Development of a Continuous Glucose Monitor Based on Microfluidics

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“The day is yours, and yours is the night.
You established the sun and moon.”
Psalm

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Chapter 1

General Introduction

1. Scope of the thesis

This thesis describes the development and application of a miniaturized system for continuous subcutaneous glucose monitoring based on microdialysis sampling coupled with microfluidic glucose analysis. The system incorporates an enzymatic microreactor (EMR), a poly(dimethylsiloxane) (PDMS) - glass microfluidic device designed for the rapid reaction of glucose with oxygen using the glucose oxidase (GOx) enzyme. Fast mixing of GOx with glucose was achieved through integration of an array of slanted microgrooves in the PDMS microreactor channel to induce chaotic mixing. In *in vitro* experiments, detection of the hydrogen peroxide produced in the reaction was performed using either electrochemical (amperometric) measurement at integrated, planar Pt microelectrodes or optical (chemiluminescence) detection with a silicon photodiode molded into the EMR. For *in vivo* application, a microdialysis probe was coupled to the EMR for the continuous measurement of subcutaneous glucose in rats.

Chapter 1 provides a general introduction to diabetes mellitus and the different types of biosensors that have been used for both intermittent and continuous glucose monitoring. “Intermittent” monitoring refers to analyses made just a few times per day using, for instance, a finger-prick approach to determine blood glucose levels. “Continuous” monitoring approaches involve subcutaneous implantation of either biosensors or microdialysis probes, which then allow for constant measurement of glucose concentrations in the interstitial fluid. The technical requirements for continuous glucose monitors (CGM)s are briefly introduced, as are the kinetics of glucose transport between the blood and the interstitial fluid. Microdialysis sampling is described, and its combination with microfluidics proposed for the miniaturization and improved performance of CGMs employing microdialysis. Soft lithography is presented for the microfabrication of microfluidic devices, as it is the main approach used in this thesis. The chapter finishes with a short summary of the broad range of applications, particularly in the life sciences, for which microfluidics (or lab-on-a-chip) is now being considered as an alternative technology to facilitate research and development.

Chapter 2 gives an overview of the physiological processes that influence continuous monitoring of subcutaneous glucose, and discusses invasive CGMs based on implantable needle-type glucose sensors or microdialysis-based glucose sampling. The development of the concept of coupling microdialysis sampling with microfluidic devices is also presented. Since CGM implantable sensors or microdialysis probes are placed in the subcutaneous interstitial space, the

physiological factors influencing glucose concentrations measured with these systems is extensively discussed. These factors include the relationship between blood and interstitial glucose levels, implantation effects, location of the sensor, and the duration of measurement periods.

Chapter 3 presents a new sensing system exploiting the GOx conversion of glucose to gluconic acid and H_2O_2 for continuous glucose monitoring. The system is based on a n enzymatic microreactor incorporating chaotic mixing channels to rapidly mix a solution containing GOx with a sample solution containing glucose. An electrochemical detection method was implemented, using thin-film Pt electrodes positioned at the end of the microreactor channel as an on-chip amperometric detector of the reaction product, H_2O_2. In this study, the glucose oxidase concentration required for optimal sensing performance is identified. The dependence of EMR operation on flow rate is characterized, and the mixing properties of microreactor channels with and without mixing grooves are compared. **Chapter 4** describes a different type of microfluidic glucose sensing system based on an EMR with integrated chemiluminescence detection. A two-reactor system was developed for the enzymatic conversion of glucose by GOx, followed by the reaction of the H_2O_2 produced with luminol to produce chemiluminescence. In both of these chapters, linear calibration curves for glucose concentrations in the clinical range of interest were obtained for *in vitro* measurements.

An *in vivo* application of microfluidic EMR is described in **Chapter 5**. The EMR was coupled with a m icrodialysis probe which was inserted into the subcutaneous tissue of anesthetized rats. The performance of the EMR for *in vivo* monitoring was evaluated by modulation of blood glucose concentrations in the rat with intravenous injections of glucose or insulin, with saline as a control. Blood glucose was measured every 15 minutes as a reference value for comparison with the EMR. The difference between glucose values observed with blood glucose sampling and EMR monitoring is also discussed.

To conclude this thesis, a su mmary of the work and future perspectives for the development of CGMs based on m icrodialysis and microfluidics are given in **Chapter 6**.

2. Introduction

Diabetes mellitus is a widespread chronic metabolic disorder in which blood glucose levels are not maintained properly due to the failure of physiological mechanisms controlling insulin levels. Insulin is a peptide hormone produced in the β cells of the

islets of Langerhans in the pancreas and functions as binding receptor to unlock the pathway for glucose penetration into cells. Therefore, insulin plays a crucial role in maintaining glucose levels in the blood. There are two main types of diabetes which are characterized by the failure of cell uptake of sufficient glucose from the blood. The hallmark of diabetes type 1 is insulin deficiency, and this type is also referred to insulin-dependent diabetes mellitus (IDDM).[1, 2] The problem with this type of diabetes is that the pancreas does not secrete enough insulin so that the tissues in the body cannot take up glucose sufficiently, leading to glucose levels which are dangerously high. This type of diabetes occurs during childhood and is thought to be caused by an autoimmune response of the body, in which the insulin-producing cells in the pancreas are actually attacked and destroyed by the body's own immune system. Type 2 diabetes is characterized by insulin resistance, a condition in which insulin reduces glucose levels less effectively.[1, 2] Also referred to as non-insulin-dependent diabetes mellitus (NIDDM), this form of the disease is more common than type 1 (about 10 % of patients have type 1 diabetes, whereas 90 % have type 2). In type 2 di abetes, the pancreas produces insulin continually, generally in what normally would be sufficient amounts. However, cells that require insulin to absorb glucose no longer respond adequately to insulin, a condition known as insulin resistance, and glucose levels rise. The liver also does not reduce its production of glucose as effectively in the presence of insulin, which also contributes to elevated glucose levels. Insufficient insulin secretion can also cause this type of diabetes. Gestational diabetes mellitus may arise during pregnancy, caused by hormonal changes which can affect insulin activation during this period. Maturity onset diabetes of the young (MODY; a genetic malfunction) and latent autoimmune diabetes in adults (LADA) are also types of diabetes which can occur in some individuals, though these forms are uncommon.[3]

According to the World Health Organization (WHO), more than 220 million people are affected with diabetes worldwide, and an estimated 1.1 million people died from diabetes-related diseases in 2005. With the number of patients growing rapidly, it is expected that there will be some 366 million diabetes patients by 2030.[4] Moreover, many people who have not been diagnosed will suffer short-term or long-term complications. The major consequences of diabetes are damage to the heart, blood vessels, eyes, kidneys and nerves. Proper management of blood glucose levels is a long-term goal to support both diabetes patients and the physicians treating them.

The conventional way in which blood glucose determinations are carried out involves the finger-prick method. Usually, diabetic patients measure their own blood

glucose up to four times per day by pricking a finger and applying the resulting drop of blood to a portable device. Extensive clinical studies have been done to manage blood glucose levels based on self-monitoring of blood glucose (SMBG).[5, 6] The trials improved the handling of hyperglycemic episodes significantly with intensified insulin treatment. However, SMBG does not provide the whole range of information required, with hypoglycemic episodes still tending to go unnoticed. Even worse, patients often fail to perform finger-prick checks according to the recommended schedule because this method is painful and tedious to perform. A more sophisticated approach to determine blood glucose levels is to identify the level of glycated hemoglobin (HbA_{1c}), which is a measure of the average plasma glucose concentration over a prolonged period of many weeks.[7, 8] This HbA_{1c} level is an overall indication of how well glycemia has been controlled the previous 8 to 10 weeks.

When compared with the conventional finger-prick method, continuous glucose monitoring provides many advantages, including a better capability to check metabolic status, gain more insight into daily glucose fluctuations and identify episodes of both hypoglycemia as well as hyperglycemia. Obviously, this method is more suited to controlling diabetes, especially in the hypoglycemic (low glucose levels) regime. Continuous glucose monitoring is often performed by using a biosensor (monitoring system) that is inserted into subcutaneous tissue to measure interstitial fluid (ISF) glucose levels. Glucose levels can be monitored over days, providing very useful information for both diabetic patients and physicians. This section provides general information about biosensors, continuous glucose monitoring, non-invasive glucose sensing, and the techniques employed for continuous glucose monitoring.

2.1. Biosensors

A biosensor is a device that recognizes a biomolecular compound of interest and can convert concentrations of the compound into analytically measurable signals.[9, 10] It consists of two components, namely a biomolecular recognition element such as an antibody, enzyme, or receptor, and a transducer to convert a chemical signal into a quantifiable electrical one (Figure 1). For example, in a biosensor designed to detect glucose, the enzyme, GOx, is generally used to catalyze the reaction of glucose and oxygen to produce H_2O_2 and gluconic acid. The glucose concentrations can be directly determined by using an oxygen sensor to measure oxygen consumption [11], a pH sensor to measure gluconic acid production [12] or an amperometric sensor to measure H_2O_2 production [13].

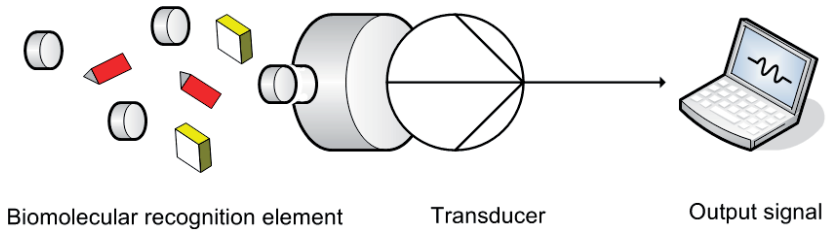

Figure 1. Schematic diagram of the general sensing mechanism on which biosensors are based.

2.1.1. Different types of biosensors

Depending on the detection technique used, biosensors can be classified as electrochemical biosensors and non-electrochemical biosensors; the latter category includes optical, calorimetric, and piezoelectric biosensors. Electrochemical biosensors are the most commonly used sensor type in monitoring and diagnostic tests in clinical analysis, due to their sensitivity, ease of use and amenability to miniaturization.[10, 14] They are based on the principle of chemical interaction between a biological recognition element and the species of interest. The electrochemical detector measures the output signal produced by the reaction process. Electrochemical biosensors can be categorized into amperometric biosensors, potentiometric biosensors or conductometric biosensors, depending on which parameter (current, potential or conductivity, respectively) is monitored. An amperometric biosensor measures the changes in current at a working electrode when oxidation or reduction take place, by applying a potential with respect to a reference electrode.[15] When substrates such as GOx, lactate oxidase, and ethanol oxidase are used to determine these respective substrates, it is the H_2O_2 produced by the enzymatic reaction that is oxidized at the working electrode to produce the current signal. High sensitivity and rapid signal response are major advantages of amperometric sensors when compared to potentiometric and conductometric biosensors. A potentiometric biosensor measures the potential difference between an indicating electrode and a r eference electrode. The sensor uses ion-selective electrodes (ISE) to detect the accumulated charge due to certain ions in a solution. One example is the pH sensitive ion-selective field-effect transistor (ISFET)-based enzyme sensor.[16] However, this approach has limitations with respect to *in vivo* applications, as sensor response depends on buffer capacity. Moreover, interfering

substances present in the sample can result in an unstable sensor signal. Conductometric biosensors based on conducting polymer coatings containing immobilized enzymes were proposed in the early 1990s.[17] Reaction of the enzyme with the desired biomolecule leads to a change in local redox potential and/or pH in the polymer matrix, which translates to a measurable change in polymer conductivity. A glucose nanobiosensor based on this concept was described in 2004 by Forzani *et al*.[18] A polyaniline layer coated with GOx formed a bridge over the 20-60 nm gap between two gold nanoelectrodes. Local oxidation of the polyaniline layer in this gap by the H_2O_2 produced in the enzymatic reaction causes an increased polymer conductivity, which is recorded by the Au nanoelectrodes. In this case, the reaction is not limited by oxygen or mediators. The concept works also when larger interdigitated arrays of Pt microelectrodes are used (see for example the sensor for urea detection in ref. [19]). The advantage of these electrodes is the ease by which they can be fabricated using a screen-printing process.

There are a variety of different non-electrochemical biosensors. Optical biosensors measure changes in light observed as a result of a biological and/or chemical reaction. Various methods for detection of luminescence, fluorescence and surface plasmon resonance (SPR) may be involved in this approach. Many technical developments and applications of optical biosensors have been reviewed elsewhere.[20, 21]

Fluorescence is a more attractive approach for optical glucose sensing, due to its exceptional sensitivity. Affinity-based sensing is generally applied in this type of method, which involves the competitive displacement of fluorescein-labeled binders with unlabelled glucose. Concanavalin A (ConA) has often been used as a glucose-specific binding protein, along with fluorescein-labelled dextran as a competitive binder.[22] The displacement of bound, labelled dextran by unlabelled glucose results in a fluorescent signal due to the fluorescein-labeled dextran which is proportional to the concentration of glucose. Unique here was that the ConA was immobilized on the inner surface of a microdialysis probe that in turn was coupled to an optical fiber for transfer of fluorescent light to a fluorimeter. Glucose diffusing over the dialysis membrane could immediately be detected in this way. This fluorescence concept was further developed with optical transducer techniques using fluorescence resonance energy transfer (FRET).[23, 24] ConA, again immobilized on the inside of a microdialysis fiber, was labelled with fluorescent rhodamine dye molecules which acted as acceptor molecules. Dextran was labelled with the donor, fluorescein isothiocyanate (FITC). When dextran was bound to the ConA in the absence of glucose, FITC, when in the excited state, could transfer energy in a

radiationless way to the rhodamine acceptor molecules of the ConA. The fluorescence of the acceptor, rhodamine, increased as a result. In the presence of glucose, the labelled dextran and ConA moved apart, resulting in a decrease of the rhodamine acceptor signal. On the other hand, an increase of the fluorescence due to FITC was observed, which occurred at a very different wavelength and thus could easily be distinguished from the rhodamine signal. The authors of ref. 44 noted, however, that there was an issue with the stability of this sensor, as ConA tended to form irreversible aggregates over a number of hours, adversely affecting sensor response.

Calorimetric biosensors operate by sensing enthalpy changes in a thermistor resulting from biochemical reactions.[25] The thermistor detects the temperature change caused by a reaction such as enzyme catalysis as a change in electrical resistance. Piezoelectric biosensors are also non-electrochemical biosensors that register changes in the resonance frequency of an oscillating piezoelectric crystal as a function of the mass of analyte adsorbed onto the crystal surface.[26]

Biosensors have several advantages over conventional analytical methods, including small size, fast response time, ease of use and cost effectiveness. Biosensors have therefore been applied and used in many research fields, such as biomedical applications, food industry, and environmental monitoring.[27]

2.1.2. Biosensors for continuous glucose monitoring

Biosensors for (continuous) glucose monitoring have been under development for more than three decades and have become an important part of diabetic therapy. The enzymatic reaction of glucose and oxygen on which most glucose monitoring is based is shown in equation 1. As shown, GOx catalyzes the reaction to produce gluconic acid and H_2O_2. The H_2O_2 produced can be detected electrochemically.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \qquad (1)$$

There are several methods that can be used to assess glucose levels in ISF, such as cotton wick, microdialysis, microfiltration, and needle-type sensors. Needle-type glucose sensors [28-35] and microdialysis-based glucose sensing systems [36-39] are commonly used for continuous glucose monitoring. The needle-type sensors use GOx immobilized on the surface of an electrode, where the reaction then takes place once the sensor is inserted subcutaneously and is exposed to ISF. The microdialysis-based sensing systems use a microdialysis probe to sample glucose from ISF, which is then transported in a flowing solution to an extracorporeal sensing system. These

continuous glucose monitors can be used either retrospectively to evaluate a personal glycemic regimen, or prospectively to monitor glucose levels in real-time, depending on the patient's requirements. (Different technical strategies for continuous subcutaneous glucose monitors are described in more detail in Chapter 2.)

2.1.3. Non-invasive glucose sensing

Non-invasive glucose sensing encompasses all those techniques which allow measurement of glucose concentrations without physical contact to the samples, unlike the implantable sensors that measure glucose in blood or ISF. Depending on the approach used, non-invasive methods can be categorized as transdermal or optical. One example of transdermal glucose determination, the GlucoWatch® (Cygnus Inc. California), is based on the principle of reverse iontophoresis.[40] This involves the movement of ions and glucose from the ISF to the skin surface when a small electric current is applied across the skin surface between two electrodes. The range of glucose concentrations that is extracted through the skin is very low (on the pM level). Glucose is converted to gluconic acid with the concomitant production of H_2O_2 by enzymatic reaction with GOx. H_2O_2 is then detected amperometrically at a platinum electrode. This device was tested *in vivo* to monitor hypoglycemia in children and licensed by the US Food and Drug Administration (FDA) in 2001.[41] Another device using a transdermal approach was the Pendra® system (Pendragon Medical Ltd., Zurich, Switzerland), which employed a watch-type device to measure the dielectric spectrum of underlying tissue using impedance spectroscopy.[42] Though this new technological approach was promising, the device exhibited poor accuracy in six subjects with type 1 diabetes.[43] Unfortunately, the Pendragon company went out of business in 2005, and watch-type sensors like the Pendra® and Glucowatch® are no longer available on the market.

Optical approaches use various light frequencies to exploit different properties of light for non-invasive glucose sensing. Near-infrared (NIR) spectroscopy has been investigated over a wide range of wavelengths (750 nm - 1400 nm), since light in this portion of the spectrum can penetrate the skin layer and potentially be used to monitor glucose.[44, 45] The extent of specific NIR absorption depends on the structure of the molecule. Though the peak intensity of glucose absorption is small in complex biological matrices, it is possible to quantify the glucose concentration by using several wavelengths and multivariate analysis. On the other hand, the use of mid-infrared (MIR) spectra (3 μm - 50 μm) yielded higher-intensity glucose peaks.[46] However, one of the drawbacks of this method is that significant

background absorption by other molecules interferes with the glucose measurement. Besides the above-mentioned optical approaches, there are many other non-invasive glucose sensing methods based on spectroscopic techniques such as Raman spectroscopy,[47] photoacoustic spectroscopy,[48, 49] polarimetry[50] and thermal emission spectroscopy [51].

Though many non-invasive techniques show great promise, in particular because of their user-friendliness, they are still too underdeveloped for clinical use in terms of accuracy and precision. In the case of transdermal sensing, many factors can negatively influence analytical results. These factors include temperature change, tissue thickness, condition of the subject (age, disease state), blood pressure, motion artifacts, sweating and microvascular blood flow variation.[52] The question then arises whether non-invasive techniques can be transferred to patients, or should just be used in well-controlled laboratory environments. In order to ensure reliable non-invasive glucose monitors, further development of standardized procedures to eliminate the influence of interfering factors and of clinical applications will have to be undertaken.

2.2. Technical requirements of continuous glucose monitors (CGM)

CGMs provide great insight into daily glucose fluctuations in diabetes patients by providing data readout continuously. This allows significant advances with respect to better diabetes management, offering important information for insulin therapy and metabolic control. However, CGMs still lack reliability and need to be further improved. There are fundamental requirements which need to be met for reliable continuous glucose measurement. Some technical and clinical aspects are highlighted below.[53-55] (The term ‘sensor’ refers here to an implantable sensor.)

- Specificity: the CGM should be able to recognize glucose in a complex medium, with fast and predictable response to changing glucose concentrations.
- Accuracy (correctness): the measurement should give a value that corresponds with high accuracy to actual glucose concentrations.
- Precision (repeatability of measurement): the measurement should be reproducible and less variable with increasing numbers of glucose measurements. A coefficient of variance having a value of 5% or less for an experiment is acceptable.

- Biocompatibility: when a sensor is implanted subcutaneously in the body, there should be no (minimal) adverse effects on the host due to toxicity or local foreign body reactions caused by the invasive sensor.
- Response time: the CGM should provide a readout signal in real time and an alarm in times of rapid glucose change.
- Stability: two factors are involved when considering stability. During sensor implantation, the sensor may be influenced by the immune response of the tissue surrounding the sensor. This might cause biofouling of the sensor and affect short-term sensor stability. It is also important that the sensor is able to measure glucose concentrations over longer periods of time under physiological conditions (days or weeks).
- Interfering substances: the most commonly occurring electroactive compounds found in the body are uric acid, ascorbic acid, acetaminophen, and L-cysteine. These substances are also oxidized at the potentials applied for H_2O_2 detection.
- Cost-effectiveness: the sensor should be fabricated inexpensively and in a reproducible manner on a large scale.
- User-friendliness: for practical applications, the CGM needs to be a patient-friendly device that is portable and easy to handle.

In order to achieve the long-awaited goal of a reliable CGM, all the above mentioned requirements should be fulfilled. The most important parameters for further development of continuous CGM are accuracy, signal stability and reliability over longer periods (days). The accuracy of a glucose monitor can be assessed by the Clarke error grid [56], the Consensus error grid [57], or the continuous glucose-error grid analysis (CG-EGA).[58] These statistical approaches are presented in detail in Chapter 2 and will therefore not be discussed further here.

In addition to satisfying the technical requirements mentioned above, it is also important to bear in mind the physiological context in which CGMs are employed. Commonly, the sensor or microdialysis probe of a CGM is placed in the extracellular space to measure interstitial glucose levels. Ideally, continuous glucose monitoring should be performed at an intravascular site to give direct measurement of glucose levels in the blood. However, this approach has serious risks associated with it, such as blood clotting (thrombosis) around the sensor or microdialysis probe of the CGM, as well as surgical issues (only a specialist can perform the surgery required for the implantation of the CGM probe). It is therefore important to elucidate the relationship between blood glucose levels and interstitial glucose levels,

since subcutaneous implantation is simpler and safer. The observed differences in glucose concentrations between these two compartments are due to glucose kinetics.

2.3. Glucose kinetics

Since the glucose sensor or microdialysis probe of a CGM is inserted directly into subcutaneous tissue, the kinetics associated with the process of glucose diffusion between blood and ISF is one of the important factors which influences those two levels. Glucose is transported from the blood to the ISF at a rate determined by the concentration gradient i.e. the blood glucose level is a major factor controlling the interstitial glucose level. Additionally, there are many other compartments that change the local interstitial glucose concentration, such as cellular glucose uptake rates, local insulin levels, blood flow and permeability of the capillary blood vessels.[55]

The subcutaneous tissue is located below two skin layers, the epidermis and dermis. The epidermis is an avascular epithelial membrane, while the dermis contains many arterioles, venules and capillaries. The blood glucose level determined by a finger-prick uses blood from capillaries in the dermis, which consists of a mixture of blood from cut arterioles and venules.[59] ISF is a medium that is composed of water, amino acids, sugars, fatty acids, coenzymes, hormones, neurotransmitters, salts, and cellular products. The sensor or microdialysis probe for continuous glucose monitoring is implanted in this interstitial space. The ISF bathes both capillaries and cells, with cell nutrients being taken up from the capillaries and made available to the cells. Cengiz *et al*.[60] described the glucose dynamics between plasma and ISF compartments using the following equation (see Figure 2):

$$dV_2G_2/dt = K_{21}V_1G_1 - (K_{12} + K_{02})V_2G_2 \qquad (2)$$

where G_1 and G_2 are capillary blood and interstitial glucose concentrations, respectively, K_{12} and K_{21} the forward and reverse flux rates for glucose transport across the capillary, K_{02} the glucose uptake into the subcutaneous tissue cells, and V_1 and V_2 the volume of capillary blood and ISF, respectively. Thus, the diffusion of glucose into the ISF is proportional to the capillary blood glucose concentration, and vice versa. Glucose that is taken up by surrounding tissue cells also directly influences the glucose concentration in the ISF.

Because glucose kinetics are closely associated with physiological conditions, interstitial glucose concentrations do not always reflect blood glucose concentrations.

In general, interstitial glucose levels correlate well with blood glucose levels under steady state conditions (no systemic challenges), if a physiological lag time between ISF and blood levels is taken into account.[60-62] (Physiological lag time refers to the time interval required for the ISF glucose concentration to reach a v alue correlating to the blood glucose level.) In the case of CGMs, this lag time can be attributed in large part to the fact that glucose is taken up by tissue/cells surrounding the sensor or microdialysis probe. Under non-steady state conditions, however, when glucose levels in the blood are rapidly increased to induce hyperglycemia, observed interstitial glucose levels do not reach blood glucose levels.[63, 64] Kaptein *et al*.[65] claimed that there are significant differences between the subcutaneous and intravenous compartments. In their ultrafiltration experiments, they observed ISF glucose levels which exhibited not just a time delay with respect to blood glucose levels, but also a different course over time; the glucose concentration versus time curves recorded for the two compartments had different shapes. Hence, the relationship between the two compartments is complex, reflecting glucose kinetics that vary significantly under non-steady-state conditions. Besides differing glucose levels in the blood and ISF, reported values for physiological lag times for glucose concentrations in the ISF with respect to the blood show little agreement under non- steady state conditions. In some instances,

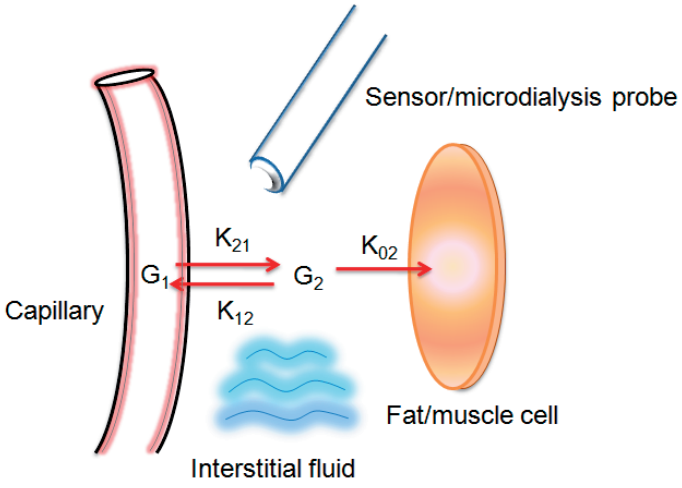

Figure 2. Model for the diffusion of glucose between blood and ISF. The glucose concentrations in the ISF and blood compartments are strongly related. (adapted from Cengiz et al., ref. [60])

ISF glucose levels lag behind those measured in blood, whereas in other instances, blood levels actually lag behind ISF levels.[66, 67] According to these authors, this variation in lag time may be explained by the so-called push-pull hypothesis. According to this hypothesis, glucose is "pushed" into the interstitial space from the blood when blood glucose levels are increased. When glucose decreases, on the other hand, the tissue/cells surrounding the sensor or microdialysis probe take up glucose, effectively "pulling" glucose from the interstitial space to the cells. This phenomenon, however, seems to be a matter of debate, as other reports have failed to support these observations.[68-70]

While progress has been made in the search for a more complete understanding of the factors governing glucose diffusion between the blood and ISF, existing hypotheses are not conclusive. In fact, experimental conditions which can critically influence observed results, such as species used, sensor type, implantation effect, implantation depth, and subject condition, vary significantly from one clinical study to the next. (This will be discussed in more detail in Chapter 2). However, it is generally agreed that invasive glucose monitoring rather than non-invasive glucose monitoring is still the most useful approach for clinical studies. The microdialysis-based sensing method is especially promising, though a number of obstacles need to be overcome to improve its reliability.

2.4. Approach to novel technology: microdialysis with microfluidics

Since microdialysis was introduced by Ungerstedt [71] in 1974 to monitor neurotransmitter release in the brain, the technique has been extensively used for research to study many other tissues and body compartments. The microdialysis probe consists of a semipermeable hollow fiber membrane which is sealed at the end, and is connected to inlet and outlet tubing.[72] The microdialysis probe is designed to mimic a blood capillary and can be placed in any target tissue of interest. The lumen of the probe is perfused with an artificial interstitial fluid (ISF; usually Ringer's solution) which is introduced from the inlet. Small pores in the membrane allow certain molecules to travel across it along the concentration gradient, from higher concentrations in the subcutaneous tissue to low or zero concentrations in the perfusing solution, known as the perfusate. However, large molecules (including proteins) cannot cross the membrane. The effective pore size defines the molecular-weight cut-off (MWCO) of a membrane; molecules having molecular weights above the MWCO are prevented from crossing the membrane because of insufficient pore size. Glucose concentrations in the perfusate collected

through the outlet reflect the concentrations in the ISF and can be quantified by a variety of bioanalytical techniques.

The molecular concentration gradient across the microdialysis membrane is affected by several factors, including the length of probe, membrane material, perfusion rate, temperature, analyte species, fraction of free analyte versus protein-bound analyte, and nature of the external medium.[73] The relative recovery is defined as follows:

$$R\ (\text{Recovery}) = C_{out}/C_{medium} \qquad (3)$$

where C_{out} is the concentration of glucose in the out flow and C_{medium} is the undisturbed concentration of glucose in the external medium (ISF in the case of subcutaneous monitoring). It should be noted that recovery is a strong function of perfusate flow rate, with C_{out} decreasing with increasing flow rate. Increased flow rates mean decreased time for diffusion of glucose across the membrane into the perfusate, and hence lower C_{out}.

Generally speaking, two types of microdialysis-based sensing systems exist. One of these, called the subcutaneous glucose monitoring system (SCGM), uses a solution-based enzymatic reaction to quantify glucose in the perfusate.[36] A Ringer's solution is perfused through the probe at a flow rate of 0.3 μL/min. The glucose which has diffused across the membrane of the probe is moved along with the Ringer's solution and transported to a extracorporeal reaction unit. GOx in solution is continuously introduced to this unit, where it is mixed with perfusate to convert glucose to gluconic acid and H_2O_2. An integrated electrode detects the produced H_2O_2 amperometrically. An example of the other type of microdialysis-based glucose sensing system is the GlucoDay®.[38] This system uses GOx enzyme immobilized on an electrode to measure the glucose concentration in the microdialysate. However, the microdialysis probe in this system is typically perfused at a high flow rate of 10 μL/min, reducing the relative glucose recovery.

The microdialysis approach has proven to be a safe and reliable technique, as tissue *in vivo* is only exposed to Ringer's solution, used to perfuse the probe. Sensor biofouling is also less of an issue because microdialysis allows the extraction of glucose from the ISF; in other words, this is a pre-filtering process that excludes protein components from the ISF. However, one drawback of microdialysis can be its low temporal resolution. The SCGM system, for example, has a relatively long physical lag time (30 min), due to the tubing connecting the probe to the external pump. The requirement for solution pumping also results in microdialysis-based

systems being somewhat bulky, though both the SCGM and GlucoDay© are portable.

The long-term goal in CGM development is to realize an autonomous system acting as an artificial pancreas. In order to achieve this goal, a fully integrated insulin delivery device including a CGM (microdialysis probe, pump and sensing system), insulin pump and operating electronics needs to be realized. Reduced system size and fast sampling time are critical criteria that require improvement in current systems. The combination of microfabrication technology and microfluidics represent a very promising opportunity to tackle issues such as glucose sampling, analysis time and overall system configuration.

3. Microfluidics

The field of microfluidics (also referred to as lab-on-a-chip) has over the past two decades experienced exponential growth in the development of both the technology itself as well as applications in the chemical and life science realms.[74, 75] The concept of microfluidic systems is defined as the science that involves design, control, manipulation and analysis of small amounts of fluids (volumes of nL or less) in a microfluidic channel network.[76] It is an interdisciplinary field that encompasses engineering, chemistry, physics, biology and bioengineering. Since very small amounts of liquids are employed in micro- or nanoscale fluidic devices, microfluidics offers many advantages over conventional methods, such as dramatically reduced usage of samples and chemical reagents, short reaction times (analysis), cost-effectiveness, increased surface-to-volume ratio and portability.

Solution flow in micrometer-dimensioned channels is typically laminar, that is, very well defined, with layers of solution moving past one another in unchanging streamlines. It is very predictable flow, in that the velocity at any one particular point in the channel remains constant over time. This is in strong contrast to turbulent flow, which occurs at higher flow rates relative to laminar flow in flow systems. As the name suggests, turbulent flow is characterized by streamlines which are not well-defined, with constantly changing velocity over time at any given point in the channel. Both laminar and turbulent flows are the result of the interplay of viscous and inertial forces in a solution flow. The Reynold's number, Re, is a dimensionless parameter describing the relationship between inertial and viscous forces in a flow system.[77] It is defined as:

$$\mathrm{Re} = \frac{\rho u L}{\mu} \qquad (4)$$

ρ is the density of the fluid, u is the linear velocity of the fluid, L is the characteristic channel dimension (e.g. diameter of a channel with circular cross-section) and μ is the dynamic viscosity of the fluid.

The use of Re makes it possible to predict the flow rate and solution conditions under which a transition from laminar to turbulent flow will occur in any given flow system. This transition is observed at Re of between 2000 and 3000, depending on the geometry of the flow conduits. For the flow rates typical of microfluidic systems, Re is generally less than 1, indicative of the well-defined, laminar flow observed in microchannels. In this regime, mixing of solutions is achieved predominantly by diffusion, a passive and slow transport process. However, because microchannel cross-sections are small, diffusion lengths can be kept short, with the corresponding diffusion times necessary for mixing on the order of sub-seconds to seconds. Use of a chaotic mixing approach, as in this thesis, augments the contact area between the solutions to be mixed, further decreasing diffusion lengths and shortening mixing times. In chaotic mixing, mixing is achieved through the incorporation of an array of microgrooves into a microchannel. Flow over the groove array assumes a helical or corkscrew pattern, in which the contact area between two adjacent solutions is increased dramatically to facilitate mixing by diffusion.[78] This microfluidic mixing technique, which is used for solution-based enzymatic reaction of glucose, is described in more detail in Chapter 3.

The technology used to fabricate interconnected microchannel networks in planar substrates originates from that used for microelectronics and later, for microelectromechanical systems (MEMS).[79] Early microfluidic devices were fabricated in silicon or glass, requiring that researchers had access to sophisticated cleanrooms. Alternative, less technology-intensive methods to replicate microchannels in polymers were introduced in the early to mid-nineties.[80] In particular, the development of a process to replicate microchannels in the silicone rubber, poly(dimethylsiloxane) (PDMS), has significantly simplified the fabrication of microfluidic devices. PDMS is a flexible material that is also optically transparent, making it a good material for many microfluidic applications. The process for making PDMS devices, known as soft lithography, can be performed for the most part outside a cleanroom environment, is easy to learn and is much less expensive than silicon or glass devices.[81] The advent of soft lithography in the nineties had a dramatic influence on microfluidics research generally, as it made microfluidics accessible to many more researchers.

A general procedure for soft photolithography for microfluidic device fabrication by replication in PDMS is shown in Figure 3. The process depicted here is the one which was used throughout this thesis. To begin, a master or mold containing the negative of the microfluidic network was made. In this thesis, the mold was formed photolithographically in a layer of photosensitive polymer which had been deposited by spin coating onto a silicon wafer. The desired layout and design of the microchannels and other features on the chip were first drawn using a computer-aided design (CAD) program. This image was then reproduced either in a thin layer of chromium deposited on a polished quartz wafer or on a high-resolution transparency sheet (resolution 3,810 dpi), to be used as a photomask. This mask was used to transfer the two-dimensional microchannel pattern to a layer of photosensitive polymer deposited on a silicon substrate. This was accomplished by exposing this layer to ultraviolet (UV) radiation (365 nm, 10 m W/cm^2) using the mask to selectively illuminate some areas of the layer to the exclusion of the rest of the layer. Though this is not shown in Figure 3, the photomask was in direct contact with the resist layer during the illumination step. The photoresist exposure step is the most critical step for defining error-free features having micrometer dimensions, and is generally done in a cleanroom or other controlled, relatively dust-free environment such as a laminar-flow hood.

Photoresist is a photosensitive material composed of a polymer, a photoinitiator and a casting solvent. Two types of photoresist, positive-type and negative-type, are used for photolithographic processes; there are many different variations of both which have been developed for a variety of applications. With positive photoresist, UV exposure causes the resist in exposed areas to become soluble, whereas unexposed resist remains insoluble upon immersion in a developer solution. Exposed areas of the resist can thus be removed upon development. The opposite is true for negative photoresist, where UV exposure causes the resist to polymerize and become insoluble, and unexposed resist remains soluble and can be removed in a developer solution. For the work presented in this thesis, a silicon wafer was used as the mold substrate, and a relatively thick positive photoresist layer was deposited on this wafer as basis for the mold. The thickness of the layer was determined after development, and was found to be around 35 μm thick. As shown in Figure 3, the mold was essentially the negative of the desired microfluidic device, consisting of raised features where microchannels were to be formed. The thickness of the patterned resist layer defined the depth of the replicated channels.

