Amperometric enzyme-based biosensors: refined bioanalytical tools for in vivo biomonitoring
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CHAPTER 4

A wireless implantable microbiosensor device for continuous glucose monitoring (CGM).

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Abstract:

Diabetes Mellitus is a chronic disease characterized by poor glucose regulation. On a long term, diabetes can lead to severe complications such as nephropathies, neuropathies, blindness and cardiovascular disorders. It is currently a leading cause of death, worldwide. With the number of diabetes patients rapidly increasing, the need for a solution to improve glucose control became crucial. Despite endless evidence that continuous glucose monitoring (CGM) leads to better diabetes management, state-of-the-art CGM devices are still hampered by poor accuracy and (very) short lifetimes. These devices rely on biosensor measurements that still require frequent calibrations, dependent on painful blood glucose measurements. Therefore, CGM devices far from replacing the burdensome blood glucose monitoring as the method of choice in diabetes management.

Here, we describe a wireless implantable microbiosensor based device (iMBD) for CGM. The glucose biosensors of our CGM device are based on needle type electrodes platinum microelectrodes. Microelectrode surfaces were functionalized, first with a permselective and after with an enzymatic hydrogel (Glucose Oxidase). All biosensors were then covered with a hollow dialysis membrane.

We assembled and characterized in vitro, various amperometric enzyme-based glucose biosensor designs. Thorough electrochemical evaluation allowed us to select the most suitable one (Pt\textsubscript{200}/Nafion/GOx/PE) to be incorporated in the wireless CGM device.

The performance of the device was evaluated in vivo by its implantation in the subcutaneous tissue of freely moving rats, for 5 days. After, all iMBD were explanted and re-calibrated to assess biofouling effects. Conversion of the in vivo CGM signals into glucose levels was performed by single and multiple point blood calibration.

The presented device was able to monitor, wirelessly continuously and in real-time subcutaneous glucose levels in freely moving animals. Additionally the device was able to detect glucose levels manipulated by pharmacological treatments. These changes were well correlated with concurrent changes in blood glucose, suggesting that the proposed approach may represent a viable option for better glucose management.
4.1- Introduction

Diabetes mellitus, commonly known as diabetes, is one of the diseases with the highest prevalence, 8% of the world population, reaching a total of 387 million worldwide. However it is estimated than an additional 46% of them maybe be undiagnosed (W.H.O 2016; Wild et al. 2004; Zimmet et al. 2001). The latest predictions by the World Health Organization estimate its prevalence to at least double by 2030 (Shaw et al. 2009). In 2014 diabetes was associated (directly or indirectly) with almost 5 million deaths (da Rocha Fernandes et al. 2015). Despite the innumerous efforts by a large scientific community devoted to study of the disease, and its key biomarker (glucose) knowledge of its etiology is still limited and no cure was found yet (Gadsby 2002; Kharroubi and Darwish 2015).

Diabetes complications arise from long periods of high levels of blood glucose (hyperglycemia). Recurring hyperglycemia can lead to severe, and even life-threatening complications. These complications include the development of cardiovascular, nephrological, retinopathic and neurologic diseases. Additionally, episodes of low blood glucose (hypoglycemia) can also have deleterious effects. Most of problems related to acute hypoglycemia arise from an inadequate supply of glucose to the brain, leading to an impairment of the cognitive function (neuroglycopenia). Its effects range from mild dysphoria to seizures, unconsciousness, brain damage (temporary or permanent), coma and even death. In order to delay/avoid diabetes complications, a good management of the disease is fundamental (Alqahtani et al. 2013; Aronoff et al. 2004; Battelino et al. 2011; Benjamin 2002; Gadsby 2002; Gerich 1993).

Although in its early stages a good management of patient diet and lifestyle is sufficient, most diabetes patients require regular blood glucose monitoring. In fact, frequent blood glucose monitoring has been described to reduce episodes of hyper-/hypoglycemia, as well as to delay and even prevent some of the diabetes related complications (Battelino et al. 2011; Block et al. 2008; Hermanides et al. 2011; McAndrew et al. 2007).

Nowadays, blood glucose monitoring is often achieved by self-monitoring of blood glucose (SMBG) (Benjamin 2002; Garg et al. 2006; Knapp et al. 2009; Penfornis et al. 2011). This type of monitoring relies almost solely on the use of hand-held glucometers, based on the “finger-prick” method. Depending on the severity of the disease, patients are required to monitor its blood levels from 3 up to 10 or more times per day. Despite significant advances in this technique, SMBG by this method is far from optimal. Non-adherence, due to inconvenience and pain, as well as poor scheduling for the measurements are amongst the main drawbacks of this method, hampering proper management of the disease (Benjamin 2002; Heinemann 2008; Penfornis et al. 2011).

In the past decades a few alternatives emerged in order to overcome the burden of SMBG with the “finger-prick” method, and improve SMBG. Significant advances in the knowledge of the disease, combined with fast advances in biosensor technology allowed the development of devices that allow real CGM (D’Archangelo 2009; DeVries 2012; Hermanides et al. 2011;
The use of CGM devices enables a more accurate picture in terms of glucose level anamnesis. Moreover, CGM devices provide trend data allowing prediction of changes in glucose levels. Additionally, these devices yield constant feedback on how multiple variables impact glucose control. Furthermore, when combined with blood glucose measurements CGM devices allow more confidence in diabetes management (Block et al. 2008; Garg et al. 2011; Garg et al. 2009). Presently, most if not all CGM devices are at least partially implantable and rely on amperometric enzyme-based biosensors (McGarraugh 2009).

