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Towards timely Alzheimer diagnosis: A self-powered amperometric biosensor for the neurotransmitter acetylcholine

Felismina T.C. Moreira [b], M. Goreti F. Sale [b], Mirella Di Lorenzo [a,*]

[a] University of Bath, Department of Chemical Engineering, Bath BA2 7AY, UK
[b] BioMark-CINTESIS/ISEP, School of Engineering, Polytechnic Institute of Porto, Portugal

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ABSTRACT

Serious brain disorders, such as the Alzheimer's Disease (AD), are associated with a marked drop in the levels of important neurotransmitters, such as acetylcholine (ACh). Real time monitoring of such biomarkers can therefore play a critical role in enhancing AD therapies by allowing timely diagnosis, verifications of treatment effectiveness, and developments of new medicines. In this study, we present the first acetylcholine/oxygen hybrid enzymatic fuel cell for the self-powered on site detection of ACh in plasma, which is based on the combination of an enzymatic anode with a Pt cathode. Firstly, an effective acetylcholinesterase immobilized electrode was developed and its electrochemical performance evaluated. Highly porous gold was used as the electrode material, and the enzyme was immobilized *via* a one step rapid and simple procedure that does not require the use of harsh chemicals or any electrode/enzyme pre-treatments. The resulting enzymatic electrode was subsequently used as the anode of a miniature flow-through membrane-less fuel cell and showed excellent response to varying concentrations of ACh. The peak power generated by the fuel cell was 4 nW at a voltage of 260 mV and with a current density of 9 μA cm^{-2}. The limit of detection of the fuel cell sensor was 10 μM, with an average response time as short as 3 min. These exciting results open new horizons for point-of-care Alzheimer diagnosis and provide an attractive potential alternative to established methods that require laborious and time-consuming sample treatments and expensive instruments.

1. Introduction

Worldwide, over 35 million people are currently diagnosed with dementia. This number is estimated to double every 20 years in aging populations, thus reaching a value of 65.7 million in 2030, and of 115.4 million in 2050 (Prince et al., 2015). The most common cause of senile dementia is the Alzheimer's disease (AD), with currently over 17 million cases worldwide (Mayeux and Schupf, 2011). AD is characterised by the progressive loss of cognitive function and personality changes. Early detection of AD, as well as the capability to distinguish it from other forms of dementia, is key to plan timely caring actions and help families intervene before the disease becomes too serious. Yet, so far, there are no definitive diagnostic tests that allow accurate and effective early detection of this condition. The diagnosis of AD occurs typically *via* extensive clinical examinations based on specific clinical diagnostic criteria that have been established in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA), known as the NINCDS-ADRDA Alzheimer's criteria (McKhann et al., 1984).

The identification of relevant biomolecules that could act as AD biomarkers, and therefore allow rapid and effective diagnosis of this disease, is of particular relevance. Recent studies have suggested that systemic signs caused by concentration changes of specific biomolecules, which include mostly the Amyloid-β and Tau proteins present in cerebrospinal fluid (CSF) and plasma, can be associated with the progression of AD (Kanai et al., 1998). A particularly promising route to diagnose AD is, however, represented by the possibility to monitor the levels of neurotransmitters, such as acetylcholine (ACh), in cerebrospinal fluid. ACh is one of the first identified neurotransmitters and is found in the peripheral and central nerve system of the brain (Cannon et al., 2004; Hou et al., 2012). The role of ACh is to transmit messages from motor nerves to muscles, especially to the heart, bladder and stomach, and ACh is involved in several functions, including cognition, memory and movement (Hasselmo and Sarter, 2010; Van der Zee and Luiten 1999). The dysfunction in ACh regulation in the brain causes neuropsychiatric disorders such as Alzheimer's disease, Parkinson's

* Corresponding author.
E-mail address: M.Di.Lorenzo@bath.ac.uk (M. Di Lorenzo).

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disease, progressive dementia, Myasthenia Gravis and schizophrenia (Davis and Berger, 1979; Tandon 1999). Considering the importance of this biomolecule, there is high interest in developing methods for its in vivo quantification (Garris, 2010). Real-time monitoring of extra-cellular concentration changes of ACh would in fact allow understanding the function of the nervous system and the association to AD, examining the degeneration of cholinergic neural systems in AD, and evaluating pharmaceuticals that affect cholinergic activity at the single cell level (Mitchell, 2004; Nguyen et al., 2010). Nonetheless, current methods to determine ACh levels in body fluids, such as ELISA (Hinman et al., 1986; Kawanami et al., 1984) and microdialysis sampling combined to offline analysis by liquid chromatography with mass spectroscopy detection (Nirogi et al., 2010; Song et al., 2012; Uutela et al., 2005), are laborious, expensive, slow and not suitable for in situ monitoring.

Electrochemical sensors are capable of fast in situ detection sensors and, therefore, offer a powerful avenue for real time diagnostics that overcomes the drawback of traditional analytical methods (Wilson and Gifford, 2005). In particular, the detection of ACh and its metabolite choline (Ch) has been successfully demonstrated with enzyme-based amperometric sensors. Depending on the specific target, the sensors use either acetylcholine esterase (AChE) or choline oxidase (ChOx), as well as both enzymes together (Garguilo and Michael, 1995; Hou et al., 2012; Mitchell 2004; Wise et al., 2002).