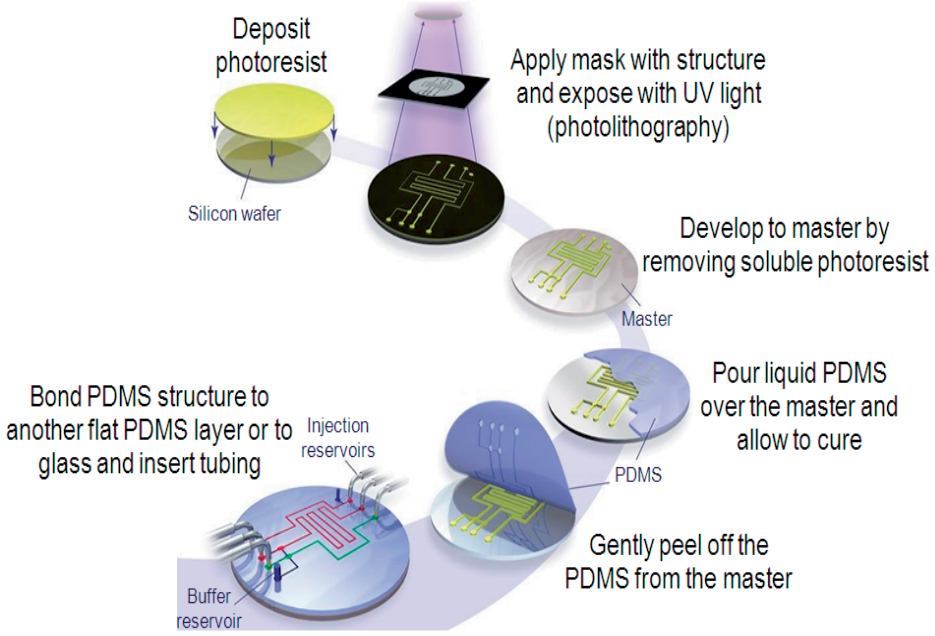

Figure 3. Schematic diagram for the soft lithography process used to make microfluidic devices in PDMS. (Illustration reprinted with permission from Sebastiaan Donders, Bionieuws 10-03-2006.)

Replication of microchannels was performed by casting a viscous liquid mixture of PDMS prepolymer and curing agent (10:1 ratio) over the mold. The mixture was allowed to cure (polymerize) at 50ºC for 4 hours. The PDMS is then smoothly peeled off from the master. This replication procedure could be carried out numerous times using the same master. Multiple PDMS chips could be formed with a single casting, since the master contained multiple designs. The resulting PDMS slab could be easily cut into smaller individual devices. To seal the microchannels, the surface of the PDMS device containing the microchannels was made hydrophilic through oxidation with oxygen plasma or UV-generated ozone. This surface was then brought into contact with either an oxidized glass or another oxidized PDMS chip containing holes (300 μm in diameter) which aligned with the ends of the channels for solution introduction or removal. The assembled chips were then placed on a preheated hot plate at 140 ºC. The hotplate was immediately turned off and the chips allowed to slowly cool down to room temperature, resulting in an irreversible

bond between oxidized surfaces. The chip was then prepared for experiments by inserting tubing connections into the inlet and outlet holes. The soft lithography process has been thoroughly reviewed elsewhere.[81-83]

3.1. Microfluidic applications

To data, an enormous number of microfluidic systems have been developed, increasing their utilization in many research fields. Many review papers have been published, offering different insights into the developments of the last decade. Verpoorte [84] reviewed the application of microfluidic chips for clinical and forensic analysis. The analytes discussed included endogenous small molecules, proteins and peptides, and nucleic acids and oligonucleotides, and their analysis in physiological samples was presented where relevant. Manz *et al*.[74, 75, 85-87] have extensively reviewed the literature several times in recent years for bioanalytical applications of microfluidic systems. These reviews cover many different fields, including cell culture, handling and analysis, clinical diagnostics, immunoassays, protein analysis, DNA separation and analysis, polymerase chain reaction and environmental monitoring. Whitesides *et al*.[88-90] have also discussed many potential applications of microfluidics in the broad area of the life sciences, including biological studies, chemical biology, biological interactions and microbiology. Microfluidics has a number of potential advantages for biosensing, including increased sensitivity, high-throughput, automation and miniaturization. For example, microfluidic-based point-of-care testing devices have opened up new possibilities to yield clinically relevant information in diagnostic applications in a simple, fast and low-cost manner.[91] Fully integrated, multifunctional devices are increasingly in demand, with many recent reports describing the development of components such as micropumps and microvalves,[92] micromixers,[78] detectors,[93] and reservoirs for existing and new lab-on-a-chip applications.

This thesis describes the development and application of a miniaturized system for continuous subcutaneous glucose monitoring based on m icrodialysis sampling coupled with microfluidic glucose analysis.

References

1. http://diabetes.niddk.nih.gov/intro/.
2. Alberti, K.; Zimmet, P.Z., Definition, diagnosis and classification of diabetes mellitus and its complications part 1: diagnosis and classification of diabetes mellitus - provisional report of a WHO consultation. *Diabet. Med.* **1998,** 15, (7), 539-553.
3. http://www.diabetesfonds.nl/.

4. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H., Global prevalence of diabetes. *Diabetes Care* **2004,** 27, (5), 1047-1053.

5. DCCT group, The dffect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **1993,** 329, (14), 977-986.

6. UKPDS Group, Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *The Lancet* **1998,** 352, (9131), 837-853.

7. Parker, K.M.; England, J.D.; Da Costa, J.; Hess, R.L.; Goldstein, D.E., Improved colorimetric assay for glycosylated hemoglobin. *Clin. Chem.* **1981,** 27, (5), 669-672.

8. Nathan, D.M.; Singer, D.E.; Hurxthal, K.; Goodson, J.D., The clinical information value of the glycosylated hemoglobin assay. *N. Engl. J. Med.* **1984,** 310, (6), 341-346.

9. Turner, A.P.F., Biochemistry: biosensors-sense and sensitivity. *Science* **2000,** 290, (5495), 1315-1317.

10. Thévenot, D.R.; Toth, K.; Durst, R.A.; Wilson, G.S., Electrochemical biosensors: recommended definitions and classification. *Biosens. Bioelectron.* **2001,** 16, (1-2), 121-131.

11. Armour, J.C.; Lucisano, J.Y.; McKean, B.D.; Gough, D.A., Application of chronic intravascular blood glucose sensor in dogs. *Diabetes* **1990,** 39, (12), 1519-1526.

12. Caras, S.D.; Petelenz, D.; Janata, J., pH-based enzyme potentiometric sensors. Part 2. Glucose-sensitive field effect transistor. *Anal. Chem.* **1985,** 57, (9), 1920-1923.

13. Shichiri, M.; Yamasaki, Y.; Kawamori, R.; Hakui, N.; Abe, H., Wearable artificial endocrine pancreas with needle-type glucose sensor. *The Lancet* **1982,** 320, (8308), 1129-1131.

14. Nakamura, H.; Karube, I., Current research activity in biosensors. *Anal. Bioanal. Chem.* **2003,** 377, (3), 446-468.

15. Gregg, B.A.; Heller, A., Cross-linked redox gels containing glucose-oxidase for amperometric biosensor applications. *Anal. Chem.* **1990,** 62, (3), 258-263.

16. van der Schoot, B.H.; Bergveld, P., ISFET based enzyme sensors. *Biosensors* **1987,** 3, (3), 161-186.

17. Contractor, A.Q.; Sureshkumar, T.N.; Narayanan, R.; Sukeerthi, S.; Lal, R.; Srinivasa, R.S., Conducting polymer-based biosensors. *Electrochim. Acta* **1994,** 39, (8-9), 1321-1324.

18. Forzani, E.S.; Zhang, H.; Nagahara, L.A.; Amlani, I.; Tsui, R.; Tao, N., A Conducting polymer nanojunction sensor for glucose detection. *Nano Lett.* **2004,** 4, (9), 1785-1788.

19. Lee, W.-Y.; Kim, S.-R.; Kim, T.-H.; Lee, K.S.; Shin, M.-C.; Park, J.-K., Sol-gel-derived thick-film conductometric biosensor for urea determination in serum. *Anal. Chim. Acta* **2000,** 404, (2), 195-203.

20. Homola, J.; Yee, S.S.; Gauglitz, G., Surface plasmon resonance sensors: review. *Sens. Actuators, B* **1999,** 54, (1-2), 3-15.

21. Rich, R.L.; Myszka, D.G., Survey of the year 2006 commercial optical biosensor literature. *J. Mol. Recognit.* **2007,** 20, (5), 300-366.

22. Schultz, J.S.; Mansouri, S.; Goldstein, I.J., Affinity sensor: a new technique for developing implantable sensors for glucose and other metabolites. *Diabetes Care* **1982,** 5, (3), 245-253.

23. Pickup, J.C.; Hussain, F.; Evans, N.D.; Rolinski, O.J.; Birch, D.J.S., Fluorescence-based glucose sensors. *Biosens. Bioelectron.* **2005,** 20, (12), 2555-2565.

24. Meadows, D.; Schultz, J.S., Fiber-optic biosensors based on fluorescence energy transfer. *Talanta* **1988,** 35, (2), 145-150.

25. Xie, B.; Hedberg, U.; Mecklenburg, M.; Danielsson, B., Fast determination of whole blood glucose with a calorimetric micro-biosensor. *Sens. Actuators, B* **1993,** 15, (1-3), 141-144.

26. Muramatsu, H.; Dicks, J.M.; Tamiya, E.; Karube, I., Piezoelectric crystal biosensor modified with protein A for determination of immunoglobulins. *Anal. Chem.* **1987,** 59, (23), 2760-2763.

27. Castillo, J.; Gáspár, S.; Leth, S.; Niculescu, M.; Mortari, A.; Bontidean, I.; Soukharev, V.; Dorneanu, S.A.; Ryabov, A.D.; Csöregi, E., Biosensors for life quality: design, development and applications. *Sens. Actuators, B* **2004,** 102, (2), 179-194.

28. Mastrototaro, J.J., The MiniMed Continuous Glucose Monitoring System. *Diabetes Technol. Ther.* **2000,** 2, (supplement 1), 13-18.

29. Diabetes Research in Children Network (DIRECNET) Study Group, The accuracy of the CGMS in children with type 1 diabetes: results of the Diabetes Research in Children Network (DirecNet) accuracy study. *Diabetes Technol. Ther.* **2003,** 5, (5), 781-789.

30. Bode, B.; Gross, K.; Rikalo, N.; Schwartz, S.; Wahl, T.; Page, C.; Gross, T.; Mastrototaro, J., Alarms based on real-time sensor glucose values alert patients to hypo- and hyperglycemia: the Guardian Continuous Monitoring System. *Diabetes Technol. Ther.* **2004,** 6, (2), 105-113.

31. Mastrototaro, J.; Shin, J.; Marcus, A.; Sulur, G., The accuracy and efficacy of real-time continuous glucose monitoring sensor in patients with type 1 di abetes. *Diabetes Technol. Ther.* **2008,** 10, (5), 385-390.

32. Garg, S.; Zisser, H.; Schwartz, S.; Bailey, T.; Kaplan, R.; Ellis, S.; Jovanovic, L., Improvement in glycemic excursions with a transcutaneous, real-time continuous glucose sensor. *Diabetes Care* **2006,** 29, (1), 44-50.

33. Garg, S.; Jovanovic, L., Relationship of fasting and hourly blood glucose levels to HbA1c values. *Diabetes Care* **2006,** 29, (12), 2644-2649.

34. Weinstein, R.L.; Schwartz, S.L.; Brazg, R.L.; Bugler, J.R.; Peyser, T.A.; McGarraugh, G.V., Accuracy of the 5-day FreeStyle Navigator continuous glucose monitoring system. *Diabetes Care* **2007,** 30, (5), 1125-1130.

35. Wilson, D.M.; Beck, R.W.; Tamborlane, W.V.; Dontchev, M.J.; Kollman, C.; Chase, P.; Fox, L.A.; Ruedy, K.J.; Tsalikian, E.; Weinzimer, S.A., The accuracy of the FreeStyle Navigator continuous glucose monitoring system in children with type 1 diabetes. *Diabetes Care* **2007,** 30, (1), 59-64.

36. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.

37. Kapitza, C.; Lodwig, V.; Obermaier, K.; Wientjes, K.J.C.; Hoogenberg, K.; Jungheim, K.; Heinemann, L., Continuous glucose monitoring: reliable measurements for up to 4 days with the SCGM1 System. *Diabetes Technol. Ther.* **2003,** 5, (4), 609-614.

38. Maran, A.; Crepaldi, C.; Tiengo, A.; Grassi, G.; Vitali, E.; Pagano, G.; Bistoni, S.; Calabrese, G.; Santeusanio, F.; Leonetti, F.; Ribaudo, M.; Di Mario, U.; Annuzzi, G.; Genovese, S.; Riccardi, G.; Previti, M.; Cucinotta, D.; Giorgino, F.; Bellomo, A.; Giorgino, R.; Poscia, A.; Varalli, M., Continuous subcutaneous glucose monitoring in diabetic patients: a multicenter analysis. *Diabetes Care* **2002,** 25, (2), 347-352.

39. Kubiak, T.; Worle, B.; Kuhr, B.; Nied, I.; Lasner, G.; Hermanns, N.; Kulzer, B.; Haak, T., Microdialysis-based 48-hour continuous glucose monitoring with GlucoDay: clinical performance and patients' acceptance. *Diabetes Technol. Ther.* **2006,** 8, (5), 570-575.

40. Garg, S.K.; Potts, R.O.; Ackerman, N.R.; Fermi, S.J.; Tamada, J.A.; Chase, H.P., Correlation of fingerstick blood glucose measurements with GlucoWatch biographer glucose results in young subjects with type 1 diabetes. *Diabetes Care* **1999,** 22, (10), 1708-1714.

41. The Diabetes Research in Children Network (DirecNet) Study Group, Accuracy of the GlucoWatch G2 Biographer and the continuous glucose monitoring system during hypoglycemia. *Diabetes Care* **2004,** 27, (3), 722-726.

42. Caduff, A.; Hirt, E.; Feldman, Y.; Ali, Z.; Heinemann, L., First human experiments with a novel non-invasive, non-optical continuous glucose monitoring system. *Biosens. Bioelectron.* **2003,** 19, (3), 209-217.

43. Wentholt, I.M.E.; Hoekstra, J.B.L.; Zwart, A.; DeVries, J.H., Pendra goes Dutch: lessons for the CE mark in Europe. *Diabetologia* **2005,** 48, (6), 1055-1058.

44. Burmeister, J.J.; Arnold, M.A.; Small, G.W., Noninvasive blood glucose measurements by near-infrared transmission spectroscopy across human tongues. *Diabetes Technol. Ther.* **2000,** 2, (1), 5-16.

45. Maruo, K.; Tsurugi, M.; Chin, J.; Ota, T.; Arimoto, H.; Yamada, Y.; Tamura, M.; Ishii, M.; Ozaki, Y., Noninvasive blood glucose assay using a newly developed near-infrared system. *IEEE J. Sel. Top. Quantum Electron.* **2003,** 9, (2), 322-330.

46. Shen, Y.C.; et al., The use of Fourier-transform infrared spectroscopy for the quantitative determination of glucose concentration in whole blood. *Phys. Med. Biol.* **2003,** 48, (13), 2023.

47. Lambert, J.L.; Morookian, J.M.; Sirk, S.J.; Borchert, M.S., Measurement of aqueous glucose in a model anterior chamber using Raman spectroscopy. *J. Raman Spectrosc.* **2002,** 33, (7), 524-529.

48. Spanner, G.; Niessner, R., Noninvasive determination of blood constituents using an array of modulated laser diodes and a photoacoustic sensor head. *Fresenius' J. Anal. Chem.* **1996,** 355, (3), 327-328.

49. Weiss, R.; Yegorchikov, Y.; Shusterman, A.; Raz, I., Noninvasive continuous glucose monitoring using photoacoustic technology-results from the first 62 subjects. *Diabetes Technol. Ther.* **2007,** 9, (1), 68-74.

50. Lo, Y.L.; Yu, T.C., A polarimetric glucose sensor using a liquid-crystal polarization modulator driven by a sinusoidal signal. *Opt. Commun.* **2006,** 259, (1), 40-48.

51. Malchoff, C.D.; Shoukri, K.; Landau, J.I.; Buchert, J.M., A novel noninvasive blood glucose monitor. *Diabetes Care* **2002,** 25, (12), 2268-2275.

52. Sieg, A.; Guy, R.H.; Delgado-Charro, M.B., Noninvasive and minimally invasive methods for transdermal glucose monitoring. *Diabetes Technol. Ther.* **2005,** 7, (1), 174-197.

53. Oliver, N.S.; Toumazou, C.; Cass, A.E.G.; Johnston, D.G., Glucose sensors: a review of current and emerging technology. *Diabet. Med.* **2009,** 26, (3), 197-210.

54. Wientjes, K.J.C., Development of a glucose sensor for diabetic patients. *Ph.D. Dissertation, University of Groningen, Groningen, The Netherlands* **2000**.

55. Koschinsky, T.; Heinemann, L., Sensors for glucose monitoring: technical and clinical aspects. *Diabetes Metab. Res. Rev.* **2001,** 17, (2), 113-123.

56. Clarke, W.L.; Cox, D.; Gonderfrederick, L.A.; Carter, W.; Pohl, S.L., Evaluating clinical accuracy of systems for self-monitoring of blood glucose. *Diabetes Care* **1987,** 10, (5), 622-628.

57. Parkes, J.L.; Slatin, S.L.; Pardo, S.; Ginsberg, B.H., A new consensus error grid to evaluate the clinical significance of inaccuracies in the measurement of blood glucose. *Diabetes Care* **2000,** 23, (8), 1143-1148.

58. Kovatchev, B.P.; Gonder-Frederick, L.A.; Cox, D.J.; Clarke, W.L., Evaluating the accuracy of continuous glucose-monitoring sensors. *Diabetes Care* **2004,** 27, (8), 1922-1928.

59. Yum, S.I.; Roe, J., Capillary blood sampling for self-monitoring of blood glucose. *Diabetes Technol. Ther.* **1999,** 1, (1), 29-37.

60. Cengiz, E.; Tamborlane, W.V., A tale of two compartments: interstitial versus blood glucose monitoring. *Diabetes Technol. Ther.* **2009,** 11, (s1), S-11-S-16.

61. Boyne, M.S.; Silver, D.M.; Kaplan, J.; Saudek, C.D., Timing of changes in interstitial and venous blood glucose measured with a continuous subcutaneous glucose sensor. *Diabetes* **2003,** 52, (11), 2790-2794.

62. Dunn, T.C.; Eastman, R.C.; Tamada, J.A., Rates of glucose change measured by blood glucose meter and the GlucoWatch Biographer during day, night, and around mealtimes. *Diabetes Care* **2004,** 27, (9), 2161-2165.

63. van der Valk, P.R.; van der Schatte Olivier-Steding, I.; Wientjes, K.-J.C.; Schoonen, A.J.; Hoogenberg, K., Alternative-site blood glucose measurement at the abdomen. *Diabetes Care* **2002,** 25, (11), 2114-2115.

64. Woderer, S.; Henninger, N.; Garthe, C.-D.; Kloetzer, H.M.; Hajnsek, M.; Kamecke, U.; Gretz, N.; Kraenzlin, B.; Pill, J., Continuous glucose monitoring in interstitial fluid using glucose oxidase-based sensor compared to established blood glucose measurement in rats. *Anal. Chim. Acta* **2007,** 581, (1), 7-12.

65. Kaptein, W.A.; Kemper, R.H.A.; Ruiters, M.H.J.; Venema, K.; Tiessen, R.G.; Korf, J., Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats. *Biosens. Bioelectron.* **1997,** 12, (9-10), 967-976.

66. Aussedat, B.; Dupire-Angel, M.; Gifford, R.; Klein, J.C.; Wilson, G.S.; Reach, G., Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am. J. Physiol. Endocrinol. Metab.* **2000,** 278, (4), E716-728.

67. Thome-Duret, V.; Reach, G.; Gangnerau, M.N.; Lemonnier, F.; Klein, J.C.; Zhang, Y.; Hu, Y.; Wilson, G.S., Use of a subcutaneous glucose sensor to detect decreases in glucose concentration prior to observation in blood. *Anal. Chem.* **1996,** 68, (21), 3822-3826.

68. Wentholt, I.M.E.; Hart, A.A.M.; Hoekstra, J.B.L.; Devries, J.H., Relationship between interstitial and blood glucose in type 1 d iabetes patients: delay and the push-pull phenomenon revisited. *Diabetes Technol. Ther.* **2007,** 9, (2), 169-175.

69. Rebrin, K.; Steil, G.M.; van Antwerp, W.P.; Mastrototaro, J.J., Subcutaneous glucose predicts plasma glucose independent of insulin: implications for continuous monitoring. *Am. J. Physiol. Endocrinol. Metab.* **1999,** 277, (3), E561-571.

70. Steil, G.; Rebrin, K.; Hariri, F.; Jinagonda, S.; Tadros, S.; Darwin, C.; Saad, M., Interstitial fluid glucose dynamics during insulin-induced hypoglycaemia. *Diabetologia* **2005,** 48, (9), 1833-1840.

71. Ungerstedt, U.; Pycock, C., Functional correlates of dopamine neurotransmission *Bull. Schweiz. Akad. Med. Wiss.* **1974,** 30, (1-3), 44-55.

72. Benveniste, H.; Hüttemeier, P.C., Microdialysis--Theory and application. *Prog. Neurobiol.* **1990,** 35, (3), 195-215.

73. Palmisano, F.; Zambonin, P.G., Ascorbic acid interferences in hydrogen peroxide detecting biosensors based on e lectrochemically immobilized enzymes. *Anal. Chem.* **1993,** 65, (19), 2690-2692.

74. Arora, A.; Simone, G.; Salieb-Beugelaar, G.B.; Kim, J.T.; Manz, A., Latest developments in micro total analysis systems. *Anal. Chem.* **2010,** 82, (12), 4830-4847.

75. Salieb-Beugelaar, G.B.; Simone, G.; Arora, A.; Philippi, A.; Manz, A., Latest developments in microfluidic cell biology and analysis systems. *Anal. Chem.* **2010,** 82, (12), 4848-4864.

76. Whitesides, G.M., The origins and the future of microfluidics. *Nature* **2006,** 442, (7101), 368-373.

77. Janasek, D.; Franzke, J.; Manz, A., Scaling and the design of miniaturized chemical-analysis systems. *Nature* **2006,** 442, (7101), 374-380.

78. Stroock, A.D.; Dertinger, S.K.W.; Ajdari, A.; Mezic, I.; Stone, H.A.; Whitesides, G.M., Chaotic mixer for microchannels. *Science* **2002,** 295, (5555), 647-651.

79. Verpoorte, E.; De Rooij, N.F., Microfluidics meets MEMS. *Proceedings of the IEEE* **2003,** 91, (6), 930-953.

80. Zhao, X.-M.; Xia, Y.; Whitesides, G.M., Fabrication of three-dimensional micro-structures: Microtransfer molding. *Adv. Mater. (Weinheim, Ger.)* **1996,** 8, (10), 837-840.

81. Xia, Y.; Whitesides, G.M., Soft lithography. *Annu. Rev. Mater. Sci.* **1998,** 28, (1), 153-184.

82. Zhao, X.-M.; Xia, Y.; Whitesides, G.M., Soft lithographic methods for nano-fabrication. *J. Mater. Chem.* **1997,** 7, (7), 1069-1074.

83. Whitesides, G.M.; Ostuni, E.; Takayama, S.; Jiang, X.; Ingber, D.E., Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.* **2001,** 3, (1), 335-373.

84. Verpoorte, E., Microfluidic chips for clinical and forensic analysis. *Electrophoresis* **2002,** 23, (5), 677-712.

85. Vilkner, T.; Janasek, D.; Manz, A., Micro total analysis systems. Recent developments. *Anal. Chem.* **2004,** 76, (12), 3373-3386.

86. Dittrich, P.S.; Tachikawa, K.; Manz, A., Micro total analysis systems. Latest advancements and trends. *Anal. Chem.* **2006,** 78, (12), 3887-3908.

87. West, J.; Becker, M.; Tombrink, S.; Manz, A., Micro total analysis systems. Latest achievements. *Anal. Chem.* **2008,** 80, (12), 4403-4419.

88. Sia, S.K.; Whitesides, G.M., Microfluidic devices fabricated in Poly(dimethylsiloxane) for biological studies. *Electrophoresis* **2003,** 24, (21), 3563-3576.

89. Weibel, D.B.; Whitesides, G.M., Applications of microfluidics in chemical biology. *Curr. Opin. Chem. Biol.* **2006,** 10, (6), 584-591.

90. Weibel, D.B.; DiLuzio, W.R.; Whitesides, G.M., Microfabrication meets microbiology. *Nat. Rev. Microbiol.* **2007,** 5, (3), 209-218.

91. Toner, M.; Irimia, D., Blood-on-a-chip. *Annu. Rev. Biomed. Eng.* **2005,** 7, (1), 77-103.

92. Unger, M.A.; Chou, H.-P.; Thorsen, T.; Scherer, A.; Quake, S.R., Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* **2000,** 288, (5463), 113-116.

93. Wang, J., Electrochemical detection for microscale analytical systems: a review. *Talanta* **2002,** 56, (2), 223-231.

Chapter 2

Physiological Processes Influence Continuous Monitoring of Subcutaneous Glucose: A Review

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Abstract

In patients with diabetes, the manifestation of long-term diabetic complications can be largely reduced by maintaining strict glycemic control. Glucose concentrations should be measured frequently to adjust therapeutic intervention, e.g. administration of insulin. Conventional self-monitoring of blood glucose with fingersticks is not an ideal method. Many hypoglycemic episodes may be missed, especially during the night. Moreover, this method is difficult to implement in some patients, including children, elderly, and people with disabilities. Patient compliance is also reduced by the fact that the method is tedious and painful. A new method is needed for convenient and continuous monitoring of glucose, which is also the prerequisite for the development of an artificial pancreas.

To date, various approaches are being used to develop continuous glucose monitors (CGM). Several CGMs based on different technologies were recently brought onto the market. However, the performance of these devices is still sub-optimal and their feasibility has been questioned. In this paper, we review the technical challenges that a C GM typically has to overcome, including the physiological influences affecting accuracy, selectivity, sensitivity, and durability.

Current data regarding CGM performance is only preliminary, as they still await evaluation in controlled clinical trials. Each type of CGM has its specific challenges. Needle-type electrodes respond rapidly but are most affected by biofouling and signal drift, especially during the night. Microdialysis-based CGMs are more stable, but these are relatively large systems and there is an inherent time delay between microdialysis sampling and analysis. The feasibility of such systems is discussed, with particular attention paid to the physiological factors that influence the reliability of the monitor in a healthcare application.

1. Introduction

Diabetes mellitus is a chronic and currently incurable disorder of metabolism in which blood glucose concentrations cannot be properly maintained due to a lack of insulin secretion or a lack of insulin action.[1, 2] Patients with diabetes often have to regulate blood glucose by therapeutic intervention, consisting of oral medication or exogenous insulin administration. Maintenance of daily glucose balance within the narrow range of physiologically acceptable concentrations requires frequent and rapid adjustments of the treatment regimen. However, the manifestation of long-term diabetic complications can be largely prevented by implementing strict glycemic control.[3] Intensified insulin therapy, however, warrants the need for the patient to monitor blood glucose levels closely. Inappropriate intervention may lead to recurrent episodes of iatrogenic hypoglycemia and further impairment of endogenous glucose control in the body.[4]

The conventional way to assess glycemic status is to measure glycated hemoglobin (HbA_{1c}) levels as an indicator of average blood glucose over a period of several weeks, and to spot-check blood glucose by the fingerstick method for evaluation of the acute state. These conventional methods, however, have several disadvantages that are limiting to the implementation of good glycemic control. Diabetic patients typically measure their own blood glucose levels multiple times per day. The frequency of glucose measurements is related to the intensity of the treatment regimen and the specific physiological responsiveness of the individual.[5] The fingerstick method is generally considered a burden because it is painful and tedious, which limits patient compliance.[6] In certain categories of patients, such as in young children, elderly, or patients with disabilities, this method is extremely difficult to implement.[7-9] As a result, the frequency of monitoring is often sub-optimal.[10] Blood glucose levels over the course of a day are very dynamic, and even during active control much information may be missed. This is especially true during the night, when checks are infrequent and there is increased risk of (severe) hypoglycemia.[11-13]

Continuous glucose monitoring is a relatively new approach aimed at improving glycemic control in patients with diabetes by providing a more convenient and complete assessment of their glycemic status. In general, a small continuous glucose monitor (CGM) is implanted in the patient's body and this device measures glucose levels frequently for prolonged periods of time, ideally up to several weeks. Depending on the exact technology, the frequency of measurements may range from once every 10 min to once every few seconds.[7, 14, 15] Recently, several different

CGMs have been brought to market. However, the performance of these devices is not yet suitable to deploy them on a large scale. Preliminary reports of positive findings still need to be corroborated by controlled trials, and it has been questioned whether the accuracy was evaluated with appropriate methods.[16-18] This review paper covers the technical challenges that the CGMs need to overcome to be suitable in healthcare applications. Particular attention is given to the physiological influences that potentially affect their selectivity, sensitivity, and durability. The impact of the implantation process, exact position of the CGM in the body, and relevance of blood-to-interstitial fluid (ISF) glucose kinetics is discussed.

2. Retrospective and prospective applications

Since the concept of the enzyme-based glucose electrode was first proposed by Clark and Lyons in 1962,[19] the development of glucose monitors has received much attention. In recent years, CGMs have proven to be a major asset in many healthcare applications, especially in diagnosis and treatment of diabetes. The aim of a CGM is to provide a continuous sequence of frequently measured glucose concentrations for prolonged periods of time. This data can be used retrospectively to establish the optimal and personal treatment regimen for the patient, to diagnose and prevent the occurrence of hypoglycemia, and to assess impact of lifestyle modifications on glycemic control.[7] In this mode, the data is usually evaluated by the physician. In addition to retrospective use, in some CGMs the data can also be used prospectively to alert the patient in real-time to take immediate corrective action. In prospective mode, the output from the CGM is primarily used by the patients themselves to assess glycemic status in real-time, although it has also been argued that CGM findings should first be confirmed by a physician to avoid therapeutic errors.[18] In this mode it is imperative that analysis is rapid and the lag time between physiological changes and corresponding sensor output is short. Patients also need to be well educated in the use of the CGM.[8]

Clinical outcome studies have suggested that the use of CGMs improve mean glycemia (mean blood glucose concentration), especially in patients with difficult-to-control diabetes.[7, 20] CGMs have also been found to effectively register hypoglycemic excursions that would otherwise go unnoticed, for example in preschool children or during the night.[9, 21] It is particularly difficult to maintain euglycemia in preschool children, because they are more sensitive to insulin, and they have variable eating habits and unpredictable levels of physical activity. In adult patients, approximately 33% of cases of hypoglycemia during the day occur

without symptoms, and 55% of cases of severe hypoglycemia occur during sleep.[13] Some studies showed that the use of CGMs effectively shortened the duration of hypoglycemic episodes, although there was no statistical improvement of metabolic control compared to frequent capillary glucose measurements.[22, 23] It was therefore concluded that CGMs are particularly useful to prevent complications from recurrent hypoglycemia. However, there is no further improvement of glycemic control compared to frequent self-monitoring with fingersticks. In addition to diabetes, CGMs are also useful in other diseases where glycemic control is at risk, as in glycogen storage disease or insulinomas.[24]

The ultimate goal of prospective CGM use in diabetes therapy is to integrate the monitor into a closed-loop system that functions as an artificial pancreas. The automated continuous dosing of insulin in such a system is based on glucose data provided by the CGM.[25, 26] Model-based predictive control algorithms are needed to convert sensory input to direct appropriate insulin delivery, because personalized control is needed that adapts to individual-specific kinetic factors, system- and physiology-based time delays, and to daily life events. Use of CGMs in prospective mode, more so than in retrospective mode, requires the devices to at least be able to detect a glucose decline of 0.6 mM in 5 min.[27] This defines lower limits for accuracy, precision and data sampling frequency. It has been reported that CGMs may incorrectly detect hypoglycemia during the night. The needle-type sensors seem to be particularly susceptible to nocturnal signal drift.[28, 29] Although this phenomenon cannot yet be explained, it may be remedied by additional and separate calibrations using fingersticks during the night.[28, 30] However, this solution reduces the level of convenience, one of the goals for CGM development in the first place.

3. Technical strategies

3.1. Needle-type electrodes

A common approach to continuously monitor glucose is by means of an implantable enzyme-coated electrode. This technique has been developed into a major analytical tool to monitor concentrations of many different biomolecules, both *in vitro* and *in vivo*.[31] Glucose is measured by coating the electrode with the enzyme glucose oxidase, which converts glucose and oxygen into gluconolactone and hydrogen peroxide. The amount of hydrogen peroxide is subsequently assayed amperometrically by oxidizing it at the electrode surface at an applied potential (usually 700 mV).[15] Enzyme-based electrodes are well suited as CGMs because

they have a high temporal resolution and a very short response delay. In addition, the size of the electrode and the potentiostat can be kept small.

The largest disadvantage of needle-type CGMs is a lack of specificity caused by the presence of other electroactive biomolecules, such as uric acid and ascorbic acid, which are oxidized at the potential used for H_2O_2 detection. A lower potential may be used when a redox mediator is incorporated in the biorecognition layer to help shuttle electrons between the enzyme and the electrode.[31] Biosensors with mediators are considered second-generation sensors, as opposed to the ones that require oxygen. Some commercially available CGMs use mediators, including the FreeStyle Navigator.[32, 33] This is also the prevailing method in teststrips for measurement of blood glucose. The working principle of both types of sensors is outlined in Figure 1. However, successful use of mediator molecules depends on their positionwith respect to the redox center of the enzyme. Mediators degrade after repeated cycling, are often chemically unstable in either reduced or oxidized form, and are prone to leaching. Moreover, mediators generally have difficulty competing with freely diffusible oxygen, and are sensitive to inhibitors.[34, 35]

For implantable biosensors, common alternative strategies to increase specificity for glucose is the affixing of a permselective layer onto the electrode to prevent

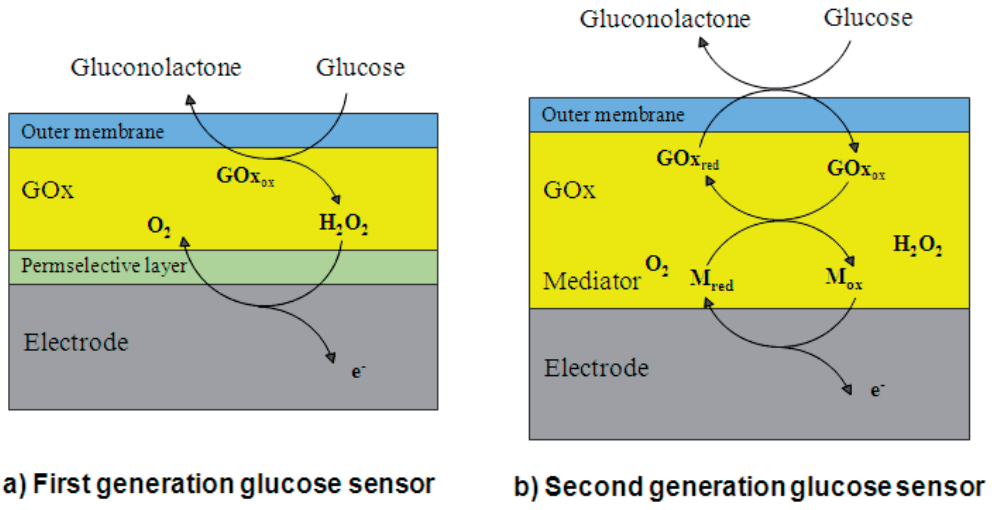

Figure 1. Schematic diagram of the enzymatic reaction on the surface of the electrode.

potential interferents from reaching the electrode surface,[34] and/or the addition of a background electrode (without glucose oxidase) for differential recording.[36] Differential recording eliminates interferences by subtracting the current caused by interferents at the background electrode from the total current generated at the working electrode. After implantation in the body, needle-type CGMs are vulnerable to biofouling, to a large extent due to the presence of enzyme in the interface between electrode and tissue. They have also been reported to suffer from signal drift, especially during the night.[29, 37]

3.2. Microdialysis probes

A different strategy is to measure glucose *ex vivo* after a small amount of ISF-related sample has been recovered from the body, for example by means of microdialysis. Microdialysis was introduced in the early 1970s to monitor neurotransmitter release *in vivo* in the brain,[38] and has since evolved into a common method in biomedical research to acquire samples derived from ISF in almost any tissue.[39, 40] The microdialysis probe, like a small catheter, is inserted into the tissue and serves to mimic a blood capillary. The probe has a semi-permeable hollow fiber at its tip that separates the tissue from the probe's lumen. The lumen of the probe is continuously perfused with an aqueous solution that resembles the ionic composition of the surrounding ISF. Small molecules, including glucose, can pass through the pores of the membrane by diffusion along their concentration gradient. Depending on the molecular weight cut-off of the membrane, most of the larger biomolecules, like proteins, are excluded from the dialysate. The dialysate emerges from the probe's outlet at a typical flow rate of 0.1 – 5.0 μL/min and can be collected for analysis. Analysis of glucose in the dialysate can be carried out by various optical and electrochemical techniques. Figure 2 shows an example of a microdialysis-based CGM.[41]

There are fewer biocompatibility issues with microdialysis compared to enzyme-coated electrodes, as contact with the tissue is made only by the semi-permeable membrane and no hydrogen peroxide leaks into the tissue. In addition, the membrane keeps proteins and cellular debris out of the dialysate, thereby reducing biofouling of the detector. However, there are also a few disadvantages. It may take up to 30 min for the sample to travel through the tubing from the probe to the detector, which creates a response delay that is unacceptable in a prospective application when the CGM should alert the patient during acute hypoglycemia.[41-44] In addition, the required pump and perfusate reservoir make such a system relatively big.

