**In vivo** “real-time” monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes

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Abstract:

Biosensors based on Pt or Pt/Ir based needle-type microelectrodes have been successfully employed for continuous in vivo real-time brain biomonitoring of key biomarkers such as glutamate and glucose.

However, when implanted, these biosensors often bend, thereby damaging its surface and degrading its bioanalytical properties. In addition, downscaling of Pt and Pt/Ir needle-type biosensors, to improve the spatial resolution and decrease tissue damage, is technically challenging.

Therefore, we investigated whether the use of the low malleability material tungsten (W), coated with a highly conductive gold (Au) layer is useful as an alternative and rigid needle base for biosensors.

Hence, we developed implantable needle-type (50 µm Ø) gold coated tungsten (W-Au) microbiosensors. We evaluated, electrochemically, by cyclic voltammetry, the ability of W-Au microelectrodes to continuously monitor changes in H₂O₂. After, we functionalized its surface first with permselective membrane(s) and after with an enzymatic hydrogel, containing Glucose Oxidase. Both enzyme loading and applied potential were optimized. The performance of functionalized W-Au microelectrodes and fully assembled biosensors was evaluated electrochemically, by amperometry. Additionally, the surface of bare and functionalized W-Au microelectrodes was characterized by imaging techniques (scanning electron microscopy).

In vivo experiments revealed that, W-Au based glucose biosensors, were able to accurately monitor, in real-time, changes in brain glucose in response to relevant pharmacological challenges.

**Keywords:** In vivo, glucose, implantable biosensors, W-Au, cyclic voltammetry, amperometry.
7.1- Introduction

Ideally, state of the art in vivo brain biomonitoring should be confined to non-invasive methods, such as positron emitting tomography (PET), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). Unfortunately, and despite its merits, these methods have severe limitations, such as 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). To suppress those limitations, the use of invasive methods is often required (Gramsbergen et al. 2004; Martínez-Valverde et al. 2016; Papadimitriou et al. 2016). The use of invasive methods, such as microdialysis, provides additional in situ information that cannot be rendered by classical non-invasive methods.

Currently, microdialysis coupled to on-line HPLC or off-line to LS-MS/MS systems, the method of choice, for invasive, in vivo brain biomonitoring. This technique allows in situ brain biomonitoring of multiple analytes, with a good spatial (≤ 1 mm) and temporal (≤ 10 min) resolution. However, microdialysis temporal resolution is still insufficient to monitor the expected fast changes in key biomarkers involved in neurophysiology and neuropathology (Cordeiro et al. 2015; Wahono et al. 2012).

Biosensors are bioanalytical tools that can combine spatial (µm) and temporal (≤ 1 s) resolution with high selectivity, rapid response time and ease of miniaturization (Turner 2013; Wang 2000; Ward 2007). These devices have been increasingly employed in experimental neuroscience for real time in vivo biomonitoring of several biomarkers such as glutamate (Burmeister et al. 2002; Oldenziel et al. 2006; Pomerleau et al. 2003; Sirca et al. 2014), choline (Baker et al. 2015; Santos et al. 2015), glucose (Ahmad et al. 2008; Fillenz and Lowry 1998; Lowry et al. 1998b; Vasylieva et al. 2011), lactate (Cordeiro et al. 2015; Vasylieva et al. 2015), pyruvate (Cordeiro et al. 2015) and reactive oxygen species (H$_2$O$_2$ and NO)(Mao et al. 2002; Santos et al. 2013). Typically, these biosensors rely on the oxidation of an electroactive product (often H$_2$O$_2$) of an enzymatic reaction (typically mediated by oxidases) at the electrode surface (Thevenot et al. 1999; Wang 1999). However, at the working potentials necessary to oxidize the electroactive molecules of interest (> 500 mV), other non-specific electroactive compounds are oxidized, resulting in non-specific electrochemical interference (Cordeiro et al. 2016). For in vivo brain biomonitoring electrochemical interference is mostly due to the oxidation of electroactive species such as dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), uric Acid (UA) and ascorbic acid (AA)(O’Neill et al. 2008; Wahono et al. 2012). Therefore permselective membranes such as Nafion and poly(phenylenediamine) (PPD) are often used to overcome these electrochemical interferences, enabling adequate biosensor selectivity (Cordeiro et al. 2016; Gerhardt et al. 1984; Wahono et al. 2012).

Despite its high spatial resolution when compared with other invasive methods, implantation of a biosensor still results in significant tissue damage (Wisniewski et al. 2000). Damage of tissue triggers a series of inflammatory processes that ultimately results in inactivation of the sensor, a process that is often referred to as “biofouling”. It has been
suggested that biofouling is one of the main reasons, to that might hinder long term biosensor implantation (Wisniewski and Reichert 2000). A possible solution to minimize biofouling may be the miniaturization of the electrode. However needle-type Pt based microelectrodes with small diameters (≤ 200 µm) easily bend upon implantation, thereby damaging the assembled layers and impairing in vivo accuracy of the biosensor.

The use of a core material such with a lower malleability coefficient, such as tungsten (W) coated with a highly conductive material (Au) will allow an efficient the miniaturization of the biosensor, hence enabling better spatial resolution. The increase in spatial resolution will result in a decrease in tissue damage upon implantation, which may enable a reduction in the biofouling.

