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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 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. In that sense, we investigated whether the use of a material with low malleability, tungsten (W), coated with a highly conductive material, gold (Au) could be as an alternative for conventional needle-type based biosensors.

Therefore, we developed implantable needle-type (50 μm $\varnothing$) gold coated tungsten (W-Au) amperometric microbiosensors. First, we evaluated electrochemically, the ability of W-Au microelectrodes (50 μm $\varnothing$) to continuously monitor changes in H_2O_2 . After, we functionalized, using a layer-by-layer assembly, the surface of W-Au microelectrodes. First with permselective membrane(s) (Nafion and Nafion-PPD) and after with an enzymatic hydrogel, containing an enzyme selective for glucose (glucose oxidase). Both the enzyme loading and the applied potential were optimized and the performance of functionalized W-Au microelectrodes and fully assembled biosensors was evaluated electrochemically. Additionally, the surface of bare and functionalized microelectrodes was also 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.

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1. Introduction

Ideally, *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, these methods have severe limitations, such as low quantitative resolution and limited temporal and/or spatial resolution [1–4]. To suppress those limitations, the use of invasive methods, such as microdialysis, is often required [5–7]. These methods can provide 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, is the method of choice, for invasive,

in vivo brain biomonitoring. This technique allows *in situ* brain biomonitoring of multiple analytes, with good spatial ($\leq 1\text{ mm}$) and temporal ($\leq 10\text{ min}$) resolution. However, this temporal resolution is insufficient to monitor the expected fast changes in key biomarkers involved in neurophysiology and neuropathology [8,9]. Moreover, although the spatial resolution of microdialysis probes is quite good, it's still insufficient to monitor changes in brain biomarkers at a single cell level.

Biosensors are bioanalytical tools that can combine spatial ($\leq 200\text{ }\mu\text{m}$) and temporal ($\leq 1\text{ s}$) resolution with high selectivity and rapid response time [10–12]. These devices have been increasingly employed in experimental neuroscience for real time *in vivo* biomonitoring of several biomarkers such as glutamate [13–16], choline [17,18], glucose [19–22], lactate [9,23], pyruvate [9] and reactive oxygen species (H_2O_2 and NO) [24,25]. Typically, these biosensors rely on the oxidation of an electroactive product (often H_2O_2) of an enzymatic reaction (typically mediated by oxidases)

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at the electrode surface [26,27]. However, at the working potentials necessary to oxidize the electroactive molecules of interest (>500 mV), other non-specific electroactive compounds are also oxidized, resulting in non-specific electrochemical interference [28]. In *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) [8,29]. Therefore, permselective membranes such as Nafion and poly(phenylenediamine) (PPD) are often used to overcome these electrochemical interferences, enabling adequate biosensor selectivity [8,28,30].

Despite its relative small dimensions, implantation of a biosensor still results in significant tissue damage and triggers foreign body reaction (FBR) [31]. It has been described that biosensor implantation generates a series of physiological processes, involved in FBR 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, for the failure of implantable biosensors, [32]. Additionally, it has been reported that the size of the implantable device, has a significant impact in the biofouling effect. In that sense, biosensor miniaturization will result in less tissue damage, thus less biofouling [33,34]. Unfortunately, conventional implantable biosensors (needle-type platinum (Pt)/carbon fiber based microelectrodes) are not easily miniaturizable. Additionally, conventional implantable biosensors can easily bend or break upon implantation, thereby damaging the assembled layers and impairing *in vivo* accuracy and applicability of the biosensor.

The use of a rigid core material, such as tungsten (W) in microelectrode assemble, can allow an efficient the miniaturization of the biosensor. Tungsten based nanoelectrodes, have been already reported [35,36]. The use of W in the construction of biosensors may enable a significant decrease in spatial resolution, thus limiting biosensor biofouling.

Due to their resistance to sheer stress and its low corrosion rates, W-based microelectrodes are the tools of choice for numerous electrophysiological techniques [37,38–40]. However, for an effective oxidation of H_2O_2 , the target electroactive molecule in implantable amperometric enzyme-based biosensors, coating of the W surface with a metal with higher conductivity, such as gold (Au), is necessary. The use of gold (Au) not only allows the quantitative monitoring of H_2O_2 [41], but also can reduce the corrosion of W based implantable microelectrodes, enhancing its biocompatibility [37].

