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Simultaneous measurements of ascorbate and glutamate *in vivo* in the rat brain using carbon fiber nanocomposite sensors and microbiosensor arrays

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ABSTRACT

Nanocomposite sensors consisting of carbon fiber microelectrodes modified with Nafion® and carbon nanotubes, and ceramic-based microelectrode biosensor arrays were used to measure ascorbate and glutamate in the brain with high spatial, temporal and chemical resolution. Nanocomposite sensors displayed electrocatalytic properties towards ascorbate oxidation, translated into a negative shift from +0.20 V to −0.05 V *vs.* Ag/AgCl, as well as a significant increase (10-fold) of electroactive surface area. The estimated average basal concentration of ascorbate *in vivo* in the CA1, CA3 and *dentate gyrus* (DG) sub regions of the hippocampus were 276 ± 60 μM (n = 10), 183 ± 30 μM (n = 10) and 133 ± 42 μM (n = 10), respectively. The glutamate microbiosensor arrays showed a high sensitivity of 5.3 ± 0.8 pA μM^{-1} (n = 18), and LOD of 204 ± 32 nM (n = 10), and $t_{50\%}$ response time of 0.9 ± 0.02 s (n = 6) and high selectivity against major interferents. The simultaneous and real-time measurements of glutamate and ascorbate in the hippocampus of anesthetized rats following local stimulus with KCl or glutamate revealed a dynamic interaction between the two neurochemicals.

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1. Introduction

Ascorbate plays important roles in the neurophysiological processes of the brain, ranging from antioxidant and enzyme co-factor to a modulator of energetic metabolism as well as glutamate and nitric oxide (•NO) dependent signaling processes [1,2]. Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system and is key to glia-neuron communication [3] and brain functions such as cognition, memory and learning. It has been suggested that changes in extracellular glutamate levels are coupled to fluctuations in extracellular ascorbate levels *via* a hetero-exchange mechanism involving glutamate transporters [4,5], in a process mediated by •NO produced upon activation of NMDA-type glutamate receptors [2]. Interestingly, deregulation in both glutamate- and ascorbate-dependent pathways appear to play a role in neurodegenerative processes such as Alzheimer's or Huntington's disease [6–9]. The monitoring of both compounds *in vivo* with high spatial and temporal resolution may offer new insights into the dynamic interplay between these two neurochemicals during excitatory neurotransmission.

Carbon nanotubes (CNTs) have been widely used for the fabrication of electrochemical sensors and biosensors owing to their unique structural, mechanical, chemical and electronic properties which offer several advantages, namely high surface-to-volume ratios, promotion of electron transfer reactions, decrease of over-potentials for various electroactive compounds, increase in sensitivity and reduction of surface fouling [10–12]. Due to their electrocatalytic properties, CNT composite films have been used to modify the surface of carbon fiber microelectrodes (CFM) for *in vivo* measurements of ascorbate in the rat brain [13,14].

Glutamate is commonly monitored *in vivo* by microdialysis [15], which allows the identification of relevant chemicals by using powerful analytical techniques such as HPLC-ECD or LC-MS-MS. However, this approach presents limitations such as the significant damage to brain tissue due to probe size that ranges between 150 and 500 μm in diameter and 1–4 mm in length [16–18], lack of spatial resolution of microdialysis probes [15], and inadequate temporal resolution to detect rapid synaptic-related events. An attractive alternate approach encompasses the use of ceramic microelectrode arrays (MEAs) coated with glutamate oxidase (GluOx) coupled to fast electrochemical techniques such as amperometry to monitor glutamate in the brain extracellular space [19,20]. These microbiosensor arrays have been successfully used for second-by-second measures, in a self-referencing mode, of tonic and potassium-evoked glutamate levels in anesthetized rodents [21,22] as

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well as tonic and evoked changes in chronic recordings in freely moving animals [23].

In this work, we have characterized the electrocatalytic oxidation of ascorbate at CFMs coated with different CNT composite films, which increased the electrochemical active surface area. These nanocomposite sensors were used for the measurement of basal levels of ascorbate in the CA1, CA3 and *dentate gyrus* (DG) sub regions of the hippocampus of urethane anesthetized rats. Moreover, the kinetic and analytical performance of the glutamate microbiosensor array was assessed *in vitro* and they were used to characterize glutamate dynamics upon potassium stimulation in the brain of anesthetized rats. Finally, the two micro(bio)sensors were assembled in an array and used for simultaneous measurement of ascorbate and glutamate *in vivo* to assess the dynamic interplay between these two neurochemicals with high spatial and temporal resolution.

