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Characterisation of a Platinum-based Electrochemical Biosensor for Real-time Neurochemical Analysis of Choline

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Abstract: A choline biosensor was characterised in detail to determine the effects of physiologically relevant parameters on the ability of the sensor to reliably detect neurochemical changes in choline. This first generation Pt-based polymer enzyme composite sensor displayed excellent shelf-life and biocompatibility with no significant decrease in choline sensitivity observed following 14 days of storage dry, or in *ex-vivo* rodent brain tissue. However, subjecting the sensor to repeated calibrations and storage over the same period resulted in significant decreases (20–70%) due to enzyme denaturation associated with the repeated calibration and storage cycles. Potential interference signals generated by the principal electroactive interferents present in the brain were minimal; typically

<1% of the choline current response at *in vivo* levels. Additionally, changing temperature over the physiologically relevant range of 34–40°C had no effect on sensitivity, while increasing pH between 7.2 and 7.6 produced only a 5% increase in signal. The limit of detection of the sensor was in the low μM range ($0.11 \pm 0.02 \mu\text{M}$), while the *in vitro* response time was determined to be less than the solution mixing time and within *ca.* 5 s, suggesting potential sub-second *in vivo* response characteristics. Finally, the sensor was implanted in the striatum of freely moving rats and demonstrated reliable detection of physiological changes in choline in response to movement, and pharmacological manipulation by injection of choline chloride.

Keywords: Choline • Acetylcholine • Biosensor • Neurochemistry • Brain

1 Introduction

Acetylcholine plays an essential role in movement, learning, memory and higher consciousness [1–2]. It is not surprising therefore that the dysregulation of the cholinergic system has been linked to a number of neurological disorders such as Alzheimer's disease [3], vascular dementia [4], schizophrenia [5] and neuromuscular disorders including Parkinson's and Huntington's disease [6]. Acetylcholine and choline have classically been studied pre-clinically in the rodent brain by means of microdialysis, which suffers from a number of limitations including low temporal resolution, and large probe size which can result in damage to the tissue at the implantation site confounding accurate analyte detection.

Electrochemical biosensors offer some key advantages *in vivo* due to their excellent temporal resolution and small size. Specifically, the ability to detect real-time cholinergic activity (and therefore an accurate readout of acetylcholine release [7–9]) in minimally perturbed tissue is vital if we are to improve our understanding of the physiological role of rapid cholinergic changes in response to activation/behaviour and neurological dysfunction. However, before such use extensive sensor characterisation is required as the mammalian brain is a hostile environment containing potential electrode poisons (e.g. proteins and lipids) and an array of electroactive interferents in high concentrations that can potentially be detected at the underlying electrochemical transducer.

The recommended criteria for establishing the properties confirming suitability for *in vivo* monitoring have been reported by Phillips and Wightman [10] and include testing sensitivity, selectivity, response time, consumption/depletion and stability.

We have recently reported the optimisation of the design of a choline biosensor for high sensitivity choline detection [11]. Here we present results on the further characterisation of this device with respect to changes in sensitivity resulting from storage, exposure to brain tissue and potential electroactive interferents such as dopamine and serotonin. The effect of physiologically relevant temperature and pH changes were also investigated, in addition to estimating the sensor's response time and limit of detection. Finally, the optimised device was employed successfully in the striatum of freely moving rats, demonstrating that the sensor can selectively detect both

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physiological and pharmacologically induced choline changes in real-time.

2 Experimental

2.1 Chemicals and Solutions

All chemicals (see below) were of analytical reagent grade or higher quality and purchased from Sigma-Aldrich Ireland Ltd (Dublin). Compounds used in the interference study were dopamine (hydrochloride), 5-hydroxytryptamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid, L-tyrosine, L-cysteine, L-tryptophan, L-glutathione (oxidized form), dehydroascorbic acid, and uric acid (potassium salt). All *in-vitro* experiments were carried out in phosphate buffered saline (PBS) solution, pH 7.4 (150 mM NaCl, 40 mM NaOH and 40 mM NaH₂PO₄). Fresh interferent solutions were prepared as required. Solutions of choline chloride ($\geq 97\%$), *o*-phenylenediamine monomer (*o*-PD, 1,2-diaminobenzene, $\geq 98\%$), bovine serum albumin (BSA, fraction V from bovine plasma), glutaraldehyde (GA, Grade 1, 25%) and polyethyleneimine (PEI, 80% ethoxylated) were always prepared fresh.

