CHAPTER 8

Summary, conclusions and outlook

Although biosensor technology has evolved tremendously, it has not reached yet its full potential. Although a variety of new devices have been reported, the amount of biosensor devices that actually made the transition from benchtop proof-of-concept to *in vivo* applications remains small. Nowadays, only a handful of sensor types are available for continuous biomonitoring of glucose in a clinically relevant environment. All biosensors designed for *in vivo* applications are electrochemical, mostly amperometric and enzyme-based. Current state-of-the art Continuous Glucose Monitoring (CGM) devices still heavily relies on this type of biosensors.

Despite the relative success of this type of biosensors in glucose biomonitoring, there are several aspects that hamper the daily use of these sensors by diabetic patients. Within this thesis I have identified, studied and hopefully contributed to a better understanding of some of those factors. Additionally I have attempted to apply these factors in the development and characterization of novel biosensors not only for CGM, but for *in vivo* biomonitoring in general.

In the first chapter (**Chapter 1**) it is explained why, there is still a need for a better CGM, despite decades of development in glucose monitoring tools in general, and biosensors in particular. Although various proof-of-concept biosensors, with multiple biorecognition elements coupled to a large array of transducers has been described, *in vivo* ~~bio~~monitoring with biosensors is still confined to electrochemical (amperometric) enzyme-based biosensors. Therefore the fundamentals of electrochemical biosensors mechanism, in particular enzyme-based ones is presented. It is concluded that a better understanding of the mechanisms underlying amperometric enzyme-based biosensors may be the key to improve the use of CGM in diabetic patients. Additionally it may enable the emergence of biosensors for continuous monitoring of other key biomarkers.

Then, in chapters 2 and 3, (**Chapters 2** and **3**) a series of experiments aimed at a better understanding of basic amperometric enzyme-based biosensors electrochemistry is described. **Chapter 2** is focused in understanding how permselective membranes (a major breakthrough in biosensor technology) enable improved selectivity of amperometric electrochemical biosensors. The surface of microelectrodes coated with various permselective membrane configurations was evaluated electrochemically and by SEM. All membranes were very effective in reducing electrochemical interference, but they also significantly reduced sensitivity towards target analyte. This effect was amplified in the cases of membrane combinations. Surface evaluation by SEM allowed the identification of an "inner polymerization" phenomena that pointed to a close relationship between (reductions) in surface availability and membrane selectivity.

In the next chapter (**Chapter 3**) we investigated how surface availability, modulated by the choice of membrane assembly, influenced the performance of amperometric enzyme-based biosensors. We found that biosensors based on permselective membranes with higher electrode active surface were more sensitive, without significant changes in their affinity for glucose. By using a model for kinetics of enzymes immobilized onto electrode

surfaces we found that I_{Max} and LRS, but not $_{app}K_M$ nor LR were dependent on biosensor surface availability. These data provide a better understanding of the relationship between enzyme kinetics and biosensors performance and the role played by surface availability. The knowledge acquired in **Chapter 2 and 3** was used to develop and characterize the biosensors described in the following chapters.

In **Chapter 4** we describe the development and characterization (*in vitro* and *in vivo*) of a novel biosensor device, for subcutaneous CGM in freely moving animal models. We evaluated *in vitro*, the performance of several designs, based on needle-type Pt based microelectrodes. We found that the use of a microdialysis membrane on biosensor assembly improved the intrinsically low LR of such biosensors. The most suitable biosensor design (PtIr/Nafion/GOx/PE) was then coupled to a wireless prototype, using a 2 channel potentiostat with a self-referencing system (Sensor and Background). The CGM wireless biosensor device was then implanted subcutaneously in freely moving rats. Its *in vivo* performance of the sensor was evaluated by submitting the animal to pharmacological challenges known to modify blood glucose levels. The described CGM was able to detect significant changes in subcutaneous glucose following intravenous administration of a glucose and insulin. We found a strong correlation in changes between blood glucose and the subcutaneous glucose levels monitored by the CGM regardless of the algorithm used to convert oxidation currents into subcutaneous glucose levels. Nevertheless, the use of multiple point blood calibration showed a higher correlation between blood and subcutaneous glucose levels. Although biofouling, due to foreign body response, had a significant deleterious impact in biosensor performance, the wireless CGM was able to accurately monitor glucose for 5 consecutive days. This prototype *i*MBD may pave the way towards a minimally invasive portable CGM.