Due to their resistance to shear stress and corrosion W-based microelectrodes are the tools of choice for numerous electrophysiological techniques, (Patrick et al. 2011). (Likhtik et al. 2006; Taşkın et al. 2011; Tseng et al. 2011). However for an effective oxidation of H$_2$O$_2$ coating of the W surface with a metal with higher conductivity, such as gold (Au) is necessary. The use of gold (Au) as conductive surface might be useful, as gold microelectrodes have been described to oxidize H$_2$O$_2$ at potentials close to those employed for platinum electrodes (Gerlache et al. 1997).

Here we describe the development and characterization of a W-Au based biosensor, suitable for in vivo brain monitoring that might enable further miniaturization. First the efficacy of W-Au microelectrodes to monitor H$_2$O$_2$ was assessed. Next an amperometric enzyme-based glucose biosensor based on the W-Au microelectrodes was developed and characterized. Finally, the ability of an implantable microbiosensor device based on W-Au biosensors, to monitor in vivo glucose levels in brain tissue was evaluated.

The present data demonstrated that W-Au based biosensors were able to monitor in vivo and in real-time, changes in brain glucose, evoked by local administration of glucose or i.v. administration of insulin.

### 7.2-Materials and Methods

#### 7.2.1- Materials

Tungsten (W) (100 µm Ø), gold (Au) (50 µm Ø) and silver wires (Ag) (250 µm Ø) were obtained from Advent Research Materials. Gold coated tungsten (W-Au) wires (50 µm Ø) were purchased from Lluma (Sweden). Silica tubes were purchased from Avantes (Appeldoorn, The Netherlands). Nafion (5% wt in aliphatic alcohols), glutaraldehyde, m- phenylenediamine (mPD), dopamine (DA), ascorboc acid (AA), uric acid (UA), 3,4- dihydroxyphenylacetic acid DOPAC, H$_2$O$_2$ (35% wt) and glucose oxidase (GOx) were purchased from Sigma (St. Louis, Missouri, USA). A phosphate buffer solution (PBS) was used containing 145 mM Na$^+$, 1.2 mM Ca$^{2+}$, 2.7 mm K$^+$, 1.0 mM Mg$^{2+}$, 152 mm Cl$^-$, and
2.0 mM PO$_4^{2-}$ in ultrapurified water, brought to pH 7.4 with sodium hydroxide and degassed before use.

### 7.2.2- Biosensor assembly

#### 7.2.2.1- Microelectrode assembly

All microelectrodes were assembled prepared according to a previously described method (Wahono et al. 2012) (Fig 1 A). All but the W microelectrodes, were assembled based on silica tubes with 75 µm ID and 150 µm OD. W microelectrodes were assembled based on silica tubes with an ID of 150 and an OD of 300 µm. The microelectrodes used in the cyclic voltammetry experiments had the following dimensions:

<table>
<thead>
<tr>
<th>Type of Electrode</th>
<th>Diameter (µm)</th>
<th>Length (mm)</th>
<th>Surface area (mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W</td>
<td>100</td>
<td>10</td>
<td>6,283</td>
</tr>
<tr>
<td>Au</td>
<td>50</td>
<td>10</td>
<td>3,147</td>
</tr>
<tr>
<td>W-Au</td>
<td>50</td>
<td>10</td>
<td>3,147</td>
</tr>
</tbody>
</table>

All W-Au based microelectrodes and biosensors characterized by amperometry had the following dimensions: 50 µm Ø x 2 mm length.

![Figure 1](image.png)

**Figure 1** - A: W-Au needle type microelectrode. B: Scheme of layer-by-layer assembly of the biosensor. C: Implantable microbiosensor device (iMBD) based on W-Au based sensor
7.2.2.2- Membrane assembly

W-Au microelectrodes were functionalized with either a Nafion membrane or with a combination of Nafion-PPD membrane as described (Cordeiro et al. 2016; Wahono et al. 2012). To assemble glucose biosensors, W-Au microelectrodes functionalized with any of the permselective membrane(s) were sub sequentially coated with an hydrogel containing GOx (0.2, 0.4, and 0.6 U/µL) reticulated with bovine serum albumin (BSA) and glutaraldehyde (GA) (Cordeiro et al. 2015).

7.2.2.3- Implantable Microsensor Device (iMBD)

For in vivo implantation, we assembled an implantable microsensor device (iMBD), based on a self-referencing principle (Cordeiro et al. 2015; Pomerleau et al. 2003; Vasylieva et al. 2015; Wahono et al. 2012). Both a glucose biosensor and a background sensor (BG) were placed in a customized microdialysis probe body (Brainlink, The Netherlands) and sealed by UV curable glue. Next a hollow fiber, to allow local compound administration, was placed between the glucose sensor and the background (BG) sensor (Fig 1 C). The MBD tip Ø was approximately 150 µm.

7.2.3- In vitro characterization

W, Au and W-Au microelectrodes were electrochemically characterized by cyclic voltammetry. W-Au microelectrodes were further characterized by amperometry. Functionalized W-Au microelectrodes and W-Au based glucose biosensors were characterized in vitro by amperometry. Additionally, W-Au microelectrodes (bared and functionalized) were characterized by imaging techniques (Scanning Electron Microscopy).

