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

De Lima Braga Lopes Cordeiro, Carlos

CHAPTER 1

Electrochemical biosensors for *in vivo* glucose biomonitoring (and beyond?)

Cordeiro, CA[1,2*]; de Vries, MG[1]; Cremers, TIFH[1,2] and Westerink, BHC[1,2]
[1]Brains On-Line BV, Groningen, the Netherlands
[2]University of Groningen, Institute of Pharmacy, Groningen, the Netherlands

1.1- Pathology and epidemiology of diabetes

1.1.1- Diabetes epidemiology

Diabetes (Diabetes Mellitus) is the 4th leading cause of death in Europe, and it is also a major risk factor for a large number of other diseases (Jönsson 2002). In 2010 estimations pointed to more than 285 million of people diagnosed with diabetes, of which 90% had type II diabetes. The age groups with most prevalence are the groups 20-79 years old (for type II) and below 20 years old (for type I). The worldwide prevalence of the disease is estimated to increase from 2.8 to 4.4 %, in all age groups for the next 30 years (W.H.O 2016; Wild et al. 2004).

Although diabetes prevalence has been increasing since the beginning of the 20th century, we witnessed, in the last few decades, to an acceleration of the rate of increase (up to 50% increase in some countries) (Wild et al. 2004). The sharp increase in prevalence, especially in the last decades, is a clear indicator that the toll of diabetes related death is likely to increase. This has led diabetes to be referred as the black plague epidemic of the 21st century (Gadsby 2002).

The increase in prevalence is expected to take place in all age groups and in all geographical areas. However, rates of prevalence increase (more than 80%) will be highest in Asia, Africa, and Latin America. The cause of most concern about these numbers is the fact that this increase will be more pronounced on the active population (between ages of 30-65).

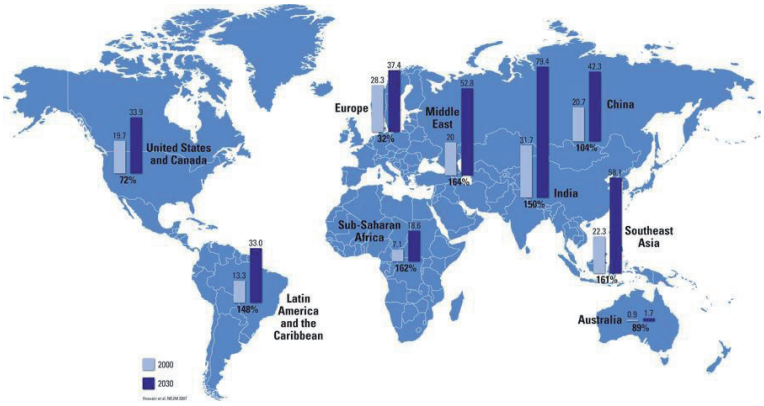


Figure 1- Worldwide diabetes prevalence. Comparison of the incidence in 2000 and predictions for 2030 (Zimmet et al. 2001).

The prevalence of diabetes, like any other pathology, directly depends on both duration and incidence. The emergence of better diagnostic tools, combined with significant advances in diabetes management are directly related with an increase in diabetes prevalence. In that sense, not only more people are aware of the disease, but the life expectancy of patients will

largely increase (W.H.O 2016).

Besides the aforementioned, there are several other known factors that influence diabetes prevalence. It has been described that amongst them, age might be the most influential one. Several studies show that prevalence increases with age, although it reaches a plateau and even declines for very old age groups (≥ 75 years). Other factors like ethnicity, socio-economic, lifestyle, obesity and country and place of residence (urban vs rural), also play a big role on diabetes epidemiology, although less significantly (Gadsby 2002).

1.1.1.1- Healthcare costs of diabetes

The worldwide incidence of diabetes and the healthcare issues associated with treatment of all patients, have a tremendous impact on world economy. In addition to the direct costs of medical expenses, one cannot exclude the significant indirect costs, due to loss of economic productivity (da Rocha Fernandes et al. 2015; Shaw et al. 2009).

It has been estimated that the total expenditure on health care on diabetes will range between 213 billion and 396 billion dollars in 2025 (King et al. 1998). This implies that by 2025 the costs associated with diabetes will range from 7-13% of the total healthcare budget, reaching up to 40% in countries where its prevalence is higher. Diabetes prevention and effective management of diabetes should be a public health priority to reduce the financial burden (Giannini et al. 2009; Jönsson 2002).

1.1.1.2- Type I diabetes

Although diabetes etiology can, nowadays, be very detailed, it can be divided into two different types: Type I and Type II.

Type I diabetes (T1DM) or IDDM (insulin-dependent diabetes mellitus) is an autoimmune form of diabetes. This type of the disease is characterized by the destruction of the β-cells of the pancreas, which are responsible for insulin production. This results in an inability of the organism to produce sufficient insulin, thus the inability of the organism to clear glucose from the blood, by its uptake by the liver and white adipose tissue. Without proper insulin treatment this type of diabetes is fatal (Fertig et al. 1995; Van Belle et al. 2011).

The onset of T1DM it is strongly correlated to genetic susceptibility. The first correlation of diabetes with genetic factors was described in 1973, specifically with the human leukocyte antigen (HLA) region (Noble and Erlich 2012). Since then, several studies corroborated and extended the close correlation of diabetes with several genes (Pociot and Lernmark 2016). Its expression depends on a certain extent on environmental factors. However its weight is limited, especially when compared to type 2 diabetes (T2DM). The onset of the disease is usually sudden and it occurs mainly during childhood or adolescence (Van Belle et al. 2011).

Despite innumerous efforts, this type of the disease cannot be prevented. Moreover, its diagnosis, mainly due to its non-specific symptoms, is problematic resulting in an

underestimation of diabetic patients. The real number of patients is believed to be about 30% larger than the official data.

1.1.1.3- Type II diabetes

Type 2 diabetes (T2DM) or NIDDM (non- insulin dependent diabetes mellitus) is characterized by high blood glucose as a consequence of an insulin resistance, often associated with moderate insulin deficiency. These abnormalities on insulin regulation have, unlike for T1DM, no autoimmune basis. Patients with NIDDM usually have higher levels of circulating insulin, due to malfunctions of insulin receptors that in turn lead to overcompensation by pancreatic β-cells. Eventually the β-cells become unable to maintain glucose homeostasis, which deregulates blood glucose levels (Olokoba et al. 2012).

The cause of this type of the disease is more complex than the ones for type T1DM. Besides a strong genetic component, environmental factors such as lifestyle and medical conditions play a major role. It has become clear that the onset of this disease has a strong hereditary genetically background (Florez 2016). This increases substantially the chances of developing this type of diabetes, and several genes have been identified as being associated to the development of type 2 diabetes. However the weight of environmental factors in the onset of T2DM is much higher when compared with T1DM.

The role of lifestyle in this type of diabetes goes beyond its onset. Nowadays, more than 50% of the diagnosed patients suffer from obesity. It is believed that changes in lifestyle can reduce the probability of onset the and even control the disease in its early stages. When diagnosed in its early phase, exercise and proper diet are effective strategies for both prevention and management of the disease (Fertig et al. 1995). Later, T2DM patients also need frequent blood glucose monitoring for an effective management of the disease (Force 2008).

1.1.1.4- Normal glucose variations

In a healthy person, blood glucose levels largely fluctuate during the day. These fluctuations depend on many factors, such as the timing of glucose supply (meals) and differential levels of glucose utilization (e.g due to physical activity) (Maggs et al. 2008). Mean blood glucose values in humans under resting conditions are between 4.4 and 6.1 mM, a state known as euglycemia. Early in the morning, however, the concentration of glucose in the blood is significantly lower. After a meal, glucose levels in the blood can increase up to 7 mM. Persistent high blood glucose levels, two hours after glucose ingestion, are a symptom of impaired glucose tolerance (≥ 7-8 mM), or diabetes mellitus (≥ 11 mM) (Association 2015).

Under certain circumstances, blood glucose concentrations can fluctuate tremendously. Intense exercise can lead to very low concentrations (hypoglycemia)(Adams 2013), whereas stress can lead to very high glucose concentrations (hyperglycemia) (Marik and Bellomo

2013). In healthy humans, physiological mechanisms are at work to maintain euglycemia, but in patients with diabetes these are less effective and may need active control by the patient (exogenous regulation).

1.1.1.5- Endogenous glucose regulation

The body tries to maintain blood glucose levels within well-defined boundaries, by means of a tight regulation. This tight control is mainly assured by the endocrine system, controlled both by hormones and by direct neuronal innervations. Insulin and glucagon are two antagonistic hormones involved in regulating the levels of circulating glucose. Glucagon promotes an increase in blood glucose by stimulating hepatic glucose production. In contrast, insulin promotes a decrease in blood glucose by stimulating glucose clearance from the blood into the liver, skeletal muscles, and adipose tissue.

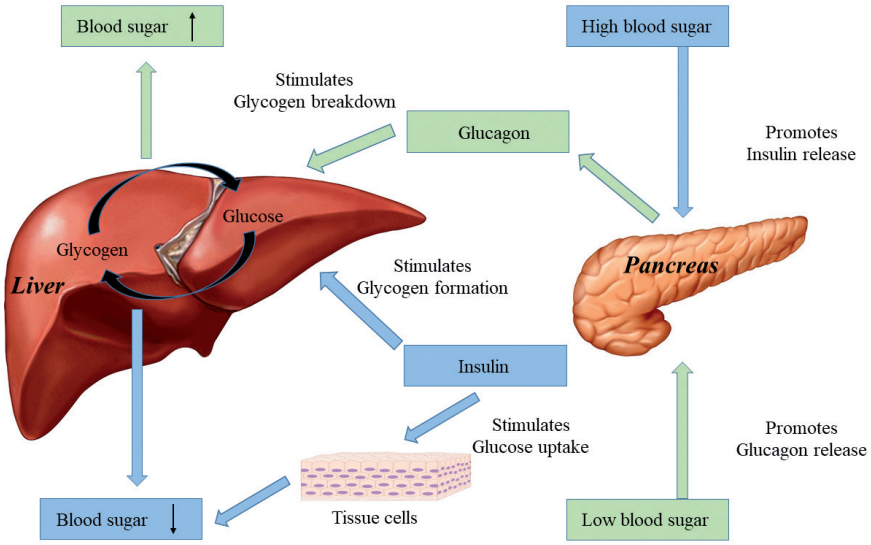


Figure 2 – Glucose endocrinous regulation diagram.

The liver, endocrine pancreas and adrenal glands are the major targets for efferent output to the periphery with regard to regulation of blood glucose. To a lesser extent, the white adipose tissue, kidneys, and skeletal muscles can also be involved in those processes. Output from the autonomic nervous system can be neuronal in nature or humoral, regulated by hormones present in body fluids.

The liver is the major organ in terms of biochemical processes, including those involved

in maintaining glucose homeostasis. Besides arterial blood the liver also receives blood directly from the intestinal tract via the portal vein. The blood from the portal vein carries not only digested nutrients (absorbed by the intestines) but also glucagon and insulin previously released by the endocrine pancreas. Although the liver has the ability to induce significant glucose release, by promoting either glycogenolysis or gluconeogenesis, most of the circulating glucose originates directly from dietary intake.

The endocrine pancreas is the source the two antagonistic hormones, insulin produced by β-cells and glucagon produced by α-cells. A third hormone, somatostatin (released by δ-cells and in lesser extent by the hypothalamus), inhibits the release of both insulin and glucagon. To a certain extent the pancreas regulates the secretion of insulin and glucagon by itself, depending on the amount of glucose that is present in the blood passing through the pancreas. However, blood glucose can be regulated by many circulating biochemical agents, as well as by humoral and neuronal output from the autonomic nervous system (Aronoff et al. 2004; Gerich 1993; Tonelli et al. 2005).

1.2- Glucose monitoring in diabetes

The body has safeguard mechanisms for tight control of blood glucose levels, but these are severely impaired in patients with diabetes. Despite all efforts, a cure for this disease is still to be found, enhancing the need of a close monitoring of blood glucose levels, for a proper management of the disease. It is widely assumed that careful glucose monitoring helps to control glucose levels and slows down progression of the disease and its related complications (Hermanides et al. 2011; McAndrew et al. 2007).

Diabetes is often diagnosed at a relatively late stage, when conservative management, is no longer possible. At this stage, pharmacological therapy by means of insulin administration is needed. (Battelino et al. 2011). The most typical pharmacotherapy for diabetes patients is insulin administration, usually achieved by subcutaneously insulin administration (Hirsch et al. 2005).

A good control of blood glucose levels of diabetic patients is clearly correlated with an increased life expectancy. It has been described to reduce the risks of developing any of the long-term vascular complications from large blood glucose. These long-term vascular complications can be divided into microvascular (retinopathies, nephropathies and neuropathies) and macrovascular diseases (severe cardio- and cerebrovascular diseases like myocardial infarcts and strokes) (Forbes and Cooper 2013).