The positive results of chapter 5 encouraged us to take a "leap of faith" and to try to bridge the gap between benchtop technology and "applicable" biosensor applications.

Sterility is a prerequisite for biomedical devices in order to be used routinely both in clinical environment and at home. Therefore, in **Chapter 5** we investigated the effect of several sterilization methods on biosensor performance, both acutely and up to one month after sterilization. Although the various sterilization methods had distinguishable effects on biosensor performance, all treatments caused a significant decrease in several key biosensor performance parameters. However, and despite such strong effects, some of the tested sterilization methods (EtOx, H_2O_2 (alone and combined with y-radiation) may allow a proper sterilization without impairing the ability the biosensor to selectively monitor glucose.

In the first chapter it is mentioned that the ability of the human body to regulate its own blood glucose levels is intrinsically related to the normal function of the endocrine system. In a diabetes patients, the ability of these mechanisms to regulate blood glucose levels is either severely impaired or, in extreme cases, absent. Therefore, diabetes patients rely on frequent glucose monitoring to maintain blood glucose levels within its "normal" range. Although glucose regulation is immediately associated to the endocrine system, new evidence points to an involvement of the CNS in glucose homeostasis. There is growing interest in the

putative ability of the brain to control blood glucose availability. Moreover, it is thought that abnormalities in brain energy metabolism may be associated with early diabetes stages.

Therefore in **Chapter 6** I describe the development and characterization of a multiplex biosensor device (MBD) for continuous and simultaneous *in vivo* biomonitoring of key biomarkers in brain energy metabolism. First we developed and characterized amperometric enzyme-based biosensors for *in vivo* biomonitoring of lactate and pyruvate. After we assembled a multiplex biosensor device comprising the most suitable pyruvate and lactate biosensors, along with the glucose biosensor (described in **Chapter 3**) and a background sensor. *In vivo* performance of the MBD was evaluated by submitting an anesthetized animal to pharmacological challenges known to induce, significant changes in blood glucose levels, as described in **Chapter 4.**

The prototype MBD was able to simultaneously and accurately monitor independently and simultaneously basal brain levels of all the target biomarkers (glucose, lactate pyruvate). Additionally, it was able to monitor, continuously, simultaneously and in real time, differential changes in glucose and lactate in response to the pharmacological challenges. Although the functionality of the pyruvate biosensors incorporated in the MBD was assessed after explanation, no significant changes in brain pyruvate were found. Nevertheless, the described MBD has proven to be a valuable tool to better understand the energetics of the brain and clarify its role on diabetes onset.

Despite the success of amperometric biosensors in brain monitoring, better spatial resolution reamains a goal in the development of new tools for experimental neuroscience. In **Chapter 7** we try to move towards the miniaturization of needle type amperometric enzyme based biosensors. Tungsten (W) is the strongest metal and microelectrodes based on tungsten might be downscaled to even a few nanometer in diameter. However, in order to use W microelectrodes as a basis for amperometric enzyme-based biosensors, its surfaces need to be coated with a highly electroactive metal, such a gold (Au). Therefore we have developed and characterized (*in vitro* and *in vivo*) biosensors based on W-Au needle type microelectrodes. We characterized the electrochemical profile of W-Au microelectrodes (bare and coated with permselective membranes) in presence of both target analytes and its putative electrochemical interfering compounds. This characterization allowed us to identify the most suitable potential to ensure continuous monitoring of H_2O_2 with high sensitivity and selectivity. These microelectrodes were then used to build glucose biosensors, whose performance was evaluated *in vitro* and *in vivo*. Amperometric enzyme-based W- Au based glucose biosensors were able to monitor, with high degree of sensitivity and selectivity, changes in glucose both *in vitro* and *in vivo*, in the brain of anesthetized rats.