7.2.3.1- Cyclic Voltammetry

Cyclic voltammetry experiments were performed in presence of H₂O₂ (0; 0.2; 0.5; 1 and 2 mM) in PBS (pH 7.4) from -0.5 to 1.2 V vs Ag/AgCl and carried out at a scan rate of 50 mV/s.

7.2.3.2- Amperometry

Microelectrode calibrations were carried out in PBS of pH 7.4 at 37°C. Bare and functionalized W- au microelectrodes were calibrated at different applied potentials (600, 700, 800 and 900 mV) using a tabletop potentiostat (Pinnacle, model 3104 Pinnacle Tech. Inc., USA) as previously described. Sensors were placed in PBS and steady state parameters (noise and baseline) were assessed after an initial equilibration period (approximately 45
In vivo “real-time” monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes

...min) when a stable current was reached. All interfering compounds (DA 2 µM; DOPAC 20 µM; UA 50 µM; and AA 200 µM) were added sequentially to a constantly stirred solution following consecutive additions of target analyte. Interfering compounds were added either after \( \text{H}_2\text{O}_2 \) (5, 10, 25, 50, 100 and 200 µM) for evaluation of the microelectrode or glucose (0.02; 0.05; 0.1; 0.2; 0.5; 1; 2; 4; 8; 16; 32 and 64 mM) for evaluation of the biosensor. The changes in oxidation currents in response to the exposure to the different analytes were used to estimate relevant microelectrode/biosensor performance parameters.

### 7.2.3.4 Scanning Electron Microscopy

W-Au microelectrodes (bare and functionalized) were visually inspected by high definition scanning electron microscopy. Biosensor tips were fixed with double sided adhesive carbon tape onto metal stubs. Observation and imaging was performed using a cold filed emission scanning electron microscope (JEOL FE-SEM 6301F) at 3 kV and a secondary electron detector (JEOL LTD. 1-2 Mushasino 3-chome, Akishama Tokyo 196).

### 7.2.4 In vivo evaluation

Male Wistar rats (350-425g) were used in the in vivo experiments. Animals were individually housed in Plexiglas cages prior to the experiment. All experiments were performed under anesthesia (isoflurane/O2). All animals were submitted to surgery to implant a jugular vein (JV) catheter (for frequent blood sampling and compound administration). The iMBD was implanted in the medial prefrontal cortex (mPFC); AP+3.4 mm; ML +0.8 mm; VD −5.0 mm relative to bregma, according to the stereotaxic atlas (Paxinos and Watson 1986).

All in vivo electrochemical measurements were performed at a constant potential of 800 mV vs Ag/AgCl. After implantation, a 2 hour period was included to allow stabilization of the electrochemical signal. Next pharmacological treatments were applied to induce changes in glucose levels, by intravenous administration of insulin (5U/kg) or intracranial administration of glucose (10 µL, 1 mM) through an injection cannula. During the experiments blood was sampled by the JV catheter at intervals of 15 min and assessed for its glucose content. Blood glucose values were obtained by colorimetric glucose strips (AccuCheck; Roche). Animals were sacrificed immediately after the experiment by i.v. administration of pentobarbital. The iMBD electrochemical signal was recorded at a rate of 10 HZ and averaged at 1 Hz.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen.

### 7.2.5 Data Analysis and statistical evaluation

Changes in oxidation current monitored in the in vitro calibrations were used to estimate
several microelectrode and biosensor performance parameters. The Selectivity Coefficient (SC) (Equation 1) was calculated using a previously described model (Cordeiro et al. 2016):

$$SC = \frac{I(\text{Analyte}, \text{nA}/[\text{Analyte}], \mu M)}{\text{H2O2 sensitivity, nA}/\mu M} \times 100$$

Equation 1

The performance parameters of the microelectrode (noise, limit of detection (LOD) and linear range sensitivity (LRS)) were estimated based on linear regression analysis. Biosensor performance parameters were calculated by non-linear regression, using a Michaelis-Menten derived kinetic model (equation 2) (O’Neill et al. 2008). The calculated parameters include limit of detection (LOD), linear Range (LR) linear range slope (LRS) Michalis-Menten constant ($K_M$), maximum current intensity ($I_{\text{MAX}}$), and the surface independent constants ($SI_{\text{app}}$, $K_M$, $I_{\text{MAX}}$). Linear and non-linear regression analysis were performed using GraphPad Prism 6.0. The LRS was calculated according to equation 3, while LOD was estimated based on equation 4.

$$I(S) = \frac{I_{\text{Max}}}{1 + \frac{KM(S)}{[S]}}$$

Equation 2

$$\text{LRS (nA/mM)} = \frac{I_{\text{Max}}}{\text{KM}}$$

Equation 3

$$\text{LOD (mM)} = 3 \times \text{Noise (nA)} \times \text{LRS (nA/mM)}$$

Equation 4

All data was presented as mean ± standard error of the mean (SEM). All calculated parameters were statistically evaluated either by One-Way (evaluation of biosensors) or Two-Way ANOVA (characterization of the microelectrode). When necessary, additional Bonferroni tests were performed. Calculated values of $p<0.05$ and $p<0.001$ were considered statistically significant and highly significant, respectively. Correlation analysis was performed using the Pearson Product Moment Correlation. All statistical analysis were performed using SigmaStat 12.0.
7.3-Results and discussion