Here, we describe the development and characterization of a W-Au based microbiosensor, for *in vivo* brain monitoring. First we evaluated the ability of W-Au microelectrodes to monitor quantitatively, H_2O_2 . After, we developed and characterized, *in vitro* and *in vivo*, an implantable amperometric enzyme-based glucose biosensor based on W-Au microelectrodes.

We demonstrated that W-Au based microbiosensors 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.

2. Materials and methods

2.1. Materials

Tungsten (W) (100 μm $\varnothing$), gold (Au) (50 μm $\varnothing$) and silver wires (Ag) (250 μm $\varnothing$) were obtained from Advent Research Materials. Gold coated tungsten (W-Au) wires (50 μm $\varnothing$) 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), ascorbic acid (AA), uric acid (UA), 3,4-dihydroxyphenylacetic acid DOPAC, H_2O_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^{4-} in ultra-purified water, brought to pH 7.4 with sodium hydroxide and degassed before use.

2.2. Biosensor assembly

2.2.1. Microelectrode assembly

All microelectrodes were assembled according to a previously described method [8] (Fig. 1A). 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 . W-Au and Au based microelectrodes/biosensors had the following dimensions, 50 μm $\varnothing$ x 2 mm length (3,147 mm² of surface area), while W electrodes had 100 μm $\varnothing$ x 2 mm length (6,283 mm² of surface area).

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 [8,28]. To assemble glucose biosensors, W-Au microelectrodes functionalized with any of the permselective membrane(s) were subsequently coated with an hydrogel containing GOx (0.2, 0.4, and 0.6 U/ μL) reticulated with bovine serum albumin (BSA) and glutaraldehyde (GA) [9].

2.2.3. Implantable microbiosensor device (iMBD)

For *in vivo* implantation, we assembled an implantable microbiosensor device (iMBD), based on a self-referencing principle [8,9,14,23]. 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. 1C). The iMBD tip was approximately 150 μm in diameter.

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 (bare and functionalized) were characterized by imaging techniques (Scanning Electron Microscopy).

2.3.1. Cyclic voltammetry

Cyclic voltammetry experiments were performed in presence of H_2O_2 (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.

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 [8,9,28]. Sensors were placed in PBS and steady state parameters (noise and baseline) were assessed after an initial equilibration period (approximately 45 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 H_2O_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

Fig. 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.

in oxidation currents in response to the exposure to the different analytes were used to estimate relevant microelectrode/biosensor performance parameters.

2.3.3. 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 field emission scanning electron microscope (JEOL FE-SEM 6301F) at 3 kV and a secondary electron detector (JEOL LTD. 1–2 Mushasino 3-chome, Akishima Tokyo 196).

2.3.4. In vivo evaluation

Male Wistar rats (350–425 g) were used in the *in vivo* experiments. Animals were group 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 permanent 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 a stereotaxic atlas [42]. All *in vivo* electrochemical measurements were performed at a constant potential of 800 mV vs Ag/AgCl. After implantation, a 2 h 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 the 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 (AccuChek; 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.

2.3.5. 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) (Eq. (1)) was calculated using a previously described model [28]:

$$SC = \frac{I(\text{Analyte}), \text{ nA} / [\text{Analyte}], \mu\text{M}}{I(\text{H}_2\text{O}_2), \text{ nA} / [\text{H}_2\text{O}_2], \mu\text{M}} \times 100 \quad (1)$$

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) [29]. The calculated parameters include limit of detection (LOD), linear Range (LR) linear range slope (LRS) Michaelis-Menten constant ($_{app}K_M$), maximum current intensity (I_{MAX}), and the surface independent constants ($SI_{app}K_M$, I_{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.

$$I(S) = \frac{I_{MAX}}{1 + \frac{KM(S)}{[S]}} \quad (2)$$

$$LRS(\text{nA/mM}) = \frac{I_{MAX}}{KM} \quad (3)$$

$$LOD(\text{mM}) = 3 \times \text{Noise (nA)} / LRS(\text{nA/mM}) \quad (4)$$

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 analyses were performed using SigmaStat 12.0.