2. Experimental

2.1. Reagents and solutions

Ascorbate and *o*-phenylenediamine (*o*-PD) were obtained from Fluka. KCl was obtained from Panreac (Barcelona, Spain). All other reagents were analytical grade and obtained from Sigma-Aldrich, unless otherwise stated. Phosphate buffered saline (0.05 M PBS) used for microelectrodes evaluations was prepared in Milli-Q water and had the following composition (mM): 10 NaH_2PO_4, 40 Na_2HPO_4 and 100 NaCl (pH 7.4). All compounds ejected into the brain were dissolved in saline (NaCl 0.9%, pH 7.4). Ascorbate oxidase (AAOx) was obtained from AppliChem (Darmstadt, Germany) and was prepared by dissolving 0.1 mg (28.8 U) of the enzyme in 1.0 mL of saline. High K^+ solution was prepared in Milli-Q water and had the following composition (in mM): 70 KCl, 79 NaCl, 2.5 $CaCl_2$. Glutamate oxidase (US Biological, Swampscott, MA) was prepared by adding 50 μL of purified H_2O to the lyophilized, purified enzyme (25 units) to obtain a final concentration of 0.5 U/μL. Carbon nanotubes (SWCNT and MWCNT) were purchased from Nano-lab (USA) and they were suspended in a 0.5% Nafion solution to a final concentration of 100 mg/mL.

2.2. Electrochemical instrumentation

All recordings were performed using a FAST16mkII potentiostat system (Quanteon, LLC., USA). Electrochemical recordings were performed in a 2-electrode electrochemical cell configuration mode consisting of the ascorbate/glutamate sensor as a working electrode and an Ag/AgCl in 3 M KCl (RE-5B, BAS Inc.) as a reference electrode. For *in vivo* experiments, the reference was replaced by a miniature pseudo-reference electrode produced by electro-oxidation of the exposed tip of a Teflon-coated Ag wire (200 μm o.d., Science Products, Germany). The holding potential for the ascorbate nanocomposite sensor was +0.05 V *vs.* Ag/AgCl, based on the voltammetry studies shown in this work and according to the values previously reported [13]. In the case of the glutamate biosensor, the holding potential was +0.70 V *vs.* Ag/AgCl, as referred in the literature [19,24,25]. Data display rate was 2 Hz for all recordings.

2.3. Ascorbate microelectrode preparation and calibration

Carbon fiber microelectrodes were fabricated as previously described [26] and the exposed tip of the carbon fiber (o.d. 30 μm) was cut to a length of 150–250 μm. Single- and multi-wall carbon nanotubes (SWCNT and MWCNT, respectively) were used to coat the exposed surface of the CFMs. In each case, a single drop of the suspension was applied onto a glassy plate and the CFM tip was immersed into the droplet for 30 s and then dried at 170^{0}C for 5 min.

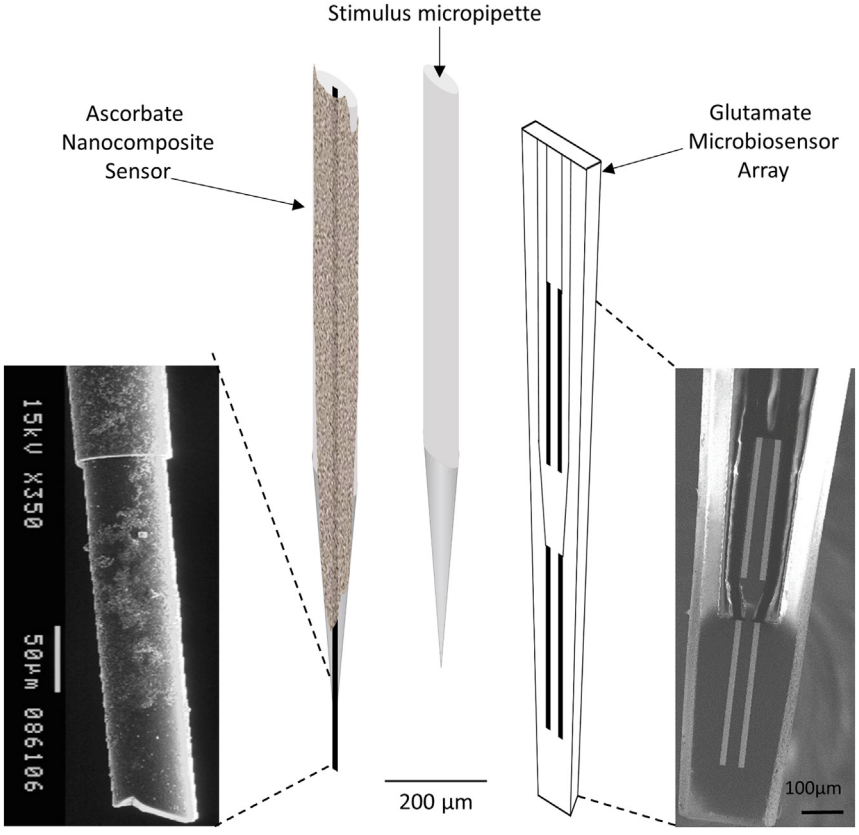

Fig. 1. Schematic representation of the array composed by the ascorbate nanocomposite microsensor (left), the glutamate microbiosensor (right) and the micropipette (center) used for local application of solutions in the extracellular space of the rat hippocampus.

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