2.2 Biosensor Preparation

Cylinder electrodes were made from Teflon-insulated platinum/iridium (Pt/Ir 90%/10%) wire (125 μm bare diameter, 175 μm coated diameter (5T), Advent Research Materials, Suffolk, UK). The electrodes were 6 cm in length and approximately 3 mm of Teflon insulation was removed from one end of the wire. This was subsequently soldered into a gold clip (*In-vitro* – Fine Science Tools, Linton, UK; *In-vivo* – Plastics One, Roanoke, VA, USA) to facilitate electrical connection to the potentiostat. The opposite end of the wire acted as the active electrode surface; a fresh disk was cut at this end before removing a 1 mm portion of the Teflon[®] to create the cylinder electrode. Poly(*o*-phenylenediamine) (PPD, 300 mM in N₂-saturated PBS) was then electrochemically grown onto the active surface and the electrode stored at 4 °C for a minimum of 3 h before addition of biosensor constituents using a previously reported dip-absorption method [11]. Briefly, electrodes were initially dipped (*ca.* 0.5 s) into methyl methacrylate (MMA, 99%) to coat the bare wire, cellulose acetate (CA, 2%, Mn *ca.* 50,000 g/mol) and a final coat of MMA. The electrodes were then sequentially dipped into choline oxidase (ChOx, Alcaligenes sp. EC 232-840-0, 500 units), BSA (1%), GA (0.5%) and PEI (2%). Each layer was allowed 5 min drying time and repeated a further 9 times. These Pt_{cyl}(PPD)(MMA)(CellAcce)(MMA)(ChOx)(BSA)(GA)(PEI) electrodes were allowed to dry at room temperature for a minimum of an hour, and stored at 4 °C until calibrated for use. Hereafter we refer to this polymer composite electrode as Pt-PC/ChOx/PC.

2.3 Biosensor Calibrations

The working electrodes (four at a time) were calibrated in a standard three-electrode glass electrochemical cell containing 20 mL PBS, unless otherwise stated. A saturated calomel electrode (SCE) was used as the reference electrode and a Pt wire served as the auxiliary electrode. A potential of +700 mV (*vs.* SCE) was applied to the working electrodes which were allowed time to settle under the influence of the applied potential until the non-faradaic current had reached a stable baseline (*ca.* 3 hrs). Calibrations were then performed by addition of aliquots of analyte into the buffer solution every 4 min. Each addition was followed by a period of brief stirring/mixing (*ca.* 20 s), with the current response subsequently measured immediately before the next injection. Temperature controlled calibrations were performed by clamping the cell in a temperature controlled water bath set at the required temperature and allowing the cell to equilibrate until the appropriate temperature was reached, as indicated by continuous monitoring of the PBS temperature using a small digital thermometer (Fisher Scientific Ireland, Ltd.).

2.4 Stability and Biocompatibility

The storage stability (shelf-life) was investigated by calibrating the same batch of biosensors on day 1 and day 14. A batch of sensors was also subjected to repeated calibrations over the same interval (days 1, 3, 7 and 14). For biocompatibility studies sensors were stored in the fridge at 4 °C in moist brain tissue and similarly subjected to calibrations on days 1 and 14.

2.5 Instrumentation and Software

Constant potential amperometry (CPA) was carried out using four-channel low noise potentiostats (*In vitro* – Biostat IV, ACM Instruments, Cumbria, UK; *In vivo* – Biostat II, Electrochemical and Medical Systems, Newbury, UK). Data acquisition was performed with either a notebook PC (*in vitro*) or Mac[®] (*in vivo*), a PowerLab interface system (ADInstruments Ltd, Oxford, UK) and LabChart[®] for Windows and Mac[®] (Version 6, ADInstruments Ltd).

2.6 Data Analysis

The graphical presentation and analysis of data was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA). All data is reported as mean \pm SEM where *n* denotes either the number of electrodes used in *in vitro* experiments, or the number of control/drug administrations in *in vivo* experiments. *In vitro* signals were background (capacitance current) subtracted. *In vivo* signals recorded from each implanted electrode were normalised to zero current according to their baseline. For experiments involving intraperitoneal choline chloride

administration the maximum amplitude (peak) of the signal was extracted from the curve obtained for each dose (including saline control). Statistical significance tests were performed using *t*-tests (two-tailed paired or unpaired where appropriate) or one-way ANOVA (with Bonferroni post-hoc analysis). Values of $P < 0.05$ were considered to indicate statistical significance.