Despite a rapid growth in proof-of-concept biosensor-based CGM devices, and even some marketed designs, CGM devices can only be considered as adjuvants in diabetes management (Peters et al. 2016; Rodbard 2016). Limited accuracy, thus poor reliability of CGM, are still the major hindrances to the use of data provided by CGM for decision making in diabetes management. Regarding this inaccuracy, there are a few factors that are common to most of the CGM. The need of frequent calibration, poor selectivity, poor biocompatibility, inducing extensive inflammatory processes and high sensor fouling are amongst the most relevant sources of CGM inaccuracy (Facchinetti et al. 2010; Group 2006; Kovatchev et al. 2008; Mazze et al. 2009; Nichols and Klonoff 2007; Vaddiraju et al. 2010). Additionally, the marketed biosensor based CGM are almost exclusively tethered to relatively bulky devices, leading to a high degree of non-compliance by diabetic patients (Garg et al. 2011). Even 30 years after its inception, there is still a need to of better, miniaturized and comfortable CGM devices.

Therefore, we developed and characterized (both in vitro and in vivo) a completely implantable wireless microbiosensor device (iMBD) for CGM. First we have evaluated the performance of several implantable needle-type amperometric enzyme-based glucose microbiosensors designs, in vitro. The most suitable design was incorporated in the assembly of the iMBD based on a self-referencing system (Cordeiro et al. 2015b; Wahono et al. 2012). The developed iMBD was fully implanted in the subcutaneous tissue of conscious, freely moving rats, for 5 days. The oxidation currents of the iMBD were converted into glucose levels using either a single or a multiple point blood glucose calibration. The ability of the iMBD to monitor changes in subcutaneous glucose was assessed by modulating pharmacologically glucose levels, by i.v. administration of glucose and insulin. After in vivo evaluation, the CGM device was explanted and its biosensors were re-evaluated in vitro, in order to determine the effect of biofouling.

### 4.2- Materials and Methods

#### 4.2.1- Materials

Glucose Oxidase (Aspergillus Niger, Type-XII, 100 kU), bovine serum albumin (BSA)
(fraction V 99%), glutaraldehyde (grade I, 25%, aqueous solution), Nafion (25% v/V in aliphatic alcohols), m-phenylenediamine, glucose, uric acid (UA), l-ascorbic acid (AA) were obtained from Sigma (St. Louis, Missouri, USA) without further purification. Regenerated Cellulose (RC; cut-off 15 kDa) and PolyEthylene (PE; cut-off 2 MDa) membranes were obtained from Brainlink, BV (Groningen, The Netherlands). Humuline RU-500 (recombinant human insulin) was purchased from Lilly (USA).

Platinum (100 and 200 µm Ø), Platinum/Iridium (125 µm Ø) and Silver wire (125 µm Ø) were purchased from Advent Research Materials (Oxford, UK). A phosphate buffer solution (PBS) was used containing 145 mM Na⁺, 1.2 mM Ca²⁺, 2.7 mm K⁺, 1.0 mM Mg²⁺, 152 mm Cl⁻ and 2.0 mM PO₄⁻ in ultrapurified water, brought to pH 7.4 with sodium hydroxide and degassed before use.

4.2.2- Biosensor assembly

The surfaces of needle-type (Pt or PtIr; 100, 200 Ø µm and 125 µm; 5 mm long) surfaces were functionalized into glucose biosensors, as previously described. All microelectrodes were first functionalized with a permselective layer, either Nafion or Nafion –PmPD (Cordeiro et al. 2016b; Cordeiro et al. 2015a). After, functionalized microelectrodes were manually coated with an enzymatic hydrogel, (glucose oxidase (GOx) (1 U/µL) reticulated with glutaraldehyde (GA) and bovine serum albumin (BSA))(Cordeiro et al. 2015a; Wahono et al. 2012). Finally a hollow dialysis membrane (either PE or RC) was applied to all glucose biosensors (Fig 1 C).

4.2.3- In vitro characterization

All biosensors were evaluated electrochemically, by in vitro calibrations. All calibrations were carried out in PBS (pH 7.4) at +700 mV vs. Ag/AgCl using a potentiostat (Pinnacle, model 3104 Pinnacle Tech. Inc., USA). 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. After, AA and UA (200 and 250 µM respectively), were added, consecutively, prior to consecutive additions of Glucose (0.02 to 25 mM). Noise and limit of detection (LOD) were calculated by linear regression, whereas linear range (LR), linear range slope (LRS), apparent Michaelis-Menten constant (appK_M) and maximum current intensity (I_{Max}) were calculated using non-linear regression (Cordeiro et al. 2015a).
4.2.4 – iMBD assembly

The iMBD device was assembled by coupling a small prototype telemetric amperostat (≤ 20 cm³) (DSI, St Paul, Minneapolis, USA) with fixed potential (+700 mV) to an implantable microbiosensor device (iMBD), using flexible leads (Fig 1 D). The device was based on the self-referencing system, thus comprised a two working electrodes (Sensor and Background) and a Reference electrode (Ag/AgCl) (Fig 1 C). After assembly, the the iMBD was calibrated in vitro. When possible (5 out of 7 devices), the iMBD was re-calibrated after its implantation.

4.2.5 - In vivo CGM evaluation

The performance of the iMBD was evaluated in vivo by implanting them in the subcutaneous tissue of rats. Male Wistar rats (350-425g) (Harlan, Horst, The Netherlands) were used in all in vivo experiments. Animals were individually housed in Plexiglas cages prior to the experiment. All animals were submitted to surgery to implant a permanent jugular vein catheter (for frequent blood sampling and compound administration) and for
subcutaneous implantation of the iMBD (Fig 1 B). All in vivo electrochemical measurements were performed at a constant potential, 700 mV vs Ag/AgCl. After the surgery, animals were allowed to recover and subcutaneous glucose levels were continuously monitored by the iMBD immediately after recovering (Fig 1 A). Blood glucose levels were monitored at least once daily, using colorimetric glucose strips (AccuChek; Roche).