3. Results and discussion

3.1. In vitro evaluation

3.1.1. Scanning electron microscopy evaluation

We used Scanning electron microscopy to evaluate the morphology of the microelectrode surface. We studied the surfaces of bare and functionalized (with Nafion, Nafion-PPD or Nafion-PPD/GOx) W-Au based microelectrodes. Our results (SI Fig. S7) show that the surface of bare W-Au microelectrodes is somewhat rough (SI Fig. S7 A1–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 (SI Fig. S7 B1–B4) or Nafion-PPD (SI Fig. S7 C1–C4) were observed. These results are in accordance with previously described experiments, in which it was shown that on

Fig. 2. Voltammograms of bare W (A), Au (B) and W-Au (C) microelectrodes in the presence of increasing H_2O_2 concentrations (0–2 mM), at a scan rate of 50 mV/s.

microelectrodes previously functionalized with Nafion, polymerization of PPD occurred on the remaining active surface after Nafion self-assembly [28].

Additionally, we haven't noticed significant differences in the surface morphology of microelectrodes functionalized with permselective membranes alone or fully assembled glucose biosensors (SI Fig. S7 D1–D4). The presence of the hydrogel in the fully assembled W-Au based glucose biosensor resulted in a slight increase in diameter, when compared with microelectrodes functionalized with a permselective membrane alone (51 vs 53 μm).

3.1.2. Characterization by of the microelectrodes by cyclic voltammetry

The electrochemical characterization of W based microelectrodes, started by performing CVs of the W, Au and W-Au based microelectrodes in presence of increasing levels of H_2O_2 . We observed in the voltammograms for W microelectrodes, two oxidation peaks, at 50 and 500 mV (Fig. 2A). These peaks, are most likely due to the two step oxidation of W by H_2O_2 , as defined by the following equations [43]:

Interestingly, we observed that increasing H_2O_2 levels, resulted in a decrease in oxidation, reaching lower values, and consequently smaller peaks, when exposed to the higher H_2O_2 levels. Additionally, we observed that the noise levels decreased with the increase in H_2O_2 , suggesting a smoother microelectrode surface [28].

The dissolution of W by H_2O_2 been long been reported [44]. Therefore, the decrease in both current and noise levels, with the increase in H_2O_2 , might be related with the dissolution of W by H_2O_2 oxidation. The W dissolution probably resulted both in a decrease in surface area and in a smoother electrode surface.

Exposing Au microelectrodes to H_2O_2 resulted in a very different electrochemical profile (Fig. 2B). Now an irreversible redox profile was observed, as previously described [41]. Our data showed a single oxidation peak (around 900 mV) followed by reduction

peak around 450 mV. However when exposed to higher H_2O_2 levels (≥ 1 mM) the oxidation peak seems to shift from 900 to 950 mV. The 900 mV peak can be attributed to the gold oxidation by H_2O_2 (900 mV) and the one observed at 450 mV, is likely due to adsorbed hydroxide ions [41]. While an increase in H_2O_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 [41]. The current observed for Au microelectrodes exposed to increasing H_2O_2 levels was lower than that of W microelectrodes, due to a significant decrease in active surface (50 vs 100 μm in $\varnothing$).

The CV's of the W-Au microelectrodes, were identical to those of Au microelectrodes (Fig. 2C). We also observed an oxidation peak around 900 mV and a reduction peak around 450 mV for W-Au microelectrodes. However, W-Au microelectrodes display much less oxidation/reduction currents when compared with Au microelectrodes. While Au is an highly conductive material, W has a lower intrinsic conductivity [45]. 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, and to the inductance effect that is exerted by the W core. Our data show that W-Au microelectrodes are suitable for monitoring changes in H_2O_2 by monitoring the changes the oxidation currents.

3.1.3. Amperometry characterization of the microelectrodes

3.1.3.1. Evaluation of bare W-Au microelectrodes. After, we evaluated the ability of W-Au microelectrodes (50 μm $\varnothing$ x 2 mm) to quantify H_2O_2 . Fig. 3A shows the increase in current of a W-Au microelectrodes polarized at 900 mV in response to consecutive additions of increasing amounts of H_2O_2 (5–500 μM).

Our results shows that the W-Au microelectrodes reached a stable baseline current within few minutes (≤ 5 min). In addition, all microelectrodes displayed fast response times ($t_{95} \leq 1$ s) and the oxidation currents were linearly correlated with the H_2O_2 concentration ($R^2 \geq 0.99$). These data indicate that W-Au microelectrodes are able to continuously monitor small changes in H_2O_2 , with a fast response time and high sensitivity.