In this work, we propose the first hybrid enzymatic fuel cell (EFC)-based sensor for the real time and in situ detection of ACh. EFCs are a particular type of fuel cells that use enzymes to catalyse the direct generation of electricity from physiological fluids under body temperature and pressure (Barton et al., 2004). As such, EFC provide an attractive possibility for implantable and wearable diagnostic devices (Leech et al., 2012) Preliminary proof-of-concept studies in this direction are highly encouraging. EFCs have been successfully implanted in living organisms, thus showing electricity generation from physiological fluids, such as blood and plasma (Castorena-Gonzalez et al., 2013; Halámková et al. 2012; MacVittie et al., 2013). Some successful examples of wearable EFCs have also been reported, such as the tattoo EFC that generates electricity from lactate in sweat (Jia et al., 2013) and the contact lens that uses the glucose in tears as fuel (Reid et al., 2015). Considering that within a specific range the power generated is proportional to the amount of substrate (the analyte) in the input solution, the most intuitive use of EFCs is as self-powered amperometric sensors for that substrate. By varying the nature of the enzyme(s) employed, several biomarkers of interest could be detected. The majority of the EFCs reported so far, uses the enzymes glucose oxidase and glucose dehydrogenase and focus on the detection of glucose (Ivanov et al., 2010). An EFC embedded into microfluidic follow-channels paper was reported for the detection of the carcinoembryonic antigen (Li et al., 2015). Successful examples of EFC applications as sensor for the detection of lactate (Katz et al., 2001), cyanide (Deng et al., 2010), mercury ion (Wen et al., 2011) and cholesterol (Sekretaryova et al., 2014) have also been reported.

The innovative fuel cell reported in the present work utilizes at the anode the enzyme acetylcholinesterase (AChE), which is immobilized onto a nanostructured electrode made of a highly porous gold (hPG) coated onto a Pt surface. The use of nanostructured electrode materials is key to improve the enzyme loading and enhance the electrochemical performance of EFCs (Ivanov et al., 2010). hPG in particular has proven to be an excellent candidate for EFCs, given its large specific surface area, biocompatibility and non-toxicity (du Toit and Di Lorenzo, 2014b). In this study, we used a rapid and simple one-step electrochemical process to immobilise AChE onto hPG that does not require complex or laborious electrode and/or enzyme pre-treatments. The first part of this study focus on investigating the electrochemical performance of the resulting electrode. Subsequently, the enzymatic electrode is tested as the anode of a flow-through membrane-less fuel cell and the current and power output is recorded for a series of ACh concentrations, thus proving its use as online sensor.

2. Experimental section

2.1. Materials

All the chemicals were of analytical reagent grade and were used without further purification. Acetylcholinesterase from *Electrophorus electricus*, 500 U, was purchased from Sigma-Aldrich. The Saturated Calomel Electrode (SCE), used as the reference electrode, was purchased from IJCambria Ltd. Platinum wire (diameter: 0.5 mm) was purchased from Cookson Precious Metals Ltd. Polydimethylsiloxane (PDMS, Dow Corning Sylgard 184) was purchased from Ellsworth Adhesives.

All aqueous solutions used were prepared using reverse osmosis purified water. The phosphate buffered saline (PBS) solution was prepared on a weekly basis and consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. The pH of the resulting solution was adjusted to a value of 7.4 with the drop-wise addition of HCl or NaOH. Glycine buffer was prepared by mixing 0.01 M of Glycine with 0.1 M of NaCl and by adjusting the pH to 7.4 by drop-wise addition of NaOH and HCl.

All potentiostatically-controlled electrochemical processes were performed using the Autolab PGSTAT128 N (Metrohm, UK) potentiostat. The moulds for the PDMS structures were 3D printed in polylactic acid using a Makerbot Replicator.

2.2. Preparation of the electrodes

The electrodes were prepared by electrodepositing a film of highly porous gold (hPG) onto Pt wires *via* a hydrogen bubbling template, as previously described (du Toit and Di Lorenzo, 2014a). Briefly, the platinum wires were immersed in an electrolyte consisting of 0.1 M $HAuCl_4$ and 1 M NH_4Cl and gold was deposited by gradually stepping down the working potential as follows: −0.7 V (vs. SCE) for 5 s; −1.5 V (vs. SCE) for 5 s; −2.5 V (vs. SCE) for 5 s and-4.0 V (vs. SCE) for 10 s. This process was performed in a three electrode set-up, with platinum as the counter electrode and SCE as the reference electrode. This set-up was also used for the immobilization of AChE onto the hPG/Pt electrode. The electrodes were immerged in a PBS buffer (pH 7.4) containing 6.25 U mL^{-1} of enzyme, and a potential of 0.6 V vs SCE was applied for one hour, according to the immobilization protocol previously established by our group (du Toit et al., 2016).

The electrochemical response of both AChE/hPG/Pt and hPG/Pt electrodes to ACh, within the range of 2- 2400 μg mL^{-1}, was evaluated by both chronoamperometry (CA) and Square Wave Voltammetry (SWV) in the conventional three-electrode set-up (SCE as the reference electrode, Pt as the counter electrode). During the test, ACh was drop-wise added to the buffer solution until the target concentration. The solution was then stirred for 30 s before the measurement. The response to ACh was evaluated in terms of current variation, $\Delta i = i_c - i_0$, where, i_c is the observed current at the set concentration and i_0 is the baseline current in the absence of substrate.

The sensitivity towards ACh was obtained from the slope, b (μA mM^{-1}), of the calibration curve and was referred to the total surface area, A (cm^2), of the electrode as:

$$sensitivity = \frac{b}{A}$$