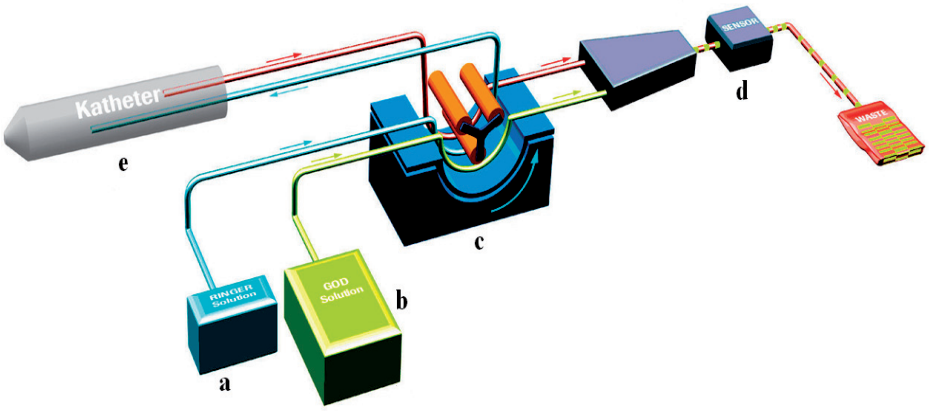

Figure 2. Schematic diagram of the Subcutaneous Continuous Glucose Monitoring (SCGM) system: a) Ringer's solution; b) glucose oxidase; c) peristaltic pump; d) sensor; e) microdialysis probe (reprinted with permission from Schoemaker et al.[41]).

3.3. Coupling microdialysis with microfluidics

In the past decade, the use of microfluidics has increased enormously in interdisciplinary research fields comprising chemical, biological, biomedical and other life science applications.[45, 46] Notable advantages of this approach include sample handling in the µL-to-pL range, fast analysis and/or reaction times and cost-effectiveness with small, disposable devices. Coupling microdialysis with microfluidics thus combines the long-term stability of microdialysis probes with substantially reduced lag times, as they occur with needle-type CGMs. Soft lithography, the replication of microchannel networks in elastomeric materials, provides a rapid-prototyping approach for the realization of devices within a few days.[47-49] Not surprisingly, a significant amount of research has been conducted in recent years on coupling microdialysis sampling with the development of microfluidic chips. This will be discussed below.

However, it is important to recognize that there is a body of literature predating the use of microfluidics which describes the use of microdialysis for glucose monitoring. In the early 1990s, for instance, Schmidt *et al*. developed a glucose monitoring system using microdialysis, in which dissolved enzyme was perfused through the hollow fiber to oxidize glucose. The resulting changes in oxygen concentration were monitored with an oxygen electrode.[50] It was demonstrated that this system could monitor changes in subcutaneous glucose concentrations *in vivo*.[50, 51] Laurell

developed a continuous glucose monitoring system where microdialysate was passed through an immobilized enzyme reactor.[52] An oxygen electrode positioned at the end of the reactor was used to monitor fluctuations in dissolved oxygen concentrations due to changing glucose concentrations in the microdialysate. This system was used to perform an *in vivo* subcutaneous glucose measurement in a patient.[53] An amperometric glucose measurement was performed by Meyerhoff *et al*. to monitor subcutaneous glucose using microdialysis in real-time.[54, 55] Other glucose sensing systems coupling microdialysis and electrochemical detection have been presented for *in vitro* and *in vivo* experiments in animals[56, 57], comparative studies of different types of microdialysis probe[58], and the evaluation of a thin layer flow cell detector for *in vivo* monitoring.[59] Glucose and lactate determination in microdialysate using microfabricated biosensors incorporated into a microfluidic system coupled to a microdialysis probe was described in 1997.[60, 61] The advantages of small-volume fluid handling systems were immediately clear, as lag times of less than 5 min between sampling and analysis were made possible using this approach. Shortly thereafter, Böhm *et al*. presented a micromachined double lumen microdialysis probe connector with incorporated sensor for on-line sampling, a diagram of which is shown in Figure 3(a).[62] Two etched silicon chips formed this device, which incorporated not only the probe connector but also a chloride sensor in a microchamber located behind the connector.

The first example of a microchip electrophoresis device coupled with microdialysis was reported in 2004 by Huynh *et al.*, who monitored an enzymatic reaction producing a fluorescent product.[63] A continuously flowing dialysate stream from a microdialysis probe was introduced into the microchip, and discrete injections into the separation channel on chip were achieved using a valveless gating approach. Fluorescence detection was used to detect separated sample components. Other examples of microchip electrophoresis for on-line analysis of microdialysate have used poly(dimethylsiloxane)(PDMS)-glass bonded devices, interfaced to microdialysis probes with silica capillary tubing. Martin's group described the use of on-chip pneumatic valve(s) for discrete plug samplings from a hydrodynamic flow channel into a separation channel (Figure 3(b)).[64, 65] The microdialysis probe was inserted directly into the sample inlet, S, and microdialysate was introduced continuously to the chip. With valve 1 normally open (NO) and valve 2 normally closed (NC), microdialysate runs from the inlet to the sample waste, SW, outlet. Periodically, valve 2 was opened, valve 1 closed, and a small amount of microdialysate was injected into the separation channel. Stimulated dopamine release from PC12 cells was monitored by microdialysis/microchip electrophoresis

in this way, using electrochemical detection. Wang *et al*. reported a segmented flow microfluidic device interfaced with a microdialysis probe in order to improve the temporal resolution of *in vivo* glucose monitoring (Figure 3(c)).[66, 67] Segmented flow was produced by flowing perfluorodecalin (oil) from one arm of a T-junction,

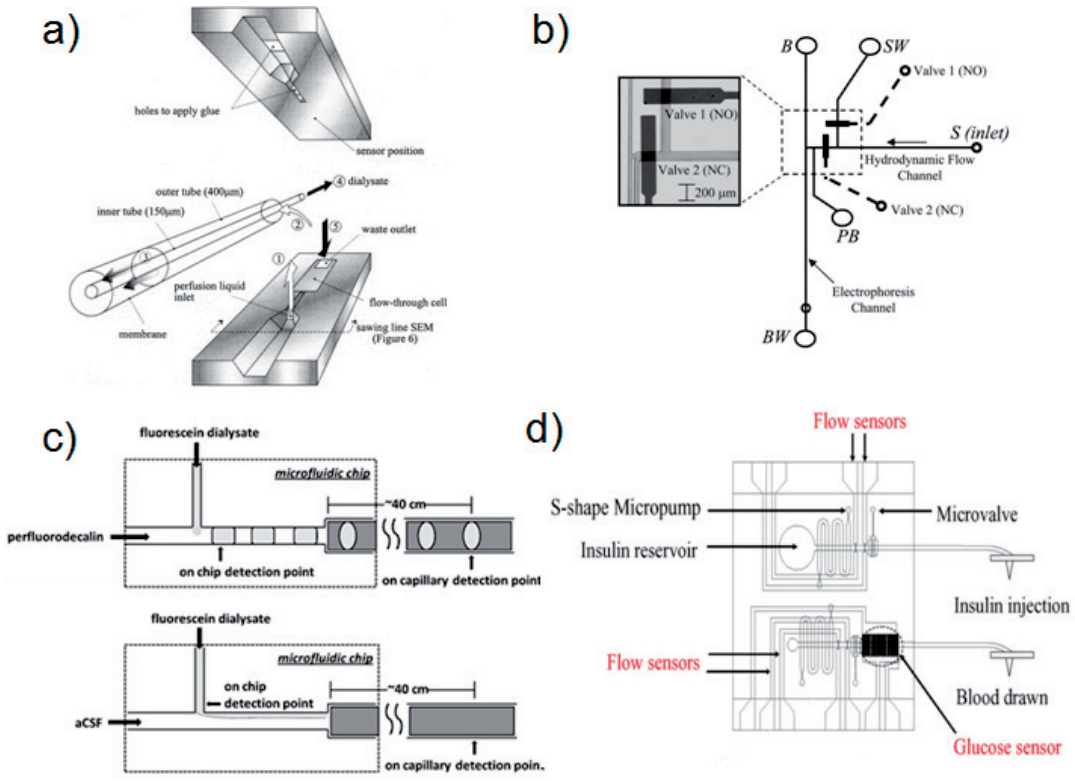

Figure 3. (a) A double lumen microdialysis probe connector micromachined in silicon. The connector has an incorporated sensor for on-line sampling (reprinted with permission from Böhm et al.[62]) (b) Poly(dimethylsiloxane)(PDMS)-based on-chip pneumatic valves for hydrodynamic flow control. (reprinted with permission from Mecker et al.[65]) (c) Microfluidic chip with a microdialysis interface based on segmented-flow (above) and continuous flow (below). (reprinted with permission from Wang et al.[66]) (d) Schematic diagram of microfluidic chip for glucose sensing and insulin injection. Micropumps, microvalves, microchannels and reservoir were incorporated to control fluids.(reprinted with permission from Huang et al.[68]).

while microdialysate from a probe was introduced continuously from a second arm. The resulting microdroplets were then directed onto a conventional fused-silica collection capillary. By adding reagents to the microdialysate containing glucose, a reaction could be performed in the droplets, producing a fluorescent signal proportional to glucose concentration which was detected at the end of the collection capillary. A couple of papers have been reviewed elsewhere for development and applications of microdialysis-microfluidic system.[69-71]

Microfluidics technology provides a route to completely integrated systems for (continuous) glucose monitoring.[72] Huang *et al*. presented a microfluidic system with compartments of control modules such as valves, pumps, fluidic channels, and reservoir and monitoring modules including glucose and flow sensors (Figure 3(d)).[68] This system is particularly interesting, since both *in vitro* results for glucose measurement and automatic insulin injection were demonstrated, with minimal amounts of reagents consumed. Hsieh *et al*. developed an on-chip microdialysis system with in-line sensing electrodes.[73, 74] A microdialysis membrane sealed a 15-μm-deep microchannel formed photolithographically in an SU-8 epoxy layer and containing electrodes for electrochemical detection. Ultimately, the device was designed to function as a microdialysis probe, with the outer surface of the membrane in direct contact with tissue, and the inner surface perfused by medium containing GOx, pumped through the microchannel. Glucose diffusing across the membrane into the microchannel reacts with the GOx to produce H_2O_2, which is then detected at the electrodes. For the studies reported in [73, 74], the outer membrane surface was placed in contact with a reservoir containing a glucose solution.

In our group, we have reported an enzymatic microreactor incorporating a chaotic mixing channel with an on-chip electrochemical detector for continuous glucose monitoring.[75] This microreactor was used in an *in vivo* application, in which a microdialysis probe was interfaced with the device for subcutaneous glucose monitoring in anesthetized rats.[Chapter 5 of this thesis] Having demonstrated the feasibility of using this type of microfluidic chip system in conjunction with microdialysis, a micro CGM combining both an enzymatic microreactor and on-chip electrochemical detector is envisioned.

4. Currently available CGMs

4.1. Commercial prototypes

To date, various approaches are being used to develop continuous glucose monitors (CGM). Several CGMs with different technologies were recently brought to market. Some of these rely on noninvasive transdermal methods to measure glucose, such as reversed iontophoresis or optical techniques. Noninvasive techniques are thoroughly reviewed by W ilkins *et al*.[76] The GlucoWatch (Cygnus Inc), based on iontophoresis,[77] was commercialized in 2001.[78] The Pendra (Pendragon Medical) uses impedance spectroscopy to measure glucose, and was introduced onto the market in 2003.[79] Although noninvasive transdermal approaches are appealing due to their high degree of convenience and user friendliness, they are still too underdeveloped in terms of accuracy.[28] Accuracy is negatively affected by variation in temperature, tissue thickness, sweating, blood pressure, motion artifacts, and microvascular blood flow variation.[80] As a result, the prospect of noninvasive CGMs is still elusive.

In contrast to the noninvasive techniques, rather promising results have been obtained with invasive methods like needle-type and microdialysis-based CGMs. In this review we therefore mainly focus on invasive techniques, such as needle-type electrodes[32, 33, 81-86] and microdialysis-based CGMs.[41-44] Characteristics of several commercially available systems are summarized in Table 1. Needle-type CGMs with glucose oxidase immobilized onto an implantable electrode are a successful approach. This technology is currently used by several CGMs, including the CGMS Gold, Guardian REAL-Time and Paradigm REAL-Time (Medtronic),[81-84] Dexcom STS (Dexcom),[85, 86] and Freestyle Navigator (Abbott Diabetes Care).[32, 33]

Commercially available CGMs based on microdialysis include the SCGM (Roche),[41, 42] and the GlucoDay (Menarini Diagnostics).[43, 44] The SCGM uses enzyme in solution rather than using immobilized enzyme on the electrode to react with glucose and produce H_2O_2. A small peristaltic pump perfuses Ringer's solution and glucose oxidase at a flow rate of 0.3 µL/min. The glucose which diffuses across the probe membrane and into the microdialysate is transported to the sensor unit. The glucose and glucose oxidase in solution are then mixed/reacted. The electrode detects the produced H_2O_2 amperometrically using an applied potential. The GlucoDay uses glucose oxidase immobilized on the working electrode. Glucose from the microdialysis fiber is conveyed to the sensor at a flow rate of 10 µL/min. This flow rate results in relatively poor glucose recovery, as a result of the reduced contact time of perfusion solution with the internal membrane surface. Compared to needle-type CGMs, the microdialysis-based approach is considered a safe technique for *in vivo* applications, as the reaction takes place outside of the body. Sensor

biofouling is also less of an issue, as microdialysis plays a role as a prefiltering tool to remove proteins and other biological complexes from the glucose sample. However, a low temporal resolution (long system lag time) and the requirement of an external pump are drawbacks of this approach. Microfluidics offers an excellent opportunity to reduce reaction and lag times and miniaturize microdialysis-based systems.

Table 1. State-of-the-art continuous glucose monitors (CGM) used for clinical studies.

Continuous glucose monitor	CGMS Gold	Guardian and Paradigm RT	Dexcom STS	FreeStyle Navigator	SCGM	Glucowatch	GlucoDay
Company	Medtronic Minimed	Medtronic Minimed	DexCom Inc.	Abbot Diabetes Care	Roche Diagnostics	Cygnus Inc.	Menarini Diagnostics
Sensor life	3 days	3 days	7 days	5 days	4 days	15 hours	2 days
Sensing technique	Needle-type	Needle-type	Needle-type	Needle-type	Micro-dialysis	Ionto-phoresis	Micro-dialysis
Signal display	Every 5 min	Every 5 min	Every 5 min	Every 1 min	Every 5 min	Every 10 min	Every 3 min
Calibration per day	4 times	2-3 times	2-3 times	1 time	1 time	1-2 times	1 time
Data interpreta-tion	Ret	Pro	Pro	Pro	Ret	Pro	Ret
Accuracy (EGA, A+B)	94%	96%	95%	98%	99%	96%	96%
Approved FDA	Yes (1999)	Yes (2006)	Yes (2007)	Yes (2008)	No	Yes (2001)	No
References	[81, 82]	[83, 84]	[85, 86]	[32, 33]	[41, 42]	[77, 78]	[43, 44]

CGM: Continuous glucose monitor; CGMS: Continuous Glucose Monitoring System; SCGM: Subcutaneous Continuous Glucose Monitoring; EGA: Error Grid Analysis; FDA: Food and Drug administration; Ret: Retrospective; Pro: Prospective

4.2. Main challenges

Technology for CGMs has been under development for over three decades, and several lines of research have produced proofs-of-principle with promising results. However, in spite of all these scientific advances, CGMs are still awaiting their widespread use in diabetes therapies. It has been acknowledged that proliferation of CGM in healthcare applications is currently hampered by a lack of reimbursement from private and government health insurance agencies, and the presence of several technical challenges that still need to be overcome.[17, 87] CGMs currently on the market should be upgraded for higher accuracy, signal stability, and reliability of longer period of days. Frequent calibrations by the fingerstick method, especially during the night, and regular replacement of the implants are still major burdens to the patient and limit applicability.

The most important criterion for reliable CGMs is that they exhibit a high degree of accuracy. The accuracy of CGMs in clinical applications is usually evaluated by means of error grid analysis. Error grid analysis takes into account that in the clinical context the required degree of accuracy is co-determined by the blood glucose concentration. In the error grid, glucose concentrations obtained from the CGM are plotted against concurrent blood glucose values obtained by the fingerstick method. Various zones in the grid are defined that are considered indicative of a certain degree of accuracy. The most common methods are the Clarke Error Grid,[88] established in 1987, and the updated version named the Consensus Error Grid (see Figure 4).[89] While the Consensus Error Grid does not have boundaries that skip risk categories, it has also been argued that this method is too forgiving when assessing accuracy during systemic hypoglycemia.[16] The Continuous Glucose-Error Grid Analysis was developed in 2004, which also takes into account that a certain time delay may exist between glucose changes in ISF compared to the blood.[90] However, the delay rate in this method is arbitrary and there currently is no consensus about the underlying physiological mechanism.[91]

The Clarke Error Grid has been used in many clinical studies to evaluate the accuracy of commercially-available CGMs. Typically, more than 93% of measurements fell in zones A or B in patients with type 1 or type 2 diabetes.[32, 92-94] Although the accuracy is sufficient, these positive findings were obtained under well controlled laboratory conditions. Accuracy was found to be much lower when patients used the CGMs at home, indicating that real-life situations and proper patient training largely affect CGM performance.[18, 93] CGMs generally have the lowest degree of accuracy when monitoring glucose during systemic hypoglycemia.[37, 95] Part of this effect may be explained by the dynamic

relationship between blood and ISF compartments under these conditions. Whatever the reason may be, it is sufficient grounds to question the reliability of CGMs in both improving glycemic control and avoiding hypoglycemia.

Another major challenge for all types of CGMs is the lag time between a change in blood glucose concentration and the measurement provided by the CGM. This lag time includes a physiological delay between the blood and the tissue site where the CGM is implanted, a phenomenon discussed in Section 5. Microdialysis-based CGMs also have considerable system delays that originate within the device itself. A wide range of lag times have been reported, ranging from 6 to 30 min.[96, 97]

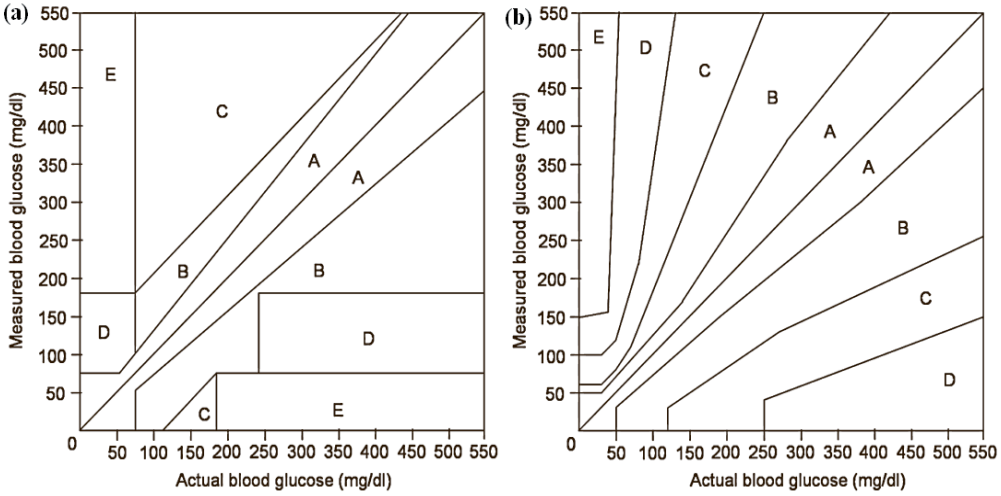

Figure 4. (a) Clarke Error Grid and (b) Consensus Error Grid, zone A: clinically accurate, zone B: benign errors, zone C: overcorrect, zone D: failure to detect, zone E: erroneous (reprinted with permission from Wentholt et al.[16]).

5. Physiological factors

5.1. Glucose in ISF versus in blood

For historical reasons, therapeutic decisions in the treatment of diabetes are made on the basis of blood glucose values. Intravascular placement of CGMs is very tempting, because it would negate issues about glucose transfer through tissues. However, there are obvious difficulties and health risks related to the placement of CGMs in the intravascular compartment for prolonged periods of time. Initial placement and then replacement of CGMs in a b lood vessel always requires the assistance of a healthcare professional, and is associated with risks for hemorrhages

and thrombosis.[98, 99] Formation of blood clots on the CGM may also reduce its lifespan and therefore necessitate frequent replacement, although Wientjes *et al*. demonstrated good long-term stability of their intravascular microdialysis-based CGM in human patients.[100] Invasive CGMs are typically placed in extracellular compartments, such as the subcutaneous adipose tissue in the abdomen or the forearm. These devices measure glucose concentrations in the ISF. Noninvasive optical techniques measure a combination of intravascular, intracellular, and extracellular concentrations. The use of either approach poses the question whether glucose concentrations in the ISF truly reflect blood glucose. It has been postulated that ISF may more adequately reflect overall tissue homeostasis compared to blood glucose measurements, which would have important clinical relevance concerning hypoglycemic symptoms and diabetic complications.[101, 102] Moreover, in a principle site for endogenous regulation of glucose homeostasis, the endocrine pancreas, ISF glucose is used by the body to adjust release of insulin and glucagon.[27] To date the dominant method has been to calibrate ISF glucose values against concurrently obtained blood glucose, with varying degrees of success, to be able to relate to existing literature on daily glycemic dynamics.

In human skin, approximately 40% of tissue volume is ISF and approximately 5% consists of blood vessels.[103] Subcutaneous adipose tissue has less ISF compared to skin, largely impacted by the degree of obesity.[104] The relationship between ISF and blood glucose is complex and not well understood. There is disagreement among the outcomes of the various studies that have investigated this relationship, which we believe to be attributable to differences in methodology. Under physiological conditions, there is a free and rapid exchange of glucose between blood and ISF.[105] During steady-state, glucose concentrations in blood and ISF will therefore be equal when glucose uptake by the tissue surrounding the CGM is negligible, and there will be a gradient when glucose is taken up by the surrounding tissue.[30] It has been reported that ISF glucose in subcutaneous adipose tissue is nearly identical to the blood[101, 106], although others reported ISF glucose to be 15-30% lower.[107-109] The blood-ISF gradient is dependent on the tissue,[101] and seems to be larger during systemic hypoglycemia.[110, 111] Conflicting reports also exist about the rate of equilibration between blood and ISF during increasing or decreasing glucose concentrations. Some studies revealed rapid equilibration[110, 112], whereas others demonstrated a delayed physiological response in the ISF by 18-30 min.[14, 94, 106, 113, 114] Moreover, Moberg *et al*. showed that the delay was larger in skeletal muscle compared to adipose tissue, and also larger during decreasing than during increasing glucose levels.[101]

There is general agreement in the literature that recovery from insulin-induced glucoprivation lasts longer in ISF compared to blood, both in healthy and in diabetic subjects.[106, 111, 115] It is interesting to note that some studies revealed glucose concentrations in ISF decrease earlier compared to the blood during insulin-induced hypoglycemia, both in rats[116] and in humans.[108] This so-called push-pull phenomenon, first described in anesthetized rats in 2000,[105] is suggestive of a dynamic relationship between blood and ISF which depends on changes in local glucose uptake. However, in diabetic dogs it was found that the delay of approximately 5-10 min was independent of circulating concentrations of insulin.[30] Diabetes may also affect the blood-ISF relationship, as hypoglycohistiosis preceded systemic hypoglycemia by approximately 15 min in diabetic rats, but not in healthy ones.[116]

The various findings described above illustrate the complexity of the relationship between glucose concentrations in blood and ISF. Especially during systemic hypoglycemia, when accuracy of measurements is most critical, ISF values obtained by any CGM have to be treated with great care. Unfortunately, it has not been possible toestablish a systematic pattern in the outcomes that could be attributed to a specific technical aspect of electrode-type or microdialysis-type CGMs. A high degree of variation between individuals has been observed with both systems.[102, 112] Therefore, it seems that the exact conditions at the tissue site of implantation may influence the outcomes.

5.2. Implantation effects

In addition to technical challenges that deal with accurate analysis of glucose concentrations, there are also physiological challenges that may affect the concentration of glucose to which the CGM is exposed. These physiological factors can be divided into acute processes that accompany CGM implantation, intermediate processes that involve wound healing, and long-term processes such as inflammation, encapsulation, and angiogenesis.[97, 117-119] In addition, these processes may also impair the performance of the device itself. The changes in tissue morphology alter diffusion parameters of the tissue, such as the endothelial barrier and tissue tortuosity. There may also be disturbances in microcirculation and local rate of metabolism. Implantation of a CGM produces signs of trauma. Therefore it is important that the tissue is given sufficient time to recover after a probe has been implanted. On the other hand, slow-onset tissue reactions may render long-term sampling impossible. It is thus expected that a certain window-of-

opportunity exists in which reliable measurements can be obtained. The goal is then to limit these reactions and maintain favorable conditions.

Implantation of a CGM into subcutaneous tissue causes acute damage to blood capillaries and cells immediately surrounding the CGM. Local supply of blood and glucose to the site of implantation is impaired.[120] On the other hand, multicellular structures that may normally form a barrier for diffusion of glucose from capillaries to more distant tissue are disrupted, allowing glucose to reach the CGM more efficiently. In the first days following CGM implantation, the tissue surrounding the CGM shows morphological changes, including signs of hemorrhage and edema.[119] At the intracellular level, major tissue disruptions have been reported 40 hours after implantation.[121] Increased rates of metabolism caused by tissue trauma and wound healing processes have been implicated in decreasing ISF glucose levels relative to the blood within the first 12 hours after CGM implantation.[97, 122] Lutgers *et al*. showed that it takes up to four days after implantation before a reasonable recovery of at least 70% is obtained.[123] This is consistent with inflammation effects, because those last for three days after CGM implantation.[124, 125] Activation of the complement system induces vasodilatation, changes the blood flow to the region, and increases vascular permeability.[126]

In chronically implanted CGMs, substantial deposits of fibrin-like polymer were found to cause a physical barrier to the diffusion of glucose, which may increase the response delay between changes in glucose concentration in the ISF compared to the blood.[127, 128] Fibrin is a sign of vascular endothelial cell damage and inflammation. A biofilm coating may form on the CGM, consisting of fibrin, proteinaceous deposits, and infiltrated granulocytes and monocytes. This biofilm hampers CGM response to changes in local glucose concentration, thereby further impairing continuous glucose monitoring in ISF. At a later stage after CGM implantation, in part stimulated by trophic factors released in the inflammatory response, angiogenesis in the damaged tissue restores local blood glucose supply but may even enhance glucose transport and thereby partially compensate negative effects caused by fibrous encapsulation.[118] The extent to which implantation disrupts the surrounding tissue is related to the shape, dimensions, and surface material of the probe as well as the exact implantation procedures.

5.3. Location of the CGM

In most applications, CGMs are implanted in subcutaneous tissue to measure glucose in the ISF (see Figure 5). The amount of glucose present in the ISF immediately surrounding the CGM depends on the concentration of glucose in the

blood, the rate of glucose diffusion from the capillary to the ISF, and the rate with which glucose is taken up by the adjacent cells. These parameters are affected by the rate of glucose metabolism at the site of implantation, the amount of insulin that is present, sensitivity of the tissue to insulin, the blood flow to the local area, and the permeability of the nearby capillaries.[27] Lutgers *et al*. showed that glucose recovery by microdialysis was negatively correlated with abdominal wall skin-fold thickness, probably due to heterogeneity in capillary density.[123] The vascular bed in the subcutaneous tissue is richly vascularized, but it contains less ISF than the skin layers above. Therefore, higher skin-fold thickness results in lower ISF glucose levels. The size of the adipocytes might affect the amount of available ISF, which would be a mechanism for the degree of adiposity to influence measurable glucose concentrations.[129] Adiposity is particularly relevant to type 2 diabetes. In addition, Korf *et al*. postulated that changes in metabolism in adipocytes surrounding the CGM also impact local glucose concentrations by modulation of local blood flow to the area.[130] During a period of increased physical activity, the CGM might register hyperglycemia even though blood glucose levels are normal.

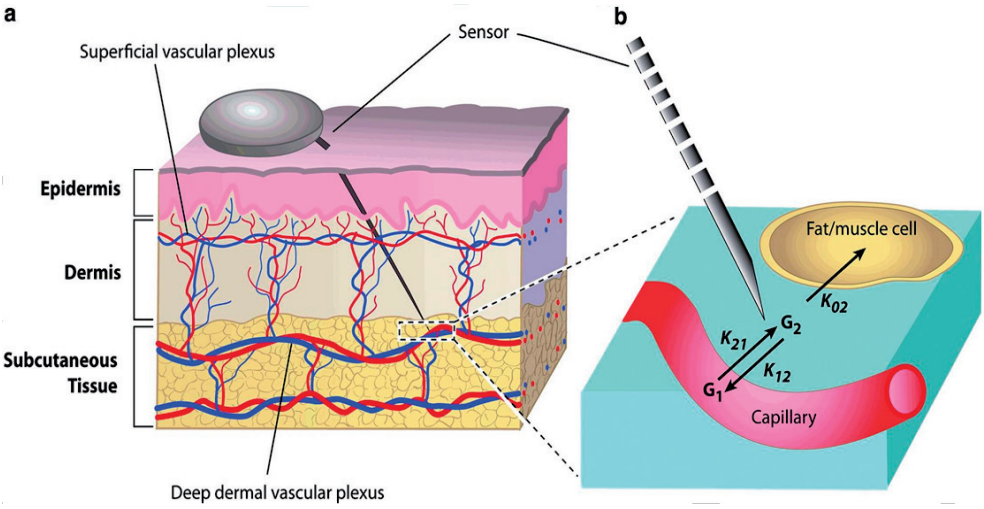

Figure 5. A close-up image of the composition of the skin and a magnified schematic view of the interstitial space: a) a sensor/microdialysis probe is inserted in the subcutaneous tissue. b) a representation of the diffusion model of blood-to-ISF; the glucose concentration in ISF is proportional to the concentration in each compartment; G_1 *and* G_2 *are capillary blood and interstitial glucose concentrations, respectively (reprinted with permission from Cengiz et al.[129]).*

Considering the factors that influence glucose concentrations in ISF, we propose here that the exact location of CGM implantation is an important factor in determining whether registered ISF glucose values will match those in the blood. Moreover, anatomical and physiological differences between species at the level of subcutaneous adipose tissue may complicate the interpretation of translational studies.[131, 132] Humans have considerably more subcutaneous fat than rats. The more fat cells, the more tortuous the path is for glucose in adipose tissue.[132] It is therefore recommended for testing purposes that CGMs be implanted in more fatty regions of the rat. Tiessen *et al*. reported that, during an oral glucose tolerance test in healthy humans, glucose values obtained by microdialysis from the loose connective tissue layer in the abdominal subcutis correlated much better with the blood than those obtained from subcutaneous adipose tissue.[133] This difference between tissues can be explained by the difference in interstitial structures, and by increased insulin-mediated glucose uptake in adipocytes. This is further supported by the increase in glucose gradient observed during systemic hyperglycemia. Papers reporting CGM performance in humans or animals are not always the same site and depth of location where CGM is implanted. Seemingly controversial findings in literature about the correlation between glucose levels in blood and ISF may very well be explained by differences in interstitial structure immediately surrounding the CGM between the various studies. Systematic investigation regarding the best site of implantation, i.e. a convenient site from which reliable glucose concentrations may be obtained, still need to be carried out.

6. Future perspectives

Implantable CGMs are instrumental in the treatment of diabetes by helping to prevent hypoglycemia, especially under conditions where self-monitoring with fingersticks is inadequate. Currently existing CGMs, however, are still hampered by a lack of accuracy, and have not yet been approved for long-term use. Future development of CGMs will be aimed at improving long-term stability and biocompatibility. It is important that the performance of CGMs is evaluated in a way that foreshadows their performance in patients. Proper test conditions should involve the use of animal models that are more analogous to humans than rats. Skin anatomy and physiology of subcutaneous ISF in pigs and dogs bear closer resemblance to humans. Humans have more adipose tissue than rats, and may not be affected to the same extent as r ats by physical and vascular factors, such as en capsulation, biofouling, and wound healing.[132]

It is also important that the accuracy of CGMs are evaluated in an appropriate way. A common approach to test *in vivo* performance is to administer a high concentration of glucose either into the peritoneal cavity or directly into the blood through a venous catheter.[134] This typically results in a CGM response that is delayed compared to rapid changes in blood glucose. CGM performance is much more accurate when glucose changes in blood are more gradual, for example following oral glucose administration.[135] Oral administration of glucose results in a more physiologically relevant response that better reflects the conditions under which CGMs are used in clinical applications.

It is our opinion that the timeframe of experimental evaluation of CGMs is an important, and yet often overlooked, factor that has major influence on the outcome. Experiments that test *in vivo* performance often last no more than a few days. Considering the large array of physiological factors that influence CGM performance after it has been implanted into the body, ranging from acute tissue damage, intermediate term wound healing and inflammation, and long-term processes such as encapsulation and angiogenesis, it should be clear that it takes considerable time before the tissue environment immediately surrounding the CGM has been stabilized. It has been suggested that a "break in" period of approximately 4-18 days should be observed before reliable glucose recordings can be obtained.[131] After that, the physiological properties of the implantation site remain stable for months. Prolonged monitoring is then only limited by deterioration of the CGM itself.

Reliable glucose monitoring with an implantable CGM for prolonged periods of time requires that the implanted device has as little interaction with the hostile environment in the body as possible. For this reason, we propose that microdialysis probes intrinsically are more resistant to biofouling than needle-type electrodes. Microdialysis probes offer the advantage that only the semi-permeable membrane forms the outer surface that comes into contact with the tissue, and the enzyme glucose oxidase and the electrode are not exposed to the hostile environment of the body. From a materials perspective, several strategies exist to use surface passivation and/or thin film modification in order to improve biocompatibility.[132, 136] Further development of these techniques can be combined with lab-on-chip technology to miniaturize all CGM components and to optimize response time and the use of reagents and solutions.

7. Summary

Continuous glucose monitoring offers potentially excellent guidance for insulin intervention therapies in the treatment of diabetes. The use of CGMs can provide accurate and continuous registration of blood glucose concentrations, both in retrospective and prospective applications. In the near future, a CGM could even be part of a closed-loop system in which the delivery of insulin is automatically adjusted based on the sensory data generated by the CGM (i.e. an artificial pancreas). In healthcare applications, the performance of CGMs currently on the market is still severely hampered by various technical challenges that to date have prevented their widespread deployment. There is particular need for higher accuracy, signal stability and reliability of longer period of days, because frequent calibrations and regular replacement of the implants are major burdens to the patient. Several physiological factors critically affect CGM performance. The most prominent factors include disturbance of the tissue in which the CGM is implanted, damage to the CGM by biofouling, and disruption of CGM function by biofilm formation and tissue encapsulation. In addition, to accurately and timely monitor changes in blood glucose levels with a device that is exposed to the ISF of abdominal or subcutaneous adipose tissue requires the CGM to be carefully positioned. The exact location, size and surface properties are very important. The glucose kinetics relationship between blood and ISF is delicate. Efforts to improve CGM performance should therefore chiefly aim at optimizing biocompatibility.

Placement of a CGM into subcutaneous tissue is probably the most practical position for general use in healthcare applications. Techniques designed for that site are much more accurate compared to transdermal techniques, and unlike intravascular methods, implantation can be carried out without the help of a physician. Moreover, in the ISF there are fewer issues with biofouling or hemostasis, and fewer risks for hemorrhages. However, placement into subcutaneous tissue warrants the need to carefully consider glucose kinetics between blood and the ISF. From reviewing the biomedical literature on this relationship, we have concluded that this is not straightforward but in fact rather complex. The present dogma is that of the push-pull theory, which states that a postprandial glucose increase in the blood is followed by a similar increase in the ISF after a short delay.[108] However, a decrease in circulating glucose (e.g. by insulin-induced clearance) would be preceded by a glucose decrease in the ISF.[116] Many studies have been reported that either support or refute the push-pull theory. Moreover, there is a great degree

of variation among studies in the reported lag times, ranging from nearly instantaneous equilibration to a delay of 45 min.[129]

From our meta-analysis, we conclude that the observed glucose kinetics between blood and ISF is largely dependent on the size and design of the CGM, CGM implantation in superficial subcutis versus loose connective tissue of deeper layers, and on t he species under investigation. Other predictive factors are degrees of adiposity and skin fold thickness, and the level of physical activity. It is therefore very difficult to make a definitive statement about the relevance of a p ush-pull phenomenon for CGM function in any particular patient. We recommend that evaluation of this phenomenon be included in the calibration procedures that are already being carried out, in particular after each placement of a new CGM implant. In the near future, further development of CGM technology is to result in higher accuracy and signal stability of these instruments. In addition to their enhanced applicability in diabetes intervention therapies per sé, technological improvements of CGMs will also enable us to examine blood-to-ISF kinetics more thoroughly, hence indirectly making the use of CGMs in patients even more reliable.