After a period of iMBD signal stabilization, glucose levels were experimentally modulated by consecutive intravenous administration of vehicle (saline 1ml/kg), glucose (20 % w/V in saline) and insulin (5U/kg) at intervals of 45 minutes, as previously described (Cordeiro et al. 2015a; Moon et al. 2013). During the period of glucose levels modulation, blood glucose was assessed at intervals of 15 minutes. Animals were sacrificed immediately after the experiment by i.v. administration of pentobarbital. The MBD electrochemical signal was acquired 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.

4.2.6- Data analysis

4.2.6.1- Biosensor performance parameters

Analytical and kinetic parameters were calculated by non-linear regression using GraphPad Prism 5.0. The calculated parameters include limit of detection (LOD), linear Range (LR) linear range slope (LRS) Michalis-Menten constant ($K_M$), maximum current intensity ($I_{MAX}$). All data were presented as Mean±standard error of the mean (SEM). All calculated parameters were statistically evaluated either by One-Way or Two- Way ANOVA. When necessary, additional Bonferroni tests were performed. 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

4.2.6.2- In vivo evaluation

The currents measured by the electrodes incorporated in the iMBD were converted into glucose levels using customized algorithms, based either on post calibration evaluation or based on single (equation 1) or multiple point blood glucose calibrations (equations 2).”
Single point blood calibration (SPBC):

\[
SC_{\text{glc}} \text{ (mM)} = \frac{[Bl_{\text{glc}} \text{ (mM)}_0 \times (S_t(nA) - PcSB(nA)) - (BG_t(nA) - PcBGB(nA))]}{(S_{t0}(nA) - PcSB(nA)) - (BG_{t0}(nA) - PcBGB(nA))}
\]

*Equation 1*

Multiple point blood calibration (MPBC):

\[
SC_{\text{glc}} \text{ (mM)} = \frac{\text{Subtraction (nA)} - \text{in vivo baseline (nA)}}{\text{In vivo biosensor sensitivity (nA} \cdot \text{mM}^{-1})}
\]

*Equation 2*

Where;

- \( SC_{\text{glc}} \) - Subcutaneous glucose (mM)
- \( Bl_{\text{glc}} \) - Blood glucose (mM)
- \( S_t \) - Sensor current (nA)
- \( BG_t \) - Background sensor current (nA)
- \( PcSB \) - Post Calibration Sensor Basal current (nA)
- \( PcBGB \) - Post Calibration Background Basal current
- \( \text{Subtraction} \) - \((S_t(nA)-PcSB(nA))-(BG_t(nA)-PcBGB(nA))\)
4.3- Results and Discussion

4.3.1- In vitro biosensor characterization

4.3.1.1- Pre Calibration

Prior to the assembly of the iMBD we evaluated a series of implantable needle type glucose biosensors, to assess which would be the most suitable for implantation. We characterized four different needle type amperometric glucose biosensors, in vitro (Pt$_{100}$/Nf/Gox/RC; Pt$_{100}$/Nf/PmPD/Gox/RC, Pt$_{200}$/Nf/Gox/PE and PtIr$_{125}$/Nf/Gox/PE).

The glucose biosensors evaluated were assembled based on different permselective layers (Nafion and Nafion-PmPD) and with different outer dialysis membrane (either RC or PE). Whilst the use of a permselective membrane enables selectivity of the sensors towards electrochemical interference (Cordeiro et al. 2016a; McMahon et al. 2004; O’Neill et al. 2008; Wahono et al. 2012), the outer membrane can both minimize biofouling effects and improve the biosensor LR (Cordeiro et al. 2015a; Koh et al. 2011; Wisniewski and Reichert 2000).

All biosensors were, as expected due to the incorporation of the permselective membranes, selective to electrochemical interference. Additionally, all biosensors responded to even the lowest glucose levels tested (50 µM). Moreover, all biosensors displayed very fast response time ($t_{95} \leq 1s$). Nevertheless, we did observe significant differences in the oxidation currents of different types of biosensors when exposed to the same glucose concentrations (Fig 2).

![Figure 2](image)

**Figure 2** – In vitro glucose calibration of the tested implantable needle type amperometric glucose biosensors. Data are mean ± SEM.

*In vitro* evaluation revealed that all biosensors displayed a linear correlation between
glucose and oxidation currents for low analyte levels (≤ 5 mM). However, for high glucose levels the linearity decreased.

Biosensors coated with Nafion alone displayed similar oxidation currents amongst them when exposed to increasing glucose levels. However, biosensors coated with Nafion in combination with PmPD displayed significantly lower oxidation currents when exposed to high glucose levels compared with all other biosensor types (p≤0.05). This can be explained by the lower surface availability of electrodes coated with membrane combinations when compared with microelectrodes functionalized with Nafion alone (Cordeiro et al. 2016a).

The use of non-linear regression methods applied to a simplified Michaelis-Menten model for biosensors (O’Neill et al. 2008) allowed us to estimate the most relevant biosensor performance parameters based on the in vitro calibrations. All biosensors displayed a very high correlation with the theoretical model (R² ≥ 0.99).