A good control of glycemic levels over several weeks can easily be traced back by measuring the levels of glycated hemoglobin (HbA1c). Diabetic patients with well-controlled glycemia have low levels of HbA1c (below 7%) and are less likely to develop long-term diabetic complications and increase their life expectancy (Alqahtani et al. 2013).

However, keeping a close control of glucose levels is a major challenge for diabetic patients. All diabetic patients require help to carry out this task, and its extent depends on

the severity of the disease. Visits to an endocrinologist for health evaluation and therapy adjustments are regular for diabetic patients.

In most of the cases, glucose must be controlled on a daily basis. Typically such control is achieved by checking blood glucose multiple times per day. In order to do so, a large number of diabetic patients perform "self-monitoring of blood glucose" (SMBG). This type of glucose monitoring is crucial for therapy adjustments, prevention of hyper- and hypoglycaemia episodes and help individuals adjust their dietary intake, physical activity. The goal of SMBG is to increase frequency blood glucose monitoring, thus improve diabetes management. It was thought that T1DM patients have a need for higher frequency of glucose monitoring than T2DM patients. However, recent studies showed that high frequency in glucose biomonitoring is beneficial to both groups of patients (Benjamin 2002; Vazeou 2011).

The most common method employed for SMBG is the "finger prick", a method that relies on instantaneous measurements of blood glucose levels, at specific time points. However, it requires frequent blood sampling. Although significantly refined over the decades, blood sampling remains a painful process and still results in non-compliance by diabetic patients. Additionally, in order to perform SMBG, it is patients need to be properly trained. Therefore SMBG is not well suited for some patient groups like children, elderly and disabled, due to its relative complexity (Heinemann 2008; Knapp et al. 2009).

Despite some improvements, SMBG is still based on the principles that emerged decades ago. Disposable biosensor based test strips are still used to analyze the glucose levels of the blood, using a glucose meter. However, over the last decades hand-held blood glucose meters have been continuously improved and nowadays blood glucose meters are more "user-friendly" and robust. The lancet mechanism has been improved, reducing the discomfort levels associated with this technique. The latest glucose meters include memory (to store blood glucose levels) and alert signs for deviations in normo- glycemia. However these developments only refined the technique and the big disadvantages, invasiveness, hence non-compliance, still remain (Krouwer and Cembrowski 2010; Tonyushkina and Nichols 2009; Yamada 2011).

Although increasing the frequency of blood glucose control, SMBG is not continuous. This limitation allows unawareness of glucose excursions, especially during the night, highly relevant for patients with large daily variations or hypoglycemia awareness. Continuous glucose monitoring would provide a better anamnesis of each patient (Poolsup et al. 2013).

An ideal *in vivo* glucose monitoring technique would be minimally invasive or even non-invasive, to maximize convenience and to increase compliance. It should enable continuous recording of the daily glucose variations for prolonged periods ($\geq$ 1 week). These envisioned new devices would allow saving the continuous data for retrospective readout, useful for the development and fine tuning of an individual therapeutic plan. Eventually, these devices would serve as input for a "closed-loop" diabetes treatment device, leading to an "artificial pancreas" (Aye et al. 2010; Wang 2008).

The development of an artificial pancreas is still a goal for scientific community, but

presently far from everyday use by diabetic patients. In theory this could be achieved by coupling a measuringdevice capable of providing a reliable continuous glucose monitoring (CGM), to a device able to selectively and accurately release insulin based on the data acquired by the first. This closed loop circuit, would be dependent on an algorithm that would instruct the device to infuse the necessary therapy to counterbalance glucose variations. The adequate algorithm would be able to predict hypo/hyperglycemia events, making the patient aware and able to take the necessary measures to regulate its glucose levels.

1.3 -Biosensors as bioanalytical tools

Biosensors are by definition analytical devices that can quantitate the amount of a specific biochemical substance, by means of a biorecognition element coupled to a transducer. In a biosensor, the biorecognition element selectively recognizes the target analyte and the transducer converts the resulting physical-chemical interactions into a measurable signal (Thevenot et al. 1999).

Biosensors are versatile bioanalytical tools that may be applicable to several different fields, ranging from biomedical applications to material sciences, chemical industry, food sciences, and even environmental applications (Serra 2011). The versatility of these devices is closely related to their intrinsic properties, which is arguably the main reason for the growing interest of these novel tools (Connolly 1995; Turner 2013). Suitable biorecognition elements are abundantly available (both in nature and produced as the result of bio-engineering) and there are numerous good ways to immobilize them onto appropriate transducers. High specificity is assured mostly by the biorecognition element and assures that the biosensor is able to recognize the target analyte in complex biological matrices. High sensitivities can be achieved in a combination of good immobilization techniques of the biorecognition element onto a transducer with high resolution. Biosensors are typically characterized by high specificity and sensitivity, fast response time (second by second), ease of use (do not require exhaustive training), compactness, and regeneration of the device (useful for continuous monitoring). It's the combination of these properties that make biosensors powerful bioanalytical devices (Kissinger 2005; Song et al. 2006).

Historically, advances in biosensor technology are driven by the ongoing interest in the fields of basic science and medical care to monitor biochemical processes in the body. And to do this with ever increasing desire for detail. There is an everlasting need for better biosensors. Biosensors that can be more accurate and precise, more analyte-specific, more durable, that can measure multiple analytes simultaneously with higher temporal and spatial resolutions, and with as little impact on the target tissue as possible (Siontorou and Batzias 2010).

Initially, biosensors used to be deployed mainly in *in vitro* and *ex vivo* approaches (e.g. to measure glucose or other biomarkers in samples of bodily fluids). But as technology evolved, it became possible to monitor biochemical processes in the body itself without the need to

extract sample material. The first implantable biosensors were still rather big and therefore could not confine their measurements to small, discrete, physiological compartments. Fortunately, biosensor technology has been evolving tremendously and current state-of-the-art biosensors can already monitor *in vivo* biochemical processes (Abel and von Woedtke 2002; Wilson and Gifford 2005).

However, despite the large number of publications regarding biosensors development and application, it seems that this technology didn't quite make the transition from "the lab" to "real world" application (Siontorou and Batzias 2010). It seems that the extensive academic work isn't being followed by industry. Although the first biosensors has been described more than 60 years ago, (in 1962, by Clarke and Lyons) (Clark 1993) the amount of biosensors commercially available is still extremely limited. There is a clear gap between academia knowledge and industry applications, hampering the widespread use of this technology.

Nowadays, where rapid information is needed, biosensors could serve exceptionally well in emergency situations, and/or in on-site field applications. The miniaturization of these devices, accompanied by an increase of sensitivity and even faster response times may lead to a dissemination of the "real" applications of these devices. Biosensor technology is a very good example where miniaturization has been applied. Ongoing research is likely to improve existing models in terms of accuracy, sensitivity, miniaturization, and increased portability, expanding the scope of biosensor applications. Biosensors could in a near future, play a big role in biomonitoring an ever growing number of key biomarkers bio-medicine. For instance, biosensors may be useful to improve diagnostics in cancer research (protein/gene recognition), hepatitis (DNA sensors for gene profiling) and even in cardiovascular diseases (recognition of PDGF and Thrombin) (Mascini and Tombelli 2008).

1.4- Geometry of biosensors

The specific application of a biosensor is the main factor in the choice of a suitable biorecognition element and its appropriate transducer. The biorecognition element ensures selective affinity towards the target analyte and largely affects the sensitivity.

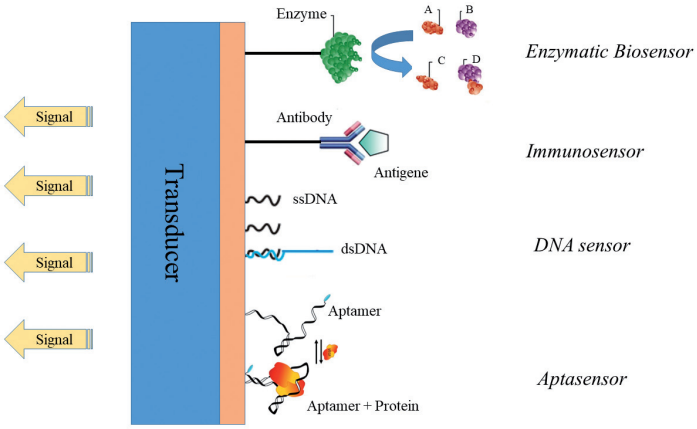


Figure 3- Schematic representation of the working mechanism of a biosensor.

1.4.1- Biorecognition elements

Biosensor selectivity is largely determined by the choice of the biorecognition element. Biorecognition elements can be divided into biocatalyst and affinity biorecognition elements. Biocatalysts use natural catalysts to effect chemical transformations with analyte consumption, like enzymes. On the other hand, affinity biorecognition elements specifically bind to individual targets or groups of structurally related targets, such as antibodies and DNA. Whole cells and tissues are generally considered to be different biorecognition elements, although their selectivity is mainly assured by enzymes present in those elements (Chambers et al. 2008).

Currently, enzymes are by far the most common biorecognition element of choice in biosensor design (Rocchitta et al. 2016; Sarma et al. 2009). However, as more fundamental research is performed, especially in terms of immobilizing new applications based on the remaining biorecognition elements are growing. These include nucleic acids (Sassolas et al. 2008), antibodies (Holford et al. 2012), whole cells (Yagi 2007) and lately, also aptamers. (Zhou et al. 2014).

1.4.2- Transducer

The transducer is the biosensor component responsible for converting the physical and/or chemical changes by the interaction between the biorecognition element and the target analyte into a quantifiable signal (Sethi 1994). The most commonly used transducers in biosensor technology are by far the electrochemical ones (Pohanka and Skladal 2008). Although the amount of biosensors based on optics (Fan et al. 2008; Ziegler) and piezoelectricity (Skládal 2016) has significantly increased, the total amount of applications is still much

lower compared to electrochemical biosensors. Other types, such as acoustic, calorimetric or mechanical transducers are also employed in biosensor assembly. However, when compared with electrochemical and even optical and piezoelectrical, its application is still residual.

1.5- Electrochemical biosensors

Electrochemistry is a surface technique characterized by small reaction volumes and minimal analyte consumption, hence, very appealing for biosensors technology. Additionally, electrochemistry is associated with relatively low cost, ease of use, simplicity of construction and possibility of online measurements. Therefore, it is easy to understand why most of the described biosensors mechanisms, involve some sort of electrochemical detection (Ronkainen et al. 2010). Electrochemical biosensors can be classified according to the various working mechanism (Bard and Faulkner 2000):

- Potentiometric: based on ion-selective electrodes or ion-sensitive field effect transistors. The output signal is generated by accumulation of ions at an ion-selective membrane.

- Impedimetric: based on changes in impedance (Z), resistance (Ω), or capacitance at the electrode surface.

- Voltammetric/amperomeric: These types of biosensors are based on changes in current at the surface of the electrode. In voltammetry a variable potential is applied, while in amperometry the applied potential remains constant.

Amperometry is the most widely used working mechanism in biosensor applications, among all of the electrochemical methods. The recurrence of this mechanism is most likely due to relative simplicity of the method and good prospects in terms of sensitivity and miniaturization.

1.5.1- Principles of amperometry

In amperometry, the current is measured by applying a constant potential to the electrode. The applied potential promotes oxidation/reduction of electroactive molecules at the electrode surface in a very sensitive way (Grieshaber et al. 2008). State-of-the art electrochemical apparatus can monitor small changes in current, down to the picoampere (pA) range (10^{-12}A) (Smith and Hinson-Smith 2002). This levels of sensitivity allows, in some cases, the detection limit to be as low1 nM for highly electroactive molecules such as hydrogen peroxide (Aziz and Kawde 2013). The relationship between the applied potential and the current generated by the redox reaction at the electrode surface is described by the Butler-Volmer equation (Bockris et al. 2000);

$$I = A.i_0.\left\{exp\left[\frac{(1-\alpha)nF}{RT}(E - E_{eq})\right] - exp\left[\frac{\alpha nF}{RT}(E - E_{eq})\right]\right\},$$

Equation 1- Buttler-Volmer equation.

Legend: I= electrode current, A; I_0 = exchange current density, A/m^2 ; E= electrode potential, V ;Eeq = equilibrium potential, V; A= electrode active surface area, m2; T= absolute temperature, K; n= number of electrons involved in the electrode reaction; F= Faraday constant; R= universal gas constant; α = charge transfer coefficient, dimensionless.

The Butler–Volmer equation is considered one of the most fundamental relationships in electrochemical kinetics. It describes how the electrical current of an electrode depends on its electrode potential. However, this equation is only valid when the electrode reaction is controlled by electrical charge transfer at the electrode surface, and not in cases when the reaction is controlled by mass transfer. Also, there are two cases when this model has limitations. In the low overpotential region (when E ≈ Eeq) and in the high overpotential region (when E << Eeq or E >> E_{eq}). Nevertheless, the utility of this equation is wide and it is still regarded as a key model in electrode kinetics.