References

1. Eisenbarth, G.S., Update in type 1 diabetes. *J. Clin. Endocrinol. Metab.* **2007,** 92, (7), 2403-2407.
2. McGarry, J.D., Banting lecture 2001. *Diabetes* **2002,** 51, (1), 7-18.
3. DCCT Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **1993,** 329, (14), 977-986.
4. Cryer, P.E., Diverse causes of hypoglycemia-associated autonomic failure in diabetes. *N. Engl. J. Med.* **2004,** 350, (22), 2272-2279.
5. Karter, A.J.; Ackerson, L.M.; Darbinian, J.A.; D'Agostino, R.B.; Ferrara, A.; Liu, J.; Selby, J.V., Self-monitoring of blood glucose levels and glycemic control: the Northern California Kaiser Permanente Diabetes registry. *Am. J. Med.* **2001,** 111, (1), 1-9.
6. Wagner, J.; Malchoff, C.; Abbott, G., Invasiveness as a barrier to self-monitoring of blood glucose in diabetes. *Diabetes Technol. Ther.* **2005,** 7, (4), 612-619.
7. Klonoff, D.C., Continuous glucose monitoring: roadmap for 21st century diabetes therapy. *Diabetes Care* **2005,** 28, (5), 1231-1239.
8. Ludvigsson, J.; Hanas, R., Continuous subcutaneous glucose monitoring improved metabolic control in pediatric patients with type 1 diabetes: a controlled crossover study. *Pediatrics* **2003,** 111, (5), 933-938.

9. Jeha, G.S.; Karaviti, L.P.; Anderson, B.; Smith, E.O.; Donaldson, S.; McGirk, T.S.; Haymond, M.W., Continuous glucose monitoring and the reality of metabolic control in preschool children with type 1 diabetes. *Diabetes Care* **2004,** 27, (12), 2881-2886.

10. Girardin, C.M.; Huot, C.; Gonthier, M.; Delvin, E., Continuous glucose monitoring: a review of biochemical perspectives and clinical use in type 1 diabetes. *Clin. Biochem.* **2009,** 42, (3), 136-142.

11. Davis, S.; Alonso, M.D., Hypoglycemia as a barrier to glycemic control. *J. Diabetes Complicat.* **2004,** 18, (1), 60-68.

12. Cryer, P.E., Hypoglycemia in diabetes: pathophysiological mechanisms and diurnal variation. *Prog. Brain Res.* **2006,** 153, 361-365.

13. DCCT Group, Epidemiology of severe hypoglycemia in the diabetes control and complications trial. *Am. J. Med.* **1991,** 90, (4), 450-459.

14. Pickup, J., Sensitive glucose sensing in diabetes. *The Lancet* **2000,** 355, (9202), 426-427.

15. Wang, J., In vivo glucose monitoring: towards `Sense and Act' feedback-loop individualized medical systems. *Talanta* **2008,** 75, (3), 636-641.

16. Wentholt, I.M.E.; Hart, A.A.M.; Hoekstra, J.B.L.; DeVries, J.H., How to assess and compare the accuracy of continuous glucose monitors? *Diabetes Technol. Ther.* **2008,** 10, (2), 57-68.

17. Wentholt, I.M.E.; Hoekstra, J.B.L.; DeVries, J.H., Continuous glucose monitors: the long-awaited watch dogs? *Diabetes Technol. Ther.* **2007,** 9, (5), 399-409.

18. Metzger, M.; Leibowitz, G.; Wainstein, J.; Glaser, B.; Raz, I., Reproducibility of glucose measurements using the glucose sensor. *Diabetes Care* **2002,** 25, (7), 1185-1191.

19. Clark, L.C.; Lyons, C., Electrode systems for continuous monitoring in cardiovascular surgery. *Ann. N. Y. Acad. Sci.* **1962,** 102, (1), 29-45.

20. Deiss, D.; Bolinder, J.; Riveline, J.-P.; Battelino, T.; Bosi, E.; Tubiana-Rufi, N.; Kerr, D.; Phillip, M., Improved glycemic control in poorly controlled patients with type 1 diabetes using real-time continuous glucose monitoring. *Diabetes Care* **2006,** 29, (12), 2730-2732.

21. Kaufman, F.R.; Austin, J.; Neinstein, A.; Jeng, L.; Halvorson, M.; Devoe, D.J.; Pitukcheewanont, P., Nocturnal hypoglycemia detected with the continuous glucose monitoring system in pediatric patients with type 1 diabetes. *J. Pediatr.* **2002,** 141, (5), 625-630.

22. Tanenberg, R.; Bode, B.; Lane, W.; Levetan, C.; Mestman, J.; Harmel, A.P.; Tobian, J.; Gross, T.; Mastrototaro, J., Use of the continuous glucose monitoring system to guide therapy in patients with insulin-treated diabetes: a r andomized controlled trial. *Mayo Clin. Proc.* **2004,** 79, (12), 1521-1526.

23. Chico, A.; Vidal-Rios, P.; Subira , M.; Novials, A., The continuous glucose monitoring system is useful for detecting unrecognized hypoglycemias in patients with type 1 and

type 2 diabetes but is not better than frequent capillary glucose measurements for improving metabolic control. *Diabetes Care* **2003,** 26, (4), 1153-1157.

24. Maran, A.; Crepaldi, C.; Avogaro, A.; Catuogno, S.; Burlina, A.; Poscia, A.; Tiengo, A., Continuous glucose monitoring in conditions other than diabetes. *Diabetes Metab. Res. Rev.* **2004,** 20, (S2), S50-S55.

25. Steil, G.M.; Panteleon, A.E.; Rebrin, K., Closed-loop insulin delivery-the path to physiological glucose control. *Adv. Drug Delivery. Rev.* **2004,** 56, (2), 125-144.

26. Bruttomesso D.; Farret A.; Costa S.; Marescotti M.C.; Vettore M.; Avogaro A.; Tiengo A.; Dalla Man C.; Place J.; Facchinetti A.; Guerra S.; Magni L.; De Nicolao G.; Cobelli C.; Renard E.; Maran A., Closed-loop artificial pancreas using subcutaneous glucose sensing and insulin delivery and a model predictive control algorithm: Preliminary studies in Padova and Montpellier. *J. Diabetes Sci. Technol.* **2009,** 3, (5), 1014-1021.

27. Koschinsky, T.; Heinemann, L., Sensors for glucose monitoring: technical and clinical aspects. *Diabetes Metab. Res. Rev.* **2001,** 17, (2), 113-123.

28. Wentholt, I.M.E.; Hoekstra, J.B.L.; Zwart, A.; DeVries, J.H., Pendra goes Dutch: lessons for the CE mark in Europe. *Diabetologia* **2005,** 48, (6), 1055-1058.

29. McGowan, K.; Thomas, W.; Moran, A., Spurious reporting of nocturnal hypoglycemia by CGMS in patients with tightly controlled type 1 diabetes. *Diabetes Care* **2002,** 25, (9), 1499-1503.

30. Rebrin, K.; Steil, G.M.; van Antwerp, W.P.; Mastrototaro, J.J., Subcutaneous glucose predicts plasma glucose independent of insulin: implications for continuous monitoring. *Am. J. Physiol. Endocrinol. Metab.* **1999,** 277, (3), E561-571.

31. Castillo, J.; Gaspar, S.; Leth, S.; Niculescu, M.; Mortari, A.; Bontidean, I.; Soukharev, V.; Dorneanu, S.A.; Ryabov, A.D.; Csoregi, E., Biosensors for life quality: design, development and applications. *Sens. Actuators, B* **2004,** 102, (2), 179-194.

32. Weinstein, R.L.; Schwartz, S.L.; Brazg, R.L.; Bugler, J.R.; Peyser, T.A.; McGarraugh, G.V., Accuracy of the 5-day FreeStyle Navigator continuous glucose monitoring system. *Diabetes Care* **2007,** 30, (5), 1125-1130.

33. Wilson, D.M.; Beck, R.W.; Tamborlane, W.V.; Dontchev, M.J.; Kollman, C.; Chase, P.; Fox, L.A.; Ruedy, K.J.; Tsalikian, E.; Weinzimer, S.A., The accuracy of the FreeStyle Navigator continuous glucose monitoring system in children with type 1 diabetes. *Diabetes Care* **2007,** 30, (1), 59-64.

34. Wang, J., Glucose biosensors: 40 years of advances and challenges. *Electroanalysis* **2001,** 13, (12), 983-988.

35. Chaubey, A.; Malhotra, B.D., Mediated biosensors. *Biosens. Bioelectron.* **2002,** 17, (6-7), 441-456.

36. Thevenot, D.R.; Sternberg, R.; Coulet, P.R.; Laurent, J.; Gautheron, D.C., Enzyme collagen membrane for electrochemical determination of glucose. *Anal. Chem.* **1979,** 51, (1), 96-100.

37. Wentholt, I.M.; Vollebregt, M.A.; Hart, A.A.; Hoekstra, J.B.; DeVries, J.H., Comparison of a needle-type and a microdialysis continuous glucose monitor in type 1 diabetic patients. *Diabetes Care* **2005,** 28, (12), 2871-2876.

38. Ungerstedt, U.; Pycock, C., Functional correlates of dopamine neurotransmission *Bull. Schweiz. Akad. Med. Wiss.* **1974,** 30, (1-3), 44-55.

39. Chaurasia, C.S., In vivo microdialysis sampling: theory and applications. *Biomed. Chromatogr.* **1999,** 13, (5), 317-332.

40. Westerink, B.H.C.; Cremers, T.I.F.H., Handbook of microdialysis: methods, applications and perspectives. *Amsterdam, Elsevier* **2007**.

41. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.

42. Kapitza, C.; Lodwig, V.; Obermaier, K.; Wientjes, K.J.C.; Hoogenberg, K.; Jungheim, K.; Heinemann, L., Continuous glucose monitoring: reliable measurements for up to 4 days with the SCGM1 System. *Diabetes Technol. Ther.* **2003,** 5, (4), 609-614.

43. Maran, A.; Crepaldi, C.; Tiengo, A.; Grassi, G.; Vitali, E.; Pagano, G.; Bistoni, S.; Calabrese, G.; Santeusanio, F.; Leonetti, F.; Ribaudo, M.; Di Mario, U.; Annuzzi, G.; Genovese, S.; Riccardi, G.; Previti, M.; Cucinotta, D.; Giorgino, F.; Bellomo, A.; Giorgino, R.; Poscia, A.; Varalli, M., Continuous subcutaneous glucose monitoring in diabetic patients: a multicenter analysis. *Diabetes Care* **2002,** 25, (2), 347-352.

44. Kubiak, T.; Worle, B.; Kuhr, B.; Nied, I.; Lasner, G.; Hermanns, N.; Kulzer, B.; Haak, T., Microdialysis-based 48-hour continuous glucose monitoring with GlucoDay: clinical performance and patients' acceptance. *Diabetes Technol. Ther.* **2006,** 8, (5), 570-575.

45. Arora, A.; Simone, G.; Salieb-Beugelaar, G.B.; Kim, J.T.; Manz, A., Latest developments in micro total analysis systems. *Anal. Chem.* **2010,** 82, (12), 4830-4847.

46. Salieb-Beugelaar, G.B.; Simone, G.; Arora, A.; Philippi, A.; Manz, A., Latest developments in microfluidic cell biology and analysis systems. *Anal. Chem.* **2010,** 82, (12), 4848-4864.

47. Zhao, X.M.; Xia, Y.N.; Whitesides, G.M., Soft lithographic methods for nano-fabrication. *J. Mater. Chem.* **1997,** 7, (7), 1069-1074.

48. Xia, Y.N.; Whitesides, G.M., Soft lithography. *Annu. Rev. Mater. Sci.* **1998,** 28, 153-184.

49. Whitesides, G.M.; Ostuni, E.; Takayama, S.; Jiang, X.Y.; Ingber, D.E., Soft lithography in biology and biochemistry. *Annu. Rev. Biomed. Eng.* **2001,** 3, 335-373.

50. Schmidt, F.; Aalders, A.; Schoonen, A., Calibration of a wearable glucose sensor. *Int. J. Artif. Organs* **1992,** 15, (1), 55-61.

51. Schmidt, F.J.; Sluiter, W.J.; Schoonen, A.J.M., Glucose concentration in subcutaneous extracellular space. *Diabetes Care* **1993,** 16, (5), 695-700.

52. Laurell, T., A continuous glucose monitoring system based on microdialysis. *J. Med. Eng. Technol.* **1992,** 16, (5), 187-193.

53. Laurell, T., Microdialysis implemented in the design of a system for continuous glucose monitoring. *Sens. Actuators, B* **1993,** 13, (1-3), 323-326.

54. Meyerhoff, C.; Bischof, F.; Sternberg, F.; Zier, H.; Pfeiffer, E., On line continuous monitoring of subcutaneous tissue glucose in men by combining portable glucosensor with microdialysis. *Diabetologia* **1992,** 35, (11), 1087-1092.

55. Meyerhoff, C.; Bischof, F.; Mennel, F.J.; Sternberg, F.; Pfeiffer, E.F., Use of the microdialysis technique in the monitoring of subcutaneous tissue glucose concentration. *Int. J. Artif. Organs* **1993,** 16, (5), 268-275.

56. Keck, F.S.; Kerner, W.; Meyerhoff, C.; Zier, H.; Pfeiffer, E.F., Combination of microdialysis and glucosensor permits continuous (on line) s. c. glucose monitoring in a patient operated device: I In vitro evaluation. *Horm. Metab. Res.* **1991,** 23, (12), 617,618.

57. Keck, F.S.; Meyerhoff, C.; Kerner, W.; Siegmund, T.; Zier, H.; Pfeiffer, E.F., Combination of microdialysis and glucosensor permits continuous (on line) SC glucose monitoring in a patient operated device. II. Evaluation in animals. *Horm. Metab. Res.* **1992,** 24, (10), 492-493.

58. Moscone, D.; Mascini, M., Microdialysis and glucose biosensor for in vivo monitoring. *Ann. Biol. Clin (Paris)* **1992,** 50, (5), 323-327.

59. Moscone, D.; Pasini, M.; Mascini, M., Subcutaneous microdialysis probe coupled with glucose biosensor for in vivo continuous monitoring. *Talanta* **1992,** 39, (8), 1039-1044.

60. Dempsey, E.; Diamond, D.; Smyth, M.R.; Urban, G.; Jobst, G.; Moser, I.; Verpoorte, E.M.J.; Manz, A.; Widmer, H.M.; Rabenstein, K.; Freaney, R., Design and development of a miniaturised total chemical analysis system for on-line lactate and glucose monitoring in biological samples. *Anal. Chim. Acta* **1997,** 346, (3), 341-349.

61. Freaney, R.; McShane, A.; Keaveny, T.V.; McKenna, M.; Rabenstein, K.; Scheller, F.W.; Pfeiffer, D.; Urban, G.; Moser, I.; Jobst, G.; Manz, A.; Verpoorte, E.; Widmer, M.W.; Diamond, D.; Dempsey, E.; deViteri, F.J.S.; Smyth, M., Novel instrumentation for real-time monitoring using miniaturized flow systems with integrated biosensors. *Ann. Clin. Biochem.* **1997,** 34, 291-302.

62. Bohm, S.; Olthuis, W.; Bergveld, P., A micromachined double lumen microdialysis probe connector with incorporated sensor for on-line sampling. *Sens. Actuators, B* **2000,** 63, (3), 201-208.

63. Huynh, B.H.; Fogarty, B.A.; Martin, R.S.; Lunte, S.M., On-line coupling of microdialysis sampling with microchip-based capillary electrophoresis. *Anal. Chem.* **2004,** 76, (21), 6440-6447.

64. Li, M.W.; Huynh, B.H.; Hulvey, M.K.; Lunte, S.M.; Martin, R.S., Design and characterization of poly(dimethylsiloxane)-based valves for interfacing continuous-flow sampling to microchip electrophoresis. *Anal. Chem.* **2006,** 78, (4), 1042-1051.

65. Mecker, L.C.; Martin, R.S., Integration of microdialysis sampling and microchip electrophoresis with electrochemical detection. *Anal. Chem.* **2008,** 80, (23), 9257-9264.

66. Wang, M.; Roman, G.T.; Schultz, K.; Jennings, C.; Kennedy, R.T., Improved temporal resolution for in vivo microdialysis by using segmented flow. *Anal. Chem.* **2008,** 80, (14), 5607-5615.

67. Wang, M.; Roman, G.T.; Perry, M.L.; Kennedy, R.T., Microfluidic chip for high efficiency electrophoretic analysis of segmented flow from a microdialysis probe and in vivo chemical monitoring. *Anal. Chem.* **2009,** 81, (21), 9072-9078.

68. Huang, C.-J.; Chen, Y.-H.; Wang, C.-H.; Chou, T.-C.; Lee, G.-B., Integrated microfluidic systems for automatic glucose sensing and insulin injection. *Sens. Actuators, B* **2007,** 122, (2), 461-468.

69. Nandi, P.; Lunte, S.M., Recent trends in microdialysis sampling integrated with conventional and microanalytical systems for monitoring biological events: A review. *Anal. Chim. Acta* **2009,** 651, (1), 1-14.

70. Perry, M.; Li, Q.; Kennedy, R.T., Review of recent advances in analytical techniques for the determination of neurotransmitters. *Anal. Chim. Acta* **2009,** 653, (1), 1-22.

71. Guihen, E.; O'Connor, W.T., Capillary and microchip electrophoresis in microdialysis: recent applications. *Electrophoresis* **2010,** 31, (1), 55-64.

72. Gravesen, P.; Raaby Poulsen, K.; Dirac, H., Lab-on-a-chip technology for continuous glucose monitoring. *J. Diabetes. Sci. Technol.* **2007,** 1, (3), 372-374.

73. Hsieh, Y.-C.; Zahn, J.D., On-chip microdialysis system with flow-through glucose sensing capabilities. *J. Diabetes Sci. Technol.* **2007,** 1, (3), 375-383.

74. Hsieh, Y.-C.; Zahn, J.D., On-chip microdialysis system with flow-through sensing components. *Biosens. Bioelectron.* **2007,** 22, (11), 2422-2428.

75. Moon, B.-U.; Koster, S.; Wientjes, K.J.C.; Kwapiszewski, R.M.; Schoonen, A.J.M.; Westerink, B.H.C.; Verpoorte, E., An enzymatic microreactor based on chaotic micromixing for enhanced amperometric detection in a continuous glucose monitoring application. *Anal. Chem.* **2010,** 82, (16), 6756-6763.

76. Wilkins, E.; Atanasov, P., Glucose monitoring: state of the art and future possibilities. *Med. Eng. Phys.* **1996,** 18, (4), 273-288.

77. Garg, S.K.; Potts, R.O.; Ackerman, N.R.; Fermi, S.J.; Tamada, J.A.; Chase, H.P., Correlation of fingerstick blood glucose measurements with GlucoWatch biographer glucose results in young subjects with type 1 diabetes. *Diabetes Care* **1999,** 22, (10), 1708-1714.

78. The Diabetes Research in Children Network (DirecNet) Study Group, Accuracy of the GlucoWatch G2 Biographer and the continuous glucose monitoring system during hypoglycemia. *Diabetes Care* **2004,** 27, (3), 722-726.

79. Caduff, A.; Hirt, E.; Feldman, Y.; Ali, Z.; Heinemann, L., First human experiments with a novel non-invasive, non-optical continuous glucose monitoring system. *Biosens. Bioelectron.* **2003,** 19, (3), 209-217.

80. Sieg, A.; Guy, R.H.; Delgado-Charro, M.B., Noninvasive and minimally invasive methods for transdermal glucose monitoring. *Diabetes Technol. Ther.* **2005,** 7, (1), 174-197.

81. Mastrototaro, J.J., The MiniMed Continuous Glucose Monitoring System. *Diabetes Technol. Ther.* **2000,** 2, (supplement 1), 13-18.

82. Diabetes Research in Children Network (DIRECNET) Study Group, The accuracy of the CGMS in children with type 1 diabetes: results of the Diabetes Research in Children Network (DirecNet) accuracy study. *Diabetes Technol. Ther.* **2003,** 5, (5), 781-789.

83. Bode, B.; Gross, K.; Rikalo, N.; Schwartz, S.; Wahl, T.; Page, C.; Gross, T.; Mastrototaro, J., Alarms based on real-time sensor glucose values alert patients to hypo- and hyperglycemia: the Guardian Continuous Monitoring System. *Diabetes Technol. Ther.* **2004,** 6, (2), 105-113.

84. Mastrototaro, J.; Shin, J.; Marcus, A.; Sulur, G., The accuracy and efficacy of real-time continuous glucose monitoring sensor in patients with type 1 diabetes. *Diabetes Technol. Ther.* **2008,** 10, (5), 385-390.

85. Garg, S.; Zisser, H.; Schwartz, S.; Bailey, T.; Kaplan, R.; Ellis, S.; Jovanovic, L., Improvement in glycemic excursions with a transcutaneous, real-time continuous glucose sensor. *Diabetes Care* **2006,** 29, (1), 44-50.

86. Garg, S.; Jovanovic, L., Relationship of fasting and hourly blood glucose levels to HbA1c values. *Diabetes Care* **2006,** 29, (12), 2644-2649.

87. Oliver, N.S.; Toumazou, C.; Cass, A.E.G.; Johnston, D.G., Glucose sensors: a review of current and emerging technology. *Diabet. Med.* **2009,** 26, (3), 197-210.

88. Clarke, W.L.; Cox, D.; Gonderfrederick, L.A.; Carter, W.; Pohl, S.L., Evaluating clinical accuracy of systems for self-monitoring of blood glucose. *Diabetes Care* **1987,** 10, (5), 622-628.

89. Parkes, J.L.; Slatin, S.L.; Pardo, S.; Ginsberg, B.H., A new consensus error grid to evaluate the clinical significance of inaccuracies in the measurement of blood glucose. *Diabetes Care* **2000,** 23, (8), 1143-1148.

90. Kovatchev, B.P.; Gonder-Frederick, L.A.; Cox, D.J.; Clarke, W.L., Evaluating the accuracy of continuous glucose-monitoring sensors. *Diabetes Care* **2004,** 27, (8), 1922-1928.

91. Wentholt, I.M.; Hoekstra, J.B.; DeVries, J.H., A critical appraisal of the continuous glucose-error grid analysis. *Diabetes Care* **2006,** 29, (8), 1805-1811.

92. Sachedina, N.; Pickup, J.C., Performance assessment of the Medtronic-MiniMed Continuous Glucose Monitoring System and its use for measurement of glycaemic control in type 1 diabetic subjects. *Diabet. Med.* **2003,** 20, (12), 1012-1015.

93. Mazze, R.S.; Strock, E.; Borgman, S.; Wesley, D.; Stout, P.; Racchini, J., Evaluating the accuracy, reliability, and clinical applicability of continuous glucose monitoring (CGM): is CGM ready for real time? *Diabetes Technol. Ther.* **2009,** 11, (1), 11-18.

94. Rossetti, P.; Porcellati, F.; Fanelli, C.G.; Bolli, G.B., Evaluation of the accuracy of a microdialysis-based glucose sensor during insulin-induced hypoglycemia, its recovery, and post-hypoglycemic hyperglycemia in humans. *Diabetes Technol. Ther.* **2006,** 8, (3), 326-337.

95. Klonoff, D.C., The need for separate performance goals for glucose sensors in the hypoglycemic, normoglycemic, and hyperglycemic ranges. *Diabetes Care* **2004,** 27, (3), 834-836.

96. Wentholt, I.M.E.; Hart, A.A.M.; Hoekstra, J.B.L.; Devries, J.H., Relationship between interstitial and blood glucose in type 1 d iabetes patients: delay and the push-pull phenomenon revisited. *Diabetes Technol. Ther.* **2007,** 9, (2), 169-175.

97. Wientjes, K.J.C.; Grob, U.; Hattemer, A.; Hoogenberg, K.; Jungheim, K.; Kapitza, C.; Schoonen, A.J.M., Effects of microdialysis catheter insertion into the subcutaneous adipose tissue assessed by the SCGM1 system. *Diabetes Technol. Ther.* **2003,** 5, (4), 615-620.

98. Renard, E., Implantable continuous glucose sensors. *Curr. Diabetes Rev.* **2008,** 4, 169-174.

99. Updike, S.J.; Shults, M.C.; Rhodes, R.K.; Gilligan, B.J.; Luebow, J.O.; Von Heimburg, D., Enzymatic glucose sensors: improved long-term performance in vitro and in vivo. *ASAIO J.* **1994,** 40, (2), 157-163.

100. Wientjes, K.J.; Vonk, P.; Vonk-van Klei, Y.; Schoonen, A.J.; Kossen, N.W., Microdialysis of glucose in subcutaneous adipose tissue up to 3 weeks in healthy volunteers. *Diabetes Care* **1998,** 21, (9), 1481-1488.

101. Moberg, E.; Hagström-Toft, E.; Amer, P.; Bolinder, J., Protracted glucose fall in subcutaneous adipose tissue and skeletal muscle compared with blood during insulin-induced hypoglycaemia. *Diabetologia* **1997,** 40, (11), 1320-1326.

102. Boyne, M.S.; Silver, D.M.; Kaplan, J.; Saudek, C.D., Timing of changes in interstitial and venous blood glucose measured with a continuous subcutaneous glucose sensor. *Diabetes* **2003,** 52, (11), 2790-2794.

103. Roe, J.N.; Smoller, B.R., Bloodless glucose measurements. *Crit. Rev. Ther. Drug Carrier Syst.* **1998,** 15, (3), 199-241.

104. Summers, L.K.M.; Clark, M.L.; Humphreys, S.M.; Bugler, J.; Frayn, K.N., The use of microdialysis to monitor rapid changes in glucose concentration. *Horm. Metab. Res.* **1999,** 31, (07), 424,428.

105. Aussedat, B.; Dupire-Angel, M.; Gifford, R.; Klein, J.C.; Wilson, G.S.; Reach, G., Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am. J. Physiol. Endocrinol. Metab.* **2000,** 278, (4), E716-728.

106. Cheyne, E.H.; Cavan, D.A.; Kerr, D., Performance of a continuous glucose monitoring system during controlled hypoglycaemia in healthy volunteers. *Diabetes Technol. Ther.* **2002,** 4, (5), 607-613.

107. Muller, M.; Schmid, R.; Nieszpaur-Los, M.; Fassolt, A.; Lonnroth, P.; Fasching, P.; Eichler, H.G., Key metabolite kinetics in human skeletal muscle during ischaemia and reperfusion: measurement by microdialysis. *Eur. J. Clin. Investig.* **1995,** 25, (8), 601-607.

108. Sternberg, F.; Meyerhoff, C.; Mennel, F.J.; Mayer, H.; Bischof, F.; Pfeiffer, E.F., Does fall in tissue glucose precede fall in blood glucose? *Diabetologia* **1996,** 39, (5), 609-612.

109. Maggs, D.G.; Borg, W.P.; Sherwin, R.S., Microdialysis techniques in the study of brain and skeletal muscle. *Diabetologia* **1997,** 40, (0), S75-S82.

110. Kulcu, E.; Tamada, J.A.; Reach, G.; Potts, R.O.; Lesho, M.J., Physiological differences between interstitial glucose and blood glucose measured in human subjects. *Diabetes Care* **2003,** 26, (8), 2405-2409.

111. Caplin, N.J.; O'Leary, P.; Bulsara, M.; Davis, E.A.; Jones, T.W., Subcutaneous glucose sensor values closely parallel blood glucose during insulin-induced hypoglycaemia. *Diabet. Med.* **2003,** 20, (3), 238-241.

112. Wientjes, K.J.C.; Schoonen, A.J.M., Determination of time delay between blood and interstitial adipose tissue glucose concentration change by microdialysis in healthy volunteers. *Int. J. Artif. Organs* **2001,** 24, (12), 884-889.

113. Kaptein, W.A.; Kemper, R.H.A.; Ruiters, M.H.J.; Venema, K.; Tiessen, R.G.; Korf, J., Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats. *Biosens. Bioelectron.* **1997,** 12, (9-10), 967-976.

114. Schmidtke, D.W.; Freeland, A.C.; Heller, A.; Bonnecaze, R.T., Measurement and modeling of the transient difference between blood and subcutaneous glucose concentrations in the rat after injection of insulin. *Proc. Natl. Acad. Sci.* **1998,** 95, (1), 294-299.

115. Monsod, T.P.; Flanagan, D.E.; Rife, F.; Saenz, R.; Caprio, S.; Sherwin, R.S.; Tamborlane, W.V., Do sensor glucose levels accurately predict plasma glucose concentrations during hypoglycemia and hyperinsulinemia? *Diabetes Care* **2002,** 25, (5), 889-893.

116. Thome-Duret, V.; Reach, G.; Gangnerau, M.N.; Lemonnier, F.; Klein, J.C.; Zhang, Y.; Hu, Y.; Wilson, G.S., Use of a subcutaneous glucose sensor to detect decreases in glucose concentration prior to observation in blood. *Anal. Chem.* **1996,** 68, (21), 3822-3826.

117. Anderson, J.M.; Rodriguez, A.; Chang, D.T., Foreign body reaction to biomaterials. *Semin. Immunol.* **2008,** 20, (2), 86-100.

118. Dungel, P.; Long, N.; Yu, B.; Moussy, Y.; Moussy, F., Study of the effects of tissue reactions on the function of implanted glucose sensors. *J. Biomed. Mater. Res. Part A* **2008,** 85A, (3), 699-706.

119. Stenken, J.; Church, M.; Gill, C.; Clough, G., How minimally invasive is microdialysis sampling? A cautionary note for cytokine collection in human skin and other clinical studies. *The AAPS Journal* **2010,** 12, (1), 73-78.

120. Clough, G.F.; Boutsiouki, P.; Church, M.K.; Michel, C.C., Effects of blood flow on the in vivo recovery of a small diffusible molecule by microdialysis in human skin. *J. Pharmacol. Exp. Ther.* **2002,** 302, (2), 681-686.

121. Clapp-Lilly, K.L.; Roberts, R.C.; Duffy, L.K.; Irons, K.P.; Hu, Y.; Drew, K.L., An ultrastructural analysis of tissue surrounding a microdialysis probe. *J. Neurosci. Methods* **1999,** 90, (2), 129-142.

122. Lourido, J.; Ederoth, P.; Sundvall, N.; Ungerstedt, U.; Nordstrom, C.H., Correlation between blood glucose concentration and glucose concentration in subcutaneous adipose tissue evaluated with microdialysis during intensive care. *Scand. J. Clin. Lab. Investig.* **2002,** 62, (4), 285 - 292.

123. Lutgers, H.L.; Hullegie, L.M.; Hoogenberg, K.; Sluiter, W.J.; Dullaart, R.P.F.; Wientjes, K.J.; Schoonen, A.J.M., Microdialysis measurement of glucose in subcutaneous adipose tissue up to three weeks in Type 1 diabetic patients. *Neth. J. Med.* **2000,** 57, (1), 7-12.

124. Gerritsen, M.; Jansen, J.A.; Lutterman, J.A., Performance of subcutaneously implanted glucose sensors for continuous monitoring. *Neth. J. Med.* **1999,** 54, (4), 167-179.

125. Onuki, Y.; Bhardwaj, U.; Papadimitrakopoulos, F.; Burgess, D.J., A review of the biocompatibility of implantable devices: current challenges to overcome foreign body response. *J. Diabetes Sci. Technol.* **2008,** 2, (6), 1003-1015.

126. Ratner, B.D.; Hoffman, A.S.; Schoen, F.J.; Lemons, J.E., Biomaterials science: An introduction to materials in medicine *Elsevier Science* **2004**, 293-338.

127. Sharkawy, A.A.; Bruce, K.; George, A.T.; Reichert, W.M., Engineering the tissue which encapsulates subcutaneous implants. I. Diffusion properties. *J. Biomed. Mater. Res.* **1997,** 37, (3), 401-412.

128. Klueh, U.; Dorsky, D.I.; Kreutzer, D.L., Use of vascular endothelial cell growth factor gene transfer to enhance implantable sensor function in vivo. *J. Biomed. Mater. Res. Part A* **2003,** 67A, (4), 1072-1086.

129. Cengiz, E.; Tamborlane, W.V., A tale of two compartments: interstitial versus blood glucose monitoring. *Diabetes Technol. Ther.* **2009,** 11, (s1), S-11-S-16.

130. Korf, J.; Tiessen, R.G.; Venema, K.; Rhemrev, M.M., Biosensors for continuous glucose and lactate monitoring. *Ned. Tijdschr. Geneeskd.* **2003,** 147, (25), 1204-1208.

131. Koschwanez, H.E.; Reichert, W.M., In vitro, in vivo and post explantation testing of glucose-detecting biosensors: current methods and recommendations. *Biomaterials* **2007,** 28, (25), 3687-3703.

132. Wisniewski, N.; Rajamand, N.; Adamsson, U.; Lins, P.E.; Reichert, W.M.; Klitzman, B.; Ungerstedt, U., Analyte flux through chronically implanted subcutaneous polyamide membranes differs in humans and rats. *Am. J. Physiol. Endocrinol. Metab.* **2002,** 282, (6), E1316-E1323.

133. Tiessen, R.G.; Rhemrev-Boom, M.M.; Korf, J., Glucose gradient differences in subcutaneous tissue of healthy volunteers assessed with ultraslow microdialysis and a nanolitre glucose sensor. *Life Sci.* **2002,** 70, (21), 2457-2466.

134. Yu, B.; Long, N.; Moussy, Y.; Moussy, F., A long-term flexible minimally-invasive implantable glucose biosensor based on an epoxy-enhanced polyurethane membrane. *Biosens. Bioelectron.* **2006,** 21, (12), 2275-2282.

135. Pickup, J.C.; Claremont, D.J.; Shaw, G.W., Responses and calibration of amperometric glucose sensors implanted in the subcutaneous tissue of man. *Acta Diabetol.* **1993,** 30, (3), 143-148.

136. Ju, Y.M.; Yu, B.; Koob, T.J.; Moussy, Y.; Moussy, F., A novel porous collagen scaffold around an implantable biosensor for improving biocompatibility. I. In vitro/in vivo stability of the scaffold and in vitro sensitivity of the glucose sensor with scaffold. *J. Biomed. Mater. Res. Part A* **2008,** 87A, (1), 136-146.

Chapter 3

An Enzymatic Microreactor Based on Chaotic Micromixing for Enhanced Amperometric Detection in a Continuous Glucose Monitoring Application

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Abstract

The development of continuous glucose monitoring systems is a major trend in diabetes-related research. Small, easy-to-wear systems which are robust enough to function over many days without maintenance are the goal. We present a new sensing system for continuous glucose monitoring based on a microreactor incorporating chaotic mixing channels. Two different types of chaotic mixing channels with arrays of either slanted or herringbone grooves were fabricated in poly(dimethylsiloxane) (PDMS) and compared to channels containing no grooves. Mixing in channels with slanted grooves was characterized using a fluorescence method as a function of distance and at different flow rates, and compared to the mixing behavior observed in channels with no grooves. For electrochemical detection, a thin-film Pt electrode was positioned at the end of the fluidic channel as an on-chip detector of the reaction product, H_2O_2. Glucose determination was performed by rapidly mixing glucose and glucose oxidase (GOx) in solution at a flow rate of 0.5 μL/min and 1.5 μL/min, respectively. A 150 U/mL GOx solution was selected as the optimum concentration of enzyme. In order to investigate the dependence of device response on flow rate, experiments with a premixed solution of glucose and GOx were compared to experiments in which glucose and GOx were reacted on-chip. Calibration curves for glucose (0 ~ 20 mM, in the clinical range of interest) were obtained in channels with and without grooves, using amperometric detection and a 150 U/mL GOx solution for in-chip reaction.

1. Introduction

Diabetes mellitus is a widespread disease causing heart disease, weight loss, blurry vision, neurological disorders, and even death.[1] Proper management of blood glucose is thus of crucial importance for diabetic patients. The conventional way blood glucose determinations are carried out involves the finger-prick method. Usually, diabetic patients measure their own blood glucose several times per day by applying a drop of blood to a portable device. However, this intermittent monitoring does not yield the full range of information necessary to effectively control glucose levels 24 hours a day. The current research trend is thus towards real-time *in vivo* monitoring over longer periods (days).[2-5] Type 1 diabetic patients in particular could benefit from a portable glucose sensor to keep their glucose values within a reasonable range. Theoretically, a portable glucose sensor could include an insulin pump in a feedback loop to serve as an artificial pancreas.