Table 1- In vitro performance parameters (Limit of Detection (LOD), apparent affinity constant (app K_M), maximum current (I_max), linear range (LR) and linear range slope (LRS). Data are Mean±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Nf/mPD/GOx/RC</th>
<th>Nf/GOx/RC</th>
<th>Nf/GOx/PES</th>
<th>Nf/GOx/PES</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Electrode</td>
<td>Platinum</td>
<td>Platinum</td>
<td>Platinum</td>
<td>Platinum/Iridium</td>
</tr>
<tr>
<td>Ø (µm)</td>
<td>100</td>
<td>100</td>
<td>200</td>
<td>125</td>
</tr>
<tr>
<td>Noise (nA)</td>
<td>0.12 ± 0.04</td>
<td>0.19 ± 0.06</td>
<td>0.21 ± 0.09</td>
<td>0.044 ± 0.008</td>
</tr>
<tr>
<td>LOD (µM)</td>
<td>26.61 ± 13.90</td>
<td>12.87 ± 5.60</td>
<td>13.73 ± 5.29</td>
<td>4.00 ± 1.24</td>
</tr>
<tr>
<td>app K_M (mM)</td>
<td>11.42 ± 1.01</td>
<td>26.41 ± 2.94</td>
<td>22.68 ± 3.76</td>
<td>8.67 ± 0.15</td>
</tr>
<tr>
<td>I_max (nA)</td>
<td>405.4 ± 16.3</td>
<td>1880.0 ± 127.8</td>
<td>1755.0 ± 168.6</td>
<td>762.3 ± 5.1</td>
</tr>
<tr>
<td>LR (mM)</td>
<td>5.71 ± 0.5</td>
<td>13.2 ± 1.5</td>
<td>11.3 ± 1.9</td>
<td>4.3 ± 0.07</td>
</tr>
<tr>
<td>LRS (nA/mM)</td>
<td>35.5 ± 16.0</td>
<td>71.2 ± 43.4</td>
<td>77.4 ± 44.8</td>
<td>87.9 ± 34.9</td>
</tr>
</tbody>
</table>

All biosensors displayed very low noise levels (≤0.2 nA). Nevertheless, the noise levels of PtIr_125/Nafion/GOx/PE were lower than all other biosensor designs (0.044 ± 0.008 vs all, p≤ 0.05). The use of an alloy in microelectrode assembly, may have resulted in a shift in the optimal oxidation potential of the electrode, thus reducing noise levels. It has been reported that Iridium has a slightly higher optimum oxidation potential for H₂O₂ (Bianchi et al. 1961).

As the LOD is a function of the noise, all biosensors displayed low LOD (≤30µM), adequate for in vivo subcutaneous glucose biomonitoring (Bindra et al. 1991; Bolinder et al. 1992; Lourido et al. 2002; Rebrin et al. 1999). As expected, PtIr_125/Nafion/GOx/PE had a
lower LOD than any other biosensor design (4.00±1.24 µM vs all, p≤ 0.001).

Besides noise and LOD, we also estimated both the apparent affinity (appK_M) constant and its derivative LR. Our results showed that biosensors based on PtIr alloys displayed the highest affinity amongst all glucose biosensors (8.67±0.15 mM vs all, p≤0.01), followed by Pt_{100}/Nafion/PmPD/GOx/RC (11.42±1.01 mM). Both Pt_{100}/Nafion/GOx/RC and Pt_{200}/Nafion/GOx/PE displayed the lowest affinities towards glucose (≥ 20 mM) very close to those observed for freely diffused enzyme. The differences observed in affinity were not unexpected. It has been described that the appK_M is dependent on some of the parameters that were modulated in biosensors assembly, such as electrode active surface and the use of an outer membranes (Cordeiro et al. 2016a; Cordeiro et al. 2015b). An increase in the active surface resulted in an increase in the appK_M due to the ability to increase the amount of enzyme immobilized per area (Cooney 2011; House et al. 2007; O’Neill et al. 2008; Rothwell et al. 2010; Tan et al. 2010). The use of an outer membrane resulted in much higher affinity constants than previously reported for implantable amperometric enzyme-based glucose biosensors (Abel and von Woedtke 2002; Cordeiro et al. 2015a; Lowry et al. 1998; O’Neill et al. 2008; Vasylieva et al. 2011; Yang et al. 2002).

The use of Nafion alone resulted in biosensor with less affinity (lower appK_M) towards glucose, but increased its linear range, when compared to those coated with Nafion-PmPD. Here too it seems that surface availability, lower for membrane combinations may have played a role. Despite having less surface, the Pt_{100}/Nafion/RC biosensors had similar LR than Pt_{200}/Nafion/PE, indicating similar affinities. Different enzyme loading and diffusion rates, mediated by the choice of outer membrane, are the reason for this phenomena. Although, the higher geometrical surface from Pt_{200}/Nafion/PE allowed an increase of enzyme immobilized in the electrode surface. However the use of a membrane with a lower cut off (15 kDa vs 2 MDa) in Pt_{100}/Nafion/RC, thus reducing glucose diffusion rate of glucose, appeared to have counterbalanced the decrease in microelectrode surface, resulting in similar affinity of both geometries.

As LR is dependent of appK_M, the results obtained for LR were similar to those obtained for appK_M. We observed that both the PtIr_{25}/Nafion/GOx/PE and Pt_{100}/Nafion/mPD/GOx/RC biosensors showed a lower linear range, 4.3±0.07 and 5.71±0.5 mM than the other designs. Both Pt_{100}/Nafion/GOx/RC and Pt_{200}/Nafion/GOx/PE glucose biosensors exhibited higher linear ranges, when compared with the previous described geometries, 13.2±1.5 and 11.3±1.9 mM, respectively.