8.1- Outlook

Although amperometric enzyme-based biosensors are already employed in *in vivo* biomonitoring, either in experimental physiology, or as diagnostic tool (in the case of the CGM devices) it is fair to assume that they haven't reached yet its true potential. There are far to few "real" applications of biosensors, when compared to the abundant proof-of-principle devices described in literature. For biosensors to be regarded as reliable bioanalytical tools, capable of providing data that can decisively impact either preclinical research and/or disease management in clinical settings, there is still a long way to go.

Nowadays, biosensors for *in vivo* biomonitoring still require miniaturization. Not only to increase its spatial resolution but also to enable better patient compliance. Biosensor miniaturization may be achieved by using new, more resistant materials in microelectrode manufacturing. However, as size does matter in terms of biosensor performance, the continuous downscale of these devices, will come with a price. As biosensors will get increasingly smaller, they will also become less sensitive. To overcome this size dependent limitation, surface modification will be necessary. The use of carbon nanotubes and metal-based nanoparticle, alone or in combination with conductive polymers or even graphene, may allow an adequate surface to area ratio in increasingly small, thus less invasive, biosensors.

However, miniaturization is not the only challenge faced by the biosensor community towards widespread application of biosensors in *in vivo* biomonitoring. As, at least in the next few decades, biosensors for in vivo biomonitoring will be most likely, invasive, better understanding of the FBR mechanism is fundamental. A deeper insight on FBR may lead to the necessary breakthrough in material sciences, enabling more favorable interactions of the biosensors with living tissues, with the obvious benefits for *in vivo* biomonitoring. Only then, biosensors can finally unleash its true potential as bioanalytical tools for in vivo biomonitoring.

Nederlandse Samenvatting

"Real-time biomonitoring" van de bloedsuikerspiegel in diabetespatiënten is een technologische uitdaging waarvoor nog geen optimale oplossing bestaat. Hoewel er reeds biosensoren in een klinische setting worden toegepast, staan lage selectiviteit en/of gevoeligheid, afstoting door het lichaam en een korte levensduur toepassing op populatieniveau in de weg. Het huidige onderzoek heeft zich daarom gericht op 1) het meer inzicht verkrijgen in de biochemische mechanismen die de eigenschappen van biosensors bepalen en 2) de ontwikkeling en optimalisatie van een elektrochemische sensor die online glucose, lactaat en pyruvaat kan meten in levend weefsel.

Het **eerste** hoofdstuk bespreekt de huidige stand van zaken in het onderzoeksveld. Aan bod komen de voor- en nadelen van bestaande klinische methoden om glucose te meten, met een focus op amperometrische biosensors op basis van enzymen. Hoofdstuk **twee** en **drie** gaan dieper in op de eigenschappen van verschillende ion-uitwisselende membranen, welke de selectiviteit van de biosensor versterken. In deze hoofdstukken blijkt dat een nafion membraan in combinatie met PPD de meest optimale *in vitro* biosensor eigenschappen bezit. Deze bevinding wordt preklinisch relevant in hoofdstuk vier, waar de gevoeligheid voor glucose en levensduur van de sensor wordt getest *in vivo*. Door koppeling aan een draadloze transponder is de sensor in staat om de bloedsuikerspiegel tenminste vijf dagen accuraat te meten, waardoor de sensor potentie heeft om te worden gebruikt in een klinische setting. Vereiste hiervoor is dat de sensor gesteriliseerd kan worden. Daarom wordt in hoofdstuk **vijf** het effect van verschillende sterilisatiemethoden onderzocht, waaruit blijkt dat - ondanks een reductie in gevoeligheid - de sensor ook na sterilisatie geschikt zou zijn om glucose te meten *in vivo*.