Fig. 3. A- Typical *in vitro* H_2O_2 calibration of a bare W-Au microelectrode at 900 mV vs Ag/AgCl. B- *In vitro* H_2O_2 calibrations of bare W-Au microelectrodes polarized at different potentials (600–900 mV vs Ag/AgCl). C- *In vitro* H_2O_2 calibrations of W-Au microelectrodes coated with Nafion polarized at different potentials (600–900 mV vs Ag/AgCl). D- *In vitro* H_2O_2 calibrations of W-Au microelectrodes coated with Nafion – PmPD polarized at different potentials (600–900 mV vs Ag/AgCl).

Moreover, we show that (Fig. 3B–D) all bare W-Au microelectrodes displayed a linear correlation between the H_2O_2 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 H_2O_2 levels, for low applied potentials (600–800 mV). However, the oxidation currents of W-Au microelectrodes polarized at 900 mV were higher ($p \geq 0.05$) than when polarized at any other potential, for high H_2O_2 levels ($\geq 100 \mu\text{M}$). These observations in accordance with the CVs data presented earlier, showing an oxidation peak at 900 mV.

Based on these calibrations and using linear regression methods, we determined various analytical parameters such as noise level, baseline current, LRS, and LOD. Despite the differences observed in the calibration curves, we found that modification of the applied potentials did not affect any of the calculated parameters, except for the LRS, which was higher for microelectrodes were polarized at 900 mV ($p \leq 0.05$).

Next, we monitored the oxidation currents of the major electrochemical interfering species DA, DOPAC, UA and AA in presence of H_2O_2 . All bare W-Au microelectrodes displayed significant oxidation currents for the interfering compounds, independently of the applied potential. However, the oxidation currents of microelectrodes in presence of UA and AA at physiological levels, were higher than in presence of DA or DOPAC (SI Fig. S1). Using a previously described model [28,29,46], we estimated the SC for all non-specific electroactive species (SI Table S1). 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 H_2O_2 oxidation [47].

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 the highest for AA, UA and DOPAC. Changes in applied potential differentially affect the SC of the major interfering compounds. Therefore, to selectively monitor the oxidation of H_2O_2 with W-Au microelectrodes, the use of permselective membranes is necessary.

3.1.3.2. Evaluation of functionalized microelectrodes. The use of permselective membranes has been described as an effective method to minimize electrochemical interference of needle type Pt based biosensors [8,28]. Therefore, we coated W-Au microelectrodes with two different permselective membrane configurations (Nafion and Nafion-PPD) and evaluated its performance in presence of H_2O_2 and the major interfering compounds.

In vitro calibration of W-Au microelectrodes functionalized with either Nafion or Nafion-PPD indicated (Fig. 3C and D) a linear correlation between the oxidation current and H_2O_2 concentrations, independently of the applied potential.

Our data shows (SI Table S1) that most of the H_2O_2 related parameters, with the exception of LRS, were independent of the coating or the applied potential. However, we found that, Nafion coated W-Au microelectrodes displayed 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 a result of the reduction of active surface due to PPD polymerization [28]. However, the reason for a higher LRS of Nafion coated microelectrodes when compared to bare electrodes is different. Interestingly, 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 Fig.

S4), which results in higher currents at potentials between 600 and 800 mV.

Functionalized microelectrodes displayed significant lower oxidation currents for all non-specific electroactive species, when compared with bare microelectrodes, (SI FigS2 and S3). 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 ($p \leq 0.001$). Additionally, 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 and AA 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 results are similar to those described in earlier studies using Pt microelectrodes functionalized with Nafion-PPD [8,28].

Overall, we observed that functionalizing W-Au microelectrodes with a permselective membrane resulted in an increase in sensitivity towards H_2O_2 when the higher potentials were applied (SI 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, as they are in the prefrontal cortex.

3.1.3.3. Evaluation of the W-Au based glucose biosensor. Next, we assembled W-Au based amperometric enzymatic glucose biosensors with the most effective permselective membrane configuration (Nafion-PPD). We fabricated biosensors with different

Fig. 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).

enzyme loadings (0.2, 0.4 and 0.6 U/μL), and evaluated their performance when polarized at several potentials (600–900 mV) (Fig. 4).