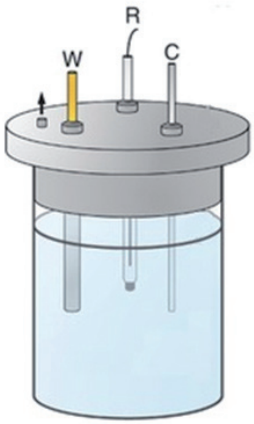
In biosensor development it is common to use both voltammetry and amperometry. Usually, voltammetry is first used to establish the optimal potential at which the redox reaction occurs most efficient. After this has be accomplished the described potential is used for amperometric measurements of the unknown samples, in order to maximize the analytical power of this technique.

Voltammetry and amperometry are usually performed using a set of 3 electrodes:

- **Working electrode**- the monitored redox reaction occurs at the surface of this electrode. In biosensor technology the surface of this electrode contains the biorecognition element.

- **Reference electrode**- this electrodes has a constant and well-known potential. The applied potential is set by the electrochemical standard potential of this electrode. In biosensor technology an Ag/AgCl reference electrode is the most common, due to its ease of miniaturization and its suitability for aqueous solutions. However, Ag/AgCl electrodes are not permanent. These type of reference electrodes need periodic regeneration and/or, replacement.

- Counter electrode- The counter electrode is used as a current sink. The use of a counter electrode prevents a current threshold by the reference electrode.



W– Working electrode
R – Reference electrode
C – Counter electrode

Figure 4 –Typical three electrode setup for amperometric/voltammetric measurements (adapted from (Ma et al. 2013)).

The use of microelectrodes (dimensions within the 10^{-6} m range) is very common in biosensor technology. Not only does it increase spatial resolution, highly relevant for *in vivo* applications, but also expands the method possibilities. It allows the possibility to work in highly resistive solutions, as they can accommodate large ohmic drops (iR), that are challenging if macroelectrodes are employed. Additionally it enables high-speed voltammetric experiments (due to the reduction of the double-layer capacitance) allowing fast electron transfer. Experimental setups that include microelectrodes often employ a two electrode setup (without counter electrode), due to the relatively low amount of current generated at the surface ($\leq 10^{-6}$ A) (Wang 1994).

1.6- Enzymes: the biorecognition element of choice

In biosensor technology, enzymes are still the biorecognition element of choice. These type of biomolecule is very appealing due to its high intrinsic selectivity, stability, and ease of immobilization onto the surface of a transducer. The first enzyme to be used in biosensors was glucose oxidase (GOx), employed in biosensors for glucose monitoring. Nowadays, GOx is arguably still the most common enzyme employed in biosensor assembly, driven by the need to have reliable blood glucose monitoring methods, for SMBG. However, as biosensors applications expanded, new enzymes became used as biorecognition elements in several biosensors. These include other oxireductase enzymes from the same class such as lactate oxidase (LOx), pyruvate oxidase (POx), glutamate oxidase (GluOx)(Cordeiro et al. 2015). Lately, other types of enzymes have been increasingly employed in biosensor technology, such as dehydrogenases (Jena and Raj 2006) and hydrolases. However, due to the rapid growth in the technology immobilization techniques, it is likely that the amount of

enzymes used in biosensor assembly to significantly grow in the future.

Enzymes are large, complex macromolecules that consist mostly of protein, associated with a co- factor. Each enzyme increases the rate of a specific chemical reaction by decreasing its activation energy. A great variety of different enzymes exists to account for the many biochemical reactions that take place inside an organism. An enzyme has specific affinity for one or just a few substrates, and catalyzes a very limited number of similar reactions. This specificity is an essential feature for the use of enzymes in biosensors.

1.6.1- Enzyme biochemistry

Despite its relatively large molecular weight (on the kDa range), only a small portion of an enzyme is involved in catalyzing the chemical reaction. This portion is called the active site. The active site typically contains an organic or inorganic co-factor, which is either directly bound or allosterically associated with the enzyme. The co-factor may have a structural or catalytic function (i.e. carries chemical groups between substrate and enzyme) (Voet and Voet 2011).

The activity of an enzyme is based on its three-dimensional structure, electrical charge, and degree of hydrophobicity vs hydrophilicity. This working principle, coined "Lock-and-Key" model by Emil Fischer in 1918, explains the nature of the interaction between enzyme and substrate. Over the years this underlying mechanisms of this interaction became clearer. It is very complex and dynamic, as the spatial configuration of the enzyme (especially the active site) is subject to change as part of its biochemical role.

1.6.2- Enzyme kinetics

The field of enzyme kinetics studies the rates of chemical processes mediated by enzymes. Despite of the important role of enzyme kinetics in overall biosensor performance, its principles are often overlooked in biosensor development.

By studying enzyme kinetics we can better understand the catalytic mechanisms. These mechanisms can be characterized by parameters such as the substrate affinity, the activity, and the turnover rate (Bisswanger 2008). Sufficient knowledge about the structure of a specific enzyme is critically important to a correct interpretation of data obtained by enzyme-based biosensors.

In biosensor technology, enzymes are often immobilized (in multiple ways) onto the microelectrode surface (Grieshaber et al. 2008). Although very effective, the immobilization process has significant negative impact on the enzyme properties (Cosnier 1999; Rocchitta et al. 2016). Unfortunately, this effect is, in my opinion, insufficiently acknowledged by the biosensor community today. A search on PubMed for four key words "enzyme biosensor electrochemical kinetics" retrieved only 133 hits. A relatively low number when compared with the number of hits when we remove the word "kinetics" (1620 hits). In the past decade,

the group headed by Prof O´Neill contributed by providing some new and helpful insights on surface enzyme kinetics (Rothwell et al. 2010).

Under certain conditions enzymatic reactions can reach saturation. This is a unique and important kinetic property that differentiates enzymatic reactions from all other types of biochemical reactions. Saturation occurs when all of the active sites of the enzymes are occupied by substrate. Once saturation has been reached, adding more substrate will not result in an increase of the reaction rate and it becomes limited by the turnover rate (Bisswanger 2008).

The fundamental principles of enzyme kinetics were first described by Victor Henri in 1902. Only after the discovery of the logarithmic scale, in 1909, Leonor Michaelis and Maude Menten repeated the experiment and related the reaction rate to the amount of substrate, wrongly naming the equation that defines the kinetics of enzymes:

$$V = \frac{Vmax[S]}{Km+[S]}$$

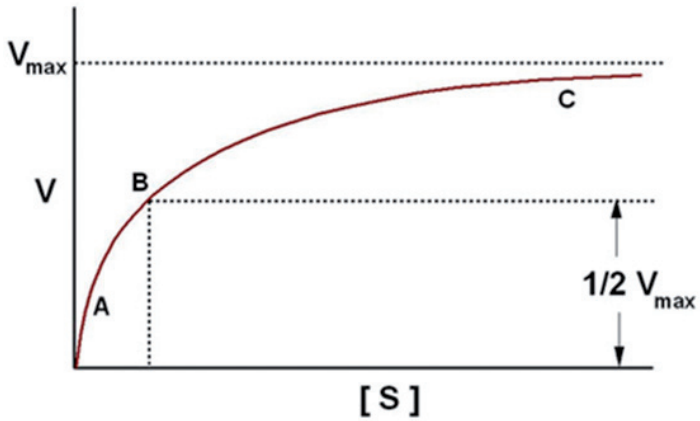


Figure 5- Michaelis-Menten equation for single substrate enzymes and its graphical representation. Legend V- reaction rate; S-Substrate ;V_{max}- maximum rate achieved by the system (when maximum substrate saturation is reached);Km- Michaelis Menten constant- substrate concentration at which the reaction rate is ½ V_{max}.

The reaction rate has a positive linear correlation with the concentration of substrate, under the assumptions that the enzyme concentration is constant and that substrate concentrations are low.

The linearity of such correlation decreases with increasing substrate concentrations.

Maximum reaction velocity (V_{Max}) is achieved asymptotically, when the substrate concentration approaches the saturation point and all enzyme molecules are bound to the substrate. The Michaelis-Menten constant (K_M) is defined as the substrate concentration at which the reaction rate is half of V_{Max}. The K_M indicates the affinity of the enzyme for its substrate.

Small values of K_M indicate a high affinity of the enzyme for the substrate, resulting in V_{Max} being reached already at low substrate concentration. Importantly, immobilized enzymes (as in biosensor development) have its intrinsic kinetic profiles significantly altered. Therefore, the affinity of immobilized enzymes, as well as other kinetic parameters is expressed as apparent constants ($appK_M$, $appV_{Max}$,)(O'Neill et al. 2008).

In the early days of kinetics research it was not possible to carry out non-linear regression analysis. Therefore it was necessary to develop linear derivations of the Michaelis-Menten model. These derivations were based on additional assumptions, and required simplification of the model to allow the various kinetic parameters to be calculated. The most important derivations that were in use for several decades are the Briggs-Haldane derivation, the Edie-Hofstee diagram, the Hannes-Wolf plot, and the standard way to calculate it, i.e. the Lineweaver-Burk linearization (Bisswanger 2008). For a long time, the Lineweaver-Burk model was widely used in enzymetic studies. According to this model, the y-intercept is equivalent to the inverse of V_{Max}, while the x-intercept represents $-1/K_M$. One of the advantages of this model was the ability of providing a quick, visual impression of the different forms of enzyme inhibition.

Nevertheless, all derivations, including the Lineweaver-Burk one, only minimize but did not solve the problem of uncertainty. All of them are prone to errors when applied experimentally. Even Linewaver-Burke linearization has its experimental limitations, as the *y*-axis takes the reciprocal of the rate of reaction – in turn increasing any small errors in measurement. The difficulty in reaching high levels of substrate [S], lead to a large extrapolation of the kinetic parameters (Dowd and Riggs 1965).

Nowadays, advances in computing systems allow analysis of experimental data from enzyme kinetics with non-linear regression, tools. These tools can determine the kinetic parameters with a higher accuracy. In that sense, advances in computing systems enabled the emergence of new mathematical models of the behavior of enzymes in membranes (Cooney 2011). The use of the new models may lead to new insights in terms of the activity of enzyme immobilized onto electrode surfaces, contributing to the optimization of biosensor performance.

1.6.3- Electrochemical enzyme-based biosensors

Although enzymes are by far the most successful biorecognition element employed in biosensors assembly, there is one group in particular that is "primus inter pares". Enzymes belonging to the oxidoreductases-group (EC1) are the most "popular", in biosensor design

(May 1999). These type of enzyme are characterized by the transferring of electrons from the electron donor to the electron acceptor. This type of enzyme require a cofactor, usually NADP or FAD, which recycle the electrons by reducing the enzyme.

In fact, despite the wide range of proof of concept biosensor designs, electrochemical (amperometric in particular) enzyme-based are still the most common type of biosensors described. And arguably, the most successful type of biosensors, especially if we confine to *in vivo* applications (Wang 1999).

Enzyme-based amperometric biosensors are classified as 1st, 2nd or 3rd generation, based on the mechanism of interaction between the enzyme and the transducer (Privett et al. 2008; Ronkainen et al. 2010; Weltin et al. 2016).

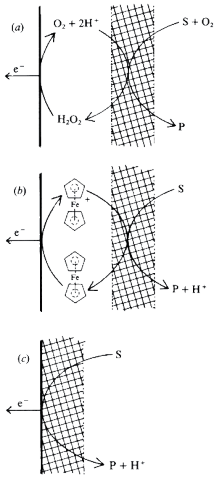


Figure 6 – Schematic representations of the proposed electron-transfer mechanisms for 1st (a), 2nd (b) and 3rd generation (c) amperometric enzyme-based biosensors.

The mechanism of 1st generation sensors is based on an indirect reduction/oxidation of one of the products of the enzymatic reaction at the electrode surface. A relatively high potential ($\geq$ 500 mV) is needed to oxidize the target electroactive analyte, typically H_2O_2. However, at such high potentials, other non-specific electroactive species are readily oxidizable, resulting in electrochemical interference, thus lowering accuracy and selectivity (Cordeiro et al. 2016; McMahon et al. 2004).

In 2nd and 3rd generation biosensors the applied potentials are much lower than those applied in 1st generation. Modifications in molecular geometry, such as the incorporation of redox mediators (2nd generation) and the implementation of "wired-enzyme" technology, through

the use of conductive polymers (3rd generation) resulted in significant improvements in its electron transfer mechanism. Besides its apparent low electrochemical interference, these geometries, unlike 1st generation, also provide oxygen independent biosensors (Putzbach and Ronkainen 2013).

