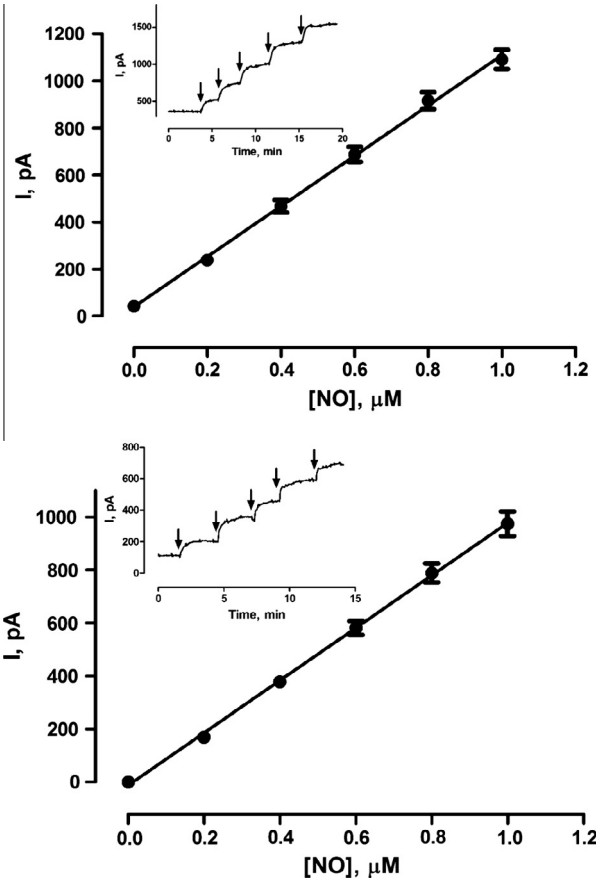

Fig. 1. A current-concentration profile for 0–1 μM NO at (top) Nafion®(1/2) PPD ($n = 38$) (bottom) Nafion®(2/1) PPD ($n = 38$) at +900 mV vs. SCE. (Inset) typical raw data traces. Arrows indicate injection of NO aliquot.

[30,41]. Cuesta and Escudero have confirmed the presence of adsorbed NO species and suggest that they are irreversibly adsorbed on Pt electrodes via two alternative mechanisms [42].

We reported previously that the Nafion®(5/2) sensor demonstrated a response time of 34 ± 3 s ($n = 14$) at room temperature (25 °C) which was deemed to be too slow for *in vivo* measurements [30]. This finding resulted in the development of these novel sensor types described within. It is well-established that due to the short half-life of NO *in vivo* [15] (10–60 s) it is critical that any sensor utilised for its electrochemical detection possess an efficient response time [15,43]. Both the Nafion®(1/2) PPD sensor and Nafion®(2/1) PPD sensor illustrated improved response times of 18 ± 2 s, $n = 38$ and 16 ± 2 s, $n = 38$ at this temperature respectively. Both sensor types demonstrated a significant improvement ($P < 0.0001$) on the Nafion®(5/2) sensor.

3.2. Effect of increasing temperature on sensitivity and response time

Since these sensors were designed with *in vivo* monitoring in mind, it was imperative to measure the effect that physiological temperature has on sensitivity and response time. Increasing the temperature from 25 °C to 37 °C resulted in a slightly increased sensitivity of 1287 ± 48 pA μM^{-1}, $n = 6$ ($R^2 = 0.99$) for the Nafion®(1/2) PPD sensor which was not significantly different ($P > 0.05$) from that recorded at 25 °C. In contrast the Nafion®(2/1) PPD sensor demonstrated a decrease in sensitivity (791 ± 41 pA μM^{-1}, $n = 6$, $R^2 = 0.99$) at the higher temperature which was not significantly different from the sensitivity recorded at 25 °C. These findings are in line with what were reported for the