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

Biosensors polarized at 600 and 700 mV (Fig. 5A and B) reached a current threshold at lower glucose levels compared with biosensors polarized at 800 and 900 mV. Moreover the oxidation currents of

Fig. 5. *In vitro* calibrations of W-Au/Nafion-PmPD/GOx biosensors loaded with different enzyme (0.2; 0.4 and 0.6 U/μL) concentrations, polarized at 600 (A), 700 (B), 800 (C) and 900 mV (D) vs Ag/AgCl. **Inserts** – *In vitro* calibrations for low glucose levels (up to 4 mM).

Fig. 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.

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 model for kinetics of enzyme immobilized onto microelectrode surfaces [9,29,48] allowed us to determine the most relevant biosensor performance parameters (LOD, I_{Max} , $\text{app}K_{\text{M}}$, 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 physiological brain glucose levels are between 0.5 and 2.5 mM [9,49], based on the LOD, all sensors should be suitable to detect brain glucose *in vivo*. Nonetheless, 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, $p \leq 0.001$).

In fact, the applied potential also had a strong influence on the I_{Max} . 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 potential for H_2O_2 Oxidation, most likely towards a higher potential.

Besides its role in I_{Max} , the applied potential also has an important role in the $\text{app}K_{\text{M}}$, 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 $\text{app}K_{\text{M}}$ ($p \leq 0.001$), thus LR, than biosensors loaded with 0.2 U/ μL . Moreover, biosensor $\text{app}K_{\text{M}}$ was also affected by the amount of enzyme loaded. Biosensors loaded with 0.6 U/ μL displayed higher $\text{app}K_{\text{M}}$ than biosensors loaded with 0.4 or 0.2 U/ μL ($p \leq 0.05$). Since the LR is dependent on the $\text{app}K_{\text{M}}$, 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_{Max} and $\text{app}K_{\text{M}}$, 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, ($p \leq 0.001$). 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 ($p \leq 0.001$). However, when polarized at 900 mV, we did not find any differences in the LRS for biosensors loaded with different enzyme concentration.

Since brain glucose levels range between 0.5 and 2.5 mM, it is concluded that polarization at 900 mV, independently of the enzyme loading, is the most suited potential for *in vivo* brain biomonitoring ($\text{LR} \geq 2$ 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 *in vivo* implantation.

In summary, our data suggest that the performance of W-Au biosensors depends both on enzyme loading and the applied potential. We demonstrated that W-Au based biosensors are able to monitor glucose *in vitro*, selectively, continuously and in real-time, within a relevant range for *in vivo* brain biomonitoring.

3.2. In vivo evaluation

Based on the *in vitro* characterization of W-Au based biosensors, we selected the W-Au/Nafion-PPD/GOx (0.6 U/ μL) biosensor configuration, polarized at 900 mV, for in evaluation *in vivo* in brain tissue. To further increase the accuracy of the sensor we implanted the W-Au based biosensor using a self-referencing system. In that sense we constructed an iMBD that included an enzymeless background sensor (BG).

Prior to implantation the iMBDs that included the W-Au glucose biosensors were evaluated *in vitro*. We observed a stable baseline currents for both the glucose and BG sensor, 20 min after the immersion of the device in PBS. Although our data (SI Fig. S5) showed that there was no cross talk between both sensors, we observed a residual oxidation current from the BG for high, non-physiological relevant glucose levels (≥ 16 mM). This current most likely due to the oxidation of H_2O_2 at the surface of the BG sensor, due to the large amount of H_2O_2 produced by enzymes immobilized on the surface of the glucose sensor. Nevertheless, this previously reported phenomenon, does not occur *in vivo*, when implanted [9].

Finally, for in vivo evaluation, the iMBD was implanted in the mPFC of anesthetized animals. After a stabilization period, brain glucose levels were modulated, by local administration of glucose or by infusing insulin intravenously (Fig. 6).

The iMDB reached stable current less than 15 min after implantation. This period was shorter than reported for any biosensor to monitor *in vivo* brain glucose [9]. The fast stabilization of this iMDB might be attributed to its improved spatial resolution, when compared with implantable self-referring biosensor devices. The higher spatial resolution resulted in lower electrode active surface, thus faster electrode stabilization but also in a reduction of tissue damage.