Electrochemical detection is commonly used for glucose analysis.[6] The advantages of electrochemical detection include sensitivity and ease of interfacing with detection electronics. Several reports incorporating electrochemical detection on a microchip have been published for the detection of glucose.[7-9] The principle of the enzymatic reaction involved is shown in Equations (1) and (2). Glucose, O_2 and glucose oxidase (GOx) react to generate hydrogen peroxide (H_2O_2) at concentrations proportional to the original glucose concentrations. The H_2O_2 is then oxidized under applied potential into O_2 and H^+, with the resulting electrons being recorded as an electrical current.

$$\text{Glucose} + O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \quad \text{(GOx: glucose oxidase)} \qquad (1)$$

$$H_2O_2 \xrightarrow{0.7\ \text{V}} 2H^+ + O_2 + 2e^- \qquad (2)$$

The reaction may be carried out by mixing the reagents in solution to produce H_2O_2 which is then subsequently detected by integrated electrodes[7, 8], or by immobilizing GOx directly onto the detection electrode surface to react with glucose in solution.[9] The electrochemical detectors in these microchips displayed fast response times and low detection limits. Immobilizing GOx certainly reduces its consumption significantly compared to devices in which GOx is added in solution. However, the major disadvantage of this approach is that the enzyme layer is prone to biofouling in *in vivo* applications, which might cause a decrease in activity over time and poor performance as a result.[10, 11]

Over the last decade, a number of glucose sensor designs have been developed and become available for continuous monitoring purposes.[12-16] Mastrototaro *et al*.[14] reported an *in vivo* needle-type continuous glucose monitoring system (CGMS), which is currently being used by consumers as a kit (MiniMed). The detector in this system is based on the reaction of immobilized glucose oxidase on the electrode with glucose. Schoemaker *et al*.[15] and Wientjes *et al*.[17] have both reported a subcutaneous continuous glucose monitoring system (SCGM) based on a microdialysis probe and using the solution-based enzymatic reaction with glucose. They reported reliable measurements in diabetes patients over periods of four days[18] up to two weeks.[19, 20] However, due to their bulky size and the need for connecting tubes between system components, these systems exhibited relatively long physical lag times of 30 min or more. Pickup *et al*.[13] reviewed the clinical use of glucose monitoring systems and introduced new technology for non-invasive glucose sensing, based on near-infrared spectroscopy and fluorescence. Wentholt *et al*.[21] reported an overview of the current applications and clinically relevant aspects of continuous glucose monitors (CGMs), with emphasis on the calibration procedure, interpretation of continuous glucose data, and some important limitations. Overall, these authors concluded that improved accuracy, reliability for longer periods of time, miniaturization, and cost-effectiveness are the main issues which need to be considered in the further development of continuous glucose monitoring systems.[21]

Chip-based microfluidic technologies are a good alternative to improve on conventional monitoring approaches such as the SCGM described above. Microfluidic systems, also termed "miniaturized total analysis systems (μTAS)"[22] or "lab-on-a-chip", are now widely used in analytical chemistry and biological applications. Advantages of these systems include dramatically reduced consumption of chemical reagents, faster reaction times and cost-effectiveness. Micro SCGM systems can thus be envisaged which exploit the solution-based reaction of GOx with glucose in nL volumes in micrometer-sized channels for glucose sensing. The overall speed of the analysis will therefore depend in part on the efficiency of mixing reagents in the microfluidic channels.

Mixing at the micrometer scale is a challenge, as flows are generally extremely well-defined and laminar. Mixing of two solution streams in a straight microchannel is possible only through means of diffusion, a passive molecular transport process which is very slow. There has therefore been an enormous amount of research done in the past decade or so on how to implement efficient mixing at the nL scale. So-

called passive micromixers are generally preferred for many micro analytical flow systems, since these elements do not require the application of an external force to achieve mixing. A large number of passive micromixers have been reported, including a planar laminar flow mixer,[23] a cross-shaped micromixer[24] and a droplet mixer.[25] The approach chosen for our work is one based on chaotic mixing, first described by S troock *et al.*[26, 27]. Mixing is achieved through the incorporation of an array of microgrooves into a microchannel. Flow over the groove array assumes a helical or corkscrew pattern, in which the contact area between two adjacent solutions is increased dramatically to facilitate mixing by diffusion. Depending on device dimensions, complete mixing can be achieved in just a few seconds. Since reaction of glucose with GOx occurs upon mixing, integrated microelectrodes positioned at the end of the mixing channel will record a signal due to the oxidation of the H_2O_2 produced.

Ultimately, the overall analysis time is determined by both the mixing/reaction time in the microchannel and the time it takes for microdialysate containing glucose to be transported from the implanted probe to the sensing component. In fact, the physical lag time of glucose transport from the probe to the sensing element in conventional systems is generally on the order of tens of minutes. A seco nd objective of miniaturization of microdialysis-based CGMs is therefore to substantially reduce the physical lag times of these systems.

The long-term goal of the present project is to realize an autonomous, portable sensing system for continuous *in vivo* glucose monitoring, based on the reaction in solution of GOx with glucose to produce H_2O_2. To accomplish this, we have designed a miniaturized glucose sensing system based on microdialysis sampling and lab-on-a-chip technology. In this system, nL amounts of sample and enzyme rapidly mix and react. As high-recovery microdialysis requires flow rates in tissue less than 1 μL/min[17], we have adapted the microreactor dimensions according to these conditions. In this paper, we describe a new application for chaotic mixing, that is, the efficient and fast mixing of GOx and glucose for reaction. To that end, either slanted or herringbone grooves were fabricated in poly(dimethylsiloxane) (PDMS) chips, and the mixing characteristics of these channels co mpared to channels without grooves. A thin-film Pt electrode at the end of the fluidic channel served as electrochemical detector of the reaction product, H_2O_2.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were analytical reagent-grade. Fluorescein and potassium iodide (KI) were purchased from Sigma-Aldrich (Germany) and used to prepare 0.002M fluorescein solution and 0.2M KI solution, respectively. Potassium ferricyanide ($K_3Fe(CN)_6$) and potassium ferrocyanide ($K_4Fe(CN)_6$) were supplied by Sigma-Aldrich (Germany) and used to prepare a 1 mM solution in a 20 mM phosphate buffer (pH = 7.2). pH was measured using a pH meter (inoLab® pH 720, WTW, Germany). The phosphate buffer was prepared by mixing solutions of $NaH_2PO_4 \cdot H_2O$ (63 mL) and $Na_2HPO_4 \cdot 2H_2O$ (100 mL) containing 0.1 M potassium nitrate (KNO_3) as a supporting electrolyte. H_2O_2 (30%) was supplied by VWR (The Netherlands) and prepared freshly every day at various concentrations in phosphate buffer. D-glucose and GOx were supplied from Merck (Germany) and used to prepare 100 mM and 5000 U/mL stock solutions, respectively. D-glucose was stored in a refrigerator at 2 ºC and used for 1 month. GOx was stored at - 20 ºC and used for 3 months. All solutions were prepared with 18 MΩ ultrapure water purified in an Arium® 611 (Sartorius Stedim Biotech, Germany).

2.2. Microfluidic chip fabrication

The first microchannels were constructed by standard microfabrication and replicated in the silicone rubber, PDMS (Sylgard 184, Dow Corning, U.S.A.). The chip layout and design were drawn using the program, Clewin (Wieweb software, Hengelo, The Netherlands). The structure on the silicon master, which served as a mold, was processed with two steps of standard photolithography (Figure 1, right column).[28] A 4-inch p-type (100) silicon wafer (Si-Mat, Germany) (525µm thickness) was employed as a substrate. The silicon wafer was first cleaned sequentially with acetone, isopropyl alcohol, and deionized water, and dried with N_2 gas. The wafer was then treated with hexamethyldisilazane (HMDS) (Sigma-Aldrich, Germany) in a vacuum desiccator for 30 min to improve adhesion of the photoresist (PR). A thick positive PR, AZ4562 (Microchemicals GmbH, Germany), was coated on the silicon wafer using a spin-coater at 1000 rpm for 3 sec and baked at 100 ºC for 30 min. The resulting PR layer was 35 µm thick. After rehydration at ambient temperature for 3 hours, the coated wafer was exposed to ultraviolet (UV) light (365 nm, 10 m W/cm^2) using a photomask printed on a high-resolution transparency to pattern the microchannels (resolution 3,810 dpi; Pro-Art BV, Groningen, The Netherlands). The exposed PR was then removed by dipping in a

developer solution (AZ351B: deionized water (DI) = 1:3, Microchemicals GmbH, Germany) and agitating the beaker for 20 m in, leaving behind PR ridges for microchannel replication. The wafer was then rinsed in DI water and dried with N_2 gas. Contrary to usual protocol, no postbake step was undertaken for this layer. Rather, the substrate was again exposed to UV light for 10 sec using a second transparency photomask to pattern the groove arrays. The patterned PR was ag ain immersed in a developer (AZ351B: DI = 1:4) solution for 6 min to form groove structures on top of the microchannel structures.

PDMS resin and a curing agent were used for microchannel replication in a PDMS slab. The two liquids were mixed at a weight ratio of 10:1 PDMS: curing agent, and the solution left to stand at ambient conditions for 20 min to remove air bubbles. Afterward, the mixture was poured over the prepared silicon master. A polycarbonate alignment piece was used together with fused-silica capillaries to form access holes to the microchannels during the replication process. Holes (300

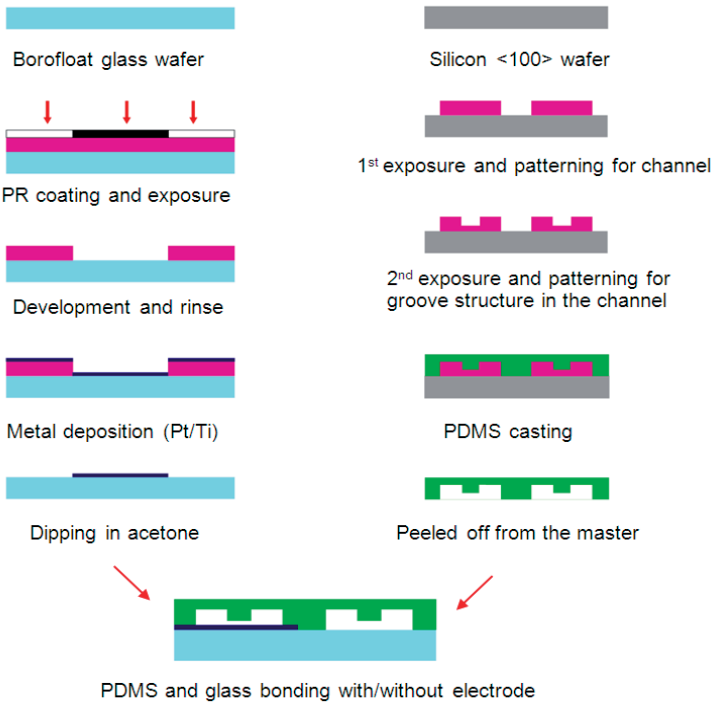

Figure 1. Fabrication process of microfluidic chip. See text for details.

μm in diameter) were drilled in the polycarbonate using a CNC machine (Sherline, USA) at points corresponding to the locations of the required inlets/outlets when this piece was aligned with the silicon master. Fused-silica capillaries were then inserted through the holes and aligned on the microchannel structures on the silicon master. The prepolymer mixture was allowed to cure against the master at 50 ºC for 4 hours.

2.3. Thin-film electrode formation

Platinum (Pt) sensing electrodes were formed on a glass wafer by a standard photolithography and lift-off process (Figure 1, left column). A 4-inch, 525-μm-thick borofloat glass wafer (Telic, USA) was used as a substrate for the electrode patterning. The glass wafer was first cleaned using a piranha solution (96% H_2SO_4: 30% H_2O_2 ratio of 3:1), then thoroughly rinsed in DI water and dried with N_2 gas. (WARNING: Piranha solution must be handled with extreme caution, as it is highly oxidizing and reacts explosively when it comes into contact with easily oxidized organic solvents. This solution must be used in a well-ventilated fumehood with absolutely no organic solvents in the vicinity. Explosive reactions also occur in the presence of trace amounts of metals such as Pt, Ag, and Mn. Metal tweezers must not be used when cleaning wafers in piranha.) The wafer was then treated with HMDS in a vacuum desiccator for 30 min. An image reversal PR 5214E (Microchemicals GmbH, Germany) was coated using a spin-coater at 2000 rpm for 20 sec, and the substrate was placed on a hotplate at 105 ºC for 50 sec to soft-bake the resist and remove solvents. Subsequently, the substrate was exposed to UV light (365 nm, 10 mW/cm^2) for 2 sec using a photomask with electrode pattern. After reversal baking of the substrate at 115 ºC for 2 min, a flood exposure was performed for 20 sec without mask to solubilize unexposed areas, resulting in a negative image of the mask. Finally, the substrate was immersed in a developer solution (AZ351B: DI water, ratio 1:4) to remove soluble PR and expose the glass surface where the electrodes were to be formed.

A lift-off process was performed to obtain Pt sensing electrodes on the patterned glass wafer. A Ti thin-film layer (20 nm) was deposited over the entire wafer by E-beam evaporation (Temescal, USA) under a high vacuum of 9×10^{-6} Torr. Ti was deposited first, as it adheres better to glass than Pt, and in turn, Pt adheres well to Ti. Subsequently, a Pt thin-film layer (150 nm) was grown by E-beam evaporation at 2×10^{-5} Torr. The glass substrate was then immersed in acetone to dissolve the remaining PR layer, thereby simultaneously lifting off the thin Pt/Ti film on top of it. The metal remained behind in the predefined electrode regions. Groups of three Pt

electrodes were patterned; the patterned electrode dimensions were 100 µm × 1500 µm and the distance between two electrodes was 30 µm.

2.4. Chip integration

The dimensions of the channel and grooves on the structured silicon master were determined using a stylus profilometer (Veeco Instrument BV, The Netherlands) before casting PDMS. There are two inlets and one outlet (waste) to the microreactor channel. The two inlet channels are half as wide as the microreactor channel after the Y-junction, to ensure a constant linear flow velocity throughout the device upon introduction of two solutions at the same flow rate. A 1:1 dilution of solutions will also result under these conditions of operation. The inlet channels are both 100 µm wide, 35 µm deep and 5 mm in length. A ruler is located along the channel to show the distance from the Y-junction. The total length of channel from the Y-junction is 22 mm, while the width and depth of the channel are 200 µm and 35 µm, respectively, and groove widths are 50 µm. The 122 grooves in the mixing

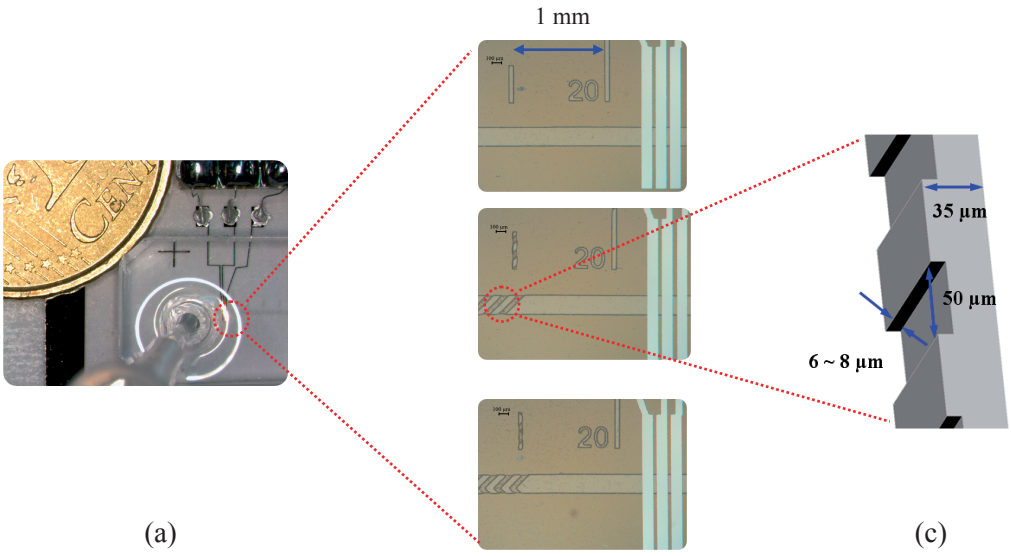

Figure 2. (a) Photograph of the detection region of a fabricated microfluidic chip. (b) Three different types of PDMS channels with integrated electrodes: from top to bottom, channels with no grooves, slanted grooves, and herringbone grooves. (c) Schematic diagram of grooves in a channel structure. Note that the grooves were 6 - 8 µm deep.

channel were 6~8 μm deep and were patterned at an angle of 45º with respect to the central axis of the channel. The total volume of the mixing/reaction channel is about 150 nL.

In order to bond the PDMS slab to the glass chip with the electrodes, the PDMS was smoothly peeled off from the master and cut. The two devices were first treated with UV-generated ozone for 15 min to oxidize the PDMS surface, which creates hydrophilic properties and improves bonding strength.[29] Subsequently, the PDMS slab and glass were immediately aligned under a microscope and brought into contact with each other. The assembled chip was then placed on a hot plate at 140 ºC and allowed to cool down to room temperature, after which the chip was irreversibly bonded. The whole bonding procedure was carried out in the cleanroom. Figure 2 shows a fabricated microfluidic chip and examples of the three different types of PDMS channels with integrated electrodes investigated in this study, containing either no grooves, slanted grooves, or herringbone grooves.

2.5. Experimental setup for fluorescence

In order to characterize mixing, fluorescence detection was used to gain quantitative information about the degree of mixing along the length of the channel. A fluorescence microscope, Model 'DMIL' (Leica Microsystems, The Netherlands), was equipped with a 10× objective, a mercury arc lamp, and a CCD camera. The mixing process was visualized using a fluorescein filter, which is made for 488 nm excitation and 518 nm emission. Fluorescence quenching was carried out for quantitative analysis using 2 mM fluorescein and 200 mM potassium iodide (KI). KI quenches the fluorescence produced by fluorescein when it is excited at 488 nm. Fluorescein and KI were introduced separately through the inlets into the Y-junction at various flow rates, and the image was captured with a CCD camera using a 20.1 ms exposure time, a gamma setting of 1.0, and a gain of 1.0. The images were acquired at different distances from the Y-junction and analyzed by determining the standard deviation (SD) of the intensity distribution across the entire channel using Lx95P image analysis software. Flow rates were controlled by syringe pumps (ProSense, The Netherlands) connected with silica capillaries to the chip inlets using 350-μm-o.d. and 250-μm-i.d. silica capillaries.

2.6. Experimental setup for electrochemical detection

Cyclic voltammetry and chronoamperometry were carried out using a potentiostat, "Electrochemical Analyzer" (CH Instrument, USA), interfaced to a computer. Two thin-film Pt electrodes were used, one as a working electrode (WE), the other as a

counter electrode (CE). The Pt electrodes were located 21 mm downstream from the Y-junction. The active area of the Pt WE was 0.02 mm^2 (200-µm-wide channel × 100-µm-wide electrode). A Ag/AgCl wire was used as a reference electrode (RE) and positioned in the reservoir at the end of the device. The RE was prepared by dipping a short piece of Ag wire (250-µm-diameter) for 5 sec into a crucible containing melted silver chloride (Sigma-Aldrich, Germany). For the glucose analysis experiments, a 1:3 flow rate ratio of glucose sample to GOx solution was introduced through the two inlets, and measurements were carried out under continuous flow conditions controlled by the syringe pumps. (For microdialysis experiments, the microdialysis probe outlet will be connected to the glucose sample inlet of the device using a simple, low-dead-volume tubing connection.) For the comparison of system response to premixed glucose-GOx solutions and direct reaction of glucose and GOx in the channel, premixed solution was prepared using a 1:3 volume ratio of 5.6 mM glucose and 150 U/mL GOx in a vial. After adding the two solutions, the vial was gently shaken 10 times and introduced in the reactor channel through both inlets approximately 5 min later after shaking. A two-position actuator switching valve (Valco Instrument Co., Inc) was adapted to alternatively introduce buffer solution and glucose sample solution to measure background and sensing signals, respectively. Once the flow rate had been set each time, the valve was switched from buffer solution to sample solution. The same solution was used

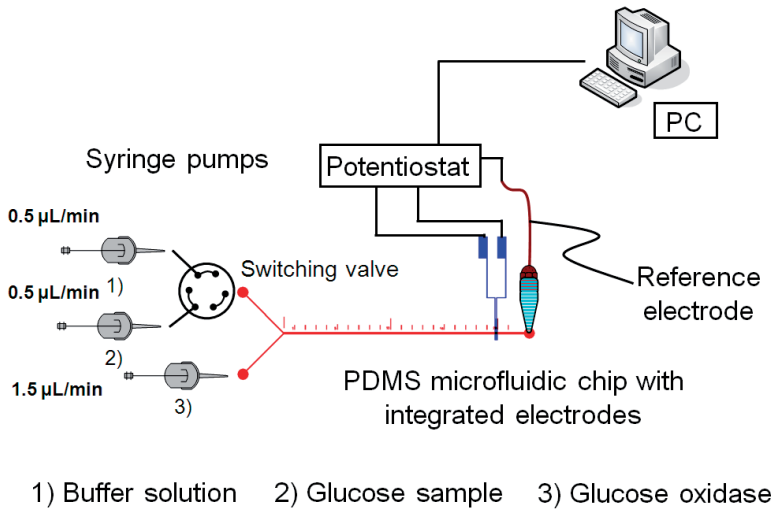

Figure 3. Electrochemical detection experimental setup.

for measurements at each flow rate, and current measurements were made continuously as flow rate was varied. All experiments were carried out at room temperature, and data were saved directly on a computer. A schematic diagram of the electrochemical detection experimental set-up is shown in Figure 3.

3. Results and discussion

3.1. Characterization of the micromixers

A fabricated micromixer was characterized by capturing images at different distances from the Y-junction using a fluorescence microscope and determining the variation in fluorescence intensity across the width of the channel. The standard deviation (SD) was calculated using the following Eq. (3)[30]:

$$SD = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \bar{x})^2} \tag{3}$$

where x_i is the gray-scale intensity value of pixel i, and $\bar{x}$ is the mean intensity value of pixels across the entire channel. Quantitative values of the SD of mixing efficiency varied between 0.5 for completely unmixed solutions (variation of intensity from 0 for KI solution to 100 % fluorescence for fluorescein) and 0 for completely mixed solutions (intensity equal across the channel).

Figure 4 shows the SD of intensity versus distance as a function of flow rate, comparing channels with slanted grooves and no grooves. Flow rates that fall in the range required to achieve high glucose recoveries in microdialysis sampling of subcutaneous tissue were considered. The results showed that grooved channels have a higher mixing efficiency compared to channels without grooves. In fact, mixing in channels with slanted grooves is almost complete at a distance of 1 cm along the channel at total flow rates ranging from 0.4 μL/min to 2 μL/min (Reynolds number = 0.08 at 1 μL/min). This result confirms that grooves formed in the channel ceiling at an oblique angle with respect to flow direction enhance the mixing rate of two adjacent solution streams. Differences in measured standard deviations of fluorescence intensity at shorter distances along the channel are most likely due to the reproducibility of the experiment itself. The depth and width of the grooves were designed based on Stroock's geometric parameters (relative groove height to channel height, $\alpha < 0.3$; channel height, h ≪ width of the channel, w) and have values of 6-8 μm and 50 μm, respectively, as shown in Figure 2 (c).[27] These initial results of mixing characterization were used as important information for further mixing and reaction experiments.

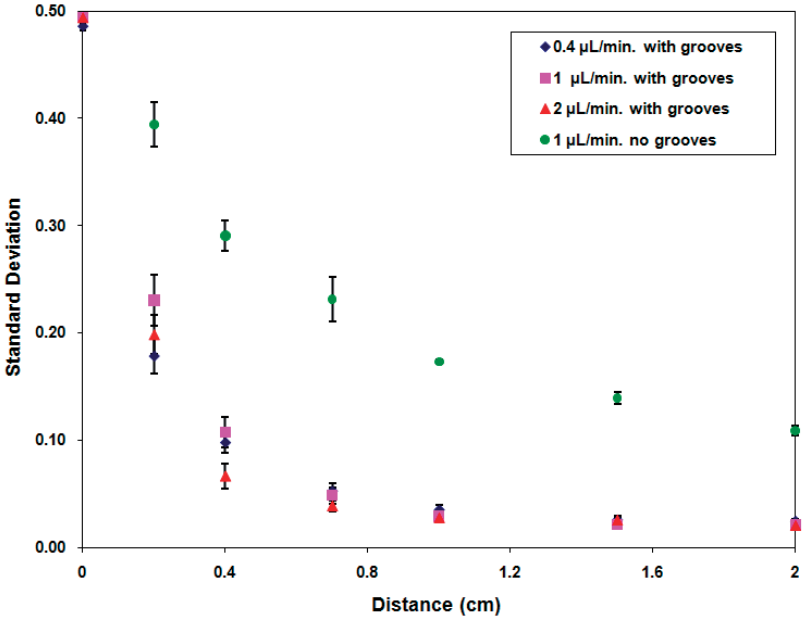

Figure 4. Comparison of microfluidic mixers having no grooves and slanted grooves as a function of distance from the Y-junction and at different flow rates (one device, n=3). The flow rate in each case is the total flow rate in the mixing channel. The flow rates of fluorescein and KI at the inlets are each half of the total flow rate.

3.2. Cyclic voltammograms of three different types of microfluidic channel

Cyclic voltammograms were carried out to check the electrochemical properties of the microfluidic chip. A ferri/ferrocyanide couple provides an ideal electrochemical model for a preliminary chip test with integrated electrodes. The redox couple ferri/ferrocyanide reaction is as follows (Eq. (4)):

$$Fe(CN)_6^{3-} + e^- \rightleftharpoons Fe(CN)_6^{4-} \qquad E^o = 0.356\ V \qquad (4)$$

Firstly, the redox curve was examined with a stationary flow when 1 mM ($K_3Fe(CN)_6$) was introduced in the channel at a scan range of -0.3 V to 0.7 V. The oxidation and reduction peaks were observed at 0.21 V (35 nA current) and 0.10 V (56 nA current) respectively, at a scan rate of 100 mV/s (data not shown). For cyclic voltammetry under continuous flow conditions, 1 mM $Fe(CN)_6^{3-}$ / 1 mM $Fe(CN)_6^{4-}$ was prepared in 20 mM phosphate-buffered solution (pH = 7.2) containing 0.1 M potassium nitrate as a supporting electrolyte. Experiments were performed at a flow

rate of 1 µL/min and a scan rate of 20 mV/s. The cyclic voltammograms recorded over an applied potential range of 0 V to 0.6 V versus Ag/AgCl are shown in Figure 5. In these early devices, the array of mixing grooves extended over the entire length of the channel, including the integrated electrodes. Higher oxidation and reduction currents were observed for channels with slanted or herringbone grooves than for unstructured channels. This was probably caused by higher local velocities over the electrode surfaces in the chaotic mixers, leading to thinner diffusion layers at these surfaces and thus improved analyte delivery and higher currents. Because of the observed, rather uncontrolled local flow effect on detector response, grooves were not structured over the electrodes in later devices used in the rest of this study. Instead, the groove patterns ended more than 1 mm upstream from the electrodes to reduce the flow effect on the electrode surface, as shown Figure 2(b). Comparing structured channels, the herringbone grooves did not yield a significant improvement in electrochemical signal with respect to the slanted grooves in the channel. It was

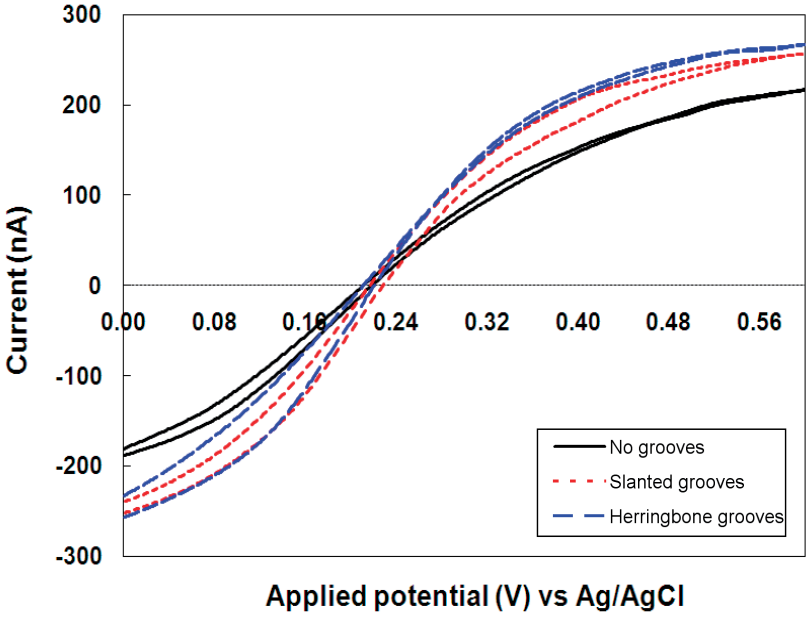

Figure 5. Comparison of cyclic voltammograms for the three different types of microfluidic channel. A 20 mM phosphate-buffered solution (pH = 7.2) containing 1 mM $Fe(CN)_6^{3-}$ /$Fe(CN)_6^{4-}$ in 0.1 M potassium nitrate was used at a flow rate of 1 µL/min and a scan rate of 20 mV/s.

therefore decided to focus further studies on comparing channels with no grooves to channels containing slanted grooves. This was because channels with slanted grooves were easier to fabricate than channels with herringbone grooves; aligning the photomask for slanted grooves in the second photolithographic step was simpler than aligning the mask for herringbone grooves.

A linear sweep voltammogram was recorded by introducing 6 mM H_2O_2 in 0.1 M KNO_3 solution, in order to decide on an applied potential for glucose measurement. The onset of oxidation current was observed at 0.3 V and showed a plateau around 0.7 V, at a total flow rate of 1 µL/min and a scan rate of 100 mV/s (data not shown). Therefore, this applied potential was selected for subsequent glucose experiments.

3.3. Optimization of GOx concentration

Prior to the optimization of GOx concentration, electrode variation from one chip to the next was characterized using a premixed solution of 20 mM glucose and GOx solution. One example of each mixer type (grooved and ungrooved) was selected, and signal variation was tested three times with each device. The results of these experiments showed that although current values varied from one device to the next, current values fell within the same range. Two devices, one with a grooved micromixer, the other an ungrooved micromixer, were selected for subsequent experiments to compare the effect of no grooves versus slanted grooves in the channel.

Since the concentration of GOx plays an important role in the reaction, various concentrations of GOx solutions were tested in the two channel types. To date, only a few papers have reported on-chip glucose sensing by mixing glucose sample and free enzyme in solution rather than using immobilized enzyme on electrode surfaces.[8, 31-33] Pijanowska *et al.*[32] developed a flow-through amperometric sensor implemented in a silicon-glass sandwich construction having a volume of 5 µL. The glucose sample was reacted in a reaction cell using only two different enzyme activities, 22 and 132 U/mL GOx, with a higher sensitivity being obtained with the latter concentration. Wang *et al.*[7] proposed a microchip capillary electrophoresis (MCE) approach for glucose sensing, in which GOx solution was injected together with glucose sample from separate reservoirs into the separation channel. A 75 U/mL GOx solution proved sufficient for these researchers to obtain a calibration curve.

Figure 6 shows the result of amperometric response arising from reaction of 20 mM glucose with GOx at different concentrations in channels with no grooves and

slanted grooves. The flow rates were set at 0.5 µL/min glucose and 1.5 µL/min GOx (1:3 mixing ratio) in order to avoid the oxygen depletion effect.[34] This is an effect which has been observed at higher concentrations of glucose for both electrochemical sensors utilizing immobilized GOx and devices based on the reaction of GOx and glucose in solution. The sensitivity of sensor response decreases because the amount of oxygen available in the solution is not sufficient for full conversion of glucose conversion to H_2O_2 with enzyme (see also Equation (1)). In effect, oxygen is depleted from the solution by the enzyme reaction. To prevent the effect of oxygen depletion in measurements employing immobilized enzymes on electrodes, electrodes have been prepared with a semi-permeable coating to reduce the amount of glucose diffusing to the electrode. Other approaches have involved the use of mediators, molecules which take on the role of oxygen in the reaction.[3, 35] In early experiments, we also observed that the signal was dramatically decreased

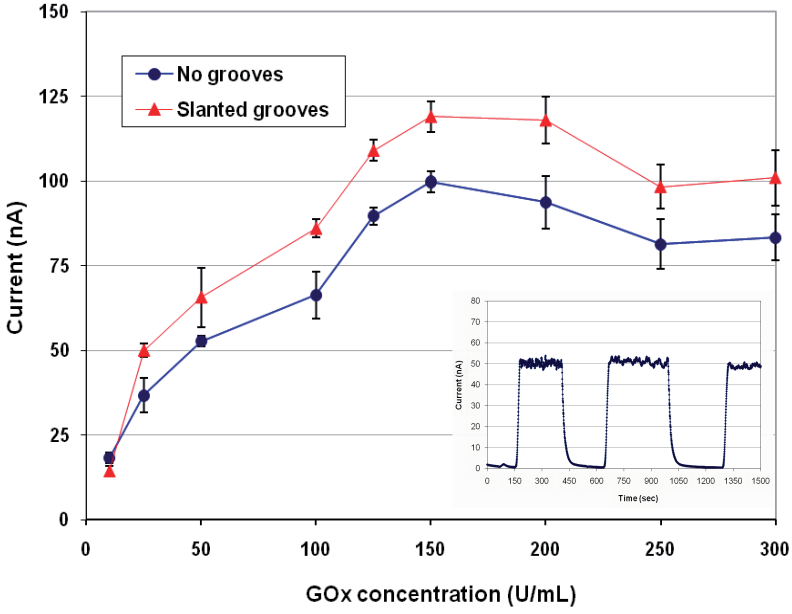

Figure 6. Amperometric response arising from reaction of 20 mM glucose with GOx at different concentrations in channels without grooves and with slanted grooves (one device, n=3). The flow rates of glucose and GOx were 0.5 µL/min and 1.5 µL/min, respectively; an example of raw data showing background signal and sensing signal is shown in the inset.

and calibration curves were not linear at higher concentrations of glucose when the mixing ratio between glucose and GOx was 1:1; instead, the signal tended to level off. It was decided to increase the flow rate of GOx with respect to glucose sample to a ratio of 3:1, diluting the effective glucose concentration in the channel by a factor of 4 to prevent oxygen depletion.

All experiments were performed three times and a repeatable output signal was observed (as shown in the inset of Figure 6). As the concentrations of GOx solution increased, oxidation current gradually increased from approximately 20 nA (10 U/mL) to reach a maximum of 100 nA and 120 nA (150 U/mL) in channels with no grooves and slanted grooves, respectively. At higher GOx concentrations, the current leveled off and then decreased in both channels with no grooves and slanted grooves. These results indicated that increasing concentrations of enzyme provided better conversion to H_2O_2 and product within a reaction time of approximately 4 sec. However, at concentrations of GOx greater than 150 U/mL, the system exhibited less stable signal, as reported elsewhere[8]; this could be due to the interaction of GOx and H_2O_2 produced. Therefore, a 150 U/mL concentration of GOx solution was chosen as an optimum condition for further experiments. Compared with slanted grooves in the channel, the absence of grooves in the channel resulted in lower current values. It can be concluded that the chaotic flow pattern results in more efficient mixing caused by transverse flow in the channel.

3.4. Comparison of system response for premixed glucose-GOx solutions and reaction of glucose and GOx in the channel

In order to investigate dependence on flow rate, the response measured for premixed glucose-GOx solutions was compared with that measured for the reaction of glucose and GOx in the mixing/reaction channel as a function of flow velocity. For the latter experiments, a 5.6 mM glucose sample and a 150 U/mL GOx solution were introduced at a 1:3 mixing ratio in a slanted grooves channel and the flow velocity was varied from 0.95 mm/s (0.4 μL/min) to 14.3 mm/s (6 μL/min). The amperometric responses for premixed solutions and reaction in the mixing channel are presented in Figure 7. There are two dominant factors in this continuous flow-through reaction system which affect detector response, namely flow velocity and reaction time. The effect of flow velocity was measured by introducing premixed solution (5.6 mM glucose sample and 150 U/mL GOx solution had already reacted to produce H_2O_2) in both inlets and varying the flow rate. The results showed that as the flow velocity increased, the recorded current values also increased gradually. It

was also observed that current values increased as a function of flow velocity when experimenting with 0.5 mM H_2O_2 solutions (data not shown). This is very different from the decreasing signal observed for GOx immobilized on electrodes at higher flow rates, which Lowry *et al.* ascribed to the removal of H_2O_2 from the electrode surface.[36] On the other hand, Wu *et al.*[37] observed currents which increased as a function of flow rate at two closely spaced oxygen sensors in a microchannel. This latter report supports our hypothesis that the observed increase in current values for premixed solutions is due to the thinner diffusion layers at the electrode surface which result at higher flow rates. These layers are stagnant, and molecules must diffuse through them to reach the electrode surface. As diffusion layers decrease in thickness, the time required for diffusion to the electrode surface also decreases, resulting in the delivery of analyte to that surface to be less limited by diffusion. Higher rates of electron exchange and thus higher recorded currents are the result.