2.7 Surgical Procedures

Male Wistar rats (3, 200–300 g; Charles River Laboratories International, Inc., UK) were anaesthetised with the volatile anaesthetic isoflurane (4% in air for induction, 1.5–3.0% for maintenance; IsoFlo[®], Abbott, UK) using a Univentor 400 Anaesthesia Unit (AgnTho's AB, Sweden). The level of anaesthesia was checked regularly (pedal withdrawal reflex). Once surgical anaesthesia was established animals were placed in a stereotaxic frame and the sensors implanted following a previously described procedure [12]. Coordinates for the striatum with the skull levelled between bregma and lambda, were: A/P +1.0, M/L ± 2.5 and D/V –6.0. A reference electrode (8T Ag wire, 200- μ m bare diameter; Advent Research Materials) was placed in the cortex and an auxiliary electrode (8T Ag wire) attached to one of the support screws. The sensors/electrodes were fixed to the skull with dental screws (Fine Science Tools GmbH) and dental acrylate (Dentalon[®] Plus, Heraeus-Kulzer, Germany). All animals were given saline (0.9%) and analgesia (Buprecare[®], AnimalCare Ltd., UK) and allowed to recover in a thermostatically controlled cage (Thermacage MkII, Datasand Limited, Manchester, UK) for several hours. They were assessed for good health according to published guidelines [13,14] immediately after recovery from anaesthesia and at the beginning of each day. All animal work was carried out with approval from the Maynooth University Research Ethics Committee, and under license in accordance with the European Communities Regulations 2002 (Irish Statutory Instrument 566/2002 – Amendment of Cruelty to Animals Act 1876).

2.8 Experimental Conditions *In Vivo*

Twenty four hours following recovery, animals were singly housed in Ratern[®] sampling cage systems (BASi, West Lafayette, IN, USA) in a temperature-controlled experimental facility with a 12 hour light/dark cycle (lights on at 07:00) with access *ad libitum* to food and water. All experiments were performed in the animal's home bowl. The implanted sensors from each animal were connected directly to the potentiostat via a six-pin Teflon[®] socket (MS363, Plastics One) using a flexible screened six core cable (363-363 6TCM, Plastics One). This arrangement allowed free movement of the animal which remained continuously connected to the instrumentation. After application of the applied potential each animal was given a further 24 h before experiments were begun in order to ensure that the background currents for the electrodes

were completely stabilised. A low-pass digital filter (50 Hz cut-off) was used to eliminate mains AC noise and all data was recorded at 40 Hz. Movement was registered using a PIR detector (Gardscan QX PIR, Gardiner Technology, Queensway, Rochdale, OL11 1TQ, UK) modified in-house with a micro-processor to enable enhancement of the resolution of the sensor thereby registering more movement. All choline chloride injections were administered intraperitoneally (i.p.) on separate days. Animals were weighed on each day and the respective doses for each animal were prepared immediately prior to injection.

3 Results and Discussion

3.1 Stability and Biocompatibility

Following the development of a new electrochemical sensor [11] it is important that the electrode-environment interactions are characterised prior to use in the target environment [10]. This is particularly important for neurochemical applications due to the complex chemical matrix of the brain which consists of surface modifying agents such as surfactants (lipids) and electrode poisons (proteins), in addition to electrocatalysts such as ascorbic acid, all of which can affect the performance of an implanted biosensor [15–16].