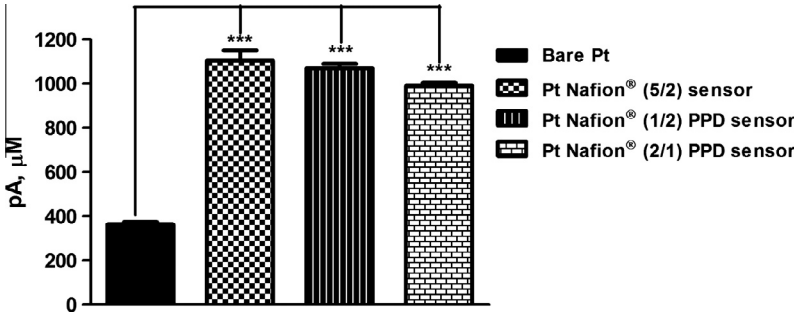

Fig. 2. Comparison of sensitivities of bare Pt ($n = 14$), Nafion®(5/2) ($n = 17$), Nafion®(1/2) PPD ($n = 38$) and Nafion®(2/1) PPD ($n = 38$) sensors to NO at +900 mV vs. SCE.

Nafion®(5/2) sensor [30]. However, they contradict findings from Shibuki where a doubling of sensitivity was recorded [44]. Similarly, Bolger et al. reported a significant increase in O_2 detection at Pt based amperometric electrodes when increasing temperature to physiological levels. This increase was attributed to the variation in solubility of O_2 gas in the membrane with temperature and it is generally recommended that the operation of any O_2 electrode should be carried out under thermostatic conditions [45]. It is unclear whether this phenomena should translate to Pt based NO electrodes and findings from our studies suggest the contrary.

In addition there was no significant improvement ($P > 0.05$) in the response time of Nafion®(1/2) PPD (14 ± 1 s, $n = 6$) or Nafion®(2/1) PPD sensor (10 ± 1 s, $n = 6$) at this elevated temperature, albeit an improved response time was recorded. It is interesting to note that no significant difference ($P > 0.05$) was observed for either sensor type against the Nafion®(5/2) sensor response time (14 ± 3 s, $n = 5$) at 37 °C which has been published previously. These findings suggest that NO permeates across the thicker Nafion®(5/2) membrane at a similar rate to the thinner Nafion®(1/2) PPD and Nafion®(2/1) PPD layers. Moreover it is important to highlight that all are in close alignment with bare Pt response times (7 ± 1 s, $n = 28$) which is very encouraging for their future deployment in animal models.

3.3. Interference investigations

In addition to validating the sensitivity towards NO, it is critical that the selectivity of the sensor has remained intact. To confirm this an extensive investigation into the effects of *in vivo* interferents on the sensors selectivity was undertaken. The mammalian brain has a large number of possible interfering species present at relatively high concentrations, (e.g., ascorbic acid (AA), uric acid (UA), and neurotransmitters including dopamine (DA), serotonin (5-HT), etc.). The concentration of NO *in vivo* has been established as around 0.1 μM [16] and when trying to detect such a small and reactive molecule in the presence of a myriad of endogenous interferents, it can prove very difficult. It is critical for the successful adoption of an electrochemical sensor *in vivo* that selectivity towards the target analyte is maintained in the presence of such interferents. AA, the most abundantly present electroactive species in the brain has a high baseline level and continuously fluctuating extracellular concentration that makes it the principle interferent for electrochemical sensors [46]. Fig. 3 demonstrates the efficacy of both sensor types at rejecting AA from the electrode surface. The decrease below baseline levels can be attributed to zero response from AA aliquots and the background current drifting below the previously recorded baseline value over the time scale of the calibration.

Fig. 4 compares the maximum current observed at 1000 μM AA for the different electrode types. Calibrations performed on bare Pt electrodes yielded a linear ($R^2 = 0.99$) sensitivity of