In 2nd generation biosensors, electron transfer from the enzyme to the electrode is mediated by acceptor/donor molecules resulting in lower resistance. Therefore a lower potential is sufficient and much of the electrochemical interference can be avoided. These mediators are often embedded into a polymeric matrix, together with the enzyme (Scheller et al. 1991). However, mediators also interact with other electroactive molecules and they are prone to leaching. Additionally, mediators are often unstable in either reduced or oxidized form, and become inoperative after multiple redox cycles. Furthermore, the difficulty to correctly assemble all of the components is a major disadvantage. It is vital to adequately align all molecules, which in practice results in poor the reproducibility of this type of biosensors.

In 3rd generation biosensors, electrons are directly transferred from the enzyme to the electrode surface through a conductive polymer that 'wires' the active center of the enzyme to the electrode surface. Enzymes are adsorbed at the surface in a (sub)-mono layer (Zhang and Li 2004). This geometry enables a low working potential and thereby achieves high specificity. The use of a single layer, however, results in less enzyme being available on the biosensor and therefore lower sensitivity. Similarly to 2nd generation biosensors, it is imperative to precisely position all of the molecular components. However, such requirement often leads to low reproducibility of these type of biosensors.

Advances in polymer technology allowed the emergence of the "so-called" permselective membranes. When applied in the assembly of 1st generation biosensors, these polymeric membranes have the ability to exclude, by charge and/or size exclusion, non-specific electroactive species. The incorporation of these membrane significantly increased the selectivity of 1st generation biosensors (Cordeiro et al. 2016), enabling its successful application in *in vivo* biomonitoring of key biomarkers (Abel and von Woedtke 2002; Cordeiro et al. 2015; Murphy 2006; O'Neill et al. 2008; Wahono et al. 2012; Wilson and Gifford 2005).

1.7- CGM state-of-the-art

Although research and development of CGMs goes back to the 1970s, the first *in vivo* glucose biosensor for CGM was only reported in 1982. It was tested in dogs, with moderate success (blood glucose trends were followed by the sensor signal) (Yoo and Lee 2010).

Since then the number of experimental CGM devices (in their different stages of development) reported in literature grew exponentially. Each different approach can be classified depending on its invasiveness and on the technology employed. These sensors can be classified by their invasiveness, as either invasive (totally implantable), minimally invasive or non-invasive (Vaddiraju et al. 2010).

Classification according to the sensing technology ranges from electrochemistry to optics, and also includes combinatory approaches. Electrochemical enzymatic biosensors are the most common and more successful type of sensors that are integrated in CGM devices. Each of the strategies employed in the development of the CGM has its own advantages and disadvantages, and is associated with its own set of technological and physiological challenges.

1.7.1- Marketed CGM devices

Over 10 billion glucose assays are performed by diabetic patients annually. Their number vastly exceeds the combined numbers of all other chemical and biochemical analyses performed by humanity. The beginning of the 21st century coincided with the release of the first CGM systems (CGMS) onto the market (DeVries 2012). Currently there are only a handful of different commercial CGMs available with both FDA and CE approval, all invasive. Two non-invasive CGM were marketed but eventually discontinued due to malfunctions.

Just like all other pioneering products these are very prone to failure for a variety of reasons that ultimately lead to lack of accuracy. Nevertheless, these CGMS try to fill a gap in glucose monitoring. All of the existing CGMS measure glucose concentrations in the subcutaneous tissue. These devices display the rate of glucose change, the trend of glucose variability, and some are equipped with alarms for high or low threshold glucose concentrations (Hermanides et al. 2011; Poolsup et al. 2013; Vazeou 2011).

Clinical trials demonstrated its efficacy in lowering HbA1c in all age groups and reducing the time spent in hypoglycemia (Garg et al. 2006). However there are still several disadvantages that discourage the use of CGMS. The main issue concerning the use of CGMS by diabetic patients is still its poor accuracy, especially in specific groups prone to suboptimal therapy implementation (e.g. children, young adults) (Riveline 2011). It has been shown that following the trend in blood glucose changes can be more helpful than to rely on the absolute values provided by the sensor at a given time-point.

In that sense, the FDA recommends that CGMS shouldn't be used to assess the blood glucose concentrations, but rather assess changes in the glycemic state. In fact, despite all the advances in CGMS technology, these devices are still only approved by regulatory agencies to act as adjuncts in insulin therapy (Nichols and Klonoff 2007). None of the CGMS were yet able to replace conventional SMBG methods. CGMS readings are required to be verified by capillary glucose measurements before a decision is made to adjust medical interventions (D'Archangelo 2008, 2009).

As a matter of fact, all marketed CGMS, apart from the recently released Freestyle Navigator Libre, still need to be frequently calibrated with blood glucose measurements. However, under "non- normal" conditions, even this device requires frequent calibration (Bailey et al. 2015). The frequency of calibration largely depends on the used sensor technology. Besides its frequency, the timing of the calibrations is as or even more important.

Glucose values entered as a calibration point at the time of the rapid increase or fall of blood glucose can lead to erroneous CGMS readings. Additionally, since less data is acquired while the patient is sleeping, night values provided by the CGMS might be less accurate than those obtained during the day (Mazze et al. 2009; Tonyushkina and Nichols 2009).

The use of algorithm that converts electrochemical signal into glucose levels that put less weight on daytime calibrations for conversion in night time values and calibrating during times of relative glucose stability, may improve CGMS accuracy. Since state of the art CGMS have an alarm system incorporated, misreading due to incorrect calibrations can easily trigger false alarms. A recent study showed that CGM data obtained during hyperglycemia is reliable, but that CGM data obtained during hypoglycemia requires confirmation by self-monitoring before compensatory actions are to be taken (Facchinetti et al. 2010; Krouwer and Cembrowski 2010).

1.7.1.1- The Guardian

The Guardian was a version of Minimed's continuous glucose monitoring system released in the early 2010's. The CGMS consists of a subcutaneously implanted needle-type amperometric enzyme electrode coupled to a portable logger. It's *in vivo* implantation time was recently expanded from three to seven days. The biosensor part of the device being is still the limiting factor for greater implantation periods (Mazze et al. 2009).

The biosensor of this CGMS consists of a first generation amperometric enzyme-based biosensor, where GOx is immobilized onto a positively charged base electrode (+0.6 V). All of the sensors incorporated in the several versions of *The Guardian* CGM system use a three electrode setup. The sensor is enclosed in flexible polymer tubing with a side "window" exposing the active electrode area that is covered by a polyurethane membrane. The purpose of this membrane is to limit glucose diffusion to ensure a linear response in the concentration range of 20-400 mg/dL, and to reduce the sensor's dependency on partial oxygen pressure (McGarraugh 2009).

In vitro, the precision is within 5% in the range of 50–350 mg/dl, and the response time (t90) is 90 s. The biosensor signal is acquired every ten seconds with the average value stored in memory every minute. The Guardian displays a measurement every five minutes, and requires two to four calibrations per day (Keenan et al. 2009).

In 2009 Medtronic, the supplier of *The Guardian,* released the "Integrated MiniMed Paradigm Real-Time". It was the first time that a Glucose Monitoring System was combined with an insulin pump to form a closed-loop system. This device uses a powerful algorithm, the "Bolus wizard calculator" that automatically translates blood glucose readings from the biosensor element into an appropriate insulin dosage to be infused by the integrated insulin pump (Bode et al. 2004; Zisser et al.2010).

Randomized controlled clinical trials in adult type-1 patients showed that patients whose illness was intensively managed, either by traditional pump-assisted therapy or the Paradigm

Real-Time lowered their HbA1c. However, there was no significant difference in the decrease in HbA1c between the two management regiments. Nevertheless the use of the Paradigm Real-Time system significantly improved the number of subjects that reached an HbA1c target (7%) or lower compared to pump therapy with SMBG (Mastrototaro and Lee 2009).

Recently, Medtronic has released the Enlite, a new CGMS device with enhanced features on the device, such as smaller sensors and a novel insertion method. However, the biosensors incorporated in the Enlite still rely on the same technology as those incorporated in the all other CGMS by Medtronic. Although some accuracy issues were found while used in intense exercise, the Enlite was considered very reliable for glucose monitoring under resting conditions (Taleb et al. 2016).

1.7.1.2- The GlucoWatch G2 Biographer

The GlucoWatch G2 Biographer from Cygnus Therapeutics used a non-invasive transdermal method, based on the principle of iontophoresis. Iontophoresis (also known as Electromotive Drug Administration; EMDA) is a method for transdermal drug application without the use of a needle. The method is based on locally increasing skin permeability by application of a small electrical current.

In the case of the GlucoWatch, a small current is passed between two skin-surface electrodes to draw ions and (by electro-endosmosis) interstitial fluid (ISF) to the skin surface and into hydrogel pads. In the hydrogel pads the glucose-containing ISF is brought into contact with a glucose oxidase biosensor. These pads contained a mixture of two hydrophilic polymers, polyethylene glycol (PEG) and polyacrilic acid, cross linked with by means of an electron beam.

The electrochemical methods required for continuous measurements were complex and consisted of applying a constant current of 3 mA for three min to achieve the reverse iontophoresis. Then, a constant potential of 0.42 mV vs Ag/AgCl was applied for seven minutes to oxidize H_2O_2. This is followed by a second cycle on a second electrode; a running average of the integrated current from both electrodes produces a glucose value every ten minutes. The concentration of glucose in the transdermally extracted fluid were proportional to the concentration of glucose in subcutaneous tissue. This device was designed with a safeguard, as it uses two sets of similar electrodes to minimize errors. It us the running average of these values of the two electrodes produces every glucose value. The latest version of the GlucoWatch used a single calibration sample (McGarraugh 2009).

The GlucoWatch was able to provide near real-time readouts of blood glucose calibration very useful for prospective glycemia analysis. Data collected can be stored to be downloaded to a computer and used for retrospective glycemia analysis. The correlation of data obtained through the GlucoWatch was similar to the CGMS, and generally good when compared with SMBG. Clinical trials showed a linear relationship between the GlucoWatch readings and serial glucose measurements. The mean absolute error between the two measurements was

15.6% and 96.8% of the data fell in the therapeutically relevant regions of the error grid analysis (Tamada et al. 1999).

Although the GlucoWatch is a non-invasive method, a significant amount of patients complained about skin irritation. It was concluded that the iontophoretic current applied caused critical disruptions of the skin surface. These complications led to the withdrawal of this product from the market. Although accuracy is one, perhaps the limiting factor for the success of a CGMS, the GlucoWatch is a good example that it is not the only parameter to take into account. Moreover, it shows that even non-invasive methods can result in lower compliances when compared with invasive methods (McGarraugh 2009).

1.7.1.3- Pendra

The *Pendra* was also a non-invasive CGM from Pendagron Medical, based on impedance spectroscopy that obtained CE approval in 2004. The *Pendra* operated by impedance, or dielectric spectroscopy. It was the first truly noninvasive device, in the sense that the tissue fluid compartment of interest (in this case the microcirculation) was not violated or extracted. The device had a power source with alternating current that induced an electromagnetic field with low frequency (1-200 MHz). This magnetic field induced changes in impedance in the skin and the layer of adipose tissue underneath, which are caused by gradients in potassium and sodium concentrations. Changes in these gradients were correlated to changes in interstitial glucose levels in those tissues. Initial encouraging clinical trial results led this product to be released to the market in 2004. However, it was withdrawn shortly after its introduction due to problems related to poor *in vivo* accuracy (McGarraugh 2009).

1.7.1.4- GlucoDay

The GlucoDay was an *ex vivo* invasive CGMS from Mennarini Diagnostics based on microdialysis. A fine hollow dialysis fiber was implanted in the subcutaneous tissue and perfused with isotonic fluid. Glucose diffuses from the tissue into the lumen of the fiber, and the perfusate containing glucose was pumped outside the body to be analyzed by a glucose oxidase-based electrochemical biosensor. The dialysis membrane needed to be replaced by a new one after an implantation lifetime of 48 hours. The dialysis membrane consists of regenerated cellulose (i.d. 0.17 mm, o.d. 0.20 mm and molecular weight cut-off of approximately 15-20 kDa) and the membrane fiber was reinforced against collapse by a pair of twisted tungsten wires (Poscia et al. 2003; Varalli et al. 2003).

The glucose biosensor has the working principle of a first generation electrochemical sensor. A platinum anode (0.4 mm ø) was melted into a glass cylinder, which was inserted into a silver tube (cathode). The electrode was then covered by three membranes: a cellulose acetate membrane, an enzymatic membrane and a polycarbonate membrane. The cellulose acetate membrane allowed diffusion of hydrogen peroxide while removing potential

interference from ascorbic and uric acid. GOx was immobilized in the enzyme membrane by cross-linking with glutaraldehyde (0.25%) in presence of glucose (5%). The polycarbonate membrane was glucose limiting in order to obtain a linear response. The sensor is placed inside a wall-jet cell connected to the electronic micro-controller for programming, signal acquisition and data storage. The Glucoday included an electronic interface for data download and 9V battery sufficient for 48 hours of data acquisition (McGarraugh 2009; Poscia et al. 2003; Varalli et al. 2003).