Understanding how sensitivity changes over time is a key consideration for *in vivo* analyte monitoring. The shelf-life is routinely used as an indicator of stability, and was established for the Pt-PC/ChOx/PC biosensor by recalibrating a batch of sensors following 14 days dry storage at 4 °C (Figure 1A). Compared to Day 1 (518 ± 56 pA/ μ M) the average change in sensitivity was a decrease of $11 \pm 6\%$ (464 ± 67 pA/ μ M, $n=4$, $P=0.64$) indicating good stability in terms of dry storage before use. Interestingly, when a batch of sensors ($n=4$) is subjected to repeated storage and calibration over the same interval decreases in sensitivity of $19 \pm 0.3\%$ (Day 3: 380 ± 21 pA/ μ M, $P > 0.05$), $48 \pm 1\%$ (Day 7: 243 ± 17 pA/ μ M, $P < 0.001$) and $70 \pm 1\%$ (Day 14: 144 ± 14 pA/ μ M, $P < 0.001$) are observed compared to Day 1 (470 ± 27 pA/ μ M), highlighting the detrimental effect of repeated calibration and storage cycles (Figure 1B). For biocompatibility testing sensors were stored in the fridge at 4 °C in moist brain tissue between calibrations on days 1 and 14 (*inset* Figure 1). A decrease of 15% (Day 14: 452 ± 69 pA/ μ M, $P=0.31$) was observed compared to Day 1 (532 ± 12 pA/ μ M, $n=4$), which is similar to that observed for dry storage, and suggests good biocompatibility characteristics.

Such decreases are not dissimilar to what is commonly observed *in vivo* where reduced sensitivity generally varies between 20% and 50%. This typically occurs over several hours immediately following biosensor implantation [17–18], and is most likely due to a combination of effects including electrode-tissue interactions [15] and continuous enzyme-substrate turnover [19–20]. Following this initial stabilisation period the signal usually settles

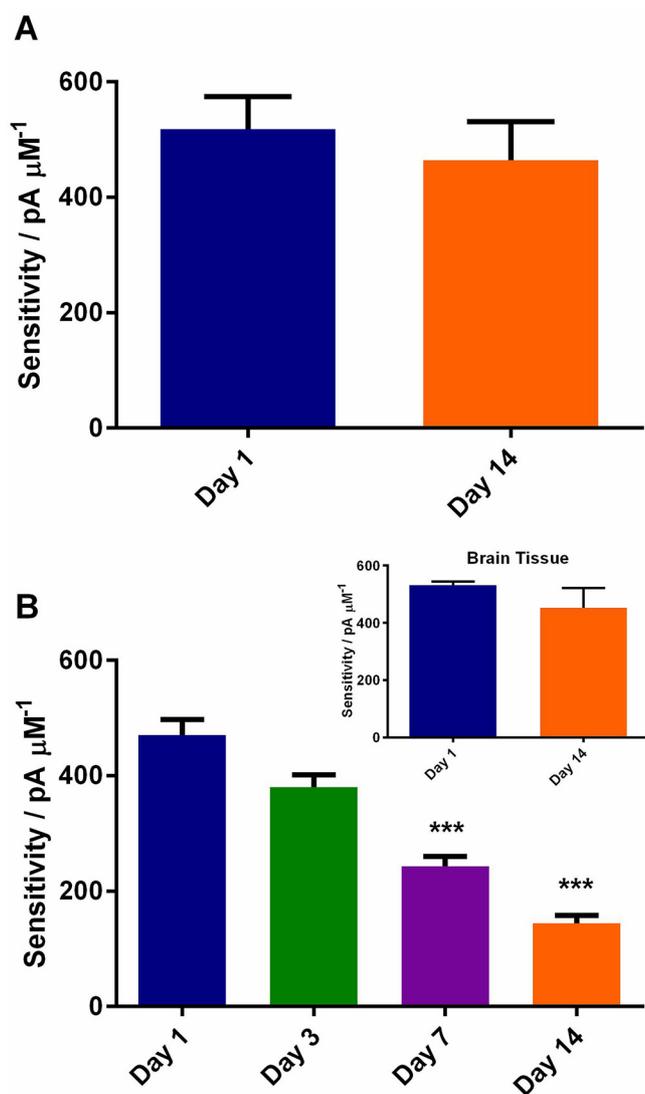


Fig. 1. (A) Comparison of the Pt-PC/ChOx/PC biosensor sensitivity following 14 days of dry storage at 4°C. (B) The effect of repeated calibrations and dry storage over the same time-period. *Inset*: The effect of 14 days storage in *ex-vivo* rodent brain tissue at 4°C. Calibrations carried out using constant potential amperometry (CPA) at +700 mV (vs. SCE) in PBS, pH 7.4. *** $p < 0.001$ compared to first calibration (Day 1).

and can remain stable for days or weeks depending on the enzyme. For this particular biosensor we have found that the baseline *in vivo* response is stable for at least 14 days [21].