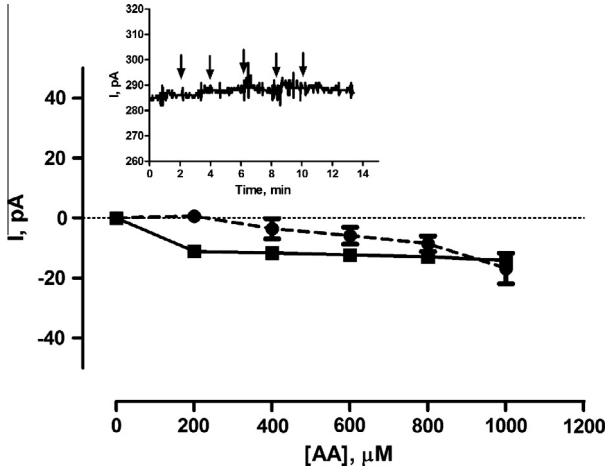

Fig. 3. A current-concentration profile for 0–1000 μM AA at Nafion®(1/2) PPD sensors ($n = 38$, solid line) and Nafion®(2/1) PPD sensors ($n = 38$, dashed line) at +900 mV vs. SCE. (Inset) typical raw data trace. Arrows indicate injection of AA aliquot.

37 ± 1 nA mM^{-1}, $n = 14$ which demonstrates clearly how difficult it would be to measure NO at an unmodified electrode. The various modifications resulted in significant decreases ($P < 0.0001$) in AA levels detected which is illustrated in Fig. 4. The maximum current demonstrated by the various sensor modifications at 1000 μM AA were −3 ± 1 pA μM^{-1}, $n = 17$ (Nafion®(5/2) sensor), −6 ± 6 pA μM^{-1}, $n = 38$ (Nafion®(1/2) PPD sensor) and −18 ± 6 pA μM^{-1}, $n = 38$ (Nafion®(2/1) PPD sensor) respectively. All calibrations resulted in non-linear responses (see Fig. 3).

Following on from the AA investigations a more in-depth look into the permselective properties of both Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors was undertaken and detailed in Table 1. All interferents investigated were compared against the responses observed for bare Pt electrodes over physiological relevant concentrations. Nafion is a poly (tetrafluoroethylene) based ionomer that contains small portions of sulfonic or carboxylic ìonic functional groups [47], the negatively charged sulfonate groups on the Nafion® layers repel negatively charged species including AA and NO_2^- but facilitate neutral species like NO to pass through the membrane to the electrode surface [48,49]. The utilisation of less Nafion® layers meant that *o*-PD had to be incorporated into the design of the sensor as a permselective film against interferents. The monomer *o*-PD has previously been implemented in sensor designs as an effective barrier against electroactive species like AA and DA [23,27].

The PPD layer was deposited by electropolymerisation in neutral electrolyte as was demonstrated by Malitesta et al. [50]. PPD can be integrated into the design of a sensor by using it either as a conducting polymer or an insulating polymer. Ariffin et al.

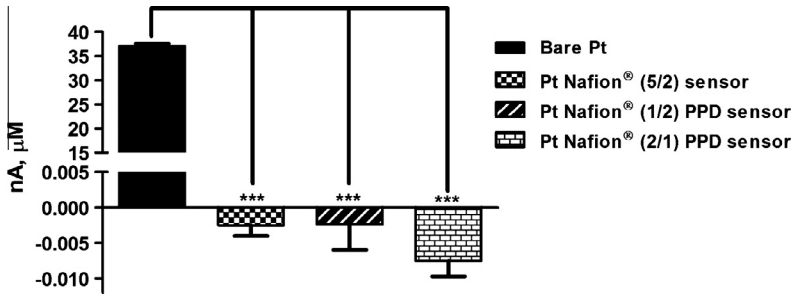

Fig. 4. Comparison of maximum current recorded at 1000 µM AA for bare Pt (n = 14), Nafion®(5/2) (n = 17), Nafion®(1/2) PPD (n = 38) and Nafion®(2/1) PPD (n = 38) sensors.

Table 1
Comparison of sensitivities of bare Pt, Nafion®(1/2) PPD (n = 38) and Nafion®(2/1) PPD (n = 38) sensors to NO at +900 mV vs. SCE and potential endogenous electroactive interferents at reported physiological concentrations.