The Glucoday also includes a programmable micro-peristaltic pump with an adjustable flow rate of either 15 or 100 μL/min. The slow flow is for sample acquisition and the fast flow is for cleaning the tubing. The disposable microdialysis probe is coupled to two reservoirs, one for the buffer and one for the waste. The whole device is contained in a pouch that can be worn on a belt. A stable perfusate flow is ensured by a pressure sensor. The Glucoday requires one calibration per day.

Randomized controlled trials with diabetic patients showed that the use of the Glucoday improves glycemic control in type-1 but not in type-2 diabetic patients (Hermanides et al. 2011; Kovatchev et al. 2008; van Bon et al. 2010).

Despite some initial commercial success, the GlucoDay and its successors disappeared from the market around 2010.

1.7.1.5- Dexcom devices.

Dexcom has commercialized several implantable CGMS. The first CGM of Dexcom series of CGMS was the STS system. Some of the issues of STS were fixed upon the released of the *Seven*. The *Seven* uses invasive technology and can be implanted up to 7 days, although some studies suggested that longer implantation periods might be possible (Garg et al. 2009; Hermanides et al. 2011; Knapp et al.2009).

All CGMS devices commercialized by Dexcom, from the STS system to the *Seven*, and lately the Gseries (G4 and G5), employ the same biosensor design.

The biosensor system isa two-electrode device with a coiled Ag/AgCl wire serving as a counter/reference electrode. The working electrode uses immobilized GOx with oxygen/hydrogen peroxide as the mediator. A mixed hydrophobic and hydrophilic polyurethane membrane is applied on top to limit the diffusion of glucose and to maximize the diffusion of oxygen. The exact structure of the polyurethane polymer was never disclosed by Dexcom, but it included small amounts of epoxy resins (McGarraugh 2009).

The biosensor was implanted in the subcutaneous tissue of the abdomen, and it required two hours for the signal to equilibrate. The CGMS needed to be calibrated once every 12 hours. (Garg et al. 2009).

A prospective real-life study showed similar glycemic benefits of using the *Seven* for patients with type-1 diabetes compared to typical SMBG methods. (Garg et al. 2011). In this study all subjects were provided guidance for adjusting insulin dose and/or food intake based

on glucose trends rate of change of glucose. Patients were initially educated on trends and pattern management while using the CGM. Although the use of the *Seven* did not improve HbA1c levels, it decreased glycemic variability when compared to gold standard methods of SMBG (Bailey et al. 2009).

The latest models released by DEXCOM are the G4 and G5. These models have significant improvements on the algorithms and user interfaces. However, these novel CGMS have yet to prove their efficacy. Nevertheless, preliminary results suggest that their performance may be improved when compared with earlier versions of CGMS delivered by DEXCOM (Christiansen et al. 2013).

1.7.1.6- Abbot Freestyle Navigator.

The latest version of the Navigator is a needle-type CGMS that can be implanted up to five days. The sensors require four calibrations at 10, 12, 24 and 72 hours after implantation. While early versions took approximately 10 hours for the stabilization, in the latest version, this period was reduced to 1 hour (McGarraugh et al. 2011).

This approach uses a classical three electrode system: a working electrode, a reference electrode and a counter electrode. The working electrode is designed as an enzyme-based (GOx) electrochemical biosensor for subcutaneous implantation. The electron transfer resistance is minimized by use of the second generation mechanisms in which the enzyme is coupled to the electrode surface with a polyvinyl-pyridine polymer and entrapped osmium, a redox mediator. This also allows the operation potential to be much lower than the one needed for oxidation of hydrogen peroxide (+40 mV vs +700 mV). The low oxidation potential increases the selectivity of the biosensors by reducing the interference from the main interfering electroactive components of the ISF (namely uric acid and ascorbic acid) (Hermanides et al. 2011; McGarraugh 2009).

In vivo tests have demonstrated a non-significant drift and consistent glucose readings from the sensor over a period of three days after implantation (Kovatchev et al. 2008; McGarraugh 2010).

Recently, Abbot Diabetes Care has released a new version of the Freestyle, the Libre®. It has been described to work accurately continuously for an unprecedented extended period (up to 14 days), without the need for regular blood glucose calibrations. However, this apparent "calibration-free" device, still requires calibration under "non-normal" conditions, such as hypoglycemia excursions and general fast changes in glucose levels (Bailey et al. 2015).

Although providing longer periods of read-out measurements, when compared to any other marketed device, early reports of its efficacy are still contradictory. While some authors show that the Libre provides reliable glucose levels, similar to exiting devices (Bonora et al. 2016), and even a positive outcome of its use in diabetic patients (Dover et al. 2016), a study questions the accuracy of the device (Schierenbeck et al. 2016).

1.7.1.7 - Reasons for criticism

Despite the continuous release of proof-of-concept glucose biosensors, the number of commercially available CGMS devices on the market has stagnated in the last decade. Additionally, none of them could yet replace the conventional methods for SMBG with complete success.

Even with all the latest developments the CGMS currently on the market are still hampered by lack of accuracy, an opinion shared by the regulatory agencies for safety of biomedical devices (e.g. FDA). The current status of CGMS in diabetes management (only to be used as adjuncts) reflects the concerns on their accuracy, thus their safety. The sources of inaccuracy vary, depending on the specific sensing technology applied. Nevertheless, they stem from the interaction between the device and the harsh environment of the body, whose deleterious effects were described as biofouling (Mazze et al. 2009; Nichols and Klonoff 2007; Poolsup et al. 2013).

In recent decades the accuracy of CGMS has been improved tremendously. However, those efforts still didn't overcome all accuracy problems. Poor accuracy on glucose levels provided by CGMS still leads to misjudgments on the real condition of diabetic patients. This, in turn, may lead to erroneous adjustments in patient therapy.

Nevertheless, the effectiveness of all of CGMS in the market was evaluated in controlled clinical trials, with diabetic patients, under proper clinical guidance. Most of the clinical trials showed that CGMS devices showed some ability to reliably monitor glucose levels. Although the exact levels of glucose provided by CGMS were slightly deviated from values from SMBG, they add useful information on the dynamics of glucose variations. Some of them even improved glycemic control of diabetic patients. However, it is acknowledge by the scientific community and regulatory agencies, that CGMS were still not fully evaluated and their effectiveness outside clinical environment remains unsure (D'Archangelo 2008, 2009; DeVries 2012; Facchinetti et al. 2010; Group 2006; Keenan et al. 2009; Kovatchev et al. 2008; Mazze et al. 2009; McGarraugh 2010; Nichols and Klonoff 2007; Poolsup et al. 2013; Vaddiraju et al. 2010).

Changes in the environment can lead to significant reduction in the accuracy of these devices. The sensing element is prone to be affected by temperature, atmospheric pressure and sudden changes in glucose of the patient (*e.g.* physical exercise). State-of-the-art CGM still need to prove their effectiveness in "real life" situations.

Additionally, all of the current CGMS have biosensors still need to be frequently replaced, because of their life span is short ($\leq$ 2 weeks). This will result in two major problems: the need to a small surgical procedure on a regular basis (for sensor implantation) and increase in the costs of the use of such devices for both health care systems and patients (Vazeou 2011). Besides the discomfort associated with frequent small surgical procedures, biosensor implantation can trigger local reactions. These can vary from skin reactions to different elements of the biosensor to local infections. Furthermore, scarring after sensor removal may be annoying for young adolescents, being a source of non-compliance in this age group

(Block et al. 2008).

Furthermore, none of the CGMS devices have yet eliminated the need for the finger prick. In fact, all still rely on frequent blood glucose measurements for calibration (often more than once per day), especially during non-euglycemic conditions. The need for calibration is due to biosensor signal drift while implanted. The drift is caused by loss of sensitivity and selectivity of the biosensor part of CGMS. Despite major improvements in algorithms, which correct for signal drift, frequent calibrations are still essential for reliable glucose measurements by the CGMS (Lodwig and Heinemann 2003).

The blood glucose levels for calibration of the CGMS need to be manually entered by patients themselves. This aspect is not trivial for some groups of diabetic patients, especially for disabled, elderly and young patients. Proper sensor use needs very good patient education. Experience improves performance as time goes by (Mazze et al. 2009).

An often disregarded negative aspect is the high costs associated to the utilization of CGMS devices. Additionally, due to its adjunct only status, these costs are only marginally reimbursed if reimbursed at all (Vazeou 2011). Even if the scientific community might be able to tackle all technological challenges, it still has to find a way to decrease the costs associated to CGMS, in order to enable a widespread of use of these devices in diabetes management.

Overall, the pain and discomfort associated with an intensive control of glucose levels still remain, even after the advent of CGMS (Heinemann 2008). However a higher degree of precision and accuracy is still necessary for CGMS to contribute for a better control of glucose levels. Although biomedical science has come a long way in the development of CGMs, there are some issues that need to be addressed. Only then CGMS will be able to replace conventional methods of monitoring glucose levels.

1.7.2- Physiological challenges of CGM biosensors

The physiological environment in which invasive *in vivo* biosensors operate is an important factor to take into account, when we discuss CGMS limitations. Glucose biosensors included in CGMS are designed to be implanted in subcutaneous adipose tissue and to measure glucose concentrations in the interstitial fluid. All the research performed with these implantable biosensors takes into account a correlation between glucose concentrations in the ISF and in the blood (Yoo and Lee 2010).

Initial efforts on biosensors research for CGM, was directed to measure glucose in the blood, similar to conventional methods (Kondo et al. 1982). However the blood is a very harsh environment for biosensors and implanting biosensors on blood vessels is not trivial. When implanted in the blood, biosensor performance decreases rapidly. The complex biochemical composition of the blood adds another problem, to selectively quantify glucose (Daniloff 1999). Moreover, poor biocompatibility of the surface materials induced infections, embolisms and in extreme cases cardiac arrests. Biosensors for *in vivo* implantation in the

blood were considered unsafe and never reached clinical trials.

To implant the biosensor in the subcutaneous adipose tissue is safer (fewer hemorrhages and lower risk of embolisms), more convenient (less complex surgeries needed), and is generally believed to provide the same information about circulating glucose. But the relationship between glucose concentrations in the ISF and in the blood is complex and dynamic, although both are considered equally useful for glycemic management (Holmäng et al. 1998; Keenan et al. 2009; Lourido et al. 2002; Mazze et al. 2009; Rosdahl et al. 1993).

Adipose tissue consists of closely packed fat cells in a meshwork of reticular collagen fibers, and is vascularized by a rich capillary network. The capillary lumen has a diameter of approximately 4–5µm. The basal membranes of the capillaries are in direct contact with the cytoplasmic membrane of the fat cells. This peculiar membrane–membrane contact is only present in adipose tissue and the central nervous system. The interstitial space is relatively small compared to other tissues. This environment forms a diffusion barrier for glucose, but not for oxygen (Facchinetti et al. 2007; Voskanyan et al. 2007).

In addition, the biochemical nature of the extracellular environment poses specific challenges for biosensors to be able to accurately measure glucose levels in the adipose tissue. The most important physiological and technological challenges are discussed below.

1.7.2.1-Selectivity

It is well know that poor selectivity of electrochemical biosensors in CGMS is one of the main causes of their inaccuracy. It is difficult to achieve good selectivity, because in the body there are many molecules with electrochemical properties similar to glucose, most prominently uric acid and ascorbic acid. Several different strategies are being used to limit the impact of interference. One of these strategies is the application of a permselective layer onto the biosensor that is permeable to the target analyte but not to non-specific electroactive species(Cordeiro et al. 2016; McMahon et al. 2004; O'Neill et al. 2008; Ronkainen et al. 2010; Wang 2008).

A second strategy to limit interference is to change the mechanism for electron transfer from the biorecognition element to the transducer, in order to reduce the applied potential. Unspecific molecules are less likely to be oxidized at a lower potential, thus an increased selectivity is achieved.

A third strategy involves the use of a background electrode for differential recording, in the so- called self-referencing system (Cordeiro et al. 2015). The background electrode is placed in the vicinity of the working electrode, and generates a signal that is proportional to the concentrations of all of the non-specific electroactive species. The current with high analyte selectivity is obtained by subtracting the background current from the working current. Of course, this technique works best if the currents are relatively low and the electrodes are positioned in close proximity of each other. It is essential that the physiological environment to which the two electrodes are exposed is as close as possible. The combination

of permselective layers with a background sensor is a promising way to achieve superior selectivity in biosensors for *in vivo* implantation.

1.7.2.2- Correlation between glucose concentrations in blood and ISF

Glucose biosensors incorporated in CGMS typically measure glucose concentrations in ISF. The working mechanism of the CGMS is based on the assumption that the ISF concentration of glucose is highly correlated with the concentration of glucose in the blood. Whether this assumption is correct is still a matter of debate.