3.2 Interference Studies

Biosensor specificity can be undermined by interference from electroactive species present in the brain. For example, ascorbic acid (AA), generally regarded as the principal electroactive interferent, can be detected with high sensitivity (*ca.* 600 pA/ μM) at bare Pt at the biosensor operating potential of +700 mV [11]. As the

AA concentration has been reported to be as high as 400 μM in the brain [22] we have used electrodeposited poly(*o*-phenylenediamine) (PPD) for the rejection of potential interferents [23–25], and have previously reported its efficiency in eliminating AA signals (<2 pA/ μM) at this polymer composite choline biosensor [11]. We have also shown that there is minimal oxygen interference with only a 1% reduction in signal observed when the concentration of oxygen is changed over physiological levels (e.g. 50–200 μM) [11], indicating that the sensor can reliably monitor choline, free from changes in current associated with *in vivo* oxygen fluctuations [21].

Here we present data for other potential interferents [26] including the neurotransmitters dopamine (DA) and 5-hydroxytryptamine (5-HT), their metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (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). The average current response for physiological levels of each molecule is presented in Table 1. All signals were found to be negligible compared to the current (3.24 ± 0.18 nA, $n = 4$) response for basal choline (*ca.* 6 μM [21]). In some cases small negative values were observed if there was no detection of the interferent, and can be attributed to slight baseline drift over the period of monitoring. The sensitivity of the biosensor as a function of DA concentration is shown in Figure 2, along with the average raw data trace for DA calibrations (*inset*). Both highlight the significant interference rejection and saturation characteristics at physiological levels, which has also previously been reported for other PPD-modified biosensors [23,27–28].

Table 1. Response of Pt-PC/ChOx/PC biosensor to various interference species found *in vivo*.

| Interferent | n | ECF Concentration (μM) | Current Response (nA, \pm S.E.M.) | Response as % of current at 6 μM Choline ^a |
|---------------|---|-------------------------------------|-------------------------------------|--|
| DA | 4 | 0.05 ^b | 0.024 ± 0.004 | 0.7% |
| 5-HT | 4 | 0.01 ^b | -0.011 ± 0.003 | 0% |
| DOPAC | 4 | 50 ^b | -0.001 ± 0.005 | 0% |
| HVA | 4 | 10 ^b | 0.014 ± 0.002 | 0.4% |
| 5-HIAA | 4 | 50 ^b | 0.003 ± 0.001 | 0.1% |
| L-Tyrosine | 4 | 100 ^c | -0.008 ± 0.003 | 0% |
| L-Cysteine | 4 | 50 ^c | 0.054 ± 0.01 | 1.7% |
| L-Tryptophan | 4 | 100 ^c | 0.028 ± 0.005 | 0.9% |
| L-Glutathione | 4 | 50 ^c | 0.026 ± 0.001 | 0.8% |
| DHAA | 4 | 100 ^c | -0.010 ± 0.008 | 0% |
| UA | 4 | 50 ^b | -0.024 ± 0.009 | 0% |

^a Estimated basal ECF concentration [21], ^b [26], ^c ECF concentrations not known, high μM values chosen

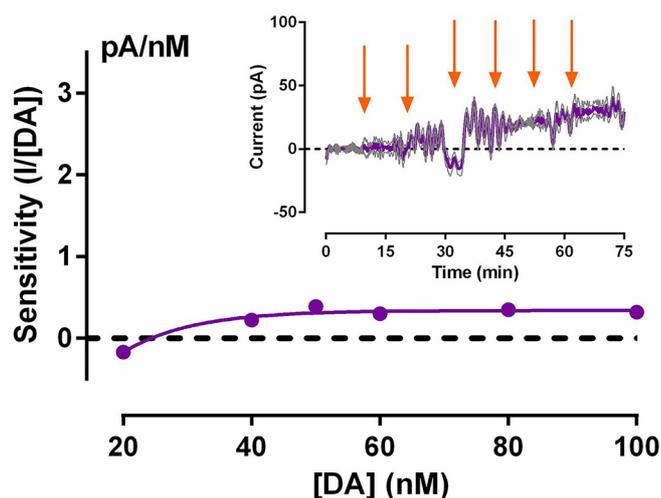


Fig. 2. The sensitivity of the Pt-PC/ChOx/PC biosensor as a function of dopamine (DA) concentration showing significant interference rejection and saturation characteristics at physiological levels. *Inset*: Average background subtracted (194 ± 11 pA, $n=4$) current-time response for DA calibrations carried out in PBS, pH 7.4, at +700 mV (*vs.* SCE). The hashed (gray) lines represent the SEM. Arrows indicate injections yielding concentrations of 20, 40, 50, 60, 80 and 100 nM DA.