Analyte	NO response (pA)			% of NO signal	
	Bare Pt	Nafion®(1/2) PPD	Nafion®(2/1) PPD	Nafion®(1/2) PPD	Nafion®(2/1) PPD
NO	352 ± 25 (n = 14)	1047 ± 34 (n = 38)	974 ± 46 (n = 38)	100	100
AA	37,063 ± 2992 (n = 14)	−6 ± 6 (n = 38)	−18 ± 6 (n = 38)	⩽1	⩽1
5-HT	15 ± 7 (n = 4)	−16 ± 20 (n = 4)	12 ± 19 (n = 3)	⩽1	⩽1
DOPAC	940 ± 170 (n = 4)	−6 ± 32 (n = 4)	−5 ± 12 (n = 4)	⩽1	⩽1
DA	9 ± 1 (n = 4)	5 ± 3 (n = 4)	8 ± 7 (n = 6)	⩽1	⩽1
Glutathione	9 ± 1 (n = 4)	2 ± 6 (n = 4)	9 ± 14 (n = 4)	⩽1	⩽1
H_2O_2	4 ± 1 (n = 3)	8 ± 2 (n = 4)	−6 ± 7 (n = 4)	⩽1	⩽1
5-HIAA	990 ± 100 (n = 4)	−6 ± 16 (n = 4)	−2 ± 6 (n = 4)	⩽1	⩽1
HVA	100 ± 10 (n = 4)	−13 ± 7 (n = 3)	6 ± 33 (n = 4)	⩽1	⩽1
NO_2^-	560 ± 110 (n = 3)	7 ± 0 (n = 4)	3 ± 1 (n = 6)	⩽1	⩽1
UA	1530 ± 50 (n = 4)	5 ± 2 (n = 4)	−11 ± 6 (n = 4)	⩽1	⩽1

incorporated it into a sensor design as a conducting polymer film, for the detection of H_2O_2 using Electrochemical Impedance Spectroscopy [51]. Utilising PPD as an insulating polymer layer has been demonstrated previously by a number of different research groups [40,52,23,53,54] due to its ability to block out any unwanted species that are larger than the *o*-PD monomer. The excellent permselective characteristics of the modified sensors are clearly apparent from Table 1. All electroactive interferents investigated displayed <1% contribution to the overall NO signal which is extremely efficient.

3.4. Sensocompatibility of Nafion® modified sensors

The mammalian brain presents a complex chemical environment that includes electrode poisons such as lipids and proteins and a tissue matrix that both restricts mass transport to the electrode surface and reacts physiologically to the presence of the sensor [55,56]. Wisniewski et al. state that the effect that the body has on the electrochemical sensor has been labelled "sensocompatibility" [57]. The material tissue interaction that results from sensor implantation is one of the major obstacles in developing viable, long term implantable sensors. This membrane biofouling is a process that starts immediately upon contact of the sensor with the brain tissue [57]. Prior to implantation of the Nafion® modified sensors, it was imperative to determine the effect of brain tissue and its component parts on the integrity of both Nafion®(1/2) PPD and Nafion®(2/1) PPD modified layers. Previously we reported a significant decrease ($P < 0.05$) of *ca.* 38% in NO sensitivity of the Nafion®(5/2) sensor over a 72 h period in 10% BSA and PEA which is in close alignment with observations reported by various other sensor publications [30,58,59]. It is worth emphasising that a significant ($P < 0.05$) drop in sensitivity was observed after the initial 24 h with no significant effect of further exposure thereafter. Interestingly both the Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors have demonstrated no significant difference ($P > 0.05$) once placed in 10% BSA or PEA for 24 h in comparison to pre-exposure sensitivities. This apparent superior ability of Nafion® PPD modified sensors over Nafion® to prevent electrode fouling may be a result of the size exclusion characteristics that the insulating PPD layer possesses. Two different structures of the insulating form of PPD have been hypothesised, as it has not been determined what the actual structure looks like. A 'ladder' like structure where the amino groups are condensed within the benzene rings adjacent to each other along the polymer chain [60] and an 'open' 1.4-substituted benzenoid–quinoid structure [61] have been hypothesised. Losito et al. have speculated that the 'open' form of PPD is the more dominant structure [62] which sterically hinders large molecules from penetrating the layer. Moreover the improved sensocompatibility demonstrated by the Nafion® PPD modified sensors over Nafion® modified sensors in terms of NO sensitivity could be due in part to the lack of hydrophobic interactions with the surrounding tissue. The Nafion® modified sensors have a dominant hydrophobic interaction with NO and more than likely the surrounding tissue, whereas the incorporated PPD layer that coats the Nafion® layer removes this effect in the novel designs. Bedioui and Griveau have reported that NO pre-concentrates on Nafion® modified surfaces by these hydrophobic interactions and the polymers hydrophilic regions may impede its diffusion to the electrode surface [63]. The inclusion of a PPD layer between the Nafion® and tissue may now allow a layer of water to form around the sensor surface which causes difficulty for proteins and lipids to be adsorbed on the surface. Nishida et al. have utilised such a technique to demonstrate improved sensocompatibility of their glucose sensor [64].

