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# *In vivo* continuous and simultaneous monitoring of brain energy substrates with a multiplex amperometric enzyme-based biosensor device

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ABSTRACT

Enzyme-based amperometric biosensors are widely used for monitoring key biomarkers. In experimental neuroscience there is a growing interest in *in vivo* continuous and simultaneous monitoring of metabolism-related biomarkers, like glucose, lactate and pyruvate. The use of multiplex biosensors will provide better understanding of brain energy metabolism and its role in neuropathologies such as diabetes, ischemia, and epilepsy.

We have developed and characterized an implantable multiplex microbiosensor device (MBD) for simultaneous and continuous *in vivo* monitoring of glucose, lactate, and pyruvate.

First, we developed and characterized amperometric microbiosensors for monitoring lactate and pyruvate. *In vitro* evaluation allowed us to choose the most suitable biosensors for incorporation into the MBD, along with glucose and background biosensors. Fully assembled MBDs were characterized *in vitro*. The calculated performance parameters (LOD, LR, LRS, $I_{MAX}$ and ${}_{app}K_M$) showed that the multiplex MBD was highly selective and sensitive (LRS $\geq$ 100 nA/mM) for each analyte and within an adequate range for *in vivo* application.

Finally, MBDs were implanted in the mPFC of anesthetized adult male Wistar rats for *in vivo* evaluation. Following an equilibration period, baseline brain levels of glucose (1.3 ± 0.2 mM), lactate (1.5 ± 0.4 mM) and pyruvate (0.3 ± 0.1 mM) were established. Subsequently, the MBDs recorded the responses of the animals when submitted to hyperglycemic (40% glucose i.v.) and hypoglycemic (5 U/kg insulin i.v.) challenges. Afterwards, MBDs were recalibrated to convert electrochemical readings into accurate substrate concentrations and to assess biofouling. The presented MBD can monitor simultaneously multiple biomarkers *in vivo*.

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## 1. Introduction

The brain has high energy demands. Despite accounting for only 2% of the total body mass, it requires up to 25% of the total glucose consumption (Brady et al., 2006; Genc et al., 2011). Its high requirements are dependent on a continuous flow of energy substrates from the circulating blood (Du et al., 2012), and the activation of discrete brain areas is directly related to increases in energy requirements and in glucose utilization (Duelli and Kuschinsky, 2001; Fox et al., 1988).

According to the classical view on brain energy metabolism, glucose is the predominant energy substrate for both neurons and glial cells, followed at lesser extent by ketone bodies and monocarboxylic acids like pyruvate and lactate (Vannucci et al., 1997). While ketone bodies are of great importance in early development stages, pyruvate and lactate seem to have a role in the adult brain (Vannucci and Simpson, 2003). Classical neuroenergetics state that lactate produced in the glycolisis is released into the extracellular fluid and metabolized to prevent damage to adjacent cells. In contrast, pyruvate is regarded solely as mediator in glucose metabolism (Sokoloff, 1977).

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Advances in the technology for monitoring neuroenergetics have provided evidence that challenges this view (Drevets et al., 2002; El Hage et al., 2011; Erlichman et al., 2008; Hertz et al., 2007). Increases in blood flow and brain glucose utilization, in response to increases in energy requirements, are not matched by parallel increases in oxygen consumption, necessary for full glucose metabolism dependency (Fillenz and Lowry, 1998; Hertz et al., 2007; Kiyatkin and Lenoir, 2012; Leegsma-Vogt et al., 2003; Lowry et al., 1998a; Lowry and Fillenz, 1997). Moreover, it was found that lactate can readily be taken up and oxidized in both neurons and astrocytes, and can even sustain neuronal activity in glucose absence (Bouzier-Sore et al., 2003). These and other insights have led to the postulation of alternative views on neuroenergetics, with the astrocyte-neuron lactate shuttle (ANLS) hypothesis the most studied (Pellerin 2010; Pellerin and Magistretti, 1994).

The ANLS states that glia cells and lactate play an unprecedented active role in brain energy metabolism. In the astrocyte, glucose is metabolized to pyruvate, which can have two fates. Whilst part of it is used to produce energy for the astrocyte itself, the remaining pyruvate is converted into lactate and released into the extracellular fluid, to be taken up by surrounding neurons (Allaman et al., 2011; Dienel, 2011; Halim et al., 2010).

Disturbances of the regulation of brain energy metabolism have been related to impairments in the cognitive processes of learning and memory (Hertz and Gibbs, 2009; Kapogiannis and Mattson, 2011) and are involved in several neuropathologies. Deregulation of either glucose levels or the lactate/pyruvate ratio are associated with neuropathologies such as epilepsy (Cloix and Hévor, 2009), meningitis (Ginsberg, 2004; Komorowski et al., 1978; van de Beek et al., 2006), ischemia (Berthet et al., 2009; Tokumaru et al., 2009) and affective disorders (Li et al., 2010; Moretti et al., 2003; Pratt et al., 2008). Additionally, there is evidence that changes in glucose and lactate brain levels affect glucose homeostasis and diabetes (Ahmad et al., 2008; Marino et al., 2011; Marty et al., 2007; McCrimmon, 2012; Ramnanan et al., 2013; Routh, 2010; Thorens, 2010; Watts and Donovan, 2010).