3.3 Temperature and pH

All of the above characterisation work was performed at room temperature (*ca.* 21 °C). However, changes in temperature may enhance or diminish the reaction rate of an immobilised enzyme and thus alter biosensor performance [29]. As such, it is important to establish the sensor response characteristics associated with temperature changes, particularly as physiological temperature variations associated with behaviour and pharmacological interventions have been reported in freely-moving animals [30–31]. While 35.5 °C to 38.8 °C has been reported to represent a typical range of physiological fluctuations in brain temperature [32], lower and higher values have been reported under certain conditions, for example 33 °C during pentobarbital anesthesia and 40 °C with psychomotor stimulants such as methamphetamine [32]. Figure 3A shows the effect on choline sensitivity of changing temperature over the range 34 to 40 °C. No significant difference was observed in the responses recorded at these two physiological extremes (34 °C: 460 ± 20 pA/ μ M, $n=16$, $P=0.95$; 40 °C: 450 ± 40 pA/ μ M, $n=19$, $P=0.97$) compared to that at 37 °C (460 ± 30 pA/ μ M, $n=16$), indicating that the sensor has the ability to accurately measure choline free from temperature induced bias, similar to a previously reported polymer modified Pt-based glucose biosensor [33].

Sensors designed for use in the *in-vivo* environment are routinely tested for their response to pH changes; usually in the range 6.8 to 8 [34–35]. As choline oxidase is susceptible to pH alteration and is inactivated in the ranges of 1 to 6 and 9 to 14 [36], a selection of pH values