However, to elucidate the effect of longer exposure times on the integrity of the Nafion® PPD layer, it was deemed more accurate to investigate the sensors AA rejection characteristics after 28 days exposure. The rationale being that a degraded Nafion® PPD layer will result in a much larger AA change as opposed to a smaller NO current change and highlight the polymers integrity in a more precise way.

Both sensor types were exposed to homogenised solutions of BSA, PEA and brain tissue for 28 days to determine sensocompatibility. Fig. 5 illustrates the effect of brain tissue treatment on AA rejection characteristics of Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors. The dashed line represent an increase in AA detection due to a deterioration in the polymer integrity following 28 days exposure to brain tissue. It is apparent from Fig. 5 that an increase in current for 0–1000 μM AA calibrations was observed after 28 days for both sensor types. In detail (see Fig. 6) the Nafion®(1/2) PPD sensor displayed significant increases ($P < 0.05$) in current for 0–1000 μM AA calibrations following exposure to 10% BSA (pre-exposure 99 ± 6 pA vs. post-exposure 369 ± 74 nA, $n = 4$), 10% PEA (pre-exposure 29 ± 13 pA vs. post-exposure 299 ± 95 nA, $n = 4$) and brain tissue (pre-exposure 30 ± 27 pA vs. post-exposure 540 ± 152 nA, $n = 4$). Similarly the Nafion®(2/1) PPD sensor displayed significant increases ($P < 0.05$) in current for 0–1000 μM AA calibrations following exposure to 10% BSA (pre-exposure −29 ± 18 pA vs. post-exposure 271 ± 48 nA, $n = 4$), 10% PEA (pre-exposure 45 ± 43 pA vs. post-exposure 396 ± 124 nA, $n = 4$) and brain tissue (pre-exposure 42 ± 32 pA vs. post-exposure 480 ± 106 nA, $n = 4$). Despite a significant difference reported for both sensor types across all treatments, the current detected is *ca.* 1% of the bare Pt response (37 ± 1 nA mM^{-1}, $n = 14$) reported earlier for 0–1000 μM AA calibrations. It is imperative to highlight that the concentration of AA encountered *in vivo* is between 200 and 500 μM. According to Fig. 5 (day 28, dashed line) this corresponds to currents of about 200 pA. However, this still has the capability of interfering with the NO signal *in vivo* but we believe that the characteristics of the PPD membrane prevent this. It is clear from Fig. 5 that the current concentration profile on day 1 (solid line) highlights the ability of the PPD polymer to facilitate the transport of AA at the lower concentrations. This feature diminishes with subsequent injections as the polymer pore size self-blocks the diffusion of incoming AA due to the entrapment of AA and its oxidised metabolite dehydroascorbic acid (DHA) within the PPD polymer matrix. We hypothesise this is what happens