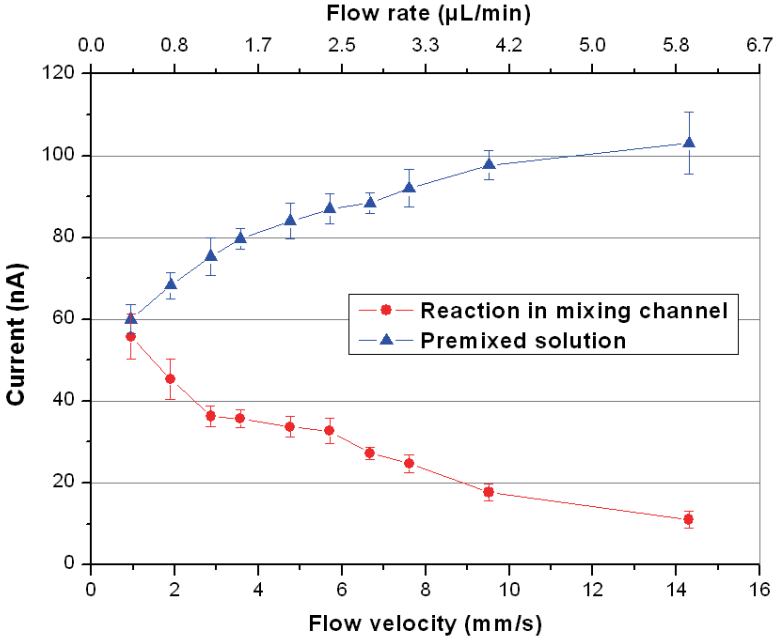

Figure 7. Amperometric response in a channel with slanted grooves, arising from a 1:3 reaction ratio of 5.6 mM glucose and 150 U/mL GOx as a function of flow velocity (from 0.4 µL/min to 6 µL/min). The response due to premixed solution (5.6 mM glucose and 150 U/mL GOx) was compared with the glucose-GOx reaction in the mixing channel (a single device was used for both curves, n=3 for each point).

In the case of glucose reaction with GOx in the mixing channel, a decreasing signal as a function of flow velocity was observed. This decrease in current is primarily due to lower H_2O_2 concentrations, the result of shorter reaction times. The maximum reaction of glucose and GOx was observed at very low flow rates (lower than 0.4 μL/min). Thus, based on reaction times of 22 sec and 4 sec (0.4 μL/min and 2 μL/min, respectively) from the Y-junction to the electrodes, the most efficient conversion of glucose with GOx can be achieved either at very low flow rates and/or in longer reaction channels.

3.5. Calibration curve

A calibration curve was obtained when a 150 U/mL GOx solution was reacted with glucose sample in a channel at a total flow rate of 2 μL/min, with a 1:3 flow rate (and thus 1:4 dilution) ratio of glucose and GOx. Figure 8 shows the amperometric response as a function of glucose concentration in channels with either no grooves or slanted grooves. The data exhibit a linear relationship between current and glucose concentrations of 0 to 20 mM, with a sensitivity of 5.3 nA/mM and 6.9 nA/mM in channels with no grooves and slanted grooves, respectively. The current density was calculated by dividing sensitivity by the active area of the working electrode, yielding 26.5 mA/M·cm^2, and 34.5 mA/M·cm^2 for channels with no grooves and slanted grooves, respectively. The linearity of the curves was tested by plotting observed current values versus predicted current values and examining the distribution of points around the resulting diagonal line (data not shown). In both cases, points were very symmetrically distributed around the line. The slope of the plot for the no-groove case was 0.9992, with an R^2 of 0.9983. In the groove case, the slope was 1.0032, with an R^2 of 0.9989. In both cases, excellent linearity was thus observed.

In our approach, the flow velocity and time for reaction of glucose sample and GOx enzyme strongly affect the sensitivity of the sensor signal, allowing flow conditions to be tuned to obtain optimal results. One of the big advantages of this microfluidic reactor approach is that by varying flow rates of glucose and GOx, it is possible not only to avoid the oxygen depletion effect without any electrode treatment but to tune the sensitivity for the application. However, continuous perfusion of glucose and GOx to the device does lead to a slightly decreased signal over time. This might be caused by GOx adsorbing either onto the microchannel or electrode surface as described elsewhere.[38] This effect will be further investigated as part of the development of systems for long-term measurement of glucose in *in vivo* applications.

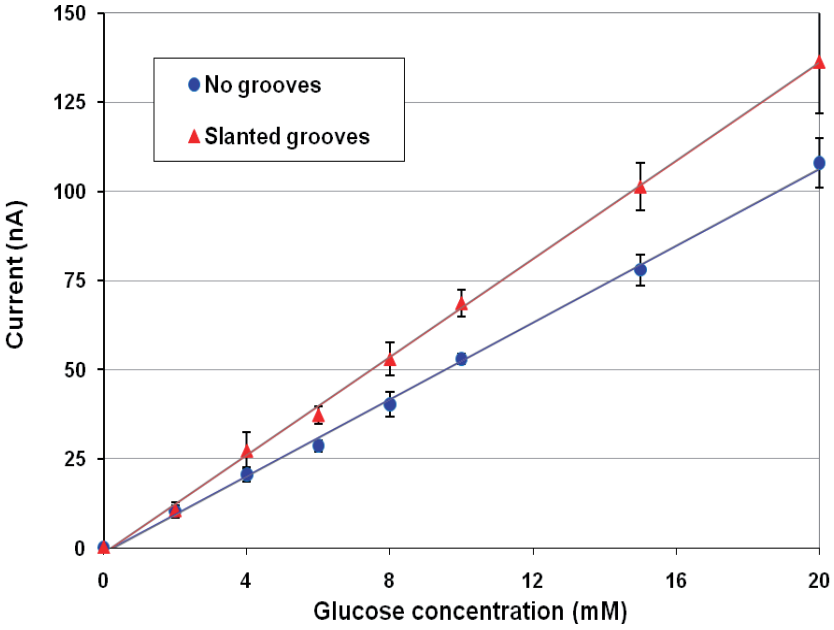

Figure 8. Calibration curves obtained using 150 U/mL GOx solution at a total flow rate of 2 µL/min (0.5 µL/min glucose: 1.5 µL/min GOx) in two channel types, without grooves and with slanted grooves (one device, n=3). Glucose concentrations were in the clinical range of interest.

4. Conclusion

We have successfully demonstrated an enzymatic glucose reactor based on chaotic mixing in a microfluidic channel network for continuous glucose monitoring. Together with another recent report describing improved lucerifase detection in a chaotic mixer[39], our example of glucose detection is one of the first examples of this type of mixer being applied to the enhancement of a biochemical reaction at the nL scale. A linear calibration curve was obtained in both microchannels with slanted grooves and no grooves, using a 150 U/mL GOx enzyme solution. Higher sensitivity was obtained with slanted groove arrays compared to micromixers with no grooves, due to enhanced mixing. The factors which determine sensitivity are flow velocity and extent of reaction. Though there is a loss of GOx due to the continuous flow to the outlet, this disadvantage is alleviated by the use of micofluidics for nL liquid handling and the application of low flow rates (2 µL/min for the optimized system).

The low flow rates used are also compatible with microdialysis sampling and are required to achieve a high recovery of glucose from the subcutaneous tissue and reduce solution consumption. The possible influence of interfering substances such as ascorbic acid, uric acid and acetaminophen on glucose determination are now under investigation, prior to testing the system *in vivo* in rats.

References

1. http://www.nlm.nih.gov/medlineplus/tutorials/diabetesintroduction/htm/index.htm.
2. Klonoff, D.C., Continuous glucose monitoring: roadmap for 21st century diabetes therapy. *Diabetes Care* **2005,** 28, (5), 1231-1239.
3. Feldman, B.; Brazg, R.; Schwartz, S.; Weinstein, R., A continuous glucose sensor based on wired enzyme technology - results from a 3-day trial in patients with Type 1 diabetes. *Diabetes Technol. Ther.* **2003,** 5, (5), 769-779.
4. Lutgers, H.L.; Hullegie, L.M.; Hoogenberg, K.; Sluiter, W.J.; Dullaart, R.P.F.; Wientjes, K.J.; Schoonen, A.J.M., Microdialysis measurement of glucose in subcutaneous adipose tissue up to three weeks in Type 1 diabetic patients. *Neth. J. Med.* **2000,** 57, (1), 7-12.
5. Tamada, J.A.; Garg, S.; Jovanovic, L.; Pitzer, K.R.; Fermi, S.; Potts, R.O., Noninvasive glucose monitoring: comprehensive clinical results. *J. Am. Med. Assoc.* **1999,** 282, (19), 1839-1844.
6. Updike, S.J.; Hicks, G.P., The enzyme electrode. *Nature* **1967,** 214, (5092), 986-988.
7. Wang, J.; Chatrathi, M.P.; Tian, B.; Polsky, R., Microfabricated electrophoresis chips for simultaneous bioassays of glucose, uric acid, ascorbic acid, and acetaminophen. *Anal. Chem.* **2000,** 72, (11), 2514-2518.
8. Wilke, R.; Büttgenbach, S., A micromachined capillary electrophoresis chip with fully integrated electrodes for separation and electrochemical detection. *Biosens. Bioelectron.* **2003,** 19, (3), 149-153.
9. Yamaguchi, A.; Jin, P.; Tsuchiyama, H.; Masuda, T.; Sun, K.; Matsuo, S.; Misawa, H., Rapid fabrication of electrochemical enzyme sensor chip using polydimethylsiloxane microfluidic channel. *Anal. Chim. Acta* **2002,** 468, (1), 143-152.
10. Gerritsen, M.; Jansen, J.A.; Lutterman, J.A., Performance of subcutaneously implanted glucose sensors for continuous monitoring. *Neth. J. Med.* **1999,** 54, (4), 167-179.
11. Wisniewski, N.; Moussy, F.; Reichert, W.M., Characterization of implantable biosensor membrane biofouling. *Fresenius' J. Anal. Chem.* **2000,** 366, (6), 611-621.
12. Maran, A.; Crepaldi, C.; Tiengo, A.; Grassi, G.; Vitali, E.; Pagano, G.; Bistoni, S.; Calabrese, G.; Santeusanio, F.; Leonetti, F.; Ribaudo, M.; Di Mario, U.; Annuzzi, G.; Genovese, S.; Riccardi, G.; Previti, M.; Cucinotta, D.; Giorgino, F.; Bellomo, A.; Giorgino, R.; Poscia, A.; Varalli, M., Continuous subcutaneous glucose monitoring in diabetic patients: a multicenter analysis. *Diabetes Care* **2002,** 25, (2), 347-352.

13. Pickup, J.C.; Hussain, F.; Evans, N.D.; Sachedina, N., In vivo glucose monitoring: the clinical reality and the promise. *Biosens. Bioelectron.* **2005,** 20, (10), 1897-1902.

14. Mastrototaro, J.J., The MiniMed Continuous Glucose Monitoring System. *Diabetes Technol. Ther.* **2000,** 2, (supplement 1), 13-18.

15. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.

16. Suzuki, H.; Tokuda, T.; Miyagishi, T.; Yoshida, H.; Honda, N., A disposable on-line microsystem for continuous sampling and monitoring of glucose. *Sens. Actuators, B* **2004,** 97, (1), 90-97.

17. Wientjes, K.J.C.; Grob, U.; Hattemer, A.; Hoogenberg, K.; Jungheim, K.; Kapitza, C.; Schoonen, A.J.M., Effects of microdialysis catheter insertion into the subcutaneous adipose tissue assessed by the SCGM1 system. *Diabetes Technol. Ther.* **2003,** 5, (4), 615-620.

18. Kapitza, C.; Lodwig, V.; Obermaier, K.; Wientjes, K.J.C.; Hoogenberg, K.; Jungheim, K.; Heinemann, L., Continuous glucose monitoring: reliable measurements for up to 4 days with the SCGM1 system. *Diabetes Technol. Ther.* **2003,** 5, (4), 609-614.

19. Wientjes, K.J.; Vonk, P.; Vonk-van Klei, Y.; Schoonen, A.J.; Kossen, N.W., Microdialysis of glucose in subcutaneous adipose tissue up to 3 weeks in healthy volunteers. *Diabetes Care* **1998,** 21, (9), 1481-1488.

20. Schoonen, A.J.M.; Schmidt, F.J.; Hasper, H.; Verbrugge, D.A.; Tiessen, R.G.; Lerk, C.F., Development of a potentially wearable glucose sensor for patients with diabetes mellitus: design and in-vitro evaluation. *Biosens. Bioelectron.* **1990,** 5, (1), 37-46.

21. Wentholt, I.M.E.; Hoekstra, J.B.L.; DeVries, J.H., Continuous glucose monitors: the long-awaited watch dogs? *Diabetes Technol. Ther.* **2007,** 9, (5), 399-409.

22. Reyes, D.R.; Iossifidis, D.; Auroux, P.-A.; Manz, A., Micro total analysis systems. 1. Introduction, theory, and technology. *Anal. Chem.* **2002,** 74, (12), 2623-2636.

23. Melin, J.; Gimenez, G.; Roxhed, N.; van der Wijngaart, W.; Stemme, G., A fast passive and planar liquid sample micromixer. *Lab Chip* **2004,** 4, (3), 214-219.

24. Wong, S.H.; Bryant, P.; Ward, M.; Wharton, C., Investigation of mixing in a cross-shaped micromixer with static mixing elements for reaction kinetics studies. *Sensors and Actuators B: Chemical* **2003,** 95, (1-3), 414-424.

25. Paik, P.; Pamula, V.K.; Fair, R.B., Rapid droplet mixers for digital microfluidic systems. *Lab Chip* **2003,** 3, (4), 253-259.

26. Stroock, A.D.; Dertinger, S.K.W.; Ajdari, A.; Mezic, I.; Stone, H.A.; Whitesides, G.M., Chaotic mixer for microchannels. *Science* **2002,** 295, (5555), 647-651.

27. Stroock, A.D.; Dertinger, S.K.; Whitesides, G.M.; Ajdari, A., Patterning flows using grooved surfaces. *Anal. Chem.* **2002,** 74, (20), 5306-5312.

28. Kim, J.; Heo, J.; Crooks, R.M., Hybridization of DNA to bead-immobilized probes confined within a microfluidic channel. *Langmuir* **2006,** 22, (24), 10130-10134.

29. Berdichevsky, Y.; Khandurina, J.; Guttman, A.; Lo, Y.H., UV/ozone modification of poly(dimethylsiloxane) microfluidic channels. *Sens. Actuators, B* **2004,** 97, (2-3), 402-408.

30. Xia, H.M.; Wan, S.Y.M.; Shu, C.; Chew, Y.T., Chaotic micromixers using two-layer crossing channels to exhibit fast mixing at low Reynolds numbers. *Lab Chip* **2005,** 5, (7), 748-755.

31. Wang, J.; Chatrathi, M.P.; Collins, G.E., Simultaneous microchip enzymatic measurements of blood lactate and glucose. *Anal. Chim. Acta* **2007,** 585, (1), 11-16.

32. Pijanowska, D.G.; Sprenkels, A.J.; Olthuis, W.; Bergveld, P., A flow-through amperometric sensor for micro-analytical systems. *Sensors and Actuators B: Chemical* **2003,** 91, (1-3), 98-102.

33. Böhm, S.; Pijanowska, D.; Olthuis, W.; Bergveld, P., A flow-through amperometric sensor based on dialysis tubing and free enzyme reactors. *Biosens. Bioelectron.* **2001,** 16, (6), 391-397.

34. Wientjes, K.J.C., Development of a glucose sensor for diabetic patients. *Ph.D. Dissertation, University of Groningen, Groningen, The Netherlands* **2000**.

35. Dixon, B.M.; Lowry, J.P.; O'Neill, R.D., Characterization in vitro and in vivo of the oxygen dependence of an enzyme/polymer biosensor for monitoring brain glucose. *J. Neurosci. Methods* **2002,** 119, (2), 135-142.

36. Lowry, J.P.; McAteer, K.; El Atrash, S.S.; Duff, A.; O'Neill, R.D., Characterization of glucose oxidase-modified poly(phenylenediamine)-coated electrodes in vitro and in vivo: homogeneous interference by ascorbic acid in hydrogen peroxide detection. *Anal. Chem.* **1994,** 66, (10), 1754-1761.

37. Wu, J.; Ye, J., Micro flow sensor based on two closely spaced amperometric sensors. *Lab Chip* **2005,** 5, (12), 1344-1347.

38. Ghosal, S., Effect of analyte adsorption on the electroosmotic flow in microfluidic channels. *Anal. Chem.* **2002,** 74, (4), 771-775.

39. Mei, Q.; Xia, Z.; Xu, F.; Soper, S.A.; Fan, Z.H., Fabrication of microfluidic reactors and mixing studies for luciferase detection. *Anal. Chem.* **2008,** 80, (15), 6045-6050.

Chapter 4

Development and Characterization of a Microfluidic Glucose Sensing System Based on an Enzymatic Microreactor and Chemiluminescence Detection

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Manuscript in preparation

Abstract

Portable systems for continuous monitoring of glucose in biomedical applications still tend to be relatively large. The use of amperometric detection has helped to reduce the volume and weight of these systems, as this mode of detection requires only small electrodes and little peripheral equipment. However, (micro)electrodes that are in direct contact with the sample may have shortened lifespans, due to electrode fouling which in turn leads to signal drift and decreased reproducibility as a result. To address these disadvantages, we have designed a new microfluidic system for glucose sensing based on chemiluminescence (CL) detection, as a route to integrated, portable systems. A two-reactor system was developed for enzymatic conversion of glucose by glucose oxidase (GOx) to H_2O_2 in the first reactor, with further reaction of H_2O_2 with luminol in the second reactor to produce light at 425 nm. A CL microfluidic chip was fabricated in poly(dimethylsiloxane) (PDMS) and bonded to a glass slide. A silicon photodiode was then cast into a second PDMS layer, which was reversibly bonded to the glass surface of the CL chip so that the diode was positioned directly over the second reactor. A chaotic mixing channel with slanted groves in its ceiling was incorporated into the first microreactor channel to enhance mixing efficiency. The performance of the fabricated CL microfluidic chip was characterized to establish optimal reaction conditions with respect to flow rate, pH, and concentrations of L/I and catalyst, HRP. To obtain a useful calibration curve for glucose sensing, the two inlet flow rates of glucose and GOx solutions were determined to be 0.5 μL/min and 1.5 μL/min, respectively. A solution consisting of 4 mM luminol/ 4 mM iodophenol in a carbonate buffer solution (pH 10.1) and containing 7 U/mL HRP was introduced at the third inlet at a flow rate of 0.5 μL/min, to react with H_2O_2 produced in the first reactor. The calibration curve obtained showed that CL light intensity was proportional to glucose concentration. The system exhibited a linear curve for current response as a function of glucose concentration in the clinically relevant range between 2 and 10 mM, with a sensitivity of 39 pA/mM ($R = 0.9963$, one device, $n=3$) and a limit of detection of 230 μM ($S/N = 3$).

1. Introduction

The main goal of glucose monitoring is to provide better information to diabetes patients and support them in the proper management of their condition. The development of continuous glucose monitoring approaches is one of the main directions in the field of glucose sensing for diabetic patients. Portability, long-term stability, and improved accuracy are the main issues in the further development of continuous glucose monitors (CGM). Over the last decade, an enormous and growing amount of research in the field of microchip-based microfluidic systems, also known as Lab-on-a-Chip, has been carried out.[1, 2] These systems make substantial reductions in the amounts of sample and chemicals used, analysis times, and reaction times possible, by applying micro- or nanoscale fluidic channels for ultra-small-volume liquid handling. Due to the fact that small amounts of solutions and chemical reagents are introduced and manipulated in microchannel networks, miniaturized, portable sensing systems can become a reality.

PDMS microfluidic systems are compatible with the use of optical components as on-chip detectors.[3, 4] PDMS is optically transparent down to wavelengths around 230 nm[5], so it can be used over a wide range of the visible spectrum in conjunction with optical detection. The major challenge is the integration of the optical detector in the PDMS microfluidic device to achieve a device with optical detection functionality. Recently, Kuswandi *et al*. reviewed optical sensing systems for microfluidic devices.[6] Various detection methods are described in which optical sensing systems are coupled and/or integrated into microfluidic devices. The detection principle varies from UV absorbance[7], to fluorescence[8], laser-induced fluorescence (LIF)[9], and chemiluminescence (CL) [10]. Detectors such as charge-coupled devices (CCD) or photomultiplier tubes (PMT) can be located either under the bottom of the fluidic chip or implanted into the device to achieve successful integration and high sensitivity.[11] Alternatively, optical fibers can be aligned in specially formed grooves or otherwise in a device to transport light to the chip from an external source, and on from the chip to an external detector. Compared to electrochemical detection, where electrodes in contact with complex samples can be subject to biofouling, optical detection methods are less prone to signal drift, because the reagents are generally not in direct contact with the detector.

CL detection has been extensively studied with respect to its incorporation in microfluidic chips.[12-18] The merit of CL detection is that it does not require an excitation source, as does fluorescence, or a monochromator for sensing the photons produced. The relatively high sensitivity and convenient instrumentation of CL have

long been popular for H_2O_2 analysis in general, with CL applications for microfluidics emerging more recently.[12] For determination of H_2O_2 concentration, the CL reaction between luminol and H_2O_2 is commonly catalyzed by Cu(II), Co(II), or horseradish peroxidase (HRP) to produce the emission of light. The main reaction principle of CL is as follows (using the example of glucose determination):

$$\text{Glucose} + 2O_2 \xrightarrow{\text{GOx}} \text{Gluconic acid} + H_2O_2 \quad (\text{GOx: glucose oxidase}) \qquad (1)$$

$$\text{Luminol} + 2H_2O_2 \xrightarrow{\text{HRP}} \text{Aminophthalate} + 4H_2O + N_2 + h\upsilon \qquad (2)$$

($h\upsilon$ is light at 425 nm wavelength)

Most CL detection systems have used a PMT located over the fluidic channel to allow very sensitive detection of the emitted light.[13-15, 18] However, PMTs need a high voltage power and an external mounting support (block). In contrast, photodiodes can be easily built into microfluidic devices and compactly integrated to provide portable sensing systems. Previously reported CL detection systems have been reported in which photodiodes have been employed as detectors. Nakamura *et al*.[19] developed a flow injection system (FIA) with CL detection based on a commercially available silicon photodiode in order to measure mM lactate concentrations in serum. Jorgensen *et al*.[16] fabricated a planar silicon photodiode detector on the back-side of a bulk micromachined microfluidic device. This microfluidic channel network consisted of an enzyme chamber, mixer and detection region. The reaction of hydrogen peroxide and luminol was performed in the microreactor and the resulting chemiluminescent light detected at the monolithically integrated photodiode at a detection limit of 5 μM H_2O_2. However, this in-house silicon photodiode required a series of complicated fabrication steps. Hofmann *et al*.[17] integrated a copper phthalocyanine–fullerene organic photodiode in a PDMS microfluidic device and demonstrated a preliminary limit of detection of 1 mM H_2O_2. Later on, this group further developed the organic photodiode with an active layer of the conjugated polymer poly(3-hexylthiophene) and [6,6]-phenyl-C61-butyric acid-methylester, a soluble derivative of C_{60}. The LOD of this device was 10 μM H_2O_2 detection.[20]

The long-term goal of this project is to realize a fully integrated system of fluidic channels, mixers, pumps and a detector for continuous glucose monitoring *in vivo*. The main idea of this approach is to retain the advantages of the Subcutaneous

Continuous Glucose Monitoring (SCGM) system[21], which is based on microdialysis sampling and has been studied for clinical use. Glucose sensing in this system exploits the reaction of glucose in the microdialysate with GOx in a second solution, with the electrochemical detection of the resulting hydrogen peroxide. This solution-based glucose monitoring principle is well suited for transfer into a microfluidic system as basis of a totally portable system for diabetes-related research. In a first approach, we demonstrated an enzymatic microreactor based on chaotic micromixing for the fast reaction of glucose with GOx, followed by the amperometric detection of hydrogen peroxide using microfabricated electrodes.[22] However, electrode-based sensing technology still has a major challenge to overcome with respect to electrode biofouling and signal drift, particular in *in vivo* applications.

As an alternative approach for this project, we have realized a new microfluidic sensing system based on CL detection. Commercially available silicon photodiodes have been used to detect the CL signal, as they are small and easily integrated into microfluidic devices. In this paper, we have designed a two-reactor system, the first for the enzymatic conversion of glucose by GOx, the second for the reaction of H_2O_2 with luminol for CL detection. A CL microfluidic chip was fabricated by PDMS/glass bonding, with a silicon photodiode fixed in a second PDMS layer directly positioned underneath the microfluidic channel. Chaotic mixing channels were incorporated in the microreactor to enhance mixing efficiency and accelerate the reactions. The performance of this system was evaluated in a series of *in vitro* tests to establish optimal reaction conditions, including flow rate, pH, and concentrations of the various reactants and catalysts.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were analytical reagent-grade. Luminol and 4-iodophenol (L/I) were purchased from Sigma-Aldrich (Germany) and used to prepare solutions of various concentrations. The L/I solutions were left for 24 hours to yield better activity and hence enhanced chemiluminescence. The solutions could be used for one month when stored at 2 ºC in a refrigerator.[23] H_2O_2 (30%) was supplied by VWR (The Netherlands) and used to prepare sample solutions in a phosphate buffer (PB) solution (pH = 7.2). The 20 mM PB solution was prepared by mixing solutions of $NaH_2PO_4 \cdot H_2O$ (63 mL) and $Na_2HPO_4 \cdot 2H_2O$ (100 mL). D-glucose was obtained from Merck (Germany) and used to prepare various glucose solutions in a PB buffer.

0.2 M carbonate buffer solution was used to prepare buffer solutions of varying pH, adjusted using 4 M NaOH. GOx and horseradish peroxidase (HRP) were supplied by Sigma-Aldrich (Germany) and used to prepare 5000 U/mL and 2500 U/mL stock solutions in ultrapure water, respectively. These solutions were stored at -20 ºC. All solutions were prepared with 18 MΩ ultrapure water purified in an Arium® 611system (Sartorius Stedim Biotech, Germany).

2.2 Fabrication of microfluidic chip

The microchannels and reactors were replicated in the elastomer, poly(dimethylsiloxane) (PDMS) (Sylgard 184, Dow Corning, Germany) using masters formed in a positive photoresist layer by standard microfabrication on a silicon wafer. The chip layout and design were drawn using the mask-design program Clewin (Wieweb software, Hengelo, The Netherlands). The structure on the silicon master was processed with two steps of standard photolithography.[22, 24] A 4-inch p-type (100) silicon wafer (Si-Mat, Germany) was employed as a substrate. The silicon wafer was first cleaned sequentially with acetone, isopropyl, and deionized water, and dried with N_2 gas. The wafer was then treated with hexamethyldisilazane (HMDS) (Sigma-Aldrich, Germany) in a vacuum desiccator for 30 min to improve photoresist adhesion to the Si wafer. A thick layer of positive photoresist (PR) AZ 4562 (Microchemicals GmbH, Germany) was then coated onto the Si wafer using a spincoater and re-hydrated at ambient temperature for 3 hours, leaving a 35-µm-thick layer. The coated wafer was exposed to ultraviolet (UV) light (365 nm, 10 m W/cm^2) using a microchannel photomask reproduced on a transparency sheet (resolution 3,810 dpi; Pro-Art bv, Groningen, The Netherlands). The exposed PR was then removed by dipping in a developer solution (AZ351B, Microchemicals GmbH, Germany) and rinsing in deionized water, to leave the ridges which form the molds for the microchannels. Subsequently, the substrate was again exposed to UV light for 10 sec using a second photomask with patterns for the groove arrays on the ridges. The patterned PR was developed once more in order to form groove structures on top of the microchannels.

The PDMS monomer solution and a curing agent were mixed at a weight ratio of 10:1 PDMS: curing agent, and the solution left to stand at ambient conditions for 20 min to allow the air bubbles introduced during the mixing process to dissipate. Afterward, the mixture was poured over the prepared silicon master. A polycarbonate alignment piece was used together with fused-silica capillaries to form access holes to the microchannels during the replication process. The holes (300 µm diameter) were drilled in the polycarbonate at the points of inlets/outlets

using a CNC machine (Sherline, USA). Finally, the microchannels on the silicon master were aligned with the 260-μm-diameter capillaries, and the mixture was cured on the master at 50 ºC for 4 hours. The PDMS was then smoothly peeled off from the master and cut into individual devices.

In order to bond the PDMS slab and glass slide (Menzel-Gläser GmbH, Germany), the glass slide was cleaned with acetone, isopropyl, and deionized water, and dried with N_2 gas. The two devices were treated with UV-generated ozone for 15 min to oxidize the PDMS and glass surfaces. Subsequently, the PDMS slab and glass slide were immediately aligned under a microscope and brought into contact with each other. The assembled chip was then placed on a hot plate at 140 ºC and cooled down to room temperature, after which the chip was irreversibly bonded. The whole bonding procedure was performed in a Class 1000 cleanroom environment.

2.3. Silicon photodiode fixation and bonding with microfluidic chip

To measure the emitted CL, a silicon photodiode was bonded to the microfluidic chip and used as a detector. There are a variety of photodiodes available that provide high sensitivity at low cost; the photodiodes used in this study were from Hamamatsu (S2387, spectral response range: 320 to 1100 nm). Chabinyc *et al*.[3] reported the integration of a microavalanche photodiode (μAPD) into a PDMS device for direct in-channel fluorescence detection. The μAPD was embedded in PDMS and placed adjacent to the microfluidic channel. In a similar manner, we fixed the silicon photodiode in a PDMS mold. A 10:1 mixture of PDMS and curing agent was cast around a silicon diode which had been placed in a Petri dish. After curing, the PDMS slab was cut to have the same outer dimensions of the microfluidic chip. The PDMS slab with fixed silicon photodiode was reversibly bonded to the outer surface of the glass slide, with careful positioning of the photodiode directly under the section of the device in which CL is generated (Figure 1(a)). The silicon photodiode could be reused simply by detaching the PDMS with diode from the microfluidic chip and re-attaching this piece to another microfluidic device.

Figure 1(d) shows a fabricated device. The device has two flow-through microreactors, the first one for the reaction of GOx and glucose, and the second one for the reaction of H_2O_2 and luminol. The length of the first reactor was 43 mm, while the width and depth of this channel were 200 μm and 35 μm, respectively. As shown in Figure 1(b), this channel had slanted grooves incorporated into the top along its entire length to enhance mixing using a chaotic mechanism. The grooves were 50 μm wide and 6~8 μm deep. The length and layout of the serpentine channel

in the second reactor was optimized to cover the entire photodiode active area to increase the detection sensitivity, as this is the channel in which CL was generated. No grooves were patterned in this second reactor. The width, length and volume of the second reactor channel were 200 µm, 16 cm and 1.2 µL, respectively. The thickness of the glass slide sealing the microchannels was 100 µm.

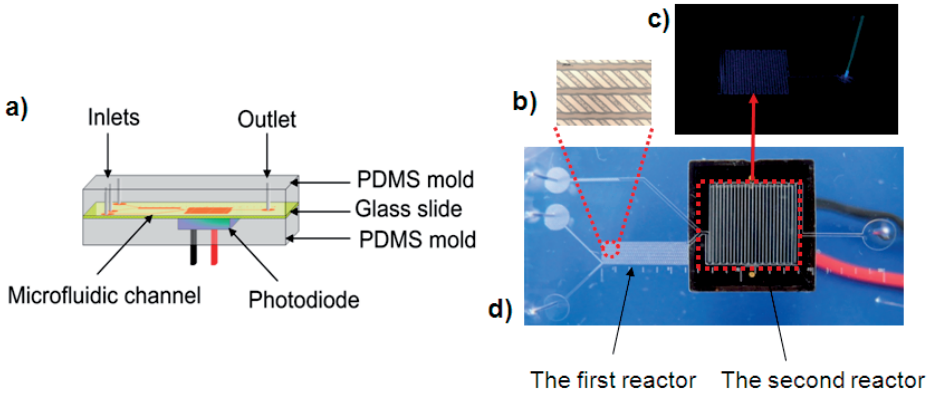


Figure 1. a) Schematic diagram of microfluidic chip. b) Groove structure incorporated into the first reactor to enhance chaotic mixing of the two solutions containing glucose and GOx. c) Emission of light at 425 nm in the second reactor upon reaction of H_2O_2 with luminol. d) A device with two flow-through microreactors.

2.4. CL experimental set-up

Figure 2 shows a schematic diagram of the CL detection experimental set-up. The syringe was connected to the chip inlet using silica capillaries (365 µm OD, 75 µm ID), and two microdialysis syringe pumps (CMA, Sweden) controlled the flow rates. Oxygen concentrations in solution were measured using 'Micro Dissolved Oxygen Electrode' (Lazarlab, Inc., CA) To obtain a calibration curve, glucose sample and GOx solution were introduced at a 1: 3 flow rate ratio using flow rates of 0.5 µL/min (Figure 2, inlet 1) and 1.5 µL/min (Figure 2, inlet 2), respectively. The flow rate of the glucose sample was chosen to be 0.5 µL/min so as to be compatible with high-recovery microdialysis sampling.[22, 25] The H_2O_2 produced and L/I were reacted at a 4:1 flow rate ratio, with a flow rate of 2 µL/min for H_2O_2 sample, and 0.5 µL/min for L/I reagent (Figure 2, inlet 3), respectively. A two-position actuator switching valve (Valco Instrument co., Inc) was used to switch between buffer

solution and glucose sample for acquisition of background and sample detector signal, respectively. The silicon photodiode was wired to a multifunctional transimpedance amplifier (10^7A/V gain) (Sglux, Germany), which converts current to voltage. A multimeter (Agilent) displayed the output signal value and the data was recorded with a LabVIEW (National Instruments, TX) program. All measurements were performed at room temperature in a light-tight metal box with black interior.

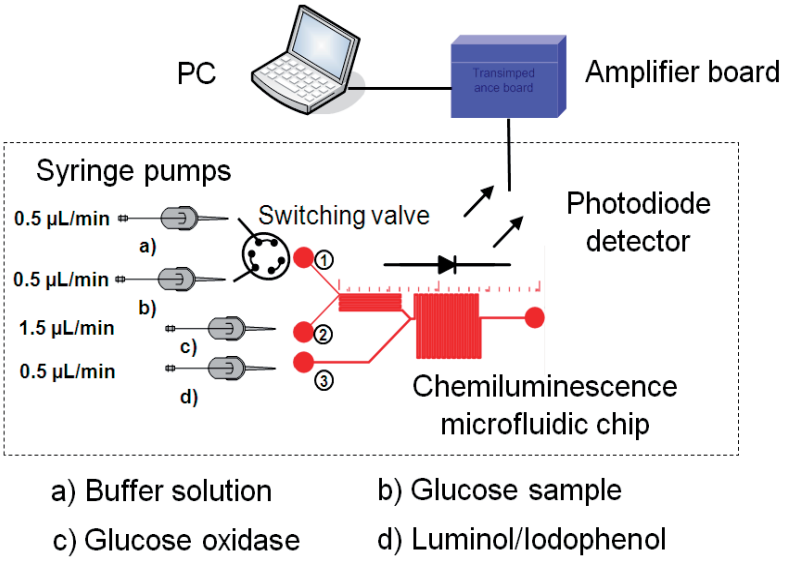


Figure 2. Chemiluminescence experimental set-up. Dashed line delineates the light-tight black box.

3. Results and discussion

3.1 Flow effect and response of L/I sample

The performance of the fabricated CL microfluidic chip was ch aracterized to establish optimal reaction conditions e.g. flow rate, pH, and concentrations of L/I and catalyst, HRP. First, the effects of flow rate and various concentrations of L/I reagent on CL response were studied.[14] The preliminary study was performed by introducing H_2O_2 at inlet 2 (Figure 2, inlet 2) and L/I at inlet 3 (Figure 2, inlet 3). The reaction of H_2O_2 and luminol causes the emission of blue light, starting at the point of flow confluence at the Y-junction and then throughout the entire second reactor, before the reaction mixture is directed to the waste outlet, as shown in

Figure 1(c). Figure 3(a) shows representative sigmoidal curves comprising background signal recorded for a 1:1 mixing ratio of buffer solution and L/I (initial parts of the curves), followed by increasing CL signal due to a 1:1 mixing ratio of 10 mM H_2O_2 sample and 4 mM L/I; the switching valve in Figure 2 was used to switch from buffer to H_2O_2 sample. The photodiode output signal was plotted as a voltage value, which was proportional to the CL intensity. The "rise time" is defined as the time it takes for the CL signal to increase from baseline (background signal) to 95% of its final value. This corresponds to the total time required for the two analytes to flow from start to finish through the second reactor to reach the outlet reservoir. A maximum CL signal is recorded when the reaction mixture fills the entire length of the long serpentine channel from the Y-junction to the outlet. The rise times are approximately 95 sec and 50 sec at a total flow rate of 1 µL/min and 2 µL/min, respectively, which corresponds to the length of the second reactor, viz., 16 cm. As the flow rate was increased from 0.4 µL/min to 4 µL/min, the CL intensity also

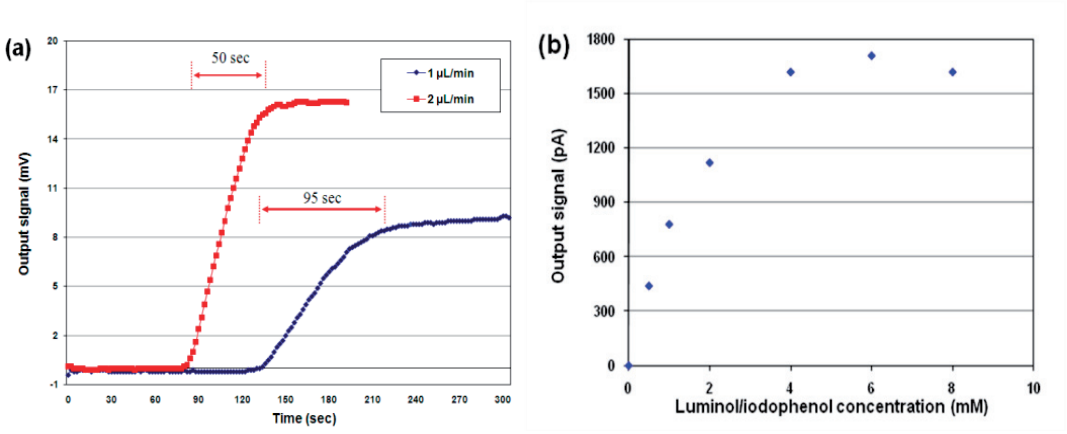


Figure 3. (a) Example of background and sensing signal arising from 1:1 mixing ratios of buffer (initial parts of the curves) or 10 mM H_2O_2 and 4 mM luminol/iodophenol (L/I). The rise times were approximately 95 sec and 50 sec at a total flow rate of 1 µL/min and 2 µL/min, respectively. The rise time was defined as the time required for the CL signal to change from baseline to 95% of the final steady-state output signal. (b) Curve of CL intensity as a function of L/I concentrations arising from the reaction of 10 mM H_2O_2 and L/I reagent at a total flow rate of 2 µL/min. L/I concentrations were always the same i.e. a value of 4 mM along the x-axis means that both luminol and iodophenol concentrations were 4 mM for this experiment.

increased (data not shown). This can be explained by the fact that the brightest emission occurs at the beginning of the reaction, which takes place in the upstream part of the channel. At higher flow rates, a larger portion of the photodiode detecting area is exposed to this bright emission as more of the initial CL reaction takes place downstream in the long serpentine channel that covers the photodiode.