Administration of pharmacologically active compounds, either glucose or insulin, resulted in significant changes in the subtracted current of the implanted W-Au based iMDB. A small (3.5 and 6%) increase in the subtracted current was observed after two consecutive injections of glucose (10 μ L, 1 M). The subtracted current raised sharply immediately after local glucose administration, reaching its maximum less than a minute after administration. A similar increase in current in response to the perfusion of 1 M of glucose was previously reported by Lowry and colleagues [50].

The administration of insulin (5U/kg, i.v.), resulted in a decrease in the subtracted current. The iMDB 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 additional 30 min. The changes in subtracted current were well correlated ($R \geq 0.85$, $p \leq 0.05$; Pearson Product Moment Correlation) with changes in circulating blood glucose. These changes are comparable to those previously described for brain glucose monitored by biosensors in response to insulin administration [9,20,50]. The decrease in brain glucose in response to insulin administration, was also observed using a different *in vivo* brain biomonitoring techniques, microdialysis [51].

4. Conclusion

The presented data demonstrated that when functionalized with permselective membranes, W-Au microelectrodes, can to monitor, with high degree of sensitivity and selectivity, changes in H_2O_2 , continuously and in real-time.

Additionally, we 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 amperometric enzyme-based glucose biosensors.

Finally, we showed that amperometric enzyme-based biosensors based on W-Au microelectrodes, can reliably monitor changes in brain glucose, evoked by local administration of glucose or i.v. administration of insulin, with suitable temporal and spatial resolution.

The use of a rigid material such as tungsten, coated with a conductive material (Au) in microelectrode assembly will allow the necessary downscale of implantable biosensors for *in vivo* brain biomonitoring. The prospective miniaturization of these devices will enable *in vivo* brain biomonitoring, with even higher spatial resolution ($\leq 5 \mu\text{m}$ $\emptyset$), less tissue damage and that are less prone to biofouling.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.snb.2018.02.116>.

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Biographies

Carlos Alberto de Lima Braga Lopes Cordeiro received his licenciatura (MSc equivalent) in Biochemistry at the Universidade do Algarve (Faro, Portugal) in 2004. In 2007, he joined the Biomonitoring and Biosensing and Biomonitoring group at the Faculty of Pharmacy of the University of Groningen to complete his MSc research project. Later (in 2008) he received an MSc in Biotechnology at the same University. In the same year he started to work as a Trial Manager for biosensing at Brains On-Line BV (Groningen, The Netherlands) where he is also a resident scientist. Since 2011 he is also Trial Manager for electrophysiology studies at the same organization. In 2018 he was awarded his PhD in the University of Groningen. His work focus on the development of biosensors for *in vivo* applications.

Ben Westerink was born in Enschede, The Netherlands in 1947. He obtained his MSc degree in Biochemistry at The TU of Delft in 1970 and later (in 1974) his PhD degree in the University of Groningen. In 1977 he was appointed as Associate Professor at the University Center for Pharmacy in the University of Groningen. In 2000 he was appointed as full professor and as head of the Biomonitoring and Sensing department at the University of Groningen. In 2002 he founded Brains On-Line. He was the scientific director of the University Center for Pharmacy, Groningen from 2004 to 2010. In 2011 he retired from his academic duties, but remained active as CSO at Brains On-Line. His extensive work has been focused on the development of analytical chemical methods (automated fluorimetry, HPLC and electrochemical detection) for neurotransmitter biomonitoring in brain tissue and body fluids. He was one the pioneers in development and application of the microdialysis technique (since 1986). He also worked on pharmacology and design of antiparkinson drugs, antidepressants and antipsychotics. Lately he worked on the development of microbiosensors for neurotransmitters and energy metabolites biomonitoring. He authored more than 230 peer-reviewed publications, whose sum of citations exceeds 10000.

Thomas I.F.H. Cremers obtained his PhD degree in the University of Groningen in 2002. His work focused in the pharmacodynamics and pharmacokinetics of SSRI augmentation strategies. In 2002 together with Ben Westerink he founded Brains On-Line, a CRO organization dedicated to CNS includes drug development and in the development of novel biomonitoring methods, such as microdialysis and micro-biosensing. He is currently the CEO of several of CRO organizations, as well as an Honorary Professor at department of Pharmaceutical Analysis of the University of Groningen.