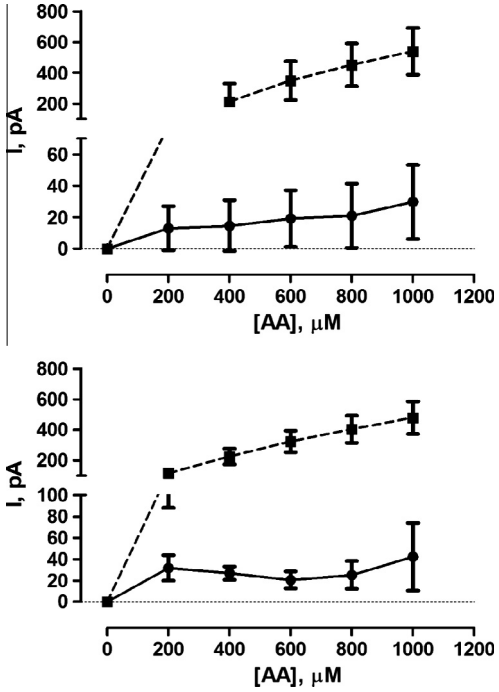

Fig. 5. A current-concentration profile for 0–1000 μM AA at (top) Nafion®(1/2) PPD sensors ($n = 4$) (bottom) Nafion®(2/1) PPD sensors ($n = 4$) at +900 mV vs. SCE. Amperometric sensocompatibility data for pre exposure (solid line) vs. 28 days post exposure (dashed line) to brain tissue.

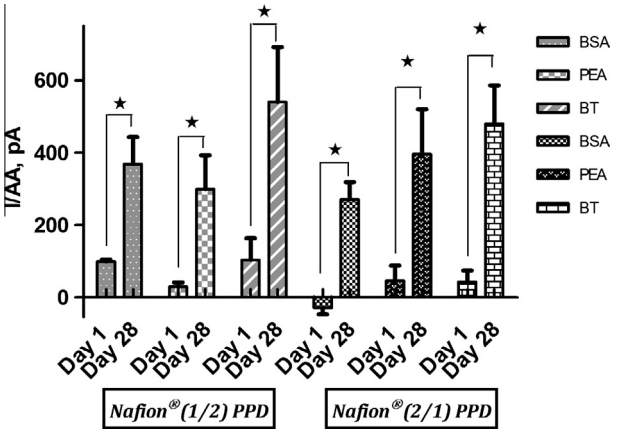

Fig. 6. Comparison of sensocompatibility data for Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors over 28 days exposure to various treatments.

in vivo so the PPD layer will self-block after initial exposure to AA and remain so for the duration of the implantation. This has been reported previously by Kirwan et al. [65] and is characteristic of PPD polymers. These findings suggest that both the Nafion®(1/2) PPD and Nafion®(2/1) PPD sensor are suitable for deployment *in vivo*.

3.5. Shelf-life of Nafion® modified sensors

After 56 days of storage at 4 °C the NO sensitivity of both Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors were investigated to determine the longevity of each design. The sensitivity of the Nafion®(1/2) PPD sensor after 56 days (859 ± 18 pA μM^{-1}, $n = 10$, $R^2 = 0.99$) was not significantly different ($P > 0.05$) in comparison to day 1 (945 ± 12 pA μM^{-1}, $n = 10$, $R^2 = 0.99$). A similar finding was reported for the Nafion®(2/1) PPD sensor where a sensitivity of 877 ± 21 pA μM^{-1}, $n = 11$, $R^2 = 0.99$ was recorded after 56 days which was not significantly different ($P > 0.05$) than the sensitivity on day 1 (728 ± 9 pA μM^{-1}, $n = 11$, $R^2 = 0.99$). This is in close alignment with work undertaken by Christodoulou et al. in which Nafion® electrodes stored in air were stable over several weeks [66]. They also corroborate previous findings from our research group whereby Nafion®(5/2) electrodes demonstrated an excellent shelf life over a 14 day period [31].

4. Conclusion

The development and characterisation of novel Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors for the electrochemical detection of NO have been discussed extensively. Important sensor parameters including sensitivity, selectivity, response time, sensocompatibility and shelf-life have been addressed comprehensively and to great success. The results described within indicate that both sensor types are suitable for deployment in physiological investigations. Future studies will involve the *in vivo* characterisation of the Nafion®(1/2) PPD and Nafion®(2/1) PPD sensors which will focus on validation of the performance and efficacy of these sensors in brain extracellular fluid.

Conflict of interest

The authors have no conflict of interest to report.

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