Implantable glucose biosensor should be able to detect changes in glucose within the media their physiological relevant levels. It has been described that resting blood glucose levels in awake freely moving rats to be around 5-6 mM. However, these levels can reach from 2 to 15 mM in cases of hypo- and hyperglycemia, respectively (Cordeiro et al. 2015a; de Vries et al. 2003; de Vries et al. 2005).

Therefore due its limited LR (4.3 and 5.75 mM respectively) Pt_{100}/Nafion/mPD/Gox/RC and PtIr_{125}/Nafion/PE were not considered suitable for implantation.
The $I_{\text{Max}}$ was also higher for Pt$_{100}$/Nafion/GOx/RC and Pt$_{200}$/Nafion/GOx/PE (1880.0±127.8 and 1755.0±168.6 nA, $p \geq 0.001$) when compared to PtIr$_{125}$/Nafion/GOx/PE and Pt$_{100}$/Nafion/mPD/GOx/RC. The low surface availability (Cordeiro et al. 2016a), due to the use of permselective membrane combinations, in the case of Pt$_{100}$/Nafion/mPD/GOx/RC and the use of an alloy for the assembly of PtIr$_{125}$/Nafion/GOx/PE can explain the low $I_{\text{Max}}$ values. Despite the different results obtained for most of the kinetic parameters, LRS was similar for Pt$_{100}$/Nafion/GOx/RC, Pt$_{200}$/Nafion/GOx/PE and PtIr125/Nafion/GOx/PE. Only the Pt$_{100}$/Nafion/PmPD/GOx/RC biosensors displayed lower LRS than the other glucose biosensors tested (35.5 ± 16.0 nA/mM vs all, $p \leq 0.05$). Once again, the low surface availability of this type of sensors resulted in lower biosensor performance.

Based on the electrochemical evaluation, both Pt$_{100}$/Nafion/GOx/RC and Pt$_{200}$/Nafion/PE designs were considered the most suitable biosensors for incorporation into the wireless CGM device. Unfortunately, preliminary experiments showed that Pt$_{100}$/Nafion/GOx/RC biosensors were not sufficiently rigid to withstand implantation. Therefore, all iMBD incorporated Pt$_{200}$/Nafion/GOx/PE biosensors in its construction.

### 4.3.1.2- Post Calibration evaluation

In order to assess biofouling effects, we have evaluated the performance of the biosensors incorporated in the iMBD in vitro following its implantation (5 days). Unfortunately, from the seven implanted devices, sensors incorporated in the iMBD were severely damaged upon explantation, and were not re-evaluated.

![Figure 3](image-url)  
**Figure 3**- Calibration of the glucose biosensor incorporated in the iMBD for CGM, before (n=7) and after (n=5) its implantation. Data are mean±SEM.
All biosensors were selective against electrochemical interference and responded with an increase in oxidation current to consecutive additions of glucose, within the entire calibration range. As observed during the pre-calibration, we found a linear correlation between glucose and the oxidation currents for low analyte concentrations (≤ 5 mM). Once again, this linear correlation was lost for higher glucose levels, resulting in a Michaelis-Menten like profile, as observed in the pre-calibration.

Despite the similarity in the calibration profile, the oxidation currents of the biosensors post implantation were significantly lower (up to 3 fold) than those obtained in the pre-calibration, for most of the calibration range (≥ 0.5 mM, p≤ 0.05), most likely due to the biofouling effect. Nevertheless, the data obtained were well correlated with the theoretical model used to estimate the most relevant biosensor performance parameters (Table 2).

Table 2- *In vitro* performance parameters of the Pt_{200}/Nafion/Gox/PE biosensors prior and post implantation. Data are mean±SEM.

<table>
<thead>
<tr>
<th></th>
<th>PreCalibration</th>
<th>PostCalibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Noise (nA)</td>
<td>0.21±0.09</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>LOD (µM)</td>
<td>13.73±5.29</td>
<td>22.45±2.80</td>
</tr>
<tr>
<td>K_{app} (mM)</td>
<td>22.68±3.76</td>
<td>14.50±0.90</td>
</tr>
<tr>
<td>I_{Max} (nA)</td>
<td>1755.00±168.60</td>
<td>337.90±10.34</td>
</tr>
<tr>
<td>LR (mM)</td>
<td>11.3±1.9</td>
<td>7.25±0.45</td>
</tr>
<tr>
<td>LRS (nA/mM)</td>
<td>77.4±44.8</td>
<td>23.30±11.43</td>
</tr>
<tr>
<td>R²</td>
<td>0.996</td>
<td>0.999</td>
</tr>
</tbody>
</table>

However, after 5 days of subcutaneous implantation, some biosensors performance parameters were significantly altered, when compared with pre-implantation values. The noise levels, along with the LOD and LRS were unaffected by iMDB implantation. No changes in noise levels suggests a high stability of all electrical connections within the iMBD, even after 5 days of implantation. One of the effects of biofouling is the degradation of the electrical connections, even for short implantation periods (Wisniewski et al. 2000). Our iMBD appears to resist to these effects since it provided continuous measurements for the whole experiment.

We observed a significant decrease in K_{app} (14.50±0.90 vs 22.68±3.76 mM, p≤0.001), thus increase in affinity, after implantation. This decrease is most likely due to a decrease in the
amount of immobilized enzymes that remained active. This would lead to a faster saturation of the enzyme, thus oxidation currents, when compared with pre-calibration performance. The significant decrease in \( I_{\text{Max}} \), further supports this hypothesis.