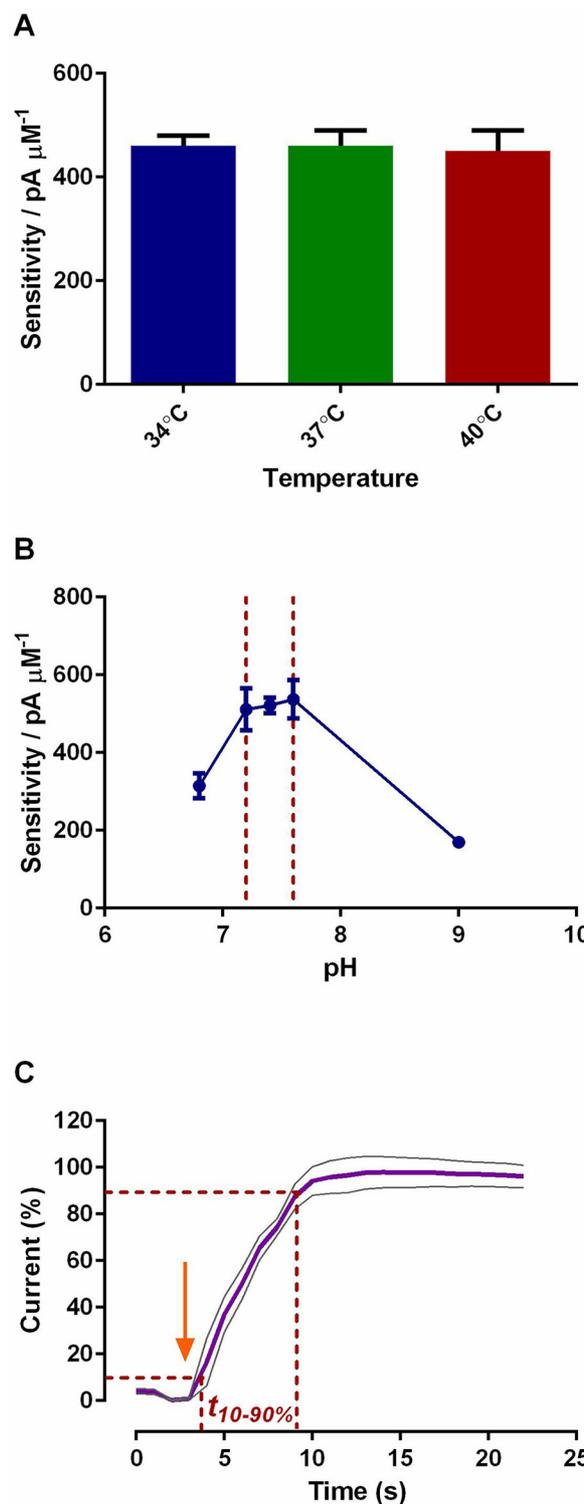


Fig. 3. The effect on Pt-PC/ChOx/PC biosensor sensitivity of changing temperature (A) and pH (B) over physiologically relevant ranges: 34 to 40 °C and 7.2 to 7.6 (hashed lines) respectively. Calibrations ($n=4$) carried out at +700 mV (*vs.* SCE) in PBS, pH 7.4. (C) A typical example of the average ($n=4$, gray lines represent the SEM) normalised current change (response time, $t_{10-90\%}$) for a choline injection (5 μ M, arrow) performed at room temperature.

(6.8, 7.2, 7.4, 7.6 and 9) were tested to determine the effect of pH on the biosensor's sensitivity. The lowest current value (Figure 3B) of 314 ± 32 pA/ μ M ($n=4$) was observed at pH 6.8. There was no significant difference ($P=0.99$, one-way ANOVA) in the signal over the physiologically relevant range of 7.2 (511 ± 54 pA/ μ M, $n=10$), 7.4 (522 ± 20 pA/ μ M, $n=17$) and 7.6 (537 ± 50 pA/ μ M, $n=10$). As expected the current dramatically decreased at pH 9 (170 ± 13 pA/ μ M, $n=4$).

3.4 Response Time and Limit of Detection

Two other important performance characteristics are the response time and limit of detection (LOD). The former is defined as the time taken for the response to rise from 10% to 90% ($t_{10-90\%}$) of the maximum amplitude for a fixed concentration step [37–38]. This is often difficult to separate from the mixing time in *in vitro* studies performed in a classical electrochemical cell such as those used here. Typical data for the Pt-PC/ChOx/PC biosensor is shown in Figure 3C and it is clear that the response is instantaneous with $t_{10-90\%}$ achieved in less than the mixing time and within *ca.* 5 s. Similar *in vitro* response times have been reported for other sensors where more rapid (typically 1 s or less) responses *in vivo* have been observed [7, 9, 33–34, 39–43]. *In vivo* data would also suggest that this is the case for this choline biosensor [21, 44]. The LOD was determined as three times the standard deviation of the baseline signal [37–38, 45]. The estimate of 0.11 ± 0.02 μ M ($n=8$) indicates that the biosensor is ideally suited to monitoring neurochemical changes associated with behavioural and/or pharmacological manipulations *in-vivo* where the choline concentration is *ca.* 6 μ M [21, 46].

3.5 In Vivo Monitoring

Disruption of the cholinergic system has been implicated in movement disorders such as Parkinson's disease (PD) and dystonia [6]. In PD a disruption of the dopamine (DA)–ACh balance, whereby DA exerts an inhibitory effect on ACh release in the striatum from its most prominent dopaminergic input, the substantia nigra pars compacta, leads to the appearance of motor symptoms [47–48]. As microdialysis has previously demonstrated the correlation between ACh and movement in the striatum [49], the choline biosensor was implanted into the striatum of freely-moving rats and over a 12 hour period the correlation between movement and the levels of choline (a proxy for ACh [8–9, 44]) were monitored. As observed with microdialysis, periods of activity were coincident with increases in choline (Figure 4A).

It is also known that dietary restriction of choline in rats has demonstrated effects on ACh release in the brain [50] due to the inability of the brain to synthesise choline. Choline used for the synthesis of ACh is sourced from the extracellular fluid and enters the brain from the systemic circulation [51]. It has been reported that 15 min after an