Figure 3(b) shows the photodiode current response arising from the reaction of 10 mM H_2O_2 and varying concentrations of L/I reagent at a total flow rate of 2 μL/min. L/I reagent was prepared at concentrations ranging from 0.5 mM to 8 mM in a 0.2 M carbonate buffer (pH 10.3) containing 20 U/mL HRP (both L and I had the same concentration). The output signal increased with increasing L/I concentration, reaching its maximum value at 4 mM. Therefore, 4 mM L/ 4 mM I was chosen for further experiments. Additionally, the reaction was monitored in the second reactor in order to check for interference of the nitrogen gas produced by the reaction. Inspection under a microscope showed that nitrogen gas bubbles were observed not in the channels but in the outlet reservoir, where nitrogen gas bubbles collected.

3.2. Effect of pH and oxygen depletion

The CL microfluidic chip was specifically designed with a two-reactor system to first allow GOx to produce H_2O_2 at an optimal pH between 5 and 7[26], before L/I is added under more alkaline conditions where the CL reaction is most efficient.[27] This current microchip design compensated for the pH discrepancy between reactions by adding an additional inlet, inlet 3 (Figure 2), for introduction of the L/I reagent after the reaction of glucose and GOx. Since luminol is not easily dissolved in solutions having a pH lower than 8, a pH range of 8.5 to 11.2 was taken into account for the experiment. Complete dissolution was observed at pH values higher than 9. For maximum activation of L/I, the solution was left for one day prior to use.[23] Figure 4 shows the effect of L/I solution pH on reaction efficiency, using 10 mM H_2O_2 and 4 mM L/ 4 mM I. The mixing ratio of H_2O_2 and L/I was 1:1 at a total flow rate of 1 μL/min and 2 μL/min; two of the three chip inlets were used (Figure 2, inlet 2) and (Figure 2, inlet 3). The maximum output signal value was obtained around pH 10.1.

The enzymatic production of H_2O_2 in the first reactor requires oxygen (see Eq. (1)), so a lack of oxygen may affect the sensitivity of our CL microfluidic system. In previous reports, the negative influence of low oxygen was alleviated by either coating polymer membrane on the electrode surface[28] or using a solution containing higher levels of dissolved oxygen to increase oxygen concentration.[29] In our microfluidic system, the amount of oxygen that is present in the GOx solution,

which is continuously supplied at a 3:1 ratio with glucose sample, is generally sufficient to allow conversion of all glucose present. Ultrapure water at room temperature has approximately 0.29 mM of oxygen, similar to concentrations used in other sensing applications.[30] In addition, PDMS is permeable to oxygen and may allow supplemental oxygenation while the fluids are traveling through the channels. We tested rate limitations of oxygen on CL output in our microfluidic system by mixing glucose sample at a 1:1 ratio with 300 U/mL GOx. The CL output signal was stable at glucose concentrations below 15 mM. However, at 15 and 20 mM the output signal decreased over time (Supplementary Information, Figure S1, at the end of this chapter). This decrease did not occur when the glucose samples were premixed with GOx well before their introduction into the microfluidic system. This indicates that oxygen does not become rate limiting at glucose concentrations below 15 mM.

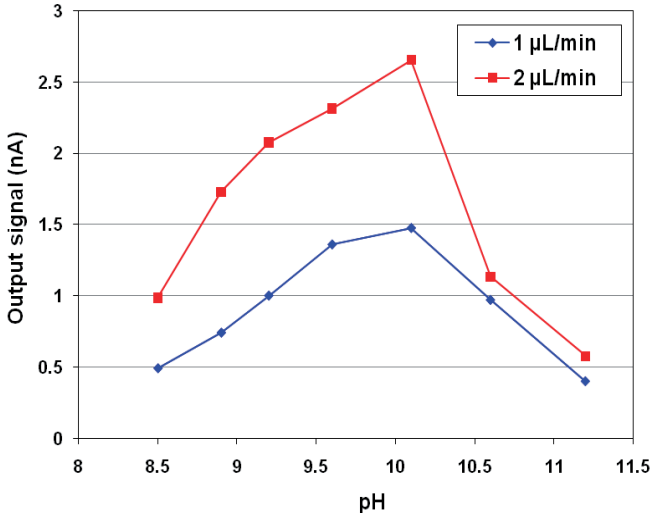


Figure 4. Effect of luminol/iodophenol buffer solution pH. Separate buffer solutions containing either 10 mM H_2O_2 or 4 mM luminol/4 mM iodophenol with 20 U/mL HRP were introduced at inlets 2 and 3, respectively, at a total flow rate of 1 µL/min or 2 µL/min.

3.3. Activities of HRP and GOx in solution

The effect of the HRP enzyme catalyst on CL signal output was further evaluated by varying the HRP concentrations in the second reactor of the microfluidic chip from 0 to 100 U/mL. The three solutions introduced at the three inlets were as follows: 1) 4

mM or 20 mM glucose samples at a flow rate of 0.5 µL/min (Figure 2, inlet 1; 2) > 1000 U/mL GOx solution at a flow rate of 1.5 µL/min (Figure 2, inlet 2); 3) 4 mM L/ 4 mM I contained HRP sample at flow rates of 0.5 µL/min or 1 µL/min (Figure 2, inlet 3). A preliminary study was performed by reacting 10 mM H_2O_2 in buffer with 4 mM L/ 4 mM I containing varying amounts of HRP in buffer at a total flow rate of 2 µL/min (inlets 2 and 3 were used). The highest output signal resulted from a 1:1 mixing ratio of H_2O_2 with L/I reagent with 20 U/mL HRP added (data not shown). Figure 5(a) shows the measured photodiode response when various concentrations of HRP in 4 mM L/4 mM I were reacted with glucose. With 4 mM glucose, the highest CL output signal was obtained at 5 U/mL HRP, whereas with 20 mM glucose, the maximum signal was observed at 20 U/mL HRP. Apparently, more HRP is required to convert all the H_2O_2 that is produced at higher glucose concentrations. Moreover, the L/I flow rate also played a defining role in the final output current value, with 0.5 µL/min producing the higher current value in the 4 mM glucose sample case, and 1 µL/min in the 20 mM glucose case. This can be explained by the fact that the amount of HRP, delivered concurrently with the L/I solution, is varied by the L/I flow rate. For 1 µL/min L/I solution, a 2:1 mixing ratio of produced H_2O_2 to L/I is achieved; for 0.5 µL/min L/I solution, a 4:1 mixing ratio of H_2O_2 to L/I is established. Thus, the dilution of H_2O_2 also influences the recorded output signal. This means that at higher L/I flow rate, a lower concentration of HRP is sufficient to generate the maximum signal. A L/I flow rate of 0.5 µL/min and 20 U/mL HRP were sufficient for good sensitivity at clinically relevant glucose concentrations. Therefore, these conditions were selected for further experiments.

It is interesting to note in Figure 5(a) that HRP concentrations higher than 20 U/mL resulted in decreasing output signal with increasing HRP concentration. This can be explained as follows, by first noting that in the absence of catalyst, the reaction speed is slow, yielding less CL light emission within the reaction channel over the photodiode active area. As the amount of catalyst increases, the reaction producing light proceeds more rapidly. This means that at a given flow rate, more light is produced further upstream from the detector. To achieve a maximum photodiode response, it is desirable to have the reaction producing a maximum amount of light directly over the photodiode's active surface. This can in effect be tuned by optimizing the amount of HRP catalyst added to the H_2O_2 solution at any given flow rate ratio. At higher catalyst amounts, the reaction takes place too far upstream in the second reactor. The amount of CL that goes undetected due to its early emission over just part of the silicon diode active surface thus increases with

the amount of catalyst involved in the reaction. (see Supplementary Information, Figure S2, at the end of this chapter).

Figure 5(b) shows the CL output signal as a function of GOx concentration between 25 and 300 U/mL. The test conditions were 4 mM L/ 4 mM I containing 20 U/mL HRP (pH 10.1), introduced at a flow rate of 0.5 µl/min (Figure 2, inlet 3); glucose sample (4 mM or 20 mM) at a flow rate of 0.5 µL/min (Figure 2, inlet 1), and GOx solution at a flow rate of 1.5 µL/min (Figure 2, inlet 2). As GOx concentration was increased, the output signal also increased up to 150 U/mL GOx solution for both 4 mM and 20 mM glucose, with the signal leveling off at higher GOx concentrations. It can be concluded that 150 U/mL GOx is sufficient for rapid conversion of all glucose, so this concentration was used for further experiments.

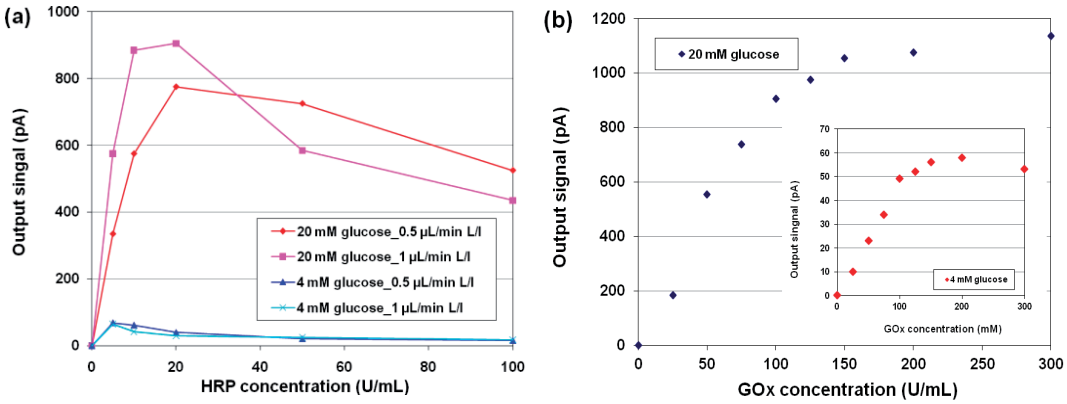


Figure 5. (a) Response to glucose recorded for various concentrations of HRP in 4 mM L/4 mM I. Conditions: 4 mM or 20 mM glucose sample (0.5 µL/min); > 1000 U/mL GOx (1.5 µL/min); and 4 mM L/ 4 mM I containing varying concentrations of HRP (0.5 µL/min or 1 µL/min). (b) Study to determine the optimal GOx concentration. Conditions: 4 mM and 20 mM glucose sample (0.5 µL/min); 4 mM L/ 4 mM I containing 20 U/mL HRP (0.5 µL/min); GOx sample (1.5 µL/min).

3.4 Calibration curve

Figure 6 shows the calibration curve of photodiode output signal as a function of glucose concentration. The test conditions were the same as described in Section 3.3, except that here 7 U/mL HRP and 150 U/mL GOx were used. The lower concentration of HRP of 7 U/mL was chosen in favor of the previously established optimum of 20 U/mL because a pilot experiment revealed that the calibration curve

was more linear and more consistent at this lower HRP concentration (data not shown). Moreover, the use of 5 U/mL HRP resulted in good linearity only at lower glucose concentrations, whereas the use of 10 U/mL HRP resulted in good linearity only at higher glucose concentrations (data not shown). Therefore it was decided to use the intermediate value of 7 U/mL instead. One set of raw data is shown in the inset in Figure 6. A linear curve was observed for current as a function of glucose concentration between 2 and 10 mM, with a sensitivity of 39 pA/mM (R = 0.9963, one device, n=3). The limit of detection was approximately 230 µM glucose concentration, based on a signal-to-noise ratio of 3 (S/N = 3). For the clinical application of monitoring subcutaneous glucose in patients with diabetes, high accuracy and precision are required only in the narrow physiological range of around 3 mM to 10 mM.[31] Thus, the linear range that we have established with our CL microfluidic system makes clinical application feasible.

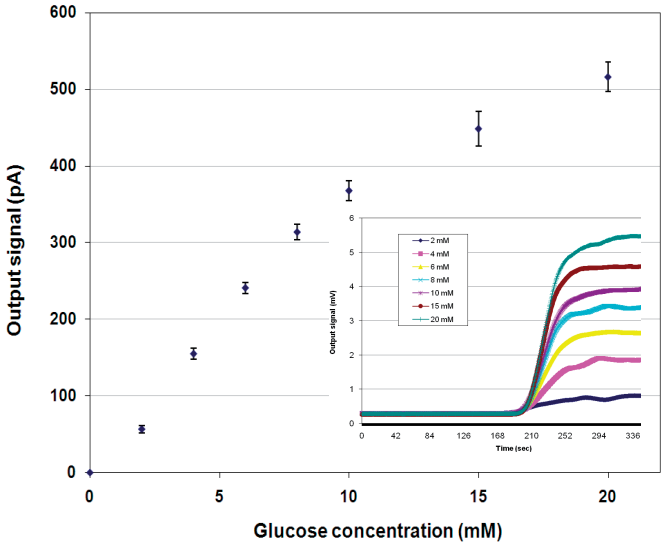


Figure 6. Calibration curve obtained using 150 U/mL GOx solution (1.5 µL/min); 4 mM L/ 4 mM I in carbonate buffer (pH = 10.1) containing 7 U/mL HRP (0.5 µL/min); glucose sample in PB (pH = 7.2) (0.5 µL/min). Raw data are shown in the inset.

4. Conclusion

We developed a n ew microfluidic system with CL detection for continuous monitoring of glucose. In a two-reactor system, glucose is converted into a CL

output signal by a silicon photodiode. In this contribution, the performance of the microfluidic system was evaluated and several important parameters were optimized to establish the best reaction conditions, including flow rate, pH, and concentrations of the various reactants and catalysts. The best performance was achieved with simultaneous flow rates of 0.5 µL/min glucose, 1.5 µL/min GOx, and 0.5 µL/min L/I solution introduced into the three respective inlet channels of the device. Minimum required enzyme concentrations were established at 150 U/mL GOx and 7 U/mL HRP, while optimal L and I concentrations are 4 mM each at a pH of 10.1. The system generated a linear calibration curve for glucose concentrations between 2 and 10 mM (R = 0.9963, one device, n=3), with an observed sensitivity of 39 pA/mM and a LOD of 230 µM (S/N=3).

The use of GOx in solution for each assay can be regarded as an advantage only if the enzyme solution itself has not been stored for a long (several days) period of time before use. Ozyilmaz *et al*. show that immobilized GOx stored at 25ºC actually loses its activity much less rapidly than GOx stored in solution at the same temperature when monitored over 20 days.[32] Use of a freshly prepared enzyme solution is thus a prerequisite for implementation of this approach, with daily replacement of this solution when operating the system over periods of several days. Furthermore, it was demonstrated that oxygen supplied via the delivery of fresh GOx solution and from diffusion through the PDMS was not rate limiting. The microfluidic system had a short reaction time of less than 1 min at a total flow rate of 2.5 µL/min (calibration curve) with a reaction volume of 1.2 µL in the second reactor. Based on these results, it can be concluded that this new CL microfluidic system is well suited for sensing glucose, and that its clinical application is feasible. A few studies have been reported on the *in vivo* use of CL systems in rabbits, in combination with microdialysis-based sample acquisition.[33, 34] However, these involved a PMT as detector, instead of the much smaller photodiode that we used here. Further research is needed to explore the application of this device as a small, portable continuous glucose monitoring system for biomedical purposes.

Acknowledgement: The authors would like to dedicate this paper to the memory of Dr. Adelbert J. M. Schoonen, who passed away May 2, 2011. We acknowledge his significant contributions to research on glucose transportation and measurement in the human body, with as ultimate goal to gain a better understanding of diabetes.

References

1. Arora, A.; Simone, G.; Salieb-Beugelaar, G.B.; Kim, J.T.; Manz, A., Latest developments in micro total analysis systems. *Anal. Chem.* **2010,** 82, (12), 4830-4847.
2. Salieb-Beugelaar, G.B.; Simone, G.; Arora, A.; Philippi, A.; Manz, A., Latest developments in microfluidic cell biology and analysis systems. *Anal. Chem.* **2010,** 82, (12), 4848-4864.
3. Chabinyc, M.L.; Chiu, D.T.; McDonald, J.C.; Stroock, A.D.; Christian, J.F.; Karger, A.M.; Whitesides, G.M., An integrated fluorescence detection system in poly(dimethylsiloxane) for microfluidic applications. *Anal. Chem.* **2001,** 73, (18), 4491-4498.
4. Verpoorte, E., Chip vision-optics for microchips. *Lab Chip* **2003,** 3, (3), 42N-52N.
5. Sia, S.K.; Whitesides, G.M., Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies. *Electrophoresis* **2003,** 24, (21), 3563-3576.
6. Kuswandi, B.; Nuriman; Huskens, J.; Verboom, W., Optical sensing systems for microfluidic devices: a review. *Anal. Chim. Acta* **2007,** 601, (2), 141-155.
7. Du, W.-B.; Fang, Q.; He, Q.-H.; Fang, Z.-L., High-throughput nanoliter sample introduction microfluidic chip-based flow injection analysis system with gravity-driven flows. *Anal. Chem.* **2005,** 77, (5), 1330-1337.
8. Pennathur, S.; Fygenson, D.K., Improving fluorescence detection in lab on chip devices. *Lab Chip* **2008,** 8, (5), 649-652.
9. Mitchell E. Johnson, J.P.L., Fundamentals and practice for ultrasensitive laser-induced fluorescence detection in microanalytical systems. *Electrophoresis* **2004,** 25, (21-22), 3513-3527.
10. Guan, Y.-X.; Xu, Z.-R.; Dai, J.; Fang, Z.-L., The use of a micropump based on capillary and evaporation effects in a microfluidic flow injection chemiluminescence system. *Talanta* **2006,** 68, (4), 1384-1389.
11. Balslev, S.; Jorgensen, A.M.; Bilenberg, B.; Mogensen, K.B.; Snakenborg, D.; Geschke, O.; Kutter, J.P.; Kristensen, A., Lab-on-a-chip with integrated optical transducers. *Lab Chip* **2006,** 6, (2), 213-217.
12. Marle, L.; Greenway, G.M., Determination of hydrogen peroxide in rainwater in a miniaturised analytical system. *Anal. Chim. Acta* **2005,** 548, (1-2), 20-25.
13. Xu, Z.-R.; Fang, Z.-L., Composite poly(dimethylsiloxane)/glass microfluidic system with an immobilized enzymatic particle-bed reactor and sequential sample injection for chemiluminescence determinations. *Anal. Chim. Acta* **2004,** 507, (1), 129-135.
14. Davidsson, R.; Genin, F.; Bengtsson, M.; Laurell, T.; Emneus, J., Microfluidic biosensing systems - Part I. development and optimisation of enzymatic chemiluminescent μ-biosensors based on silicon microchips. *Lab Chip* **2004,** 4, (5), 481-487.

15. Kamidate, T.; Kaide, T.; Tani, H.; Makino, E.; Shibata, T., Effect of the geometry of microfabricated flow reactors on chemiluminescent detection of epinephrine with lucigenin. *Luminescence* **2001,** 16, (5), 337-342.

16. Jorgensen, A.M.; Mogensen, K.B.; Kutter, J.P.; Geschke, O., A biochemical microdevice with an integrated chemiluminescence detector. *Sens. Actuators, B* **2003,** 90, (1-3), 15-21.

17. Hofmann, O.; Miller, P.; Sullivan, P.; Jones, T.S.; deMello, J.C.; Bradley, D.D.C.; deMello, A.J., Thin-film organic photodiodes as integrated detectors for microscale chemiluminescence assays. *Sens. Actuators, B* **2005,** 106, (2), 878-884.

18. Fukuba, T.; Aoki, Y.; Fujii, T., Microbial activity assay in deep-sea environment using a microfluidic device. *Proceedings of μTAS 2009* **2009**, 2010-2012.

19. Nakamura, H.; Murakami, Y.; Yokoyama, K.; Tamiya, E.; Karube, I.; Suda, M.; Uchiyama, S., A compactly integrated flow cell with a chemiluminescent FIA system for determining lactate concentration in serum. *Anal. Chem.* **2001,** 73, (2), 373-378.

20. Wang, X.; Hofmann, O.; Das, R.; Barrett, E.M.; deMello, A.J.; deMello, J.C.; Bradley, D.D.C., Integrated thin-film polymer/fullerene photodetectors for on-chip microfluidic chemiluminescence detection. *Lab Chip* **2007,** 7, (1), 58-63.

21. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.

22. Moon, B.-U.; Koster, S.; Wientjes, K.J.C.; Kwapiszewski, R.M.; Schoonen, A.J.M.; Westerink, B.H.C.; Verpoorte, E., An enzymatic microreactor based on chaotic micromixing for enhanced amperometric detection in a continuous glucose monitoring application. *Anal. Chem.* **2010,** 82, (16), 6756-6763.

23. Price, D.; Worsfold, P.J.; Fauzi, R.; Mantoura, C., Determination of hydrogen peroxide in sea water by flow-injection analysis with chemiluminescence detection. *Anal. Chim. Acta* **1994,** 298, (1), 121-128.

24. Kim, J.; Heo, J.; Crooks, R.M., Hybridization of DNA to bead-immobilized probes confined within a microfluidic channel. *Langmuir* **2006,** 22, (24), 10130-10134.

25. Menacherry, S.; Hubert, W.; Justice, J.B., In vivo calibration of micro-dialysis probes for exogenous compounds. *Anal. Chem.* **1992,** 64, (6), 577-583.

26. Bankar, S.B.; Bule, M.V.; Singhal, R.S.; Ananthanarayan, L., Glucose oxidase -- an overview. *Biotechnol. Adv.* **2009,** 27, (4), 489-501.

27. Hayashi, K.; Sasaki, S.; Ikebukuro, K.; Karube, I., Highly sensitive chemiluminescence flow injection analysis system using microbial peroxidase and a photodiode detector. *Anal. Chim. Acta* **1996,** 329, (1-2), 127-134.

28. Dixon, B.M.; Lowry, J.P.; O'Neill, R.D., Characterization in vitro and in vivo of the oxygen dependence of an enzyme/polymer biosensor for monitoring brain glucose. *J. Neurosci. Methods* **2002,** 119, (2), 135-142.

29. Böhm, S.; Pijanowska, D.; Olthuis, W.; Bergveld, P., A flow-through amperometric sensor based on dialysis tubing and free enzyme reactors. *Biosens. Bioelectron.* **2001,** 16, (6), 391-397.

30. Laurell, T.; Drott, J.; Rosengren, L., Silicon wafer integrated enzyme reactors. *Biosens. Bioelectron.* **1995,** 10, (3-4), 289-299.

31. Cryer, P.E.; Davis, S.N.; Shamoon, H., Hypoglycemia in diabetes. *Diabetes Care* **2003,** 26, (6), 1902-1912.

32. Ozyilmaz, G.; Tukel, S.S.; Alptekin, O., Activity and storage stability of immobilized glucose oxidase onto magnesium silicate. *J. Mol. Catal. B: Enzym.* **2005,** 35, (4-6), 154-160.

33. Fang, Q.; Shi, X.T.; Sun, Y.Q.; Fang, Z.L., A flow injection microdialysis sampling chemiluminescence system for in vivo on-line monitoring of glucose in intravenous and subcutaneous tissue fluid microdialysates. *Anal. Chem.* **1997,** 69, (17), 3570-3577.

34. Liu, W.; Zhang, Z.J.; Liu, H.S., Droplet-based micro-flow chemiluminescence system for in vivo glucose determination by microdialysis sampling. *Anal. Sci.* **2005,** 21, (4), 413-416.

Supplementary information:

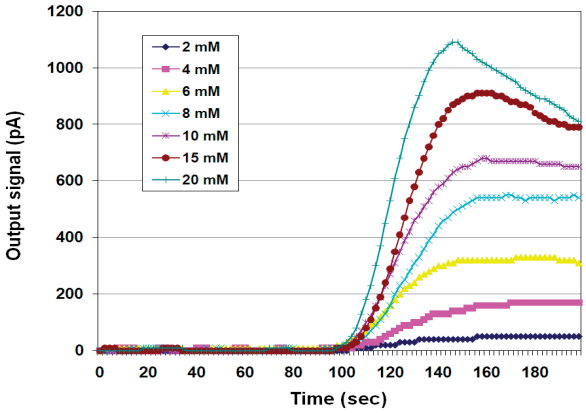
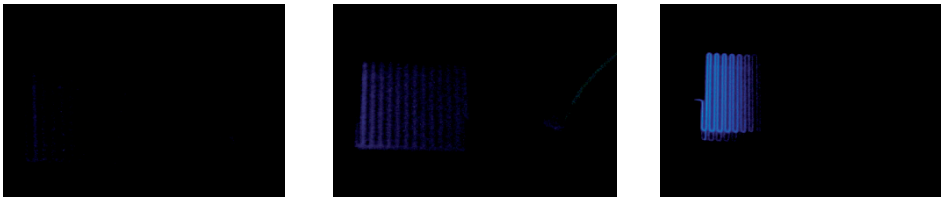


Figure S1. Oxygen depletion effect in high-concentration glucose samples. 15 mM and 20 mM glucose samples exhibited CL signals that, once having reached a maximum, decreased over time. Photodiode signal was generated by mixing glucose and 300 U/mL GOx solution in a 1:1 mixing ratio, while 4 mM L/ 4 mM I was infused into the third inlet.



(a) (b) (c)

Figure S2. Effect of HRP concentration. CL light was emitted in the second reactor by the reaction of 10 mM H_2O_2 and 4 m M L/ 4 m M I containing different concentrations of HRP (a) 0 U /mL, (b) 20 U /mL and (c) 100 U/mL. As the concentration of catalyst is increased from 0 U/mL to 100 U/mL, the faster the reaction proceeds, with maximum light emission occurring earlier in the second microreactor. The amount of CL detected therefore decreases at higher HRP concentrations, once an optimum value for this parameter has been passed.

Chapter 5

In Vivo Application of an Enzymatic Microreactor Coupled with Microdialysis for Continuous Monitoring of Subcutaneous Glucose in Rats

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Manuscript in preparation

Abstract

Continuous glucose monitoring provides additional insight into how blood glucose levels vary over the course of a day or days, important information for diabetes patients and physicians from both a diagnostic and prognostic point of view. However, optimized methodologies and further technical improvements are still needed. We present an application of a n ewly-designed microfluidic chip, incorporating an enzymatic microreactor (EMR) for continuous glucose monitoring in rats. The EMR is a microfluidic device designed for the rapid reaction of glucose with glucose oxidase in nL volumes of solution. The hydrogen peroxide produced is detected with integrated microelectrodes. In this study, the EMR is coupled to a microdialysis probe which is inserted into the subcutaneous tissue of anesthetized rats. The performance of the EMR was evaluated by modulating blood glucose concentrations with intravenous injections of 20 % glucose (w/v) or 5 U/kg insulin, with saline as a co ntrol. Dynamic changes in glucose levels were recorded, with a 1.5 mM (15 %) increase and an 8.6 mM (85%) decrease modulated by glucose and insulin administrations, respectively. Blood glucose was frequently measured colorimetrically using the Accu-Chek® method as a reference value for comparison with results obtained using the EMR. Differences between glucose values determined by blood sampling and EMR monitoring are also discussed. This is the first example of the use of a chaotic mixing approach in a microfluidic device for an *in vivo* glucose sensing application. Based on *in vitro* and *in vivo* results, we conclude that the feasibility of using the coupled microfluidics-microdialysis system for continuous subcutaneous glucose monitoring has been successfully demonstrated in rats.

1. Introduction

Continuous glucose monitoring is a relatively new approach to assist patients with diabetes mellitus to maintain blood glucose concentrations within near-normal levels.[1] The manifestation of well-known diabetic complications such as retinopathy, nephropathy and neuropathy can be prevented by using intensified-insulin therapy to lower baseline glucose levels.[2, 3] This treatment regimen, however, predisposes patients to frequently experience episodes of severe hypoglycemia.[4] Hypoglycemia is the main limiting factor for good glycemic control in diabetes therapy, and should be therefore be avoided.[5] Continuous glucose monitoring provides great insight into glucose levels throughout the day compared to the intermittent measurements by the finger-prick method. It can be used retrospectively to assess blood glucose during the night or perhaps as a tool for the physician in addition to patient anamnesis.[6] It can also be used prognostically to warn the patient of developing hypoglycemia and, when integrated into an artificial pancreas, to direct automatic adjustment of insulin dosing.[7]

In vivo microdialysis is a powerful technique for continuous monitoring of subcutaneous glucose.[8] The microdialysis probe consists of a semipermeable hollow fiber membrane which is sealed at the end, and is connected to inlet and outlet tubing. The microdialysis probe is designed to mimic a blood capillary and can be placed in any target tissue of interest. The lumen of the probe is perfused with an artificial interstitial fluid (ISF; usually Ringer's solution) which is introduced from the inlet. Small pores in the membrane allow certain molecules to travel across it along the concentration gradient, from higher concentrations in the subcutaneous tissue to low or zero concentrations in the perfusing solution, known as the perfusate. However, large molecules (including proteins) cannot cross the membrane. Glucose concentrations in the perfusate collected through the outlet reflect the concentrations in the ISF and can be quantified by a variety of bioanalytical techniques.

Microdialysis-based and needle-type sensing systems have both proven to be biocompatible and safe for the patient. However, using microdialysis to sample glucose from subcutaneous tissue has advantages over implanting a needle-type probe directly in this tissue. Microdialysis is a very effective sample pretreatment approach, since the probes are made of low-molecular-weight cut-off materials which do not allow the passage of large molecular species like proteins or proteases from tissue into the perfusate. This facilitates detection of glucose in the microdialysate collected at the outlet, since the microdialysis process has prevented

many interfering species from entering the sample. This is particularly advantageous for electrochemical detection of glucose, since the detection electrodes are far less prone to biofouling in the relatively clean microdialysate matrix than in more complex physiological fluids. Since the sensing electrodes are not in direct contact with cells/tissue, a higher degree of analytical accuracy can be obtained compared to needle-type sensors.[9]

However, microdialysis does require external components such as pumps for liquid transportation, which can make the system somewhat large and bulky. System lag time, that is, the time delay between dialysis of analyte and actual analysis result caused by the length of connecting tubing between probe and analytical device, is another disadvantage of this technique. Low perfusion flow rates on the order of hundreds of nL to just a few μL per minute are often not compatible with the internal volumes (tens of μL or more) of connecting tubing in these systems. Transport of microdialysate from the probe to the analysis component in the system can take many minutes as a result. The implanted probe is also much bigger than a needle-type sensor. Despite these potential disadvantages, however, microdialysis sampling has proven to be an attractive alternative to implanted needle-type sensors.

Among those microdialysis-based sensing systems reported,[10-13] the Subcutaneous Continuous Glucose Monitoring (SCGM) system[11] appears to be a good candidate to monitor glucose in patients with diabetes. Microdialysate containing glucose is mixed with reagent solution containing glucose oxidase (GOx), which catalyzes the oxidation of glucose to hydrogen peroxide (H_2O_2) and gluconolactone. The amount of H_2O_2 can be detected amperometrically through oxidation at a detection electrode, with the amount of current produced being directly proportional to the H_2O_2 concentration and thus also to the original amount of glucose in the sample. This approach for glucose sensing is unusual, in that most electrochemical glucose sensors employ GOx immobilized to the electrode for detection of H_2O_2. While more than adequate for short-term experiments, sensors based on immobilized GOx tend to lose sensitivity and exhibit a drift in response over longer periods of time as the enzyme slowly loses its catalytic activity. A solution-based glucose reaction with GOx in a continuously flowing system has the advantage that fresh enzyme is continuously provided, circumventing sensor drift and sensitivity loss. Free GOx in solution also loses its activity over a period of several days, so it is important to use freshly prepared GOx solution in this application, and to replace the GOx reagent daily for multi-day experiments.

A prototype of the SCGM has been studied for clinical use and shown to be reliable for continuous glucose monitoring over longer periods of days.[14] However, the overall configuration of this system is also rather bulky, and system lag times are long (over 30 min). Thus, the concept of a miniaturized SCGM is an approach with good prospects for reducing system size and lag time while retaining the advantages of the solution-based reaction of GOx with glucose for glucose sensing.

Chip-based microfluidic technologies provide an excellent alternative for conventional glucose monitoring approaches such as the SCGM mentioned above. The advantages of microfluidic systems include dramatically reduced consumption of chemical reagents, enhanced cost-effectiveness and substantially shortened reaction times. Micrometer-sized fluidic channels enable the solution-based reaction of GOx and glucose in nL volumes. The nL volumes characteristic of microfluidic devices are also compatible with microdialysis sampling, which is generally carried out at low-microlitre or even sub-microlitre per minute flow rates. The lower the flow rate, the higher the recovery of glucose from the subcutaneous tissue being sampled.

To date, significant research efforts have been expended on coupling microdialysis sampling with microfluidic devices. Several recent reviews discuss the possible integration of microdialysis with microfluidic detection components for on-line measurement of dialysate.[15-17] There have been few reports, however, of microchip-based sensing systems linked with microdialysis for continuous glucose monitoring. Hsieh *et al*. developed an on-chip microdialysis system with an in-line sensing electrode.[18, 19] A microdialysis membrane was integrated directly into the fluidic channel in order to reduce system dead volumes such that lag times were just a couple of minutes. The membrane sealed a 15-µm-deep microchannel formed photolithographically in an SU-8 epoxy layer. This channel contained two microfabricated Pt electrodes and a Ag/AgCl pellet electrode. Ultimately, the device was designed to function as a microdialysis probe, with the outer surface of the membrane in direct contact with tissue, and the inner surface perfused by medium containing GOx, pumped through the microchannel. Glucose diffusing across the membrane into the microchannel reacts with the GOx to produce H_2O_2, which is then detected at the electrodes. For the studies reported in [18, 19], the outer membrane surface was placed in contact with a reservoir containing a glucose solution. In this configuration, the system exhibited a very high microdialysis

recovery of 99 % at a 0.5 μL/min flow rate, and fluctuating glucose concentrations in the clinically relevant range were tracked using electrochemical detection.

The aim of this study is to evaluate the performance of a new microfluidic system coupled to microdialysis sample acquisition for continuous monitoring of subcutaneous glucose in rats. Recently, we reported a new enzymatic microreactor (EMR) incorporating a chaotic mixing channel with microfabricated electrodes for the continuous-flow measurement of glucose.[20] The mixing channel had an array of small, slanted parallel grooves formed in its top surface along the length of the channel. Chaotic mixing of two side-by-side flowing solution streams is induced when the solutions flow over the groove array, as flow is forced to turn towards the sidewall of the channel in the direction of the grooves. Solution streams rapidly begin to twist around each other in a helical fashion, and the flow pattern becomes chaotic. This in turn enlarges the contact area between solutions, reducing diffusion lengths and increasing mixing rates as a result. In the EMR, a solution containing glucose was mixed with a solution containing glucose oxidase, with the resulting H_2O_2 detected amperometrically at the end of the channel. The EMR with slanted-groove array demonstrated superior mixing and hence enhanced glucose detection sensitivity compared to a non-grooved EMR.