Despite its importance has been previously described, post-implantation evaluation is very uncommon. Although a few studies reported the effects of biofouling through post-implantation evaluation (Calia et al. 2009; Vasylieva et al. 2011; Wahono et al. 2012), only one has evaluated the effect of biofouling on biosensor affinity (Cordeiro et al. 2015b). In that study, an increase in \( appK_M \) was observed following acute implantation (8 hours) of needle type biosensors in the brain. The differences in duration and site of implantation may be the reason for the discrepancies in the effect of implantation of \( appK_M \).

As a derivative of \( appK_M \), we also observed a decrease in LR (11.3±1.9 vs 7.25±0.45 mM, \( p \leq 0.05 \)). Previously, a decrease in LR, to levels underneath the complete pathophysiological range, would imply lower accuracy to monitor increases in glucose levels. However, advances in non-linear regression analysis and algorithm construction regarding biosensor technology (Ford et al. 2016; O’Neill et al. 2008), allow an accurate glucose monitoring with these biosensors, accommodating the loss in LR.

Besides a decrease in \( appK_M \), we also observed a decrease in \( I_{\text{Max}} \) after implantation. The values obtained on the post-calibration are more than 4-fold lower than those obtained in the pre-calibration (1755.0±168.6 vs 337.90±10.34 nA, \( p \leq 0.001 \)). The decrease in \( I_{\text{Max}} \) is closely related to the decrease in the amount of active enzymes, revealed by the decrease in \( appK_M \). However, since the magnitude of the decrease in this parameter largely exceeds the decrease in \( appK_M \), enzyme deactivation/degradation it may not be the only factor to affect \( I_{\text{Max}} \). It has been reported that one of the biofouling effects implies the adsorption of molecules of low molecular weight onto the electrode surface. This adsorption can result in a significant decrease in electrode active surface, thus biosensors sensitivity. As \( I_{\text{Max}} \) is dependent on the electrode active surface (Cordeiro et al. 2016a), it is possible that the striking decrease in \( I_{\text{Max}} \) may be due to a cumulative effect of enzyme inactivation and reduction in surface availability.

Despite a decrease in \( I_{\text{Max}} \) and \( appK_M \), we found no differences in the LRS before and after implantation (77.4±44.8 vs 23.30±11.43 nA/mM). The non-significantly different values in LRS can be easily explained by the decrease in LR after implantation. In other words, although the LRS was similar, due to a decrease in LR, biosensor sensitivity was effectively lower after implantation. Nevertheless the LRS after implantation is higher than the sensitivity of previously described glucose biosensor, successfully implanted in the subcutaneous tissue of animal and human subjects. (Ahmad et al. 2008; Calia et al. 2009; Daniloff 1999; Palmisano et al. 2000; Preidel et al. 1993; Rocchitta et al. 2013; Thomé-Duret et al. ; Vasylieva et al. 2011; Wilson and Gifford 2005).

Finally, we also did not observe any differences in the LOD estimated from the post-calibration, when compared with values obtained prior to the implantation (13.73±5.29 vs 22.45±2.8 µM). These data suggest that the biosensors were able to monitor small changes
in subcutaneous glucose levels, accurately.

Taken together, the post-calibration data assurers that the results obtained by the implanted iMBD were reliable. Despite a sharp decrease in its performance, the biosensors incorporated in the iMBD were able to accurately monitor subcutaneous glucose levels. Nevertheless, our evaluation revealed a strong influence of biofouling, triggered by the foreign body reaction, on overall biosensor performance. Although the biosensors were designed to diminish biofouling, by the incorporation of an outer membrane, its performance was still significantly affected by its implantation.

4.3.2- In vivo iMBD evaluation

To assess the in vivo performance of the iMBD for CGM, we implanted a series of devices (n=7) in the subcutaneous tissue of male Wistar rats (Figure 1 B). The devices were implanted for 5 days. During the course of the experiments, we evaluated the ability of the iMBD to monitor changes in subcutaneous glucose levels, by i.v. administration of glucose and insulin.

4.3.2.1- In vivo stability

The iMBD was turned on to start high frequency data acquisition (10 Hz), as soon as the animals recovered from surgery. The currents obtained by the iMBD were converted in subcutaneous ISF glucose levels, using the traditional single point calibration algorithm (Choleau et al. 2002).
Our data (Figure 4) showed that all the iMBDs provided continuous measurements for the entire duration of the experiment. However, we observed a marked decrease in subcutaneous glucose levels, that started immediately upon the start of the experiment. The decrease in current followed a similar pattern during the first 48 hours post-implantation, for all iMBD. We observed, a sharp decrease immediately after the start of the experiment that tapered until reaching a stable steady-state signal.

While blood glucose levels remained fairly constant during the 5-day implantation period, subcutaneous glucose levels provided by the iMBD were only well correlated with blood glucose levels after the stabilization period of the device. The low correlation between the subcutaneous levels provided by the iMBD and blood glucose for the first 48 hours post implantation, despite the use of a membrane with high biocompatibility, is apparently the result of a foreign body reaction (FBR) (Koschwanetz and Reichert 2007; Wisniewski et al. 2000).

Acute FBR also implies a severe alteration of local homeostasis (Anderson et al. 2008; Ward 2008). This abnormal homeostasis is due to the rupture of local blood vessels and it results in large fluctuations in the content respective levels of the existing analytes. It has been described a significant increase in local glucose consumption, at the early stages of FBR, especially during the strong initial inflammatory response. An increase in glucose consumption is directly coupled to an increase in local glucose. Although this high

**Figure 4** - *In vivo* subcutaneous converted glucose levels, averaged hourly, measured by the CGM device compared with blood glucose levels during the 5 days of CGM implantation. Data are mean±SEM
consumption converted by macrophages can lead to an increase in $\text{H}_2\text{O}_2$ (Koh et al. 2011), the use of a self-referencing system eliminates this source of inaccuracy. Therefore, the initial high glucose provided by the iMBD may also be due to a “truly” high glucose concentration on the implant site. A lower decrease in biosensor LRS upon explanation ($\leq 3$ fold after 5 days of implantation) when compared with the difference between blood and subcutaneous glucose further support the measured high local glucose levels.