In fact several studies revealed that the relationship between glucose concentrations in the blood and in the ISF is dynamic and very complex. It has been reported the concentration of glucose in adipose ISF is lower than in the blood during steady-state conditions. The extent of this difference is affected by the physiological conditions of the tissue immediately surrounding the biosensor. These conditions include the rate of glucose utilization in the tissue, the degree of vascularization, local blood flow, tissue damage caused by implantation, and any tissue responses to the implanted device (Lourido et al. 2002; Schoonen and Wientjes 2003).

Studies evaluating the relationship between plasma glucose (PG) and interstitial glucose (IG) have provided conflicting results. Some investigators demonstrated the presence of a glucose concentration gradient between blood and ISF during steady-state conditions between 20% and 110%. Additionally, it was found that during dynamic conditions, the equilibration time delay between PG and IG can range from 2-3 minutes to 45 minutes. The use of a wide variety of techniques and methods used, as well as differences in subjects/species, in very different as experimental conditions, eg, glucose/insulin clamp possible may be the reasons for such highly variability in the ratio. Nevertheless, most studies have demonstrated not a constant, but a dynamic interstitial to plasma glucose gradient (the IG/PG ratio). This implies that the IG/PG relationship is not trivial. A deeper understanding is required if subcutaneous glucose sensing is to become an even more accurate surrogate for PG measurements (Bolincier et al. 1992; Keenan et al. 2009; Lourido et al. 2002; Rebrin et al. 1999; Regittnig et al. 2003; Rosdahl et al. 1993; Schaupp et al. 1999; Voskanyan et al. 2007).

It is well known that, during both steady-state and hyperglycemic conditions, the glucose levels in the ISF are significantly lower than the corresponding venous plasma values under resting conditions (adipose tissue 3.2 mM, plasma 5.3 mM and adipose tissue 7.3 mM, plasma 9.9 mM respectively). However, in case of hypoglycemia, a clear decrease in IG/PG ratio has been reported. Nonetheless, the extent of the decrease and the associated time lag is controversial. Several investigators have noted that recovery from hypoglycemia takes longer in IG compared with PG. However a study concerning the ISF glucose dynamics during insulin-induced hypoglycemia showed the same decrease in IG/PG ration but no delay in recovery from hypoglycemia. These finding may have important implications for

the development of better algorithms for the calibration of the biosensors incorporated in the CGMS (Rebrin et al. 1999; Regittnig et al. 2003; Schaupp et al. 1999).

Regardless, shortly after insertion of biosensors into the subcutaneous adipose tissue, the collected samples are not in a steady state with the surrounding tissue. This is due to the trauma caused by the implantation that results in the disruption of local blood vessels, cells, and capillaries. Biosensor implantation results in physiological artifacts of the measurements, due to inflammation and wound healing process. These processes in turn, have a negative impact on biosensor performance.

It was reported that stable glucose values in the ISF were only obtained 4-6 h after probe/sensor insertion. The implantation procedure induces changes in trans-capillary exchange of glucose between the blood and ISF, with significant implications on local glucose levels. Additionally, it has been suggested that device implantation by itself, may also have a significant impact on local glucose levels, especially for hypoglecemia events, due to the "push-pull" effect. However this correlation is not entirely understood. Nevertheless, it is clear that physiological changes in blood glucose directly, as well as those triggered by device implantation affect the IG/PG ratio, with clear implications in the output of CGMS measurements. (Schoonen and Wientjes 2003; van der Valk et al. 2002).

The wound healing processes that are triggered following sensor/probe implantation may lead to additional local glucose consumption, with implications in the trans-capillary exchange. The implantation of sensor/probe for glucose measurements affects not only glucose availability in the tissues but the sensor/probe itself. Inflammation due to implantation is known to affect both probes (changes in recovery rates) and sensors (changes in sensor performance). A good understanding of all these phenomena is crucial to interpret CGM in the ISF.

1.7.2.3- Foreign body response

Biofouling, defined as reduced biosensor performance caused by the interaction with the tissue, is the most important reason for the failure of biosensors upon implantation. Biofouling starts immediately after implantation, and is causally related to the physiological processes that are involved in wound healing, known as foreign body response (FBR). Wound healing has four stages in chronological order: homeostasis, inflammation, repair and scar formation. Biofouling can be characterized by the effect (mostly adverse), of the adhesion of proteins and other biological material to the sensor surface (Cordeiro et al. 2015; Wisniewski et al. 2000; Wisniewski and Reichert 2000).

Membrane biofouling starts immediately upon contact of the sensor with the body. Upon implantation, cells, proteins and other biological components adhere to the surface, impregnating the pores of the membrane. These phenomena results in impaired analyte diffusion by protein adhesion. The adhering proteins involved in membrane fouling are thought to be involved in the modulation of long term cellular and encapsulation response by

the body. It is known that encapsulation characteristics such as layer thickness, vascularization and permeability can be controlled through membrane porosity/topology. In spite of the distinction between biofouling and encapsulation it is difficult to differentiate them. Both effects are intertwined and retard access of the sensor to analyte (Wisniewski et al. 2000; Wisniewski and Reichert 2000).

Biosensor failure due to biofouling can be divided into two categories: component-based failure and biocompatibility based-failure. Component-based failure is mainly characterized by failure due to disconnection of leads, physical rupture of the membranes, and electrical short. Biocompatibility-based failure includes degradation of membranes and enzyme, membrane biofouling, encapsulation, and electrode passivation.

Both types of biofouling lead to the same outcome, a continuous decline in sensor signal, and ultimately its failure.

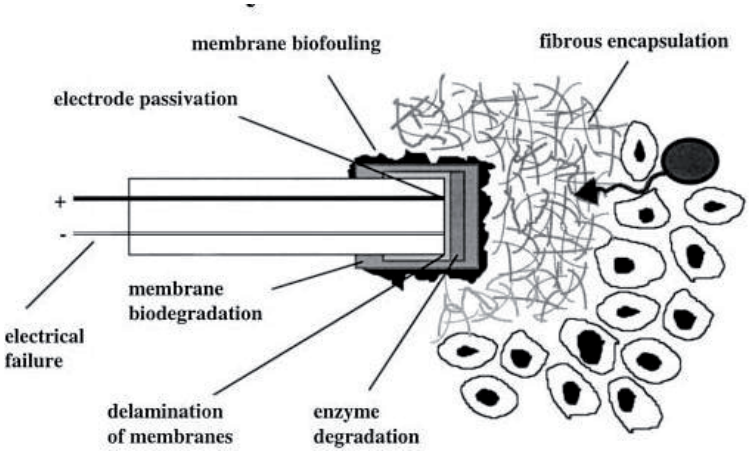


Figure 8 – Schematic representation of the different biofouling effects in sensor failure (Wisnieski, 2000).

Up to date, there are no evidences that point to a convincing ranking in the order of the biofouling issues leading to *in vivo* sensor failure. There is not a consensus on which of the issues described above has a bigger impact on biosensor longevity, when implanted. In fact, the relative importance of each of the problems is dependent on the design and construction of a particular sensor.

Most of the efforts made for improving biosensor performance is focused on increasing their biocompatibility levels (Onuki et al. 2008). Biocompatibility can be defined as the capability of a prosthesis implanted in the body to exist in harmony with tissue without causing deleterious changes. It is believed that an increase in biosensor biocompatibility may reduce the FBR, thus reduce biofouling and hence increase the lifetime and accuracy of biosensors when implanted (Soto and Schoenfisch 2015).

Recently some strategies have been developed, aiming to increase biocompatibility of implanted biosensors. These include surface modifications to reduce protein adsorption; assembly of hydrogels employing adhesion ligands and growth factors; local drug delivery strategies and physical modification strategies (Koh et al. 2011). However, biosensor biocompatibility has not yet been fully understood. Despite several advances, a reliable solution remains elusive.

In this thesis I tried to deepen the knowledge on the working mechanisms of amperometric enzyme-based biosensors, focusing on those assembled on a "modified" 1st generation technology. Based on the newly acquired knowledge, I tried to develop and characterize a series of amperometric biosensors, for in vivo biomonitoring of key biomarkers in diabetes management and etiology. Additionally, we tried to show that the application of these type of biosensors can reach beyond the classical continuous glucose monitoring.

1.8- Bibliography

Abel, P.U., von Woedtke, T., 2002. Biosensors for in vivo glucose measurement: can we cross the experimental stage. Biosensors and Bioelectronics 17, 1059-1070.

Adams, O.P., 2013. The impact of brief high-intensity exercise on blood glucose levels. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy 6, 113-122.

Alqahtani, N., Khan, W.A.G., Alhumaidi, M.H., Ahmed, Y.A.A.R., 2013. Use of Glycated Hemoglobin in the Diagnosis of Diabetes Mellitus and Pre-diabetes and Role of Fasting Plasma Glucose, Oral Glucose Tolerance Test. International Journal of Preventive Medicine 4(9), 1025-1029.

Aronoff, S.L., Berkowitz, K., Shreiner, B., Want, L., 2004. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectrum 17(3), 183-190.

Association, A.D., 2015. 2. Classification and Diagnosis of Diabetes. Diabetes Care 38(Supplement 1), S8-S16. Aye, T., Block, J., Buckingham, B., 2010. Toward Closing the Loop: An Update on Insulin Pumps and Continuous Glucose Monitoring Systems. Endocrinology and metabolism clinics of North America 39(3), 609-624.

Aziz, M.A., Kawde, A.-N., 2013. Nanomolar amperometric sensing of hydrogen peroxide using a graphite pencil electrode modified with palladium nanoparticles. Microchimica Acta 180(9), 837-843.

Bailey, T., Bode, B.W., Christiansen, M.P., Klaff, L.J., Alva, S., 2015. The Performance and Usability of a Factory-Calibrated Flash Glucose Monitoring System. Diabetes Technology & Therapeutics 17(11), 787-794.

Bailey, T., Zisser, H., Chang, A., 2009. New Features and Performance of a Next-Generation SEVEN-Day Continuous Glucose Monitoring System with Short Lag Time. Diabetes Technology & Therapeutics 11(12), 749-755.

Bard, A.J., Faulkner, L.R., 2000. ELECTROCHEMICAL METHODS Fundamentals and Applications, 2nd ed. John Wiley & Sons.

Battelino, T., Phillip, M., Bratina, N., Nimri, R., Oskarsson, P., Bolinder, J., 2011. Effect of Continuous Glucose Monitoring on Hypoglycemia in Type 1 Diabetes. Diabetes Care 34(4), 795-800.

Benjamin, E.M., 2002. Self-Monitoring of Blood Glucose: The Basics. Clinical Diabetes 20(1), 45-47. Bisswanger, H., 2008. Enzyme Kinetics: Principles and Methods. Wiley-VCH.

Block, C.D., Manuel-y-Keenoy, B., Van Gaal, L., 2008. A Review of Current Evidence with Continuous Glucose Monitoring in Patients with Diabetes. Journal of Diabetes Science and Technology 2(4), 718-727.

Bockris, J.O.M., Reddy, A.K.N., Gamboa-Aldeco, M., 2000. Modern Electrochemistry 2A Fundamentals of Electrodics, 2nd ed. Kluwer Academic/Plenum Publishers.

Bode, B., Gross, K., Rikalo, N., Schwartz, S., Wahl, T., Page, C., Gross, T., Mastrototaro, J., 2004. Alarms Based on Real-Time Sensor Glucose Values Alert Patients to Hypo- and Hyperglycemia: The Guardian Continuous Monitoring System. Diabetes Technology & Therapeutics 6(2), 105-113.

Bolincier, J., Ungerstedt, U., Arner, P., 1992. Microdialysis measurement of the absolute glucose concentration in subcutaneous adipose tissue allowing glucose monitoring in diabetic patients. Diabetologia 35(12), 1177-1180.

Bonora, B., Maran, A., Ciciliot, S., Avogaro, A., Fadini, G.P., 2016. Head-to-head comparison between flash and continuous glucose monitoring systems in outpatients with type 1 diabetes. Journal of Endocrinological Investigation 39(12), 1391-1399.

Chambers, J.P., Arulanandam, B.P., Matta, L.L., Weis, A., Valdes, J.J., 2008. Biosensor Recognition Elements. Curr. Issues Mol. Biol. 10, 1-12.

Christiansen, M., Bailey, T., Watkins, E., Liljenquist, D., Price, D., Nakamura, K., Boock, R., Peyser, T., 2013. A New-Generation Continuous Glucose Monitoring System: Improved Accuracy and Reliability Compared with a Previous-Generation System. Diabetes Technology & Therapeutics 15(10), 881-888.

Clark, L.C., 1993. Guest Editorial. Biosensors and Bioelectronics 8(1), iii-vii.

Connolly, P., 1995. Clinical diagnostics opportunities for biosensors and bioelectronics. Biosensors and Bioelectronics 10(1), 1-6.