7.3.1- In vitro evaluation

7.3.1.1- Scanning Electron Microscopy evaluation

Scanning electron microscopy was applied to evaluate the morphology of the microelectrode surface. We studied the surfaces of W-Au based microelectrodes, bare as well as functionalized with Nafion, Nafion-PPD or Nafion-PPD/GOx. Our results (Fig S7) show that the surface of bare W-Au microelectrodes is somewhat rough (Fig S7 A1 to A4). However after its functionalization with permselective membranes this roughness disappeared. No significant differences between the surfaces of W-Au microelectrodes covered with either Nafion (Fig S7 B1 to B4) or Nafion-PmPD (Fig S7 C1 to C4) were observed. These results are in accordance with previously described experiments in which it was shown that on microelectrodes previously functionalized with Nafion, polymerization of PPD occurred on the remaining active surface after Nafion self-assembly (Cordeiro et al. 2016).

In addition, we noticed few differences between the morphology of the surface of the microelectrodes functionalized with permselective membranes and the fully assembled glucose biosensors (Fig S D1 to D4). Due to the presence of the hydrogel the diameter of the W-Au glucose sensor was somewhat larger than those of the microelectrodes coated with the permselective membrane alone. Our data showed that the diameter of a fully assembled biosensor to be approximately 53 µm.

7.3.1.2- Cyclic Voltammetry characterization

For an electrochemical characterization of W based microelectrodes, we first performed CVs of the W, Au and W-Au based microelectrodes were recorded in absence and presence of increasing levels of H$_2$O$_2$. The results show that W microelectrodes made of the core metal, displayed two oxidation peaks at 50 and 500 mV (Fig 2 A). Interestingly we observed that increasing H$_2$O$_2$ levels, resulted in a decrease in oxidation current for both peaks, reaching lower values when exposed to the highest H$_2$O$_2$ levels. The high noise levels that were observed is explained by the rough surface of the electrodes.
Exposing the Au microelectrodes to $\text{H}_2\text{O}_2$ produced a very different electrochemical profile (Fig 2 B). Now an irreversible redox profile was observed, as previously described (Gerlache et al. 1997). Our data showed first a single oxidation peak (around 900 mV) followed by reduction peak around 450 mV. However when exposed to higher $\text{H}_2\text{O}_2$ levels ($\geq$ 1mM) the oxidation peak seems to shift from 900 to 950 mV. While an increase in $\text{H}_2\text{O}_2$ resulted in higher currents for the oxidation peak, we observed the opposite effect for the reduction peak, as increasing analyte concentration resulted in lower reduction currents. These results are in line with existing literature data (Gerlache et al. 1997).

It is evident that W microelectrodes displayed a very different electrochemical profile, when compared to Au microelectrodes In addition Au microelectrodes responded to $\text{H}_2\text{O}_2$ with much lower currents when compared to W microelectrodes, also due to a significant decrease in active surface.

The CV’s of the W-Au microelectrodes, displayed an electrochemical profile that was very similar to the profile of Au microelectrodes (Figure 2-C). As observed for Au microelectrodes, we observed an oxidation peak around 900 mV and a reduction peak around 450 mV. However, W-Au microelectrodes display much less oxidation/reduction when compared with Au microelectrodes. Although Au is highly conductive, W has a lower intrinsic conductivity (Sankar et al. 2014). As W is the core material of the microelectrode, electron transfer on the W-Au wire can be hampered by the less favorable electrical intrinsic properties of W such as lower conductivity and higher resistivity.

Additionally the W-Au electrodes have higher resistance than pure W wires, due to the low thickness of Au, both also due to the inductance effect that is exerted by the W core.
Nevertheless, our data show that W-Au microelectrodes are suitable for monitoring changes in H$_2$O$_2$ by monitoring the changes the oxidation currents.

7.3.1.3- Amperometry characterization of the microelectrodes

7.3.1.2.1- Evaluation of bare W-Au microelectrodes

After, the response of the bare W-Au micro electrode (50 µm Ø x 2 mm) to H$_2$O$_2$ was determined. Fig 3A shows the effects of consecutive additions of increasing amounts of H$_2$O$_2$ (5 to 500 µM) potential of the electrode was set at 900 mV.

![Figure 3](image)

**Figure 3** - A- Typical *in vitro* H$_2$O$_2$ calibration of a bare W-Au microelectrode at 900 mV vs Ag/AgCl. B- *In vitro* H$_2$O$_2$ calibrations of bare W-Au microelectrodes polarized at different potentials (600-900 mV vs Ag/AgCl). C- *In vitro* H$_2$O$_2$ calibrations of W-Au microelectrodes coated with Nafion polarized at different potentials (600-900 mV vs Ag/AgCl). D- *In vitro* H$_2$O$_2$ calibrations of W-Au microelectrodes coated with Nafion –PmPD polarized at different potentials (600-900 mV vs Ag/AgCl).

