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Glutamate microbiosensors based on Prussian Blue modified carbon fiber electrodes for neuroscience applications: In-vitro characterization

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ABSTRACT

Herein we report a Prussian Blue modified carbon fiber electrode (CFE/PB) to be used in microbiosensors for glutamate monitoring in physiological applications as an alternative to the classical Pt and Pt-Ir transducers. Their low dimensions (~250 μm CFE length and ~10 μm diameter) are advantageous for measuring in living tissues. In addition, PB-modified microelectrodes allow the detection of enzyme-generated hydrogen peroxide at a low applied potential (~0.0 V against SCE), contrasting the high potential used in many previous designs (~0.7 V), decreasing the endogenous interference contributions. Moreover, the electrosynthesized polymer, poly-*o*-phenylenediamine (PoPD), was used to improve biosensor stability and selectivity. CFE/PB was conveniently characterized using impedance, Raman and XPS spectroscopies. Optimization of the fabrication procedure and analytical conditions is described, including activation of CFE/PB, enzyme enrichment, cross-linking, stabilization and anti-interference. A range of analytical parameters were also characterized such as sensitivity, limit of detection, linear range, and enzymatic loading. Finally, an optimized biosensor displaying a linear sensitivity of 135 ± 2 nA μM^{-1} cm^{-2} (n = 3), LOD of <2 μM, linear range up to 150 μM and effectively free of interference, is proposed as a suitable candidate for in-vivo glutamate monitoring in the central nervous system.

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1. Introduction

L-Glutamate is an important neurotransmitter in the mammalian central nervous system (CNS) [1–3] where approximately 90% of all neurons use this amino acid as a primary messenger molecule. In contrast, other transmitters, e.g. acetylcholine, noradrenaline, dopamine, histamine and 5-hydroxytryptamine only account for a small percentage of neurotransmission. Recent discoveries have demonstrated that glutamatergic neurotransmission is mediated by a dynamic interaction between neurons and astrocytes [4]. Although glutamate concentration inside the synaptic cleft is elevated (ca. 100 mM), its baseline concentration in the extracellular space is relatively low (ca. 2–40 μM), depending on, inter alia, the brain region and state of anesthesia [5,6]. Glutamatergic neurotransmission is involved in normal brain function including cognition, memory and learning process and in several neurological disorders such as schizophrenia, Parkinson's disease, epilepsy, amyotrophic lateral sclerosis and stroke [2,7,8]. Traditional analytical methods such as microdialysis, coupled with high performance liquid chromatography or electrophoresis, have been used widely in neurochemical and food analysis applications [9,10]. Nevertheless, during recent decades, biosensors have challenged these techniques because of their excellent analytical properties, such as high time and spatial resolution, high sensitivity, selectivity and reproducibility, low expense, etc. Previous studies have shown that biosensors with low glutamate sensitivity (ca. 10 μA mM^{-1} cm^{-2}) are not adequate for neurochemical applications [6,11]. Thus, there is an urgent need to increase glutamate sensitivity for detection of very low concentration changes in brain extracellular fluid (ECF).

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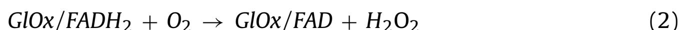
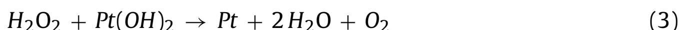
Glutamate biosensors have been developed mainly by using two different enzymes: glutamate dehydrogenase (Gldh) [12–14] and glutamate oxidase (GlOx) [15–22]. Although Gldh activity can be sensed by monitoring NADPH or NADH, the most common enzyme used for biosensor in physiological applications is GlOx, which involves FAD-catalyzed oxidation (see Eqs. (1) and (2)) and the formation of H_2O_2 [23].

$$L\text{-glutamate} + H_2O + GlOx/FAD \rightarrow \alpha\text{-ketoglutarate} + NH_3 + GlOx/FADH_2 \quad (1)$$

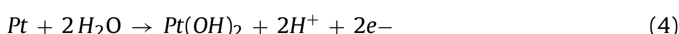


$$GlOx/FADH_2 + O_2 \rightarrow GlOx/FAD + H_2O_2 \quad (2)$$

The most common material for detecting H_2O_2 is platinum [18–20,23,24]. H_2O_2 oxidation has been shown to be catalyzed by the presence of platinum hydroxides (Pt-$(OH)_2$) on the surface of the electrode (Eqs. (3) and (4)):



$$H_2O_2 + Pt(OH)_2 \rightarrow Pt + 2H_2O + O_2 \quad (3)$$



$$Pt + 2H_2O \rightarrow Pt(OH)_2 + 2H^+ + 2e- \quad (4)$$

In addition, the availability of commercial Pt and Pt alloy wires with low dimensions (25 μm diameter), makes the use of this material in neuroscience applications very attractive. Nevertheless, the high applied potential often used with Pt to oxidize H_2O_2 (~0.7 V vs. SCE) produces biofouling and interference by electroactive species [23,25–27].

