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Research paper

Development of a microelectrochemical biosensor for the real-time detection of choline

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ABSTRACT

Here we describe the development of a first generation biosensor for the detection of brain extracellular choline, investigating important considerations for in-vivo monitoring such as sensor sensitivity, O_2 interference and selectivity. Extensive optimisation of choline biosensor designs resulted in a biosensor with excellent sensitivity towards choline (0.54 ± 0.03 nA/μM). Oxygen interference studies demonstrate a 1% reduction in current at 50 μM O_2 when compared to atmospheric O_2 levels (200 μM), indicating that the sensor can be used for reliable choline monitoring, free from changes in current associated with physiological O_2 fluctuations. A negligible sensitivity of 0.0021 ± 0.0002 nA/μM n = 8 was achieved utilising poly-phenylenediamine (PPD) as a permselective membrane for interference rejection of ascorbic acid (AA), the most physiologically important endogenous electroactive species present in the brain. The optimised biosensor when implanted into the striatum of a freely moving rat successfully detected local perfusions of choline demonstrating the sensors ability to detect choline in-vivo.

1. Introduction

Acetylcholine (Ach) is a major excitatory neurotransmitter implicated in many neurological disorders such as Alzheimer's disease and Parkinson's disease [1,2]. Its real-time measurement has proven difficult due to its rapid hydrolysis by acetylcholine esterase. Hence the measurement of its precursors and metabolite choline has proven advantageous [3–5]. The primary technique utilised for Ach measurement has been microdialysis, which, despite recent advances such as using segmented flow [6], remains limited by its spatial and temporal resolution. The development of choline biosensors for the indirect measurement of Ach has become a necessary tool for its detection due to the difficulties associated with multi-enzyme approaches required for direct Ach detection.

The utilisation of first generation choline biosensors incorporating choline oxidase has been reported previously [4,5]. These sensors rely on an enzymatic process involving the catalysis of choline oxidation to glycine betaine with betaine aldehyde as an intermediate and molecular oxygen as the primary electron acceptor [7], resulting in the production of the signal generating H_2O_2 as shown in Eq. (1):

$$(CH_3)_3N^+ - (CH_2)_2 - OH + 2O_2 + H_2O \xrightarrow{\text{ChOx}} (CH_3)_3N^+ - CH_2 - COOH + 2H_2O_2 \quad (1)$$

Biosensor mediated analyte detection offers a number of advantages over classical microdialysis approaches, including their small size and ability to allow real-time detection of an analyte making them an attractive option for neurochemical monitoring in-vivo. This approach however, still suffers from its own limitations. For example, the incorporation of an oxidase enzyme means these sensors are subject to interference from in-situ fluctuations of both the co-substrate (molecular oxygen) and from electroactive species present in the extracellular fluid (ECF) also detected at the high (+700 mV) oxidation overpotential [8].

We have recently reported the in-vivo characterisation of a novel choline biosensor [9] and here we present the detailed development of this biosensor in-vitro, encompassing extensive sensitivity optimisation, oxygen interference and choline selectivity confirmation.

Excellent choline sensitivity is a critical criteria for the physiological monitoring of choline due to its relatively low concentration in the ECF (approximately 6 μM). The effect of oxygen variability on sensor performance was also determined in an attempt to eliminate any potential interference once implanted in a freely moving

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animal. In addition, a permselective membrane was used in order to reduce the interference contribution from the highly abundant and electroactive species Ascorbic Acid (AA). Finally, implantation of the sensor alongside a microdialysis probe validated that the sensor can detect changes in choline concentrations around the active surface when implanted in living tissue.

2. Materials and methods

2.1. Chemicals and solutions

The *o*-phenylenediamine (*o*-PD, 1,2-diaminobenzene, ≥98%), styrene (Sty, 99%), methyl methacrylate (MMA, 99%), cellulose acetate (Mn ~50,000 g/mol), Nafion® (5 wt% in lower aliphatic alcohols in H_2O), choline oxidase (ChOx; from Alcaligenes sp., EC 232-840-0, 1 KU), bovine serum albumin (BSA, fraction V from bovine plasma), glutaraldehyde (Grade 1, 25%), polyethyleneimine (PEI, 80% ethoxylated), NaCl (SigmaUltra), NaH_2PO_4 (Sigma, A.C.S. reagent), NaOH (SigmaUltra), choline chloride (≥97%), were obtained from Sigma-Aldrich Ireland Ltd (Dublin). In-vitro electrochemical experiments were conducted in a phosphate buffered saline (PBS) solution, pH 7.4; NaH_2PO_4 (40 mM), NaOH (40 mM), NaCl (150 mM). Choline chloride (0.1 M), *o*-PD monomer (300 mM in N_2-saturated PBS), BSA (0.1–1%), glutaraldehyde (0.1–1%) and PEI (1–2%) were always prepared fresh.