Our results shows that the W-Au microelectrodes reached a stable baseline current within a few minutes ($\leq$ 5 min). In addition the electrodes responded fast ($t_{95} \leq 1$ s) to consecutive additions of increasing H$_2$O$_2$. The oxidation currents were linearly correlated with the
H₂O₂ concentration (R² ≥ 0.99). These data indicate that W-Au microelectrodes are able to continuously monitor small changes in H₂O₂, with a fast response time and high sensitivity. Data presented in Figure 3-B to D shows that all bare W-Au microelectrodes displayed a linear correlation between the H₂O₂ concentration and oxidation current, when the applied potential was varied between 600 and 900 mV. We did not find differences in the oxidation currents of microelectrodes exposed to increasing H₂O₂ levels, for low applied potentials (600-800 mV). However, the oxidation currents of W-Au microelectrodes polarized at 900 mV were higher (p ≥0.05) than when polarized at any other potential, for high H₂O₂ levels (≥ 100 µM). These observations in line with the CV presented earlier, that showed an oxidation peak at 900 mV.

Based on these calibrations and using linear regression methods, we estimated various analytical parameters such as noise level, baseline current, LRS, and LOD. Despite the differences observed in the calibration curves, we found that that modification of the applied potentials did not affect any of the calculated parameters, except for the LRS (p≤0.05).

Next, we monitored oxidation currents of the major electrochemical interfering species DA, DOPAC, UA and AA in presence of H₂O₂. All bare W-Au electrodes displayed significant oxidation currents for the interfering compounds independently of the applied potential. However, the oxidation currents in presence of UA and AA in its physiological levels, were higher than DA or DOPAC (SI Fig 1). Using a previously described model (Cordeiro et al. 2016; M. and Buck 1996; O’Neill et al. 2008), we estimated the SC for all non-specific electroactive species (SI Table 1). The SC for DA is 10 fold higher when compared to the SC of the other interfering compounds, independently of the applied potential. Interestingly, we observed that the SC of DA was lower at 900 mV, when compared to lower applied potentials (600-800 mV). This is explained by the fact that the oxidation peak of DA on Au surfaces occurs at a lower potentials than H₂O₂ oxidation (Zachek et al. 2008).

Manipulation of the applied potential significantly affected the SC for all other interfering species. However, while an increase in potential resulted in a decrease in SC for DA, we observed the opposite effect for all other species. The SC at 900 mV was always the highest for AA, UA and DOPAC. It is concluded that changes in applied potential differentially affects the SC of the major interfering compounds. Therefore, to selectively monitoring the oxidation of H₂O₂, the use of permselective membranes is necessary.

7.3.1.2.2- Evaluation of functionalized W-Au microelectrodes

The use of permselective membranes has been described as an effective method to minimize electrochemical interference of needle type Pt based biosensors (Cordeiro et al. 2016; Wahono et al. 2012). Therefore, we coated W-Au microelectrodes with two different permselective membrane configurations (Nafion and Nafion-PPD) and evaluated its performance in presence of H₂O₂ and the major interfering compounds.
In vitro calibration of W-Au microelectrodes functionalized with either Nafion or Nafion-PPD indicated (Figure 3 C and D) a linear correlation between the oxidation current and $\text{H}_2\text{O}_2$ concentrations, independently of the applied potential.

Our data shows (SI Table 1) that most of the $\text{H}_2\text{O}_2$ related parameters, except LRS, were independent of the coating or the applied potential. We found that, Nafion coated W- Au microelectrodes had higher LRS, than microelectrodes coated with Nafion-PPD (for all potentials) and bare microelectrodes (for 600 and 700 mV) ($p \leq 0.05$). The higher LRS of Nafion coated microelectrodes when compared to electrodes coated with Nafion-PPD is due to reduction of active surface due to PPD polymerization (Cordeiro et al. 2016). However, the reason for a LRS when compared to bare electrodes is different. We found a shift in the oxidation peaks of the CV profile of Nafion coated W-Au microelectrodes when compared with bare W-Au microelectrodes (SI Figure 4), that results in higher currents at potentials between 600 and 800 mV.

Functionalized microelectrodes displayed significant lower oxidation currents when compared with bare microelectrodes, for all investigated non-specific electroactive species (SI Figures 2 and 3). These lower oxidation currents implied significant changes in the respective SC.

Functionalized microelectrodes display lower SCs for all interfering compounds at all tested potentials when compared with the SC obtained for bare microelectrodes ($p \leq 0.001$). We observed significant differences in the SC of some interfering compounds, between microelectrodes coated with Nafion and Nafion-PPD. While no differences were observed in the SC for AA and DOPAC, at any applied potential, we found that SC for both UA were lower in Nafion-PPD coated microelectrodes than in those coated with Nafion only. ($p \leq 0.05$ and $p \leq 0.001$ respectively). These result are in agreement with earlier studies using Pt microelectrodes (Cordeiro et al. 2016; Wahono et al. 2012).