In recent years, our group has been working with Prussian Blue (PB) modified carbon fiber microelectrodes to detect enzyme-generated H_2O_2 at a low applied potential, ~0 V vs. SCE. With this approach we developed a high-sensitivity and selective transducer for H_2O_2 detection using a Prussian Blue-modified microsensor, and have reported glucose and lactate biosensors with very low dimensions (ca. 10 μm diameter) and with excellent in-vitro and in-vivo responses [24,28–32]. Nevertheless such biosensors were developed to detect glucose and lactate, which have a high basal concentration in brain ECF (0.5–1 mM). In the present paper, we optimize our previous biosensor design to detect glutamate with a significantly lower basal concentration (<50 μM). This low concentration makes the optimization of enzymatic loading, linear range, sensitivity and selectivity critical for its future applications in neuroscience studies. Thus, polyethyleneimine (PEI) was used as protein immobilization agent and stabilizer [24,33–35]. Other important steps during biosensor assembly, including the dip-coating procedure and cross-linking with glutaraldehyde solution, have been revised. In addition, poly-*o*-phenylenediamine (P*o*PD) has been employed to improve the stability and selectivity of these novel biosensors [24–26]. Finally, using the present approach, a novel glutamate microbiosensor is reported with excellent properties (very low dimensions, high sensitivity at low applied potentials, and excellent anti-interference properties) for future neuroscience applications.

2. Materials and methods

2.1. Reagents and solutions

The enzyme L-glutamate oxidase (GlOx) from *Streptomyces* sp. (X119-6) was purchased as a lyophilized powder from Yamasa Corporation (Japan), glutaraldehyde 25% (Gluth) was obtained from Sigma Chemical Co. and they were stored at −21 °C until use. Bovine serum albumin (BSA, fraction V) was also obtained from Sigma. All chemicals, including *o*-phenylenediamine (*o*-PD), glutamate, polyethyleneimine (PEI), KCl, $FeCl_3$, $K_3[Fe(CN)_6]$, HCl (35%w/w), H_2O_2 (30% w/v), phosphate buffer saline tablets (PBS, pH 7.4 containing 0.1 M NaCl) and test interference species were obtained from Sigma and used as supplied. PBS stock solutions were prepared in doubly distilled water (18.2 MΩ cm, Millipore-Q), and stored at 4 °C when not in use. A stock 1 M solution of glutamate was prepared daily. The PEI solutions used were prepared by dissolving PEI at X% w/v in H_2O (X being 1.2, 2.4, 4.8 and 7.5). A cross-linking solution of glutaraldehyde (in the range 0–0.4% w/v), was prepared in PBS and supplemented with 1% w/v BSA. Monomer solution of 300 mM *o*-PD was prepared using 48.6 mg of *o*-PD and 7.5 mg of BSA in 1.5 mL of N_2-saturated PBS and sonicated for 15 min. A 100 U/mL solution of GlOx was prepared by dissolving 8 mg in 0.5 mL of PBS and then stored at 4 °C. Interference solutions were prepared in water just before use and, if necessary, pH was adjusted to 7.4. Carbon fibers (10 μm diameter), glass capillaries, and 250 μm internal diameter Teflon-coated copper wire were obtained from Word Precision Instruments Inc., and silver epoxy paint was supplied by Sigma. N_2 (high grade, $O_2 \leq 2$ ppm) was supplied by Air Liquide.

2.2. Instrumentation and software

Experiments were computer controlled with data-acquisition software EChem™ for CV and Chart™ for constant potential amperometry. The data-acquisition system used was e-Corder 401 (EDAQ) and a low-noise and high-sensitivity potentiostat, Quadstat (EDAQ). To electro-deposit and activate the PB [24,31,36], a custom-made Ag/AgCl/saturated KCl reference electrode and platinum wire auxiliary electrode were used.

EIS experiments for microsensor characterization were conducted with an Autolab PGSTAT 20 potentiostat and a FRA module from EcoChemie, computer controlled by their General Purpose Electrochemical System (GPES) and FRA software, respectively. For detailed PB characterization, PB was deposited according previous reported methods [24,31] onto titanium foils. Raman spectra were recorded with a HORIBA HR-800-UV microscope. X-ray photoelectron spectroscopy (XPS) measurements were obtained using a Phoibos-100 spectrometer working in the pass energy constant mode and using the Mg Kα as excitation source. The binding energy scale was referenced at 284.5 eV for the C 1s peak of some minor contamination of carbon on the electrode films. Field emission scanning electron micrographs (FE-SEMs) were obtained using a HITACHI S-4800 microscope for bare CFEs and CFE-based glutamate microbiosensors.

2.3. Microbiosensor construction based on CFE/PB

Carbon fiber electrodes (CFEs) where constructed as described previously [24,31]. Briefly, a carbon fiber (diameter 10 μm) was attached to Teflon-coated copper wire (diameter 250 μm) using high purity silver paint, and dried for 1 h at 80 °C. A borosilicate glass capillary was pulled to a tip using a vertical microelectrode puller (Needle/Pipette puller, Model 750, David Kopf Instruments, California, USA). After drying, the carbon fiber was carefully inserted into the pulled glass capillary tube under a microscope, leaving 2–4 mm of the carbon fiber protruding at the pulled end. Subsequently, the carbon fiber was cut to the desired length (250 μm), using a microsurgical scalpel. At the stem end of the capillary tube, the copper wire was fixed by casting with non-conducting epoxy glue; the carbon fiber was also sealed into the capillary mouth, using the same epoxy glue. Subsequently, CFEs were dried again for 1 h and were optically and electrochemically inspected before use.

Then a PB layer was electrodeposited onto CFEs using cyclic voltammetric (CV) methodology, applying 3 cyclic scans within the limits of −0.2 to +0.4 V at scan rate of 0.1 V s^{-1} in a solution containing 1.5 mM $K_3[Fe(CN)_6]$ and 1.5 mM $FeCl_3$ in the background solution (0.1 M KCl and 0.1 M HCl). These CFE/PBs were cleaned in doubly distilled water and activated by applying another 50 cycles in de-aerated electrolyte solution (0.1 M KCl and 0.1 M HCl), using the same protocol. Before being used, the CFE/PBs were cleaned again in doubly distilled water for several seconds. Finally, the PB film was tempered at 100 °C for 1 h.

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