Cooney, M.J., 2011. Kinetic Measurements for Enzyme Immobilization. In: Minteer, D.S. (Ed.), Enzyme Stabilization and Immobilization: Methods and Protocols, pp. 207-225. Humana Press, Totowa, NJ.

Cordeiro, C.A., de Vries, M.G., Cremers, T.I.F.H., Westerink, B.H.C., 2016. The role of surface availability in membrane-induced selectivity for amperometric enzyme-based biosensors. Sensors and Actuators B: Chemical 223, 679-688.

Cordeiro, C.A., de Vries, M.G., Ngabi, W., Oomen, P.E., Cremers, T.I.F.H., Westerink, B.H.C., 2015. In vivo continuous and simultaneous monitoring of brain energy substrates with a multiplex amperometric enzyme-based biosensor device. Biosensors and Bioelectronics 67, 677-686.

Cosnier, S., 1999. Biomolecule immobilization on electrode surfaces by entrapment or attachment to electrochemically polymerized films. A review. Biosensors and Bioelectronics 14(5), 443-456.

D'Archangelo, M.J., 2008. New Guideline Supports the Development and Evaluation of Continuous Interstitial Glucose Monitoring Devices. Journal of Diabetes Science and Technology 2(2), 332-334.

D'Archangelo, M.J., 2009. Unlocking the Potential of Continuous Glucose Monitoring: A New Guideline Supports the Development of Continuous Glucose Monitoring Devices. Journal of Diabetes Science and Technology 3(2), 363-365.

da Rocha Fernandes, J., Ogurtsova, K., Linnenkamp, U., Guariguata, L., Seuring, T., Zhang, P., Cavan, D., Makaroff, L.E., 2015. IDF Diabetes Atlas estimates of 2014 global health expenditures on diabetes. Diabetes Research and Clinical Practice 117, 48-54.

Daniloff, G.Y., 1999. Continuous Glucose Monitoring: Long-Term Implantable Sensor Approach. Diabetes Technology & Therapeutics 1(3), 261-266.

DeVries, J.H., 2012. Continuous glucose monitoring: coming of age? European Journal of Endocrinology 166(1), 1-4.

Dover, A.R., Stimson, R.H., Zammitt, N.N., Gibb, F.W., 2016. Flash Glucose Monitoring Improves Outcomes in a Type 1 Diabetes Clinic. Journal of Diabetes Science and Technology.

Dowd, J.E., Riggs, D.S., 1965. A Comparison of Estimates of Michaelis-Menten Kinetic Constants from Various Linear Transformations. Journal of Biological Chemistry 240(2), 863-869.

Facchinetti, A., Sparacino, G., Cobelli, C., 2007. Reconstruction of Glucose in Plasma from Interstitial Fluid Continuous Glucose Monitoring Data: Role of Sensor Calibration. Journal of Diabetes Science and Technology 1(5), 617-623.

Facchinetti, A., Sparacino, G., Cobelli, C., 2010. Modeling the Error of Continuous Glucose Monitoring Sensor Data: Critical Aspects Discussed through Simulation Studies. Journal of Diabetes Science and Technology 4(1), 4-14.

Fan, X., White, I.M., Shopova, S.I., Zhu, H., Suter, J.D., Sun, Y., 2008. Sensitive optical biosensors for unlabeled targets: A review. Analytica Chimica Acta 620(1–2), 8-26.

Fertig, B.J., Simmons, D.A., Martin, D.B., 1995. Therapy for Diabetes. Diabetes in America, 2nd ed. Nationl

Institutes of Health, Bethesda.

Florez, J.C., 2016. Leveraging Genetics to Advance Type 2 Diabetes Prevention. PLoS Med 13(7), e1002102. Forbes, J.M., Cooper, M.E., 2013. Mechanisms of Diabetic Complications. Physiological Reviews 93(1), 137-188.

Force, U.S.P.S.T., 2008. Screening for Type 2 Diabetes Mellitus in Adults: U.S. Preventive Services Task ForceRecommendation Statement. Annals of Internal Medicine 148(11), 846-854.

Gadsby, R., 2002. Epidemiology of diabetes. Advanced Drug Delivery Reviews 54(9), 1165-1172.

Garg, S., Zisser, H., Schwartz, S., Bailey, T., Kaplan, R., Ellis, S., Jovanovic, L., 2006. Improvement in Glycemic Excursions With a Transcutaneous, Real-Time Continuous Glucose Sensor. A randomized controlled trial 29(1), 44-50.

Garg, S.K., Voelmle, M.K., Beatson, C.R., Miller, H.A., Crew, L.B., Freson, B.J., Hazenfield, R.M., 2011. Use of Continuous Glucose Monitoring in Subjects With Type 1 Diabetes on Multiple Daily Injections Versus Continuous Subcutaneous Insulin Infusion Therapy. A prospective 6-month study 34(3), 574-579.

Garg, S.K., Voelmle, M.K., Gottlieb, P., 2009. Feasibility of 10-Day Use of a Continuous Glucose-Monitoring System in Adults With Type 1 Diabetes. Diabetes Care 32(3), 436-438.

Gerich, J.E., 1993. Control of glycaemia. Baillieres Clinical Endocrinology and Metabolism 7(3), 551-586. Giannini, C., Mohn, A., Chiarelli, F., 2009. Technology and the issue of cost/benefit in diabetes.
Diabetes/Metabolism Research and Reviews 25(S1), S34-S44.

Grieshaber, D., MacKenzie, R., Vörös, J., Reimhult, E., 2008. Electrochemical Biosensors - Sensor Principles and Architectures. Sensors (Basel, Switzerland) 8(3), 1400-1458.

Group, D.i.R.C.N.D.S., 2006. Evaluation of Factors Affecting CGMS Calibration. Diabetes Technology & Therapeutics 8(3), 318-325.

Heinemann, L., 2008. Finger Pricking and Pain: A Never Ending Story. Journal of diabetes science and technology (Online) 2(5), 919-921.

Hermanides, J., Phillip, M., DeVries, J.H., 2011. Current Application of Continuous Glucose Monitoring in the Treatment of Diabetes. Diabetes Care 34(Supplement 2), S197-S201.
Hirsch, I.B., Bergenstal, R.M., Parkin, C.G., Wright, E., Buse, J.B., 2005. A Real-World Approach to Insulin Therapy in Primary Care Practice. Clinical Diabetes 23(2), 78-86.

Holford, T.R.J., Davis, F., Higson, S.P.J., 2012. Recent trends in antibody based sensors. Biosensors and Bioelectronics 34(1), 12-24.

Holmäng, A., Müller, M., Andersson, O.K., Lönnroth, P., 1998. Minimal influence of blood flow on interstitial glucose and lactate-normal and insulin-resistant muscle. American Journal of Physiology - Endocrinology and Metabolism 274(3), E446-E452.

Jena, B.K., Raj, C.R., 2006. Electrochemical Biosensor Based on Integrated Assembly of Dehydrogenase Enzymes and Gold Nanoparticles. Analytical Chemistry 78(18), 6332-6339.

Jönsson, B., 2002. Revealing the cost of Type II diabetes in Europe. Diabetologia 45(7), S5-S12.

Keenan, D.B., Mastrototaro, J.J., Voskanyan, G., Steil, G.M., 2009. Delays in Minimally Invasive Continuous Glucose Monitoring Devices: A Review of Current Technology. Journal of Diabetes Science and Technology 3(5), 1207-1214.

King, H., Aubert, R.E., Herman, W.H., 1998. Global Burden of Diabetes, 1995–2025: Prevalence, numerical estimates, and projections. Diabetes Care 21(9), 1414-1431.

Kissinger, P.T., 2005. Biosensors—a perspective. Biosensors and Bioelectronics 20(12), 2512-2516.

Knapp, P.E., Showers, K.M., Phipps, J.C., Speckman, J.L., Sternthal, E., Freund, K.M., Ash, A.S., Apovian, C.M., 2009. Self-Monitoring of Blood Glucose with Finger Tip Versus Alternative Site Sampling: Effect on Glycemic Control in Insulin-Using Patients with Type 2 Diabetes. Diabetes Technology & Therapeutics 11(4),219-225.

Koh, A., Nichols, S.P., Schoenfisch, M.H., 2011. Glucose Sensor Membranes for Mitigating the Foreign Body Response. Journal of Diabetes Science and Technology 5(5), 1052-1059.

Kondo, T., Ito, K., Ohkura, K., Ito, K., Ikeda, S., 1982. A Miniature Glucose Sensor, Implantable in the Blood Stream. Diabetes Care 5(3), 218-221.

Kovatchev, B., Anderson, S., Heinemann, L., Clarke, W., 2008. Comparison of the Numerical and Clinical Accuracy of Four Continuous Glucose Monitors. Diabetes Care 31(6), 1160-1164.

Krouwer, J.S., Cembrowski, G.S., 2010. A Review of Standards and Statistics Used to Describe Blood Glucose Monitor Performance. Journal of Diabetes Science and Technology 4(1), 75-83.

Lodwig, V., Heinemann, L., 2003. Continuous Glucose Monitoring with Glucose Sensors: Calibration and Assessment Criteria. Diabetes Technology & Therapeutics 5(4), 572-586.

Lourido, J., Ederoth, P., Sundvall, N., Ungerstedt, U., Nordström, C.H., 2002. Correlation between blood glucose concentration and glucose concentration in subcutaneous adipose tissue evaluated with microdialysis during intensive care. Scandinavian Journal of Clinical and Laboratory Investigation 62(4), 285-292.

Ma, W., Ying, Y.-L., Qin, L.-X., Gu, Z., Zhou, H., Li, D.-W., Sutherland, T.C., Chen, H.-Y., Long, Y.-T., 2013. Investigating electron-transfer processes using a biomimetic hybrid bilayer membrane system. Nat. Protocols 8(3), 439-450.

Maggs, D., MacDonald, I., Nauck, M.A., 2008. Glucose homeostasis and the gastrointestinal tract: insights into the treatment of diabetes. Diabetes, Obesity and Metabolism 10(1), 18-33.

Marik, P.E., Bellomo, R., 2013. Stress hyperglycemia: an essential survival response! Critical Care 17(2), 1-7.

Mascini, M., Tombelli, S., 2008. Biosensors for biomarkers in medical diagnostics. Biomarkers 13(7-8), 637-657.

Mastrototaro, J., Lee, S., 2009. The Integrated MiniMed Paradigm Real-Time Insulin Pump and Glucose Monitoring System: Implications for Improved Patient Outcomes. Diabetes Technology & Therapeutics 11(s1), S-37-S-43.

May, S.W., 1999. Applications of oxidoreductases. Current Opinion in Biotechnology 10(4), 370-375.

Mazze, R.S., Strock, E., Borgman, S., Wesley, D., Stout, P., Racchini, J., 2009. Evaluating the Accuracy,

Reliability, and Clinical Applicability of Continuous Glucose Monitoring (CGM): Is CGM Ready for Real Time? Diabetes Technology & Therapeutics 11(1), 11-18.

McAndrew, L., Schneider, S.H., Burns, E., Leventhal, H., 2007. Does Patient Blood Glucose Monitoring Improve Diabetes Control?: A Systematic Review of the Literature. The Diabetes Educator 33(6), 991-1010.

McGarraugh, G., 2009. The Chemistry of Commercial Continuous Glucose Monitors. Diabetes Technology & Therapeutics 11(s1), S-17-S-24.

McGarraugh, G., 2010. Alarm Characterization for a Continuous Glucose Monitor That Replaces Traditional Blood Glucose Monitoring. Journal of Diabetes Science and Technology 4(1), 49-56.

McGarraugh, G., Brazg, R., Weinstein, R., 2011. FreeStyle Navigator Continuous Glucose Monitoring System with TRUstart Algorithm, a 1-Hour Warm-up Time. Journal of Diabetes Science and Technology 5(1), 99-106.

McMahon, C.P., Killoran, S.J., Kirwan, S.M., O'Neill, R.D., 2004. The selectivity of electrosynthesised polymer membranes depends on the electrode dimensions: implications for biosensor applications. Chemical Communications(18), 2128-2130.

Murphy, L., 2006. Biosensors and bioelectrochemistry. Current Opinion in Chemical Biology 10(2), 177-184.
Nichols, J.H., Klonoff, D.C., 2007. The Need for Performance Standards for Continuous Glucose Monitors. Journal of diabetes science and technology (Online) 1(1), 92-94.

Noble, J.A., Erlich, H.A., 2012. Genetics of Type 1 Diabetes. Cold Spring Harbor Perspectives in Medicine 2(1), a007732.

O'Neill, R.D., Rocchitta, G., McMahon, C.P., Serra, P.A., Lowry, J.P., 2008. Designing sensitive and selective polymer/enzyme composite biosensors for brain monitoring in vivo. TrAC Trends in Analytical Chemistry 27(1), 78-88.