Functionalizing W-Au microelectrodes with a permselective membrane resulted in an increase in sensitivity towards $\text{H}_2\text{O}_2$ when the higher potentials were applied (Table S1). The use of PPD in combination with Nafion results in a slight but significant decrease in sensitivity when compared with Nafion coated microelectrodes. However, it enhances microelectrode selectivity to some of the major non-specific electroactive species, like UA and especially DA.

For implantation in brain tissue with low DA levels, both Nafion and Nafion-PPD coated microelectrodes seem well suited. However, a Nafion-PPD membrane should be used when DA levels are relevant.

7.3.1.2.3- Evaluation of W-Au based glucose biosensors

Next, we assembled W-Au based amperometric enzymatic glucose biosensors; Nafion combined with PPD was used, as it was most effective combination of permselective membranes (W-Au/Nafion-PPD/GOx). We fabricated biosensors with different enzyme
loadings (0.2, 0.4 and 0.6 U/µL), and evaluated their performance when polarized at several potentials (600 to 900 mV).

**Figure 4** - A- Typical *in vitro* glucose calibration of a W-Au/Nafion-PmPD/GOx (0.6 U/µL) biosensor at 900 mV vs Ag/AgCl exposed to increasing concentrations of glucose (20 µM to 64 mM). **Insert** – Change in oxidation currents in response to the addition of low glucose levels (up to 2 mM).

All W-Au glucose biosensors displayed a non-linear increase in oxidation current in response to its exposure to increasing glucose levels response to glucose in the wide dynamic range tested (20 µM to 64 mM) (Figure 4). Our results (Figure 5) shows that the calibration profiles were dependent both on the enzyme loading and the applied potential.

Biosensors polarized at 600 and 700 mV (Figure 5 A and B) reached a current threshold at lower glucose levels compared with biosensors polarized at 800 and 900 mV. Moreover the oxidation currents of biosensors polarized at 800 and 900 mV levels were higher than for biosensors polarized at 600 and 700 mV for high glucose levels (≥ 8 mM).
The use of a previously described model for kinetics of enzyme immobilized onto microelectrode surfaces (Calia et al. 2009; Cordeiro et al. 2015; O’Neill et al. 2008) allowed us to estimate the most relevant biosensor performance parameters (LOD, I_max^app, appK_M, LR and LRS). Our data (SI Table 2) showed that both enzyme loading and applied potential have a significant influence on most but not all biosensor performance parameters.

Despite the differences in calibration profiles, the LOD of the biosensors were independent of both the applied potential and enzyme loading. All of the W-Au microelectrodes displayed an LOD between 96.32 and 222.84 µM. As the expected brain glucose levels are between 0.5 and 2.5 mM (Cordeiro et al. 2015; Duelli and Kuschinsky 2001), based on the LOD, all sensors should be suitable to detect brain glucose in vivo.

W-Au biosensors loaded with the highest enzyme concentrations (0.6 U/µL) displayed higher I_max than any other biosensors configuration, when polarized at the highest potentials (800 and 900 mV, p≤ 0.001).

Additionally, the applied potential had also a strong influence on the I_max^app. Biosensors polarized at the highest potentials displayed higher I_max than biosensors polarized at lower potentials, independently of the amount of enzyme. The loading of the enzyme might have shifted, as observed for the permselective membranes, the optimal H_2O_2, most likely towards a higher potential.

Besides its role in I_max^app, the applied potential also has an important role in the appK_M^app.
especially for biosensors loaded with more enzyme. When polarized at 900 mV, biosensors loaded with 0.6 and 0.4 U/µL displayed a higher \( \text{app} K_m \) (p≤0.001), thus LR, than biosensors loaded with 0.2 U/µL. Moreover, biosensor \( \text{app} K_m \) was also affected by the amount of enzyme loaded. Biosensors loaded with 0.6 U/µL displayed higher \( \text{app} K_m \) than biosensors loaded with 0.4 or 0.2 U/µL. Since the LR is dependent on the \( \text{app} K_m \), differences in the affinity constant dependent on both parameters, imply differences in the LR.

Furthermore, we found that both applied potential and enzyme loading influence biosensor LRS. This fact is not surprising, since the LRS is dependent on both \( I_{\text{max}} \) and \( \text{app} K_m \), kinetic parameters that are dependent on the applied potential and enzyme loading. Biosensors polarized at the lowest potential displayed, independently of the amount of enzyme loaded, display higher LRS than when polarized at any other potential, (p≤0.001). As the potential increases, we observed a decrease in LRS. Additionally we found that for applied potentials lower than 900 mV, the LRS of biosensors loaded with 0.4 U/µL was higher when compared with biosensors loaded with either 0.2 or 0.6 U/µL (p≤0.001). However, when polarized at 900 mV, we did not find any differences in the LRS for biosensors assembled with different enzyme concentration.

Regarding the fact that brain glucose levels vary between 0.5 and 2.5 mM, it is concluded that biosensors polarized at 900 mV, independently of the enzyme loading, were the most suited for \textit{in vivo} brain biomonitoring (LR ≥ 2 mM). Biosensors loaded with 0.6 U/µL, and polarized at 800 mV (7.5 ± 0.36 mM) also displayed a LR that seems adequate for \textit{in vivo} implantation.

In summary, our data suggest that the performance of W-Au biosensors depends both on enzyme loading and the applied potential.