2.2. Working electrode preparation

5T Teflon®-coated Pt/Ir (90%/10%) disc electrodes or cylinder electrodes (125-μm bare diameter, 175-μm coated diameter, Advent Research Materials, Suffolk, UK) of aprx. 6 cm in length were utilised in this study. 3 mm of Teflon® insulation was stripped from the wire and soldered onto a gold clip (Fine Science Tools GmbH, Heidelberg, Germany) to facilitate an electrical connection to the potentiostat while the opposite end of the wire was freshly cut to form an active surface disc for the biosensor. All cylinder electrodes had an additional 1.0 ± 0.1 mm of Teflon stripped away (Fig. 1a). Poly(*o*-phenylendiamine) (PPD) was electrochemically grown onto the active surface and stored at 4 °C for a minimum of 3 h before biosensor constituent addition. The active surface of each electrode was coated with biosensor constituents using a dip absorption method. Briefly, electrodes were dipped for 0.5 s into monomeric immobilisers MMA or Styrene, followed by incorporation of Nafion® (5%) or cellulose acetate (2%) and then consecutively dipped into ChOx (50 or 500 U/ml), BSA (0.1–1%), glutaraldehyde (0.1–1%) and PEI (1–2%) for a total of 10 layers. 5 min drying incubations were conducted between each layer (Fig. 1b). Sensors were prepared in batches of four, typically taking three days to manufacture. Prior to biosensor utilisation, the sensors were kept at room temperature for a minimum of one hour or stored at 4 °C until calibrated. Oxygen measurements were conducted using a freshly cut 5T Teflon®-coated Pt/Ir (90%/10%) disc electrode (125-μm bare diameter, 175-μm coated diameter, Advent Research Materials, Suffolk, UK) of aprx. 6 cm in length. Teflon® insulation was stripped from one end of the wire and soldered into a gold clip (Fine Science Tools GmbH, Heidelberg, Germany).

2.3. Instrumentation

A standard three-electrode glass electrochemical cell was used for all sensor calibrations (choline: 0–3 mM and oxygen 0–240 μM) in 20 mL PBS. The reference electrode was a saturated calomel electrode (SCE), while bare Pt wire was utilised as the auxiliary electrode. Constant potential amperometry (CPA; +700 mV (choline), −650 mV (O_2)) performed in all electrochemical experiments were conducted using a custom designed low-noise potentiostat, Biostat IV, ACM Instruments, Cumbria, UK; with a notebook PC (in-vitro) or Mac® a PowerLab interface system (ADInstruments Ltd., Oxford, UK) and LabChart® for Windows and Mac® (Version 6, ADInstruments Ltd).

2.4. Data analysis

Data presented as mean ± standard error (SEM) while *n* refers to the number of sensors used in each study. Data analysis was conducted with the commercial package of Prism (version 6.04; GraphPad Software, Inc., CA, USA). Unpaired *t*-tests (two-tailed) were also calculated where appropriate using Prism. $P < 0.05$ values were deemed statistically significant. Michaelis-Menten non-linear regression analysis (Eq. (2)) was used to provide enzyme kinetic parameters V_{MAX}, K_M, Linear Region Slope (LRS) and K_MO_2. V_{MAX} is the maximum current value at enzyme-substrate saturation. The Michaelis constant K_M is the concentration of substrate that gives half of the V_{MAX} response. LRS was obtained from linear regression analysis which is valid up to half the K_M value. K_MO_2 is the concentration of O_2 that gives half the V_{MAX} response to choline. The CAD programme SolidWorks and online CAD programme Tinkercad were used for schematic design.

$$\nu = \frac{V_{\max}[S]_0}{[S]_0 + K_M} \quad (2)$$

2.5. Sensor calibrations

The working electrodes were calibrated at room temperature. They were allowed time to settle under the influence of the applied potential until the non-faradaic current had reached a stable baseline. When this was achieved, calibrations were performed by addition of aliquots of analyte into the buffer solution. For all calibrations additions were made every 4 min into the buffer which was stirred briefly (20 s) after the addition of each aliquot. The current response was taken immediately before the next injection.

2.6. Monitoring dissolved oxygen

Biosensor and oxygen sensor (−650 mV vs SCE) data were recorded simultaneously. The PBS was de-aerated by vigorously purging the solution with N_2 (BOC Ireland, average O_2 content 2 ppm, maximum content 5 ppm) for at least 30 min before recording began [10]. A N_2 atmosphere was maintained over the cell during biosensor and oxygen sensor settling. Aliquots of analyte were introduced into the buffer and stirred briefly. The N_2 source was removed to facilitate the natural air equilibration of the solution where the O_2 concentration was taken as 100% at air saturation (200 μM).

Some experiments were carried out under continuous forced convection by the introduction of either air (from a RENA air pump) or N_2 into the buffer. Aliquots of analyte were introduced during N_2 saturation and monitored during the transition to air saturation. The use of either natural air equilibration, or forced convection is clearly indicated in the text.

2.7. Surgical procedures

As described in our previous work [9], anesthesia was achieved in 200–300 g male wistar rats (Charles River Laboratories International, Inc., UK) using the volatile anaesthetic isoflurane (4% in air) for induction and maintenance (1.5–3.0%; IsoFlo®, Abbott, UK) using a Univentor 400 Anaesthesia Unit (AgnTho's AB, Sweden). Anaesthetised animals were anchored into a stereotaxic frame and the sensors implanted following a previously described procedure