Olokoba, A.B., Obateru, O.A., Olokoba, L.B., 2012. Type 2 Diabetes Mellitus: A Review of Current Trends. Oman Medical Journal 27(4), 269-273.

Onuki, Y., Bhardwaj, U., Papadimitrakopoulos, F., Burgess, D.J., 2008. A Review of the Biocompatibility of Implantable Devices: Current Challenges to Overcome Foreign Body Response. Journal of Diabetes Science and Technology 2(6), 1003-1015.

Pociot, F., Lernmark, Å., 2016. Genetic risk factors for type 1 diabetes. The Lancet 387(10035), 2331-2339.

Pohanka, M., Skladal, P., 2008. Electrochemical biosensors – principles and applications. Journal of Applied Biomedicine 6, 57-64.

Poolsup, N., Suksomboon, N., Kyaw, A.M., 2013. Systematic review and meta-analysis of the effectiveness of continuous glucose monitoring (CGM) on glucose control in diabetes. Diabetology & Metabolic Syndrome 5, 39- 39.

Poscia, A., Mascini, M., Moscone, D., Luzzana, M., Caramenti, G., Cremonesi, P., Valgimigli, F., Bongiovanni, C., Varalli, M., 2003. A microdialysis technique for continuous subcutaneous glucose monitoring in diabetic patients (part 1). Biosensors and Bioelectronics 18(7), 891-898.

Privett, B.J., Shin, J.H., Schoenfisch, M.H., 2008. Electrochemical Sensors. Analytical Chemistry 80(12), 4499-4517.

Putzbach, W., Ronkainen, N., 2013. Immobilization Techniques in the Fabrication of Nanomaterial-Based Electrochemical Biosensors: A Review. Sensors 13(4), 4811.

Rebrin, K., Steil, G.M., van Antwerp, W.P., Mastrototaro, J.J., 1999. Subcutaneous glucose predicts plasma glucose independent of insulin: implications for continuous monitoring. American Journal of Physiology - Endocrinology and Metabolism 277(3), E561-E571.

Regittnig, W., Ellmerer, M., Fauler, G., Sendlhofer, G., Trajanoski, Z., Leis, H.-J., Schaupp, L., Wach, P., Pieber, T.R., 2003. Assessment of transcapillary glucose exchange in human skeletal muscle and adipose tissue. American Journal of Physiology - Endocrinology and Metabolism 285(2), E241-E251.

Riveline, J.P., 2011. Is continuous glucose monitoring (CGM) for everyone?: To whom should CGM be prescribed and how? Diabetes & Metabolism 37, Supplement 4, S80-S84.

Rocchitta, G., Spanu, A., Babudieri, S., Latte, G., Madeddu, G., Galleri, G., Nuvoli, S., Bagella, P., Demartis, M., Fiore, V., Manetti, R., Serra, P., 2016. Enzyme Biosensors for Biomedical Applications: Strategies for Safeguarding Analytical Performances in Biological Fluids. Sensors 16(6), 780.

Ronkainen, N.J., Halsall, H.B., Heineman, W.R., 2010. Electrochemical biosensors. Chemical Society Reviews 39(5), 1747-1763.

Rosdahl, H., Ungerstedt, U., Jorfeldt, L., Henriksson, J., 1993. Interstitial glucose and lactate balance in human skeletal muscle and adipose tissue studied by microdialysis. The Journal of Physiology 471, 637-657.

Rothwell, S.A., Killoran, S.J., O'Neill, R.D., 2010. Enzyme Immobilization Strategies and Electropolymerization Conditions to Control Sensitivity and Selectivity Parameters of a Polymer-Enzyme Composite Glucose Biosensor.

Sensors (Basel, Switzerland) 10(7), 6439-6462.

Sarma, A.K., Vatsyayan, P., Goswami, P., Minteer, S.D., 2009. Recent advances in material science for developing enzyme electrodes. Biosensors and Bioelectronics 24(8), 2313-2322.

Sassolas, A., Leca-Bouvier, B.D., Blum, L.J., 2008. DNA Biosensors and Microarrays. Chemical Reviews 108(1), 109-139.

Schaupp, L., Ellmerer, M., Brunner, G.A., Wutte, A., Sendlhofer, G., Trajanoski, Z., Skrabal, F., Pieber, T.R., Wach, P., 1999. Direct access to interstitial fluid in adipose tissue in humans by use of open-flow microperfusion. American Journal of Physiology - Endocrinology and Metabolism 276(2), E401-E408.

Scheller, F.W., Schubert, F., Neumann, B., Pfeiffer, D., Hintsche, R., Dransfeld, I., Wollenberger, U., Renneberg, R., Warsinke, A., Johansson, G., Skoog, M., Yang, X., Bogdanovskaya, V., Bückmann, A., Zaitsev, S.Y., 1991. Second generation biosensors. Biosensors and Bioelectronics 6(3), 245-253.

Schierenbeck, F., Franco-Cereceda, A., Liska, J., 2016. Accuracy of 2 Different Continuous Glucose Monitoring Systems in Patients Undergoing Cardiac Surgery: Intravascular Microdialysis Versus Subcutaneous Tissue Monitoring. Journal of Diabetes Science and Technology.

Schoonen, A.J.M., Wientjes, K.J.C., 2003. A Model for Transport of Glucose in Adipose Tissue to a Microdialysis Probe. Diabetes Technology & Therapeutics 5(4), 589-598. Serra, P.A., 2011. Biosensors - Emerging Materials and Applications. InTech.

Sethi, R.S., 1994. Transducer aspects of biosensors. Biosensors and Bioelectronics 9(3), 243-264.

Shaw, J.E., Sicree, R.A., Zimmet, P.Z., 2009. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Practice 87(1), 4-14.

Siontorou, C.G., Batzias, F.A., 2010. Innovation in biotechnology: moving from academic research to product development—the case of biosensors. Critical Reviews in Biotechnology 30(2), 79-98.

Skládal, P., 2016. Piezoelectric biosensors. TrAC Trends in Analytical Chemistry 79, 127-133.

Smith, J., Hinson-Smith, V., 2002. Product Review: The Potentiostat: Electrochemistry's Utility Player. Analytical Chemistry 74(19), 539 A-541 A.

Song, S., Xu, H., Fan, C., 2006. Potential diagnostic applications of biosensors: current and future directions. International Journal of Nanomedicine 1(4), 433-440.

Soto, R.J., Schoenfisch, M.H., 2015. Preclinical Performance Evaluation of Percutaneous Glucose Biosensors: Experimental Considerations and Recommendations. Journal of Diabetes Science and Technology 9(5), 978-984.

Taleb, N., Emami, A., Suppere, C., Messier, V., Legault, L., Chiasson, J.-L., Rabasa-Lhoret, R., Haidar, A., 2016. Comparison of Two Continuous Glucose Monitoring Systems, Dexcom G4 Platinum and Medtronic Paradigm Veo Enlite System, at Rest and During Exercise. Diabetes Technology & Therapeutics 18(9), 561-567.

Tamada, J.A., Garg, S., Jovanovic, L., et al., 1999. Noninvasive glucose monitoring: Comprehensive clinical results. JAMA 282(19), 1839-1844.

Thevenot, D.R., Toth, K., Durst, R.A., Wilson, G.S., 1999. ELECTROCHEMICAL BIOSENSORS: RECOMMENDED DEFINITIONS AND CLASSIFICATION. Pure Appl. Chem 71(12), 2333-2348,.

Tonelli, J., Kishore, P., Lee, D.-E., Hawkins, M., 2005. The regulation of glucose effectiveness: how glucose modulates its own production. Current Opinion in Clinical Nutrition & Metabolic Care 8(4), 450-456.

Tonyushkina, K., Nichols, J.H., 2009. Glucose Meters: A Review of Technical Challenges to Obtaining Accurate Results. Journal of diabetes science and technology (Online) 3(4), 971-980.

Turner, A.P.F., 2013. Biosensors: sense and sensibility. Chemical Society Reviews 42(8), 3184-3196.

Vaddiraju, S., Burgess, D.J., Tomazos, I., Jain, F.C., Papadimitrakopoulos, F., 2010. Technologies for Continuous Glucose Monitoring: Current Problems and Future Promises. Journal of Diabetes Science and Technology 4(6), 1540-1562.

Van Belle, T.L., Coppieters, K.T., Von Herrath, M.G., 2011. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. Physiological Reviews 91(1), 79-118.

van Bon, A.C., Hermanides, J., Koops, R., Hoekstra, J.B.L., DeVries, J.H., 2010. Postprandial Glycemic Excursions with the Use of a Closed-Loop Platform in Subjects with Type 1 Diabetes: A Pilot Study. Journal of Diabetes Science and Technology 4(4), 923-928.

van der Valk, P.R., van der Schatte Olivier-Steding, I., Wientjes, K.-J.C., Schoonen, A.J., Hoogenberg, K., 2002. Alternative-Site Blood Glucose Measurement at the Abdomen. Diabetes Care 25(11), 2114-2115.

Varalli, M., Marelli, G., Maran, A., Bistoni, S., Luzzana, M., Cremonesi, P., Caramenti, G., Valgimigli, F., Poscia, A., 2003. A microdialysis technique for continuous subcutaneous glucose monitoring in diabetic patients (part 2). Biosensors and Bioelectronics 18(7), 899-905.

Vazeou, A., 2011. Continuous blood glucose monitoring in diabetes treatment. Diabetes Research and Clinical Practice 93, S125-S130.

Voet, D., Voet, J., 2011. Biochemistry. Wiley.

Voskanyan, G., Keenan, D.B., Mastrototaro, J.J., Steil, G.M., 2007. Putative Delays in Interstitial Fluid (ISF) Glucose Kinetics Can Be Attributed to the Glucose Sensing Systems Used to Measure Them Rather than the Delay in ISF Glucose Itself. Journal of Diabetes Science and Technology 1(5), 639-644.

W.H.O, 2016. Global Report on Diabetes. World Health Organization, Switzerland.
Wahono, N., Qin, S., Oomen, P., Cremers, T.I.F., de Vries, M.G., Westerink, B.H.C., 2012. Evaluation of permselective membranes for optimization of intracerebral amperometric glutamate biosensors. Biosensors and Bioelectronics 33(1), 260-266.

Wang, J., 1994. Analytical Electrochemistry. VCH Publishers.
Wang, J., 1999. Amperometric biosensors for clinical and therapeutic drug monitoring: a review. Journal of Pharmaceutical and Biomedical Analysis 19(1–2), 47-53.

Wang, J., 2008. In vivo glucose monitoring: Towards ‘Sense and Act’ feedback-loop individualized medical systems. Talanta 75(3), 636-641.

Weltin, A., Kieninger, J., Urban, G.A., 2016. Microfabricated, amperometric, enzyme-based biosensors for in vivo applications. Analytical and Bioanalytical Chemistry 408(17), 4503-4521.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., 2004. Global Prevalence of Diabetes. Estimates for the year 2000 and projections for 2030 27(5), 1047-1053.

Wilson, G.S., Gifford, R., 2005. Biosensors for real-time in vivo measurements. Biosensors and Bioelectronics 20(12), 2388-2403.

Wisniewski, N., Moussy, F., Reichert, M.W., 2000. Characterization of implantable biosensor membrane biofouling. Fresenius’ Journal of Analytical Chemistry 366(6), 611-621.

Wisniewski, N., Reichert, M., 2000. Methods for reducing biosensor membrane biofouling. Colloids and Surfaces B: Biointerfaces 18(3–4), 197-219.

Yagi, K., 2007. Applications of whole-cell bacterial sensors in biotechnology and environmental science. Applied Microbiology and Biotechnology 73(6), 1251-1258.

Yamada, S., 2011. Historical Achievements of Self-Monitoring of Blood Glucose Technology Development in Japan. Journal of Diabetes Science and Technology 5(5), 1300-1306.

Yoo, E.-H., Lee, S.-Y., 2010. Glucose Biosensors: An Overview of Use in Clinical Practice. Sensors 10(5), 4558.

Zhang, W., Li, G., 2004. Third-Generation Biosensors Based on the Direct Electron Transfer of Proteins. Analytical Sciences 20(4), 603-609.

Zhou, W., Jimmy Huang, P.-J., Ding, J., Liu, J., 2014. Aptamer-based biosensors for biomedical diagnostics. Analyst 139(11), 2627-2640.

Ziegler, K.J., Developing implantable optical biosensors. Trends in Biotechnology 23(9), 440-444.

Zimmet, P., Alberti, K.G.M.M., Shaw, J., 2001. Global and societal implications of the diabetes epidemic. Nature 414(6865), 782-787.

Zisser, H., Wagner, R., Pleus, S., Haug, C., Jendrike, N., Parkin, C., Schweitzer, M., Freckmann, G., 2010. Clinical Performance of Three Bolus Calculators in Subjects with Type 1 Diabetes Mellitus: A Head-to-Head-to- Head Comparison. Diabetes