The FBR is the result of an immune response triggered by inflicted tissue trauma, in this case iMBD implantation device (Wang et al. 2015). It can be divided into acute (up to a couple of days/weeks) and chronic phases (weeks to years). The initial decrease in measured glucose levels is probably related to the acute phase of the FBR, due to the relatively short implantation time. This acute response includes strong inflammatory processes. These inflammatory processes have been associated with events that reportedly decrease in biosensor sensitivity. Those events can be diverse and can include the recruiting of proteolytic enzymes to the tissue damage site, decrease in pH and high concentration of reactive oxygen species. Additionally, the acute FBR also includes the adhesion of proteins and cells onto the implanted material, in this case the iMBD, which creates a diffusional barrier. The sum of these FBR-triggered events leads to the presently observed biofouling. Biofouling effects such as electrode passivation, membrane dilapidation and biodegradation and fibrous encapsulation, might explain the high subcutaneous glucose levels measured by the iMBD, during the initial phase of the experiment.

Nonetheless, the development of better algorithms, based on an extended database from further in vivo experiments, will most likely increase the accuracy of the subcutaneous glucose values provide the iMBD for CGM. An algorithm able to correct for the variations in glucose levels provided by the iMBD will allow a better correlation between blood and subcutaneous glucose levels, and even reduce the in vivo stabilization time.

**4.3.2.2- In vivo biomonitoring of dynamic changes in glucose with the iMBD**

Besides evaluation of the ability of the iMBD to monitor glucose continuously, we also tested whether the device was able to pick up fast changes in glucose levels. Therefore, after stabilization of the iMBD (at least 40 hours after implantation), we induced hyperglycemia and hypoglycemia states to the animals with the iMBD, by i.v. administration of glucose (20% m/V) and Insulin (5U/Kg). Saline administrations (1 mL/Kg, i.v.) were used as control. The absolute glucose levels (both from blood and ISF) as well their relative changes are depicted in Figure 5 (A-C).

The currents provided by the individual sensors within the iMBD, following in vivo stabilization, were 81.9±14.9 nA and 33.7±4.2 nA for sensor and BG, respectively. The subtracted current was 53.2±15.7 nA, which was converted into 5.9±0.3 mM, and 8.0±1.8 mM of glucose, using either single (Equation 1) or multiple point blood calibration (Equation 2), respectively. Basal blood glucose levels upon iMBD stabilization were 6.2±0.5 mM. No
differences were observed between blood and subcutaneous glucose levels provided by the iMBD, regardless of the algorithm employed for the conversion. Additionally, these levels are well within the range described for both blood (de Vries et al. 2003; de Vries et al. 2005) and subcutaneous glucose levels in awake freely moving rats (Jamali et al. 2002).

Systemic administration of the vehicle had no effect in blood nor subcutaneous glucose levels. However, consecutive administration of glucose and insulin evoked significant changes in both blood and subcutaneous glucose. After glucose administration, blood glucose increased rapidly from 6.5±0.2 to 12.2±2.1 mM, reaching its highest value, 15 minutes after glucose administration. Subcutaneous glucose levels started to increase almost immediately after glucose administration, reaching its highest levels 7.2±0.2 mM (using a SPBC) and 10.1±1.9 (using a MPBC) 20 minutes after administration. Although changes in glucose were noticeable faster by the iMBD than those observed in the blood, we observed a lag time of 5 minutes between the highest blood and subcutaneous glucose levels. Additionally, our data show significant differences between glucose blood and subcutaneous glucose levels provided by the iMBD using a SPBC but not for the levels obtained through MPBC.

After reaching its maximum, blood glucose decreased sharply reaching basal levels 15 minutes after. Then, blood glucose remained unchanged until insulin administration. Instead, subcutaneous glucose levels decrease gradually at a much slower rate than blood glucose, reaching basal levels nearly 25 minutes after reaching its maximum level, just before insulin administration.

Both subcutaneous and blood glucose levels decreased in response to insulin administration; sharply for blood glucose and gradually for subcutaneous glucose. Blood glucose decreased rapidly from 6.2±0.2 to 3.3±0.3 mM, 15 minutes after insulin administration and remained fairly stable until the end of the experiment (120 min after saline administration). Once again, changes in subcutaneous glucose were noticeable faster when compared to those observed in the blood. Subcutaneous glucose started decrease immediately after insulin administration. However, subcutaneous glucose decreased at a much slower rate when compared with blood glucose, reaching its lowest level 2.9±0.7 mM at the end of the experiment.

Although we observed a significant lag time (10 min) between changes in blood and SG following insulin administration, no differences were observed in the absolute values, nor in the magnitude of changes in glucose.
Figure 5 - *In vivo* glucose levels provided by the iMBD following administration of vehicle (saline), and pharmacologically active compounds (glucose and insulin). A- Subcutaneous glucose levels provided by the iMBD using a single point blood calibration signal conversion algorithm B- Subcutaneous glucose levels provided by the iMBD using multiple point blood calibration signal conversion algorithm. C- Changes in subcutaneous glucose levels using a multiple point blood calibration method. Data are mean±SEM.