Biomonitoring of glucose, lactate and pyruvate is fundamental for understanding and treatment of these pathologies. The existing technology allows us to understand that glucose and lactate levels are higher than pyruvate levels, both in humans and animal models. While glucose and lactate are within the millimolar range (between 1 and 2 mM) (Ahmad et al., 2008; Gramsbergen et al., 2004; Leegsma-Vogt et al., 2001; Lin et al., 2009; Lowry et al., 1998a; Rocchitta et al., 2013), pyruvate brain levels are significantly lower (circa 200 μM) (Schulz et al., 2000; Wagner et al., 2005). However, these values depend on many factors, most related to the analytical method employed, each with their advantages and drawbacks.

State of the art technology for brain biomonitoring include invasive and non-invasive techniques. Ideally, biomonitoring of target analytes should be performed by non-invasive methods such as positron emitting tomography (PET), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). However, these methods have severe limitations characterized by low quantitative resolution and limited temporal and/or spatial resolution (Byrnes et al., 2014; Haller et al., 2014; Lang et al., 2014; Li et al., 2013). Therefore invasive methods such as microdialysis and microbiosensors are needed for additional *in situ* information, such as basal levels and dynamic changes of each analyte in a discrete brain area.

Although microdialysis allows biomonitoring of multiple analytes with a high spatial resolution (mm), it still lacks the needed temporal resolution to monitor the expected fast changes in brain energy metabolism. Biosensors can combine spatial (μm) and temporal ($\leq 1$ s) resolution with high selectivity, rapid response time and ease of miniaturization. These features make these devices very appealing for *in vivo* biomonitoring of brain energy biomarkers.

A wide variety of biosensors have been successfully used for *in vivo* biomonitoring (Wang, 1999; Wilson and Gifford, 2005). However, in biomedical applications, experimental neuroscience in particular, amperometric enzyme-based biosensors have arguably been the most successful. Amperometric enzyme-based biosensors rely on the oxidation of an electroactive product (often $H_2O_2$) of an enzymatic reaction (typically mediated by oxidases) at the electrode surface (Thévenot et al., 1999). However, at the working potentials necessary to oxidize the electroactive molecules of interest ($>500$ mV), other electroactive compounds are prone to be oxidized, resulting in non-specific electrochemical interference (Lowry et al., 1998b; Wahono et al., 2012). The incorporation of permselective membranes (e.g. Nafion and Poly (Phenylenediamine) (PPD)) is often used to overcome electrochemical interference (Moatti-Sirat et al., 1994; Moussy et al., 1993; O'Neill et al., 2008). Besides increasing biosensor selectivity, these membranes minimize electrode passivation, a biofouling effect (Wisniewski et al., 2000). Although its impact is bigger in chronic cases, biofouling can be observed even in acute implantations (Koschwanez and Reichert, 2007; Wisniewski et al., 2000; Wisniewski and Reichert, 2000).

These type of biosensors were successfully applied for *in vivo* biomonitoring of neurotransmitters (Mitchell, 2004; Oldenziel et al., 2006; Pomerleau et al., 2003; Wahono et al., 2012) and energy biomarkers (Ahmad et al., 2008; Calia et al., 2009; Gramsbergen et al., 2004; Leegsma-Vogt et al., 2003; Lowry et al., 1998b; Roche et al., 2011; Vasylieva et al., 2011). Simultaneous monitoring of glucose and lactate has already been successfully described, even coupled to a telemetric device (Rocchitta et al., 2013). Unfortunately, the device did not allow the necessary spatial resolution needed for biomonitoring in a discrete brain area. The attempt of combining glucose, lactate and pyruvate biosensors into a single device, for *in vivo* real-time simultaneous monitoring of these analytes within a discrete brain area is an original application.

Here we describe the development and characterization of a novel multiplex biosensor device (MBD) for *in vivo* real-time continuous and simultaneous *in vivo* monitoring of glucose, lactate and pyruvate. *In vitro* electrochemical evaluation allowed us to choose the most suitable lactate and pyruvate biosensors to be incorporated in MBD, along with a glucose biosensor. Fully assembled MBDs were electrochemically evaluated *in vitro* prior to assess its suitability for *in vivo* implantation. After the MBDs were implanted in the medial prefrontal cortex (mPFC) of anesthetized adult Wistar rats. Following an equilibration period, animals were submitted to hyperglycemia (40% glucose i.v.) and hypoglycemia (5 U/kg insulin i.v.) challenges. Subsequently, the MBDs were explanted and recalibrated to convert electrochemical readings into accurate glucose, lactate and pyruvate levels and to assess biofouling.

## 2. Materials and methods

### 2.1. Materials

Glucose oxidase (GO*x*) (EC 1.1.3.4) from *Aspergillus niger* and Lactate oxidase (LO*x*) (EC 1.13.12.4) from *Pediococcus sp*, bovine serum albumin (BSA), glutaraldehyde (GA), *m*-phenylenediamine (*m*PD), glucose, (L)-lactic acid, sodium pyruvate, ascorbic acid (AA), uric acid (UA), dopamine (DA) and 3.4-dihydroxiphenylacetic (DOPAC) were purchased from Sigma-Aldrich (Schnelldorff, Germany). Pyruvate oxidase (POx) (PYO 311) (EC 1.2.3.3) was

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