### 7.3.2- \textit{In vivo} evaluation

Based on the present results we selected the W-Au/Nafion-PPD/GOx (0.6 U/µL) biosensor polarized at 900 mV, for \textit{in vivo} evaluation in brain tissue.

To further increase the accuracy of the sensor we applied a self-referencing system. In that sense the \( i \text{MBD} \) that included an enzymeless background sensor (BG).

Prior to implantation the glucose sensors were evaluated \textit{in vitro}. We observed a stable baseline currents for both the glucose 20 min after the immersion of the device in PBS. Our data (SI Figure 5) showed that that there was no cross talk between both sensors. However, we observed a residual oxidation current from the BG for high, non-physiological relevant brain glucose levels (≥ 16 mM). This is due to the large amount of \( \text{H}_2\text{O}_2 \) produced by enzymes immobilized on the glucose sensor surface, and it has been reported to have no influence when implanted (Cordeiro et al. 2015).

Finally, the \( i \text{MBD} \) was implanted in the mPFC of anesthetized animals. After a stabilization period, we modulated brain glucose levels, by intrathecal administration of glucose (1 M) or by infusing insulin (5U/Kg) intravenously.
In vivo “real-time” monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes

Figure 6- Monitoring glucose in vivo with W-Au based iMBD. A- Stabilization period B- Changes in subtracted currents following local glucose administration. C- Changes in subtracted currents following insulin administration (5U/Kg, i.v); Insert - Changes in blood glucose levels.

After implantation, the iMBD reached stable current less than 15 minutes after implantation. This period was shorter than reported for any biosensor to monitor in vivo brain glucose (Cordeiro et al. 2015). The fast stabilization of this iMBD might be attributed to an improved spatial resolution. The higher spatial resolution resulted in lower electrode active surface, thus faster electrode stabilization but also in a reduction tissue damage.

A small (3.5 and 6%) increase in the iMBD subtracted current was observed following two consecutive injections of glucose (10 µL, 1 M). The current raised sharply immediately after the administration, reaching its maximum less than a minute after local glucose injection. A similar fast sensor response after perfusion of 1M of glucose, was reported by Lowry and colleagues (Lowry et al. 1998a).

Additionally, we observed a decrease in the subtracted current, after insulin was administration (5U/kg, i.v.). The iMBD subtracted current started to decrease, at a slow rate, immediately after insulin administration, reaching 90% of its basal levels, approximately 30 min after administration. After reaching its lowest levels, the subtracted current remained stable for an additional 30 min. The changes in subtracted current were well correlated (R ≥ 0.85, p≤ 0.05; Pearson Product Moment Correlation) with changes in the decrease in circulating blood glucose. These changes are comparable to those previously described for brain glucose monitored by biosensors in response to insulin administration (Cordeiro et al. 2015; Lowry et al. 1998a; Vasylieva et al. 2011). A decrease in brain glucose after insulin administration was also observed using other brain biomonitoring techniques, such as
microdialysis (de Vries et al. 2005).

### 7.4-Conclusion

The presented data demonstrated that W-Au microelectrodes, when functionalized with permselective membranes, are able to monitor with high degree of sensitivity and selectivity, changes in $\mathrm{H}_2\mathrm{O}_2$. We also demonstrated that, with appropriate polarization and enzyme loading, functionalized W-Au microelectrodes can be used as a platform for the assembly of sensitive and selective glucose biosensors. Finally, we demonstrated that W-Au based biosensors are able to monitor changes in brain glucose, evoked by local administration of glucose or i.v. administration of insulin.

The use of a core of the strongest metal, coated with a thin layer of a highly conductive material (Au) may allow a successful downscale of implantable, needle type biosensors. The prospective downscaling of these devices will enable *in vivo* brain biomonitoring, with even higher spatial resolution ($\leq 5 \mu \text{m } \Omega$), hence (even) less tissue damage.
7.5-Bibliography


7.6- Supplementary Material

7.6.1-Oxidation currents of non-specific electroactive species

**Figure S1** - Changes in oxidation current of bare W-Au electrodes exposed to physiologically relevant levels of the major non-specific electroactive species.

**Figure S2** - Changes in oxidation current of Nafion coated W-Au electrodes exposed to physiologically relevant levels of the major non-specific electroactive species.

**Figure S3** - Changes in oxidation current of Nafion-PPD coated W-Au electrodes exposed to physiologically relevant levels of the major non-specific electroactive species.
7.6.2- *In vitro* voltammetry evaluation.

**Figure S4**- Voltammograms of bare Au and W-Au microelectrodes and Nafion coated W-Au microelectrodes in presence of $\text{H}_2\text{O}_2$. 

*In vivo* “real-time” monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes
7.6.3- *In vitro* evaluation of the performance of W-Au microelectrodes.

Table S1- Performance parameters of bare (A) and functionalized W-Au microelectrodes (B-Nafion, C- Nafion-PmPD) polarized at different potentials (600 to 900 mV vs Ag/AgCl).