Our data show that the iMBD was able to monitor changes in subcutaneous glucose, within the same magnitude as those observed in the blood. Additionally the changes in both blood and subcutaneous glucose following administration of glucose and insulin, are in accordance with those previously reported, despite the differences in biomonitoring methods (de Vries et al. 2003; de Vries et al. 2005; Moon et al. 2013). However, although a relationship between blood and ISF glucose has been established, the debate on how these two parameters are related is ongoing. While some authors report the same magnitude in the changes in blood and subcutaneous glucose, lower subcutaneous glucose levels when compared with blood glucose have also been reported (Bolinder et al. 1992; Garg et al. 2006; Holmång et al. 1998; Lourido et al. 2002; Pescia et al. 2003; Rebrin et al. 1999; Rosdahl et al. 1993; Thennadil et al. 2001).

Despite good correlation in the magnitude of changes, we observed some lag when comparing the kinetics of blood and subcutaneous glucose. Although the time resolution for
blood glucose levels is much lower when compared with subcutaneous glucose (1 sample per 15 minutes vs 10 Hz), it is still higher than that typically CGM devices used by diabetic patients (Benjamin 2002; Hermanides et al. 2011; Penfornis et al. 2011; Vazeou).

The lag times observed after glucose and insulin administrations are not significantly different from those reported for other biosensor based CGM (Keenan et al. 2009; Mazze et al. 2009; Thennadil et al. 2001; Vaddiraju et al. 2010; Wentholt et al. 2008). Interestingly, we observed that, in the case of the proposed CGM device, the lag time seems to be dependent of the direction of the changes in glucose. The lag time is longer (10 min) when glucose levels decrease compared when they increase (5 min). As for the differences in magnitude, there is a large variation in reported lag times observed in CGM devices. The lag times can range from 5 to 30 minutes and depend on several factors. These factors include size of the device, biosensor dimension and geometry, site of implantation and even biomaterials used in its construction (Bolinder et al. 1992; Daniloff 1999; Facchinetti et al. 2007; Keenan et al. 2009; Lodwig and Heinemann 2003; Rebrin et al. 1999; Regittnig et al. 2003; Schaupp et al. 1999; Thennadil et al. 2001). Moreover, the combinations of these factors make the lag times largely variable amongst different CGM. Nevertheless, our data suggest that the proposed iMBD is able to monitor fast changes in glucose, with an adequate lag time. Further in vivo characterization of the iMBD will most likely provide better insights on the iMBD behavior, and may result in an improvement of the algorithm used in the conversion of subtracted currents into subcutaneous glucose.

4.3.2.3- Modelling the iMBD output

The recent focus on the use of complex modeling of in vivo data provided by biosensor based CGM is thought to largely improve the accuracy and in vivo lifetime of these devices (Facchinetti et al. 2010; Mazze et al. 2009; McGarraugh et al. 2011). However, due to the innumerous device dependent parameters involved, it seems that choice of algorithm may be dependent on the device itself. Our data further supports this idea.

For the proposed iMBD, the choice of algorithms had significant implications on the output of the CGM iMBD. The oxidation currents provided by the iMBD were converted into subcutaneous glucose levels, according to a classical SPBC approach, but also using a novel MPBC method. The use of a SPBC resulted in lower amplitude of changes in subcutaneous glucose when compared to those obtained using a MPBC.
Both correlation analysis (Table 3) showed a significant positive correlation ($R \geq 0.5$, $p \leq 0.05$) between subcutaneous and blood glucose levels using a MPBC. We have also obtained a positive correlation between blood glucose and subcutaneous glucose levels converted using a SPBC method. However, the correlation was only significant when we applied the less stringent method (Spearman Rank Order) but its correlation coefficient was lower than for MPBC conversion algorithm.

Although the use of a SPBC algorithm allowed us to have a good correlation between blood and subcutaneous glucose levels, the use of a MPBC resulted in a better correlation. Although the use of MPBC has been reported to lead to lower accuracy when compared with SPBC (Mahmoudi et al. 2014), our data suggests the opposite. The use of a third calibration point is likely to be the reason for a better performance of the MPBC, when compared with SPBC method.

Nevertheless our correlation levels are still hampered by the existence of lag times for changes in glucose levels. An increase in blood sampling frequency during experimental conditions combined with the development of an algorithm that would also take into account the lag time may result in an even better correlation between subcutaneous and blood glucose.

### 4.5- Conclusion

We developed and characterized a fully implantable wireless iMBD, for CGM. Initially, we assembled and characterized a series of needle type amperometric enzyme based glucose biosensors. In vitro evaluation revealed that the Pt$_{200}$/Nafion/GOx/PE biosensor design was the most suitable for incorporation in the wireless iMBD.

The proposed iMBD, based on a self-referencing system, was fully implanted for a period of 5 days in the subcutaneous tissue of awake, freely moving rats. Despite a significant decrease in biosensor performance, due to foreign body reaction related biofouling, the iMBD was able to monitor continuously, subcutaneous glucose levels for 5 consecutive days. During this period, changes in subcutaneous glucose levels, modulated by pharmacological

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Table 3 – Analysis of correlation between blood and subcutaneous glucose, using a Single Point Blood Calibration (SPBC) or a Multiple Point Blood Calibration (MPBC) algorithm.
challenges, obtained by the iMBD were well correlated with changes in blood glucose levels. The use of a multiple point blood calibration algorithm provided a better correlation between subcutaneous and blood glucose, than the single point blood calibration algorithm. Nevertheless, additional in vivo characterization of the wireless iMBD will most likely further improve iMBD accuracy, expanding its already satisfactory in vivo lifetime.
4.6- Bibliography


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