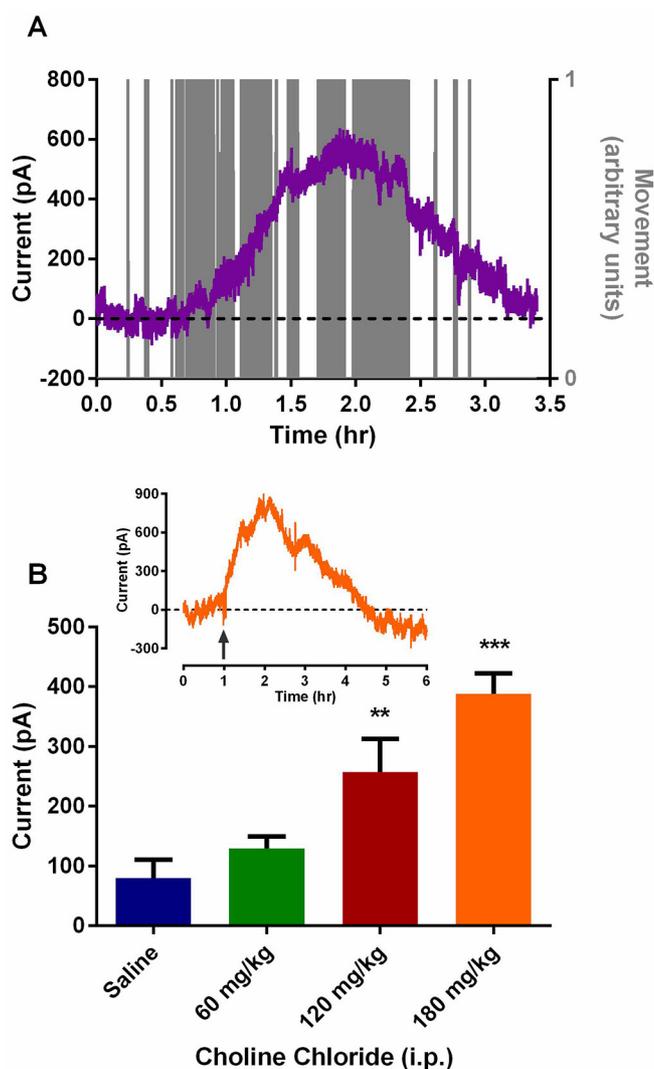


Fig. 4. (A) A typical example of changes in the Pt-PC/ChOx/PC biosensor signal recorded in the striatum of a freely-moving rat using CPA at +700 mV. The gray lines represent simultaneously monitored movement. (B) The effect of systemic intraperitoneal (i.p.) choline chloride administration on the striatal Pt-PC/ChOx/PC biosensor signal (peak current). ** $p < 0.01$, *** $p < 0.001$ compared to saline control. *Inset*: A typical example of the response resulting from administration of a 180 mg/kg dose (arrow indicates the point of injection).

i.p. injection of 20 mg/kg choline chloride an elevation in choline is detectable in brain microdialysate [52]. As such, we investigated the effect of i.p. injections of increasing concentrations of choline chloride on the biosensor signal recorded in the striatum (Figure 4B). All data was taken 15 min post-injection and compared to saline control (79 ± 31 pA, $n=10$). The biosensor response increased for all concentrations in a dose-dependent manner (60 mg/kg: 130 ± 20 pA $n=4$, $P > 0.05$; 120 mg/kg: 258 ± 55 pA $n=6$, $P < 0.01$; 180 mg/kg: 388 ± 35 pA $n=8$, $P < 0.001$) confirming the ability of the sensor to detect dynamic physiological choline fluctuations in freely-moving animals.

4 Conclusions

Characterisation of a sensitivity optimised polymer composite choline biosensor was performed to determine its suitability for *in-vivo* neurochemical detection of choline. The sensor successfully detected choline with no significant decrease in sensitivity over a two-week period when stored at 4 °C dry or in *ex-vivo* rodent brain tissue. Subjection to repeated calibrations and storage (dry) over a similar period resulted in significant decreases due to enzyme denaturation associated with the repeated calibration and storage cycles. Where repeated calibrations/use are an operational factor such decreases would need to be accounted for when using *in vitro* calibration data to determine concentration changes over time. The ability of the Pt-PC/ChOx/PC biosensor to selectively detect choline was also tested in the presence of the most physiologically relevant electroactive species. The interference signal contributions from these species were found to be negligible compared to the current response for basal choline *in vivo*, which is unaltered by the interference rejection layer [11]. In addition, physiologically relevant temperature and pH changes had minimal effect on the sensor performance, which, when taken with a calculated low μM detection limit and rapid response time, suggests ideal suitability for *in vivo* brain choline monitoring. This was supported by *in vivo* experiments in freely-moving animals where changes were recorded in response to systemic i.p. injections of choline chloride and physiological activity in the striatum.

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