<table>
<thead>
<tr>
<th></th>
<th>Bare</th>
<th>Nafion</th>
<th>Nafion-PPD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>600 mV</td>
<td>700 mV</td>
<td>800 mV</td>
</tr>
<tr>
<td></td>
<td>n=6</td>
<td>n=6</td>
<td>n=6</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>SEM</strong></td>
<td><strong>Mean</strong></td>
<td><strong>SEM</strong></td>
</tr>
<tr>
<td>Noise (nA)</td>
<td>0.10</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Baseline (nA)</td>
<td>30.26</td>
<td>11.68</td>
<td>32.02</td>
</tr>
<tr>
<td>LRS (nA/µM)</td>
<td>0.15</td>
<td>0.03</td>
<td>0.16</td>
</tr>
<tr>
<td>LOD (µM)</td>
<td>2.03</td>
<td>0.73</td>
<td>1.05</td>
</tr>
<tr>
<td>SC DA</td>
<td>1924.65</td>
<td>377.28</td>
<td>2333.49</td>
</tr>
<tr>
<td>SC DOPAC</td>
<td>35.74</td>
<td>7.68</td>
<td>124.16</td>
</tr>
<tr>
<td>SC UA</td>
<td>173.33</td>
<td>21.03</td>
<td>384.53</td>
</tr>
<tr>
<td>SC AA</td>
<td>118.49</td>
<td>8.77</td>
<td>175.45</td>
</tr>
</tbody>
</table>
### 7.6.4 *In vitro* evaluation of the performance of W-Au based glucose biosensors.

**Table S2** - Performance parameters of W-Au/Nafion-PmPD/GOx biosensors loaded with different enzyme concentrations (0.2; 0.4; 0.6; and 0.8 U/µL) at different applied potentials (600-900 mV).

<table>
<thead>
<tr>
<th></th>
<th>0.2 U/µL</th>
<th></th>
<th>0.4 U/µL</th>
<th></th>
<th>0.6 U/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>600 mV</td>
<td>700 mV</td>
<td>800 mV</td>
<td>900 mV</td>
<td>600 mV</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=4</td>
<td>n=5</td>
<td>n=5</td>
<td>n=10</td>
</tr>
<tr>
<td>LOD (µM)</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>0.2 U/µL</td>
<td>177,45</td>
<td>127,29</td>
<td>35,82</td>
<td>0,66</td>
<td>72,90</td>
</tr>
<tr>
<td>0.4 U/µL</td>
<td>5,29</td>
<td>0,36</td>
<td>7,56</td>
<td>0,69</td>
<td>11,72</td>
</tr>
<tr>
<td>0.6 U/µL</td>
<td>0,24</td>
<td>0,09</td>
<td>0,98</td>
<td>0,42</td>
<td>4,33</td>
</tr>
<tr>
<td>LRS (nA/mM)</td>
<td>21,76</td>
<td>1,48</td>
<td>1,17</td>
<td>0,71</td>
<td>2,71</td>
</tr>
<tr>
<td>LRS (nA/mM)</td>
<td>0,10</td>
<td>0,02</td>
<td>0,26</td>
<td>0,07</td>
<td>0,88</td>
</tr>
<tr>
<td>LR (mM)</td>
<td>0,12</td>
<td>0,05</td>
<td>0,49</td>
<td>0,21</td>
<td>2,16</td>
</tr>
</tbody>
</table>

**In vivo** "real-time" monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes.
7.6.5- *In vitro* iMBD evaluation.

Figure S5 - Typical *in vitro* calibration of the implantable biosensor device (iMBD).

7.6.6- *In vivo* iMBD evaluation.

Figure S6 - Experimental setup for *in vivo* evaluation of the W-Au based iMBD.
**7.6.7- Scanning Electron Microscopy evaluation**

![Figure S7](image)

**Figure S 7-** SEM evaluation of bare W-Au microelectrodes (A1 to A4) and W-Au microelectrodes coated with Nafion (B1 to B4), Nafion-PmPD (C1 to C4) and Nafion-PmPD/GOx (0.4 U/µL) (D1 to D4). Figure S7 A1 to A4 show details of bare W-Au microelectrodes. Figures A1 and A2 show a detail of the cross section of a bare W-Au microelectrode magnified 2500x and 10000x respectively. Figures A3 and A4 show a detail of the surface of a bare W-Au microelectrode magnified 2500x and 10000x respectively. Figures B1 and B2 show a detail of the cross section of a W-Au microelectrode coated with Nafion magnified 2500x and 10000x respectively. Figures B3 and B4 show a detail of the surface of a W-Au microelectrode coated with Nafion magnified 2500x and 10000x respectively. Figures C1 and C2 show a detail of the cross section of a W-Au microelectrode coated with Nafion-PmPD magnified 2500x and 10000x respectively. Figures C3 and C4 show a detail of the surface of a W-Au microelectrode coated with Nafion-PmPD magnified 2500x and 10000x respectively. Figures D1 and D2 show a detail of the cross section of a W-Au microelectrode coated with Nafion-PmPD magnified 2500x and 10000x respectively. Figures D3 and D4 show a detail of the surface of a W-Au microelectrode coated with Nafion-PmPD magnified 2500x and 10000x respectively.

*In vivo* “real-time” monitoring of glucose in the brain with an amperometric enzyme-based biosensor based on gold coated tungsten (W-Au) microelectrodes