Hoofdstuk **zes** beschrijft de ontwikkeling een multiplex biosensor die simultaan glucose, lactaat en pyruvaat meet. Omdat de hersenen een belangrijke rol spelen in de regulatie van de bloedsuikerspiegel wordt door middel van deze multiplex sensor het verband tussen perifere en centraal metabole processen bestudeerd. Een interessante bevinding in dit hoofstuk is dat de pyruvaatconcentratie in de hersenen constant blijft, ongeacht sterke fluctuaties in de glucosespiegel. In het hoofdstuk **zeven** wordt de ontwikkeling van een mini-biosensor op basis van een goud-gecoate tungstenelektrode beschreven. *In vitro* en *in vivo* experimenten laten zien dat ook deze elektrode accuraat glucose kan meten, wat potentie biedt tot minder invasieve biomonitoring in diabetespatiënten.

List of Abbreviations

ΔE_p- Difference in peak potential
AA- Ascorbic Acid
ANLS- Astrocyte-neuron lactate shuttle
ANOVA- Analysis of variance
BG- background sensor
BSA- Bovine serum albumin
CGM- Continuous glucose monitoring
CGMS- Continuous glucose monitoring system
CH- clorohexidine
CV- cyclic voltammetry
DA- Dopamine
DOPAC- 3-4-dihydroxyphenylacetic acid
DNA- Deoxyribonucleic acid
E.C. - Enzyme commission number
ECF- extracellular fluid
EtOx- Ethylene oxide.
FAD - Flavin adenine dinucleotide
FBR- Foreign body response
FDA- U.S. Food and Drug Administration GA- Glutaraldehyde
GABA- γ-aminobutiric acid
GOx- Glucose oxidase
GluOx- Glutamate oxidase
HBA1c - Glycated hemoglobin
HLA- Human Leukocyte Antigen
HPLC – High-performance liquid chromatography.
I_p – Current on the peak Potential
ID- Inner diameter
IDDM – Insuline-dependent Diabetes Mellitus
ISF- Interstitial fluid
IG- Interstitial glucose
IPA- Isopropyl alcohol
i.v.- Intravenous
J_{Max}- Maximum movement of solutes (from Fick's law)
kDa- KiloDalton
K_M- Michaelis-Menten constant
$_{app}K_M$- Apparent Michaelis-Menten constant
LBL- layer-by-layer
LC-MS – Liquid chromatography-mass spectrometry

LOD - Limit of detection
LOx- lactate oxidase
LR- Linear Range
LRS- Linear range sensitivity
MBD- Microbiosensor device
iMBD- Implantable microbiosensor device
MPBC – Multiple point blood calibration
MRI- Magnetic resonance imaging
MRS- Magnetic resonance spectroscopy
nA- Nanoampere (10^{-9} A)
NADP - Nicotinamide adenine dinucleotide phosphate
NIDDM- Non Insuline-dependent Diabetes Mellitus
NMR- Nuclear magnetic resonance spectroscopy
OPPy- Overoxidized polypyrrole
OD- Outer diameter
pA- PicoAmpere (10^{-12} A)
PAN- Polyacylonitrile
PBS – Phosphate buffer saline
PDGF- Platelet-derived growth factor
PE- Polyether sulfone
PEG- Polyethylene Glycol
PET- Positron-emission tomography
mPFC- medial prefrontal cortex
PG- Plasma Glucose
POx- Pyruvate oxidase
PPD- Poly(phenylenediamine)
P*m*PD- Poly(m-phenylenediamine)
P*o*PD- Poly(o-phenylenediamine)
PreC – Pre calibration
PostC or PC – Post calibration
RC- Regenerated Cellulose (when applied to membranes)
RC- Rejection Coefficient (when applied to biosensor performance parameters)
SAL- Sterilization assurance level
SAM- Self-assembled monolayer
SC- Selectivity Coefficient
SEM- Scanning electron microscopy (when applied to imaging)
SEM- Standard error of the mean (when applied to statistics)
SI I_{Max}- Surface independent maximum current

$SI_{app}K_M$ – Surface independent Michaelis-Menten constant
SMBG- Self-monitoring of blood glucose
SPBC – Single point blood calibration
T1DM- Type I Diabetes Mellitus
T2DM- Type II Diabetes Mellitus
UA- Uric Acid
UV- ultraviolet
V_{Max}- Maximum reaction rate
W.H.O- World Health Organization

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“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.”

Marie Curie

“Never, never, never give up!”

Winston Churchill.

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Curriculum Vitae

Biography

Carlos Cordeiro was born in Vila do Conde, Portugal in 1982. From 1990 until 2001 he was a competitive swimmer. He was the twice individual portuguese age groups national champion (1996 and 1998) and once 2nd division national champion by teams (1999). He started his undergraduate studies in 2000 at the Universidade do Algarve. He finished his licenciatura (MsC equivalent) in Biochemistry at the Universidade do Algarve (Faro, Portugal) in 2005. In 2006, he started an MsC in Biotechnology at the same University. In 2007 he was granted an Erasmus Mobility Scholarship and joined the Biomonitoring and Biosensoring group at the Faculty of Pharmacy of the Rijksuniversiteit Groningen to carry out his MsC research project. Later (in 2009) he received his MsC in Biotechnology at Universidade do Algarve (Faro, Portugal), with honors. In 2008 he started his Ph.D. at the Rijksuniversiteit Groningen in also in the department of Biomonitoring and Sensoring. Additionally, he started to work as Trial Manager and Scientist at Brains On-Line BV (Groningen, The Netherlands). Since 2011 he is also Trial Manager for *in vivo* electrophysiology studies at the same organization. In 2014 he joined the business development team at Brains On-Line. His main focus is the development of biosensors for in vivo applications. However, his work and scientific interests also comprise electrochemistry, neurochemistry and neuropharmacology,as well as surface chemistry and biotechnology, especially enzyme technology.

Publications

Moon B-U, de Vries MG, Cordeiro CA, Westerink, BHC, Verpoorte, E. Microdialysis-Coupled Enzymatic Microreactor for *in Vivo* Glucose Monitoring in Rats. Analytical Chemistry 2012; 85 (22) 10949-55.

Cordeiro CA, de Vries MG, Ngabi W, Oomen PE, Cremers TIFH, Westerink, BHC. *In vivo* continuous and simultaneous monitoring of brain energy substrates with a multiplex amperometric enzyme-based biosensor device. Biosensors and Bioelectronics 2015; 67 677-686.

Cordeiro CA, de Vries MG, Cremers TIFH, Westerink, BHC The role of surface availability in membrane-induced selectivity for amperometric enzyme-based biosensors. Sensors and Actuators B: Chemical 2016; 223, 679-688

Cordeiro CA, de Vries MG, Cremers TIFH, Westerink, BHC. The impact of surface availability, modulated by the choice of permselective membrane, on the performance of implantable amperometric enzyme-based biosensors. Submitted to Electrochimica Acta, 2017.

Cordeiro CA, Sias A, Koster T, Westerink, BHC, Cremers TIFH. In vivo real-time brain biomonitoring with enzyme-based microbiosensors based on gold coated tungsten (W-Au)

microelectrodes. Submitted to Sensors and Actuators B:Chemical, 2017.

Patents

PT2895071 (T) — 2017-06-27
Rod Shaped Implantable Biosensor
Co-Inventors: Thomas IFH Cremers
Carlos Alberto de LBL Cordeiro

Grants

2007- Erasmus mobility Grant, granted by the Fundação para a Ciência e Tecnologia (Portugal). This grant, awarded by merit, was intended for the completion of the MsC in Biotechnology. research project in a selected international academic Institution (University of Groningen).

2016- FLAG-ERA Joint Translational Call Grant-Graphene Flasghip “Graphtivity” Project. Project Manager/Scientist at Brains On-Line, BV, in an European consortia involving also the Ruhr-Universität Bochum (Germany), KU Leuven (Belgium), The Italian Institute of Technology (Italy), Centre National de la Recherche Scientifique (France) and the International Centre of Biodynamics (Romania).