Here we show the *in vivo* application of the EMR with slanted-groove array for continuous monitoring of subcutaneous glucose. Glucose sampling is achieved using a microdialysis probe, the outlet of which is coupled to one of the EMR inlets to enable analysis of glucose in the microdialysate. The monitoring system was first thoroughly characterized *in vitro* with respect to glucose recovery (probe) and glucose response (EMR). To demonstrate the performance of EMR *in vivo*, a microdialysis probe was inserted subcutaneously into a rat. Glucose concentrations in the ISF were then monitored in response to intravenous injections of saline, glucose and insulin, with frequent sampling of blood for blood glucose analysis as a reference. Microdialysate glucose levels obtained using the EMR were compared with blood glucose levels. The observed differences between glucose concentrations measured in ISF using microdialysis sampling and in blood are also discussed.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were analytical-reagent grade. Ascorbic acid was obtained from Merck (Germany). Uric acid and acetaminophen were purchased from Sigma-Aldrich (Germany). Pyrrole was purchased from Sigma-Aldrich and used to prepare

a 200 m M solution in phosphate-buffered saline solution (PBS). The PBS was prepared in-house by mixing Na_2HPO_4 and NaH_2PO_4 in water to final concentrations of 0.04 M and 0.01 M, respectively. NaCl was added to this buffer to a final concentration of 0.10 M. Ringer's solution, also prepared in-house, was used as the microdialysis infusion solution, and had a composition of 147.0 mM NaCl, 3.0 mM KCl, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$. Saline solution (0.9%, sterile) was purchased from Baxter and stored at 2 ºC. Insulin (recombinant human insulin, 100 IE/mL) was purchased from Eli Lilly (Indianapolis, U.S.) and stored at 2 ºC. D-glucose and GOx were supplied by Merck (Germany) and used to prepare 1 M and 5000 U/mL stock solutions, respectively. D-glucose was stored in a refrigerator at 2 ºC while GOx was stored at - 20 ºC. All solutions were prepared with 18 MΩ·cm ultrapure water purified in an Arium® 611 system (Sartorius Stedim Biotech, Germany).

2.2. Fabrication of microfluidic chip

The fabrication of the microchannel in polydimethylsiloxane (PDMS) by replication using a st ructured silicon wafer as master was described in detail in a previous paper.[20] Briefly, a 4-inch, p-type, polished (100) silicon wafer (Si-Mat, Germany) (525 µm) was employed as a substrate. After wafer cleaning, it was treated with hexamethyldisilazane (HMDS) (Sigma-Aldrich, Germany) to improve adhesion of the photoresist. The structure on the silicon master was p rocessed in a thick photoresist layer with two steps of standard photolithography. A thick layer of positive photoresist AZ 4562 (Microchemicals GmbH, Germany) was co ated onto the silicon wafer using a sp in coater, and then re-hydrated at ambient temperature for 3 hours. The coated wafer was exposed to ultraviolet (UV) light (365 nm, 10 mW/cm^2) using a photomask made on a transparency sheet (resolution 3,810 dpi; Pro-Art bv, Groningen, The Netherlands) to structure the ridges which are the negative of the microchannels. The exposed photoresist was then removed by dipping in a developer solution and rinsed in deionized water. Subsequently, the substrate was again exposed to UV light for 10 s using a photomask to structure the grooves on top of the microchannel-ridges and developed. For microchannel replication in PDMS, a mixture of PDMS resin and curing agent (Sylgard 184, Dow Corning, U.S.) was prepared and cast onto the silicon master. The mixture was allowed to cure at 50 °C for 4 hours. Once hardened, the PDMS slab was peeled off of the silicon master. Microfabricated thin-film platinum (Pt) electrodes were obtained from microLIQUID (Arrasate – Mondragón, Spain). These electrodes were

formed on a 4-inch Pyrex glass wafer using a standard photolithography and lift-off process. Groups of threes electrodes were prepared onto one wafer (active area 100 μm x 1500 μm). The PDMS slab was cut into individual devices for bonding to the glass chip with electrodes. After UV-ozone treatment, the PDMS slab and glass chip with electrodes were immediately aligned under a microscope and brought into contact with each other to form an irreversible bond. The whole bonding procedure was done in a cleanroom.

In order to prevent protein adsorption and reaction with interfering substances, the thin-film Pt electrodes were coated by electrodeposition of overoxidized polypyrrole (PPy). A solution of 200 mM PPy was prepared in 100 mM PBS (pH 7.4, composition as above) solution and used for electrode coating.[21] The PPy solution was first bubbled for 20 min with nitrogen (N_2) gas in order to lower the oxygen concentration in solution. The electrodes were then coated by introducing the PPy solution into the microfluidic channel and applying a constant potential of 0.85 V for 5 min. Subsequently, the PPy was overoxidized by maintaining a potential of 0.85 V for 6 hours in PBS solution. The chip was then cleaned with ultrapure water.

2.3. Evaluation of *in vitro* microdialysis recovery

A cannula microdialysis probe (Brainlink B.V., Groningen, The Netherlands) was used in this study. The shaft was made of concentric fused silica tubes (not rigid but quite flexible), and the semi-permeable membrane at the tip made of polyacrylonitrile (PAN). This material has a 45-50 kDa molecular-weight cut-off. Membrane lengths of 1 or 3 cm (ID of 250 μm, OD of 340 μm) were used for this study. The inflow of perfusion solution was down the center of the concentric shafts, with outflow up the peripheral channel. In order to evaluate *in vitro* microdialysis recovery, relative glucose recovery was determined using a colorimetric method. Prior to microdialysis use, the probe was cleaned with 70% ethanol and ultra-purified water. A 10 mM glucose sample solution was prepared in DI water in a beaker as a reference solution, and a probe was immersed in this glucose solution. The probe was perfused with Ringer's solution using a syringe pump which was connected to the inlet of the probe with silica tubing. The glucose solution was stirred and maintained at 37°C. Microdialysate samples were collected for 30 min in vials at the outlet of the probe at various perfusion flow rates (0.3 μL/min - 20 μL/min). For the quantitative colorimetric determination of glucose recovery in the microdialysate, 6 μL dialyste samples were mixed with 50 μL of glucose reagent (CMA, Sweden) and allowed to react for one hour in a 60-well plate. This reaction is catalyzed by peroxidase and yields the red-violet colored quinoneimine. The

amount of compound formed is measured photometrically at 546 nm wavelength and is proportional to the glucose concentration. After reaction, the 60-well plate was placed on a Microplate reader (Molecular Device, CA) and SoftMax Pro Software displayed the absorbance. Relative recovery was expressed as percent of beaker glucose content.

2.4. *In vitro* evaluation of performance of microdialysis probe coupled to EMR

Figure 1 shows a schematic diagram of the experimental set-up used for *in vitro* evaluation of the microdialysis probe coupled to the EMR. The microdialysis probe was perfused with Ringer's solution at 0.5 µL/min. The resulting microdialysate was then transported from the probe outlet to one of the EMR inlets. A 150 U/mL GOx solution was introduced at 1.5 µL/min into the other EMR inlet. The length of the mixing channel in the EMR was 10 cm long.

Glucose was detected electrochemically as described previously.[20] Two thin-film Pt electrodes were used as working and counter electrodes, respectively. A Ag/AgCl electrode was used as a reference electrode and prepared by using a silver wire (Ag) (250 µM diameter, Sigma-Aldrich). The Ag wire was immersed in 1 M HCl solution saturated with NaCl. The AgCl layer was coated by connecting the wire to the positive terminal of a 9-V battery for 10 min to oxidize silver at the surface of the wire to form AgCl. With respect to the electrochemical detection, signal fluctuation and damping noise originating from pulsation of the syringe pumps were observed in the pilot experiments. To alleviate this problem, a pulsation-dampening set-up was introduced into our continuous measurement system, as shown elsewhere.[22] Briefly, a 20-µL air bubble was entrapped in each gastight syringe next to the piston. Because air is far more compressible than liquid, this bubble served as a cushion capable of absorbing piston pulsation without transfer of this pulsation to the surrounding liquid. The syringe pumps used for the remaining experiments were from Harvard Apparatus Ltd. (UK).

The *in vitro* EMR evaluation was performed by injecting varying volumes of 1 M stock glucose solution into the beaker, which contained a known amount of water, to achieve different concentrations of glucose (2.1 mM, 5.6 mM, 10.1 mM, 15.0 mM, 20.6 mM). The microdialysis probe was immersed in this glucose solution, which was kept at 37ºC and stirred by placing on a stirring hotplate. (Note that the chip and tubing were not heated.) The H_2O_2 produced by the reaction between glucose and GOx (150 U/mL) was oxidized at the integrated microelectrode at an applied potential of 700 mV (CH Instrument, U.S.) with respect to Ag/AgCl. The resulting

current was recorded as a function of glucose concentration. The output current value was displayed on a PC. The data acquisition rate was every 2 sec.

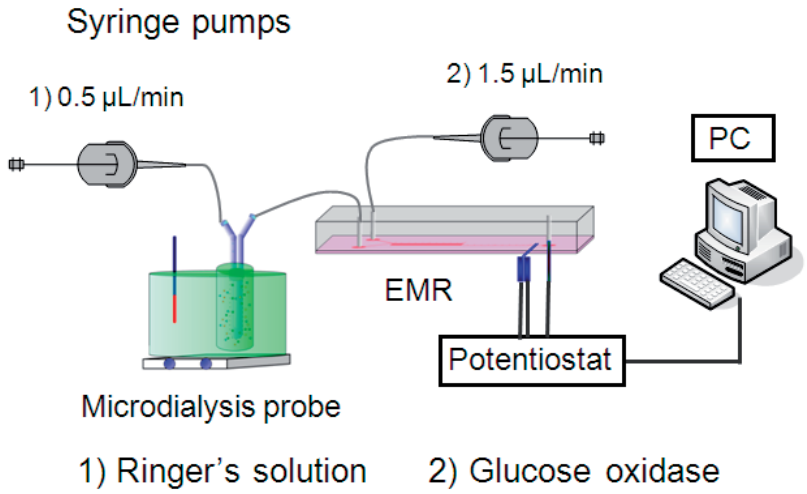

Figure 1. Experimental set-up used for the evaluation of the in vitro performance of the microdialysis probe coupled to the EMR.

2.5. Experimental set-up and procedure for *in vivo* glucose monitoring

This study was approved by the Institutional Animal Care and Use C ommittee (IACUC) of the University of Groningen. Male Wistar rats (350-410 g, Harlan, Horst, The Netherlands) were singly housed in Plexiglas cages (30 x 30 x 40 cm) in a room in which light (12 h:12 h light cycle; lights on at 07: 00) and temperature (21 ± 1 °C) were controlled. The animals had free access to fresh water and standard rodent chow. The animals were acclimated for one week before surgery and the start of the experiments.

On the day of an experiment, the rat was anesthetized with isoflurane gas (5% isoflurane in O_2 to induce anesthesia, followed by 2% isoflurane in O_2 to maintain an anesthetized state). The level of anesthesia was monitored and maintained at an appropriate level throughout the surgery and experimental procedures. A cat heter was placed in the jugular vein to accommodate the collection of blood samples. A 10-cm segment of silicone tubing (0.50 mm ID; 0.90 mm OD) was inserted into the right jugular vein. The catheter was exteriorized from the neck and fitted onto a 21-gauge stainless steel (0.80 mm OD; 40 mm length) connector. The catheter was kept

patent by infusing 30 μL of heparin (20 U/mL in saline) every 15 minutes (immediately after collecting each blood sample). A second incision was made in the dorsal part of the thorax and a microdialysis probe (1 mm open surface) was placed in a deep subcutaneous tissue layer. Both wounds were carefully closed with suture.

In vivo glucose monitoring in the rats was started immediately after surgery. During the experiments, the animals were kept under anesthesia, body temperature was maintained at 37°C, and dehydration was prevented by hourly subcutaneous injections of saline at a site distant from the microdialysis probe. The microdialysis probe was perfused with Ringer's solution at a flow rate of 0.5 μL/min. The outlet of the probe was connected to one of the EMR inlets by 10 cm of silica tubing (0.005 in ID; 0.02 in OD) (see Figure 1). GOx (150 U/mL) was continuously introduced by a second syringe pump to the other EMR inlet at a flow rate of 1.5 μL/min. The connection between this syringe pump and the EMR was realized using a 30-cm-long piece of silica tubing. The microelectrode signal in the EMR was allowed to stabilize for 90 min. After a stable signal had been established, glucose concentrations were modulated by consecutive intravenous injections of saline (1 mL; control), 20% (w/v) glucose in saline (1 mL), and 5 U/kg insulin in saline (1 mL), administered at one-hour intervals. Samples for determination of blood glucose levels were collected (70 μL) via the jugular vein at 15 min intervals. Loss of fluid volume in the animals was compensated by replacement with saline injected hourly. Blood glucose was co lorimetrically quantitated with an Accu-Chek® analyzer (Roche Diagnostics, Switzerland). After completion of the experiment, the rats were immediately sacrificed with 20% Euthasol® (AST Beheer BV, The Netherlands).

2.6. Data analysis

The acquired *in vivo* raw data was further processed for EMR evaluation and comparison with blood glucose values (Microsoft® Excel 2007). Data points obtained using the EMR were displayed as 1-min average values of raw data. The dynamic change of glucose levels was plotted with respect to baseline levels. In order to transform EMR signal into "blood" glucose values, a one-point *in vivo* calibration was performed using an average blood glucose value obtained at steady state, after saline solution administration but before administration of glucose.[23] A conversion factor, k, was first calculated by equating the product of k multiplied by EMR current at steady state, i_{EMRss}, to the average blood glucose value at steady-state, $[BG]_{ss}$:

$$k \cdot i_{EMRss} = [BG]_{ss} \quad (1)$$

All measured values of i_{EMRss} were then transformed to blood glucose concentrations through multiplication by k. The maximum values of transformed EMR signal and experimentally determined blood glucose concentrations achieved by injection of glucose were compared for statistical significance. The statistically significant difference was analyzed by one way ANOVA, followed by a Bonferroni-Holm's post-hoc test. The significance was set at $p < 0.05$.[24]

3. Results and Discussion

3.1. *In vitro* interference study

Electroactive molecules can diffuse through the microdialysis membrane and be oxidized electrochemically at the surface of the bare Pt working electrode, thus increasing the output current and interfering with glucose determination. The most electroactive interfering substances present in physiological samples are ascorbic acid, uric acid and acetaminophen.[25] The extent to which these substances could adversely affect observed signal must therefore be addressed prior to *in vivo* glucose analysis. Much effort has been spent to eliminate the effect of interfering substances on sensor response.[26] Permselective coating layers such as nafion, polypyrrole (PPy)[27], overoxidized PPy[21], polyphenylenediamine[28] and cellulose acetate[29] have been coated on bare electrode surfaces to prevent undesirable contributions to the measured current.

In this study, the effects of interfering substances on electrochemical measurements in the microfluidic chip made with bare electrodes were compared with electrodes coated with overoxidized PPy. Separate samples containing 5.6 mM glucose and either 150 µM ascorbic acid, 500 µM uric acid or 150 µM acetaminophen were prepared. The concentrations chosen for the interfering species are typical maximum values in physiological sample.[11] These solutions were introduced into the channel and the resulting current compared with that produced by a 5.6 mM glucose sample alone. The results, shown in Figure 2, indicate that all three electroactive substances increase electrode response by less than 10 % when a bare electrode was used. With the PPy-coated electrode, the interference was decreased to less than 5 % in all cases, with uric acid exhibiting almost no measurable interference. Interestingly, the bare electrode shows less interference compared to previous electrochemical biosensor reports.[30, 31] This could possibly

be explained by a high enzymatic conversion of glucose in the reaction channel. The enzyme is continuously converting glucose as the reaction mixture travels along the mixing channel to the sensing electrode. In fact, the current produced for a 5.6 mM glucose solution is equivalent to that measured for an approximately 3 mM H_2O_2 sample (data not shown). The contribution to overall electrode signal from interfering substances is thus relatively small.

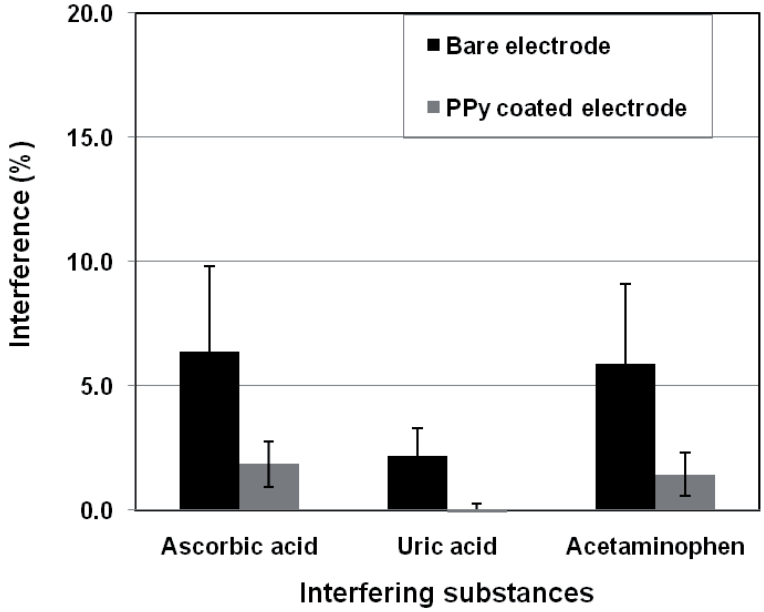

Figure 2. Interference study (n = 4 devices). The current recorded for solutions containing 5.6 mM glucose with 150 µM ascorbic acid, 500 µM uric acid or 150 µM acetaminophen added was compared to that of a solution containing only 5.6 mM glucose. Observed differences are expressed as a percentage (%).

3.2. *In vitro* microdialysis probe recovery

In vitro microdialysis recovery was characterized as a function of flow rate and probe length. The molecular weight cut-off of the semipermeable microdialysis membrane dictates the extent to which different molecular species can diffuse across it. A high-precision syringe pump is used to transport the analyte compounds which have diffused over the membrane into the microdialysate to the probe outlet. When the analyte concentration reaches equilibrium across the membrane, 100 % relative recovery is established. The relative recovery is thus defined as[32]:

$$\% \text{ Recovery} = C_{out} / C_{beaker} \times 100\ (\%) \qquad (1)$$

where C_{out} is the concentration of glucose in dialysate and C_{beaker} the undisturbed concentration of glucose in the stirred medium in which the microdialysis probe is immersed.

It is well known that the relative recovery is increased when flow rate is decreased. Relative recovery can be regulated by adjusting perfusion flow rate and length of probe membrane; recoveries of 100 % are also possible. The effects of flow rate and probe length were investigated in this study, since these are the parameters that dictate *in vitro* recovery.

Figure 3 shows the *in vitro* recovery of glucose at different flow rates with microdialysis probes having lengths of 1 cm and 3 cm. The results showed that as the flow rate was increased, recovery was decreased for both probe lengths, due to

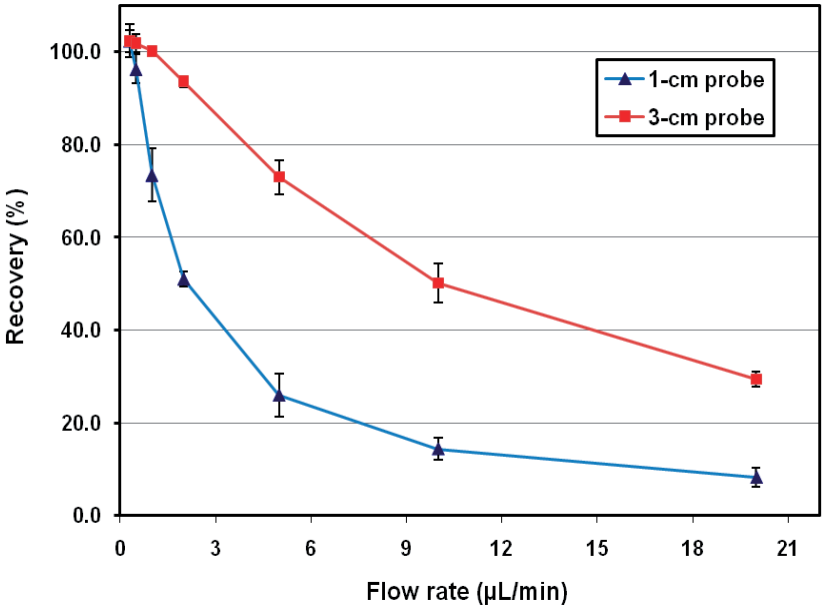

Figure 3. In vitro recovery of glucose at flow rates between 0.3 and 20 µL/min (n=3 probes, 3 experiments per probe). The recovery of 1-cm probe and 3-cm probe were obtained and compared as a function of flow rate.

reduced times for diffusion of glucose over the membrane. Comparing the 3-cm probe to the 1-cm probe, it is clear that the 3-cm probe showed higher recovery at higher flow rates. This is because of the greater contact area with medium afforded by the longer membrane, resulting in more molecules diffusing through the membrane. The recovery for the 1-cm probe was, however, still very good at a flow rate of 0.5 μL/min with a value of 95%, while the longer microdialysis probe exhibited a 100% recovery at this flow rate. Damage to cells and tissues during probe insertion for *in vivo* application is greater for the longer probe, however. The 1-cm probe was thus deemed more appropriate with respect to minimizing the adverse side effects of probe insertion. In addition to optimal recoveries, flow rates of less than 1 μL/min also result in a reduced requirement for perfusion medium. This is in keeping with the use of a microfluidic sensing device in this application to reduce reagent consumption for the eventual development of a portable monitoring system. Therefore, a 1-cm-long membrane and a flow rate of 0.5 μL/min allowing for nearly 100 % recovery were chosen for further experimentation.

3.3. Calibration of the EMR coupled to the microdialysis probe

Prior to performing an *in vitro* evaluation of the EMR coupled to a microdialysis probe, possible backpressure effects caused by connection to the probe which could result in altered flow rates were considered. When tubing, microchip, and microdialysis probe are all coupled, perfusion fluid might be lost at the EMR inlets, a weak point in the system which could be subject to leakage upon applied pump flow (see Figure 1). Theoretically, the expected pressure drop over the system can be calculated using the Poiseuille equation.[33] This pressure drop was calculated to be approximately ~10 kPa under the given conditions, described in Section 2.4. Possible changes in set flow rates due to backpressure were then experimentally determined for the pumps operating individually, and for the pumps coupled to the chip and microdialysis probe (see Figure 1). Each experimental configuration was run for 30 min at a perfusion flow rate of 0.5 μL/min and/or 1.5 μL/min. The liquid collected at the tubing outlet of the pumps or chip outlet during this time was weighed, and the flow rate was calculated. The pumps delivering Ringer's solution and GOx reagent were tested separately and found to operate at their set flow rates of 0.5 μL/min and 1.5 μL/min, respectively, with no observed backpressure effects. There were also no adverse effects noted when the two pumps were coupled directly to the two inlets of the EMR chip and operated at their set flow rates (total flow rate at chip outlet was 2.0 μL/min, as expected). This changed, however, when the

Ringer's solution pump (0.5 µL/min) was connected to the microdialysis probe, which in turn was connected to one inlet of the EMR, while the other pump introduced buffer at a flow rate of 1.5 µL/min to the other chip inlet.The results showed an approximate 5 % loss of fluid at a total flow rate of 2 µL/min when two pumps were operated simultaneously under these conditions.

Figure 4 shows the result for the *in vitro* calibration of an EMR in line with a microdialysis probe as a function of glucose concentration. The experimental set-up depicted in Figure 1 was employed for these measurements. Changes in electrode current in response to glucose concentrations up to 20.6 mM were measured. A linear relationship between current and glucose concentration was shown in the range between 2.1 mM and 20.6 mM, with a sensitivity of 7.8 ± 1.0 nA/mM (n = 6 devices, 1 experiment per device, as shown in the inset of Figure 4) and correlation coefficient R = 0.9966. An example of a recorded current-time trace is shown in the

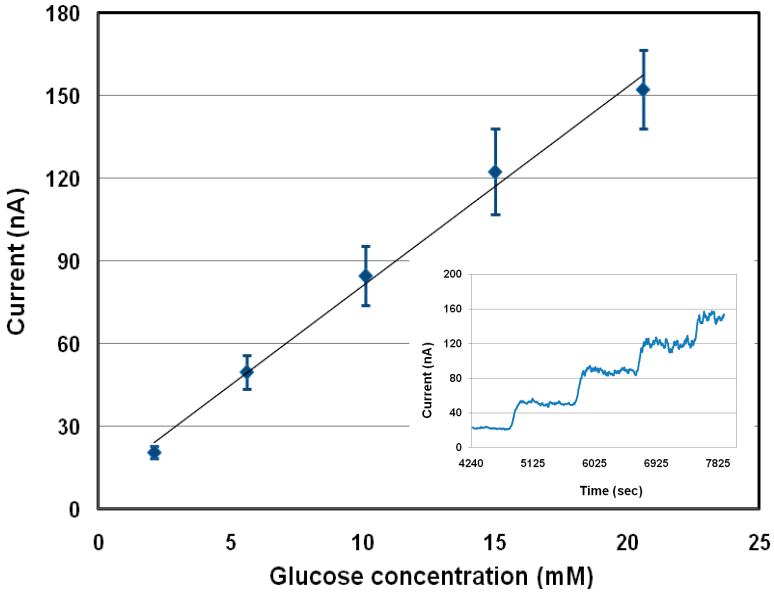

Figure 4. In vitro calibration of the EMR coupled to a 1-cm long microdialysis probe (n = 6 devices). An example of a recorded current-time trace is shown in the inset, exhibiting current values that increase stepwise over time as the glucose concentration in the beaker was increased. Current was recorded for 15 min at each concentration. Each value of current in the calibration curve is an average of data acquired over a 15-min period.

inset, exhibiting current values that increase stepwise over time as the glucose concentration in the beaker was increased. Current was recorded for 15 min at each concentration. This result confirms that the microfluidic chips were reproducible from one device to the next. During the *in vitro* evaluation, the physical lag time, or delay between microdialysis and analytical signal, was estimated to be about 18 to 19 min (time after concentration change at which the steady-state current had increased by 10%), due mainly to the tubing connecting the probe to the chip inlet and the dead volume of the microdialysis probe. In principle, this physical lag time can be reduced through further miniaturization of the sensing system to bring it closer to the probe insertion site.

3.4. *In vivo* study

The performance of the *in vivo* glucose monitoring microsystem in rats was evaluated by modulating blood glucose concentrations with intravenous injections of glucose or insulin, using saline as a control. In a previous study in humans, a rapid increase in blood glucose concentrations was achieved by administration of an oral glucose load (75 g glucose), and a rapid decrease was achieved by intravenous insulin administration (6-40 units).[34] Capillary blood samples were collected every 15 min for 3-5 hours and glucose was analyzed by different commercial glucose monitors. In rats, fast and robust changes in blood glucose can be induced by intraperitoneal injection of 1g/kg glucose[35] or intravenous injection of 5 U/kg insulin.[36] Here we have chosen to modulate glucose levels by consecutively injecting saline (1 mL/kg), 200 mg/kg glucose, and 5 U/kg insulin intravenously at one-hour intervals.

The effect of these treatments on glucose concentrations in the blood and in subcutaneous ISF are shown in Figure 5. Blood glucose levels are expressed as change in glucose concentration over time relative to the baseline concentration within the same animal. Recorded EMR current, which reflects subcutaneous ISF glucose concentrations, was calibrated to *in vivo* blood glucose values by a one-point calibration against blood glucose at baseline (an average of blood glucose concentrations at 3:00 and 3:15).[23] Continuous registration of subcutaneous ISF glucose was converted into one-minute averages, and expressed as change in glucose concentration over time relative to the baseline concentration within the same animal. Arrows indicate the time points of the saline, glucose and insulin administrations.

Baseline blood glucose concentration was measured to be 10.2 ± 2.3 mM by using the Accu-Chek® analyzer. These relatively high baseline values can be attributed to

the effects of anesthesia. Our approach does not allow us to directly compare glucose levels in blood and ISF at steady-state. The correlation between glucose in blood and ISF has often been debated. In some studies it has been shown that under steady-state conditions ISF glucose levels were similar to blood glucose in healthy subjects.[37, 38] However, others reported ISF glucose to be ~15-30% lower.[39, 40] Despite this, we have chosen to report our measured ISF glucose signal trends in terms of baseline blood glucose concentration, as it is generally accepted that ISF glucose trends will mirror blood glucose trends, though with a physiological lag time. As seen in Figure 5, the physiological lag time in our experiments is between 5 and 9 minutes, which agrees with the literature.[41]

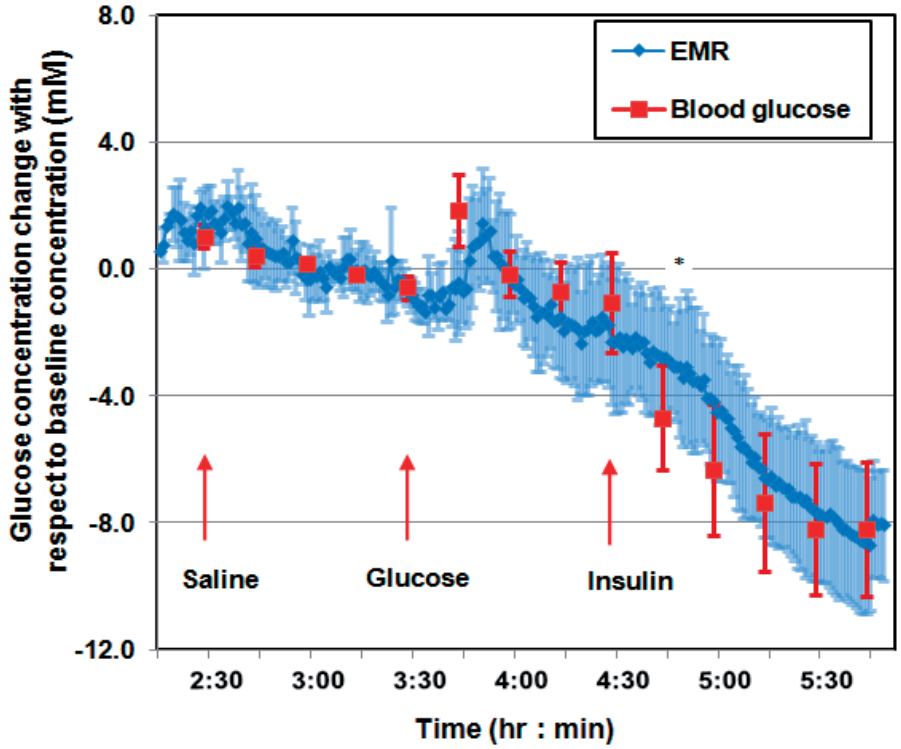

*Figure 5. Dynamic changes in subcutaneous glucose concentration with respect to the baseline concentration by administration of glucose-modulating substances (n = 5 rats) Arrows indicate consecutive administrations (saline, glucose and insulin) at one-hour time intervals. Plotted EMR data points are 1-min averages of the recorded data and blood glucose are every 15 min intervals. * indicates a statistically significant difference compared to baseline at 3:30. ($p < 0.05$; ANOVA)*

Approximately 5 min after glucose injection, but not after saline administration, ISF glucose values started increasing to a maximum increase of 1.5 mM (15%). In contrast, ISF glucose decreased by 8.6 mM (85%) in total after insulin administration, starting about 15 min after insulin injection. These dynamic changes in glucose levels can be explained by the different mechanisms that exist for glucose kinetics in subcutaneous tissue.[42] When glucose is injected into the jugular vein, it is quickly spread to all capillaries. Glucose is then transported from the blood to the interstitial fluid of the subcutaneous tissue and ISF glucose rapidly
equilibrates with the blood. Insulin is also rapidly distributed to all capillaries after being injected into the jugular vein. However, insulin affects glucose concentrations in an indirect manner, namely by activating glucose transporter proteins, mainly GLUT4, that clear glucose into muscle cells and adipocytes. In this way, glucose is removed from the ISF into cells surrounding the microdialysis probe.

The relationship between ISF and blood glucose is complex and not well understood. Under physiological conditions there is free and rapid exchange of glucose between blood and ISF.[35] However, there will be a gradient between blood and ISF when glucose is taken up by the tissue surrounding the microdialysis probe.[43] It has been reported that the blood-ISF gradient seems to be larger during systemic hypoglycemia[44, 45], however, our results indicate that the amplitude in blood and in subcutaneous ISF is identical.

Here we demonstrated a physiological lag time between blood and ISF of approximately 5-9 min, independent of the direction of glucose change. Conflicting reports exist in the literature about the rate of equilibration between blood and ISF during increasing or decreasing glucose concentrations, ranging from rapid equilibration[39, 45] to a delayed response in ISF by 18-30 min.[38, 46, 47] The relationship between blood and ISF is dynamic, and depends on the species,[43] the tissue in which the microdialysis probe is implanted[37, 48], and on the diabetic state.[49] Moreover, ISF glucose levels are affected by the act of probe implantation. Insertion of the probe into adipose tissue causes vasoconstriction at the site of implantation, and damages cells and capillaries surrounding the probe. Wientjes *et al*.[50] showed that ISF glucose levels are lower than blood glucose during the first 12 hours after implantation. Agreement between ISF and blood is expected to improve after tissue trauma has been mostly cleared and angiogenesis of new capillaries into the implantation site has been allowed to enhance delivery of glucose to the tissue.[51]

We would like to reiterate that our measurement was started within a couple of hours after probe insertion, and our results may reflect a shorter delay caused by

disruption of the endothelial barrier, a longer delay caused by the presence of tissue trauma and cellular debris, as well as relatively low ISF levels caused by local inflammation and wound healing processes. The focus of this present study is the *in vitro/in vivo* evaluation of a new microfluidic glucose sensing system incorporating microdialysis sampling. Fluids in the *in vivo* environment are very complex and contain many potentially interfering substances. The use of microdialysis as a sampling technique in this *in vivo* monitoring approach could thus be quite appealing for lab-on-a-chip glucose sensing applications.

For further research, it would be interesting to study freely moving rats instead of anesthetized rats with the EMR-based system. Since the chemicals used for anesthesia affect rat metabolism, it is not an optimal situation in which to study the relationship between BG and ISF. Ultimately, microtechnology could enable the development of portable systems small enough to allow (small) animal studies under conditions in which the animals can move normally. Along with improved microdialysis techniques, microfluidics-based systems should enable increasingly reliable long-term glucose-monitoring studies in both animal and human subjects.

4. Conclusion

We have successfully demonstrated an *in vivo* application of a microfluidic EMR coupled with microdialysis for continuous subcutaneous glucose monitoring. This is the first example of the use of a chaotic mixing approach in a microfluidic device for an *in vivo* glucose sensing application. The results obtained show the feasibility of this microfluidic glucose sensing system, and its potential for further miniaturization and integration into an autonomous system incorporating micropumps, solution reservoirs and energy source. The advantage of this microfluidic glucose sensing system compared to commonly using immobilized enzyme sensors is the fact that the sensing reaction occurs in solution, which circumvents the necessity of enzyme immobilization and subsequent slow enzyme deactivation. Instead, a continuous supply of fresh, active enzyme for the sensing reaction is provided, made possible by the strongly reduced GOx volumes consumed in the chip. With the reaction of GOx and glucose, a significant amount of H_2O_2 is produced along the grooved mixing channel. Moreover, the amount of H_2O_2 production can be tuned by simply modifying the length of the mixing channel or alternatively, the flow rate. Several important factors have been taken into account in the current microfluidic chip design, as described in Moon *et al.*[20].

Factors such as the concentration of GOx, an optimal GOx-glucose mixing ratio to prevent oxygen depletion and the use of longer channel lengths to increase glucose conversion to H_2O_2 and thus improve detection sensitivity were considered in this earlier work. For the studies presented here, it was important to characterize flow in the device once coupled to the microdialysis probe, to avoid induced pressure differences and ultrafiltration effects. As already mentioned, the use of microdialysis is another advantage in terms of analyte sampling, as the sample collected and reacted in the EMR is relatively clean compared to the fluid *in vivo*.

For the evaluation of the EMR, electrodes coated with a PPy layer were shown to exhibit a less than 5% difference in measured current in the presence of added interferents (ascorbic acid, uric acid, acetaminophen) compared to a solution containing only 5.6 mM glucose. *In vitro* microdialysis probe recovery was approximately 95 % at a flow rate of 0.5 µL/min. In tissue, there are disruptions and capillary wound healing in the cell layers surrounding the microdialysis probe. Those processes in the vicinity of the probe affect glucose transport to the probe membrane and hence glucose recovery. However, it is possible to reach a glucose concentration equilibrium between the dialysate and nearby capillaries through adjustment of the flow rate, as shown in adipose tissue model.[32] The EMR coupled with microdialysis showed a good correlation coefficient in the concentration range of 2.1 mM to 20.6 mM (n=6 devices). In the *in vivo* animal experiments, dynamic changes in glucose levels of about 1.5 mM (15 %) increase and an 8.6 mM (85 %) decrease, modulated by glucose and insulin administrations, respectively, were observed. The trends in measured EMR levels correlated well with trends in blood glucose levels. Comparison of pre- and post-EMR evaluation data indicated that no significant biofouling of the detection electrodes had taken place during *in vivo* monitoring experiments (data not shown). It should be noted that since the animal study was performed on anesthetized rats, the glucose levels measured in ISF and blood cannot directly be compared. Under anesthetic, the glucose levels in ISF and blood are dependent on the glucose kinetics, location of the probe, up-take of glucose by cells and the condition of the animal itself. This can lead to quite different absolute glucose concentrations in the two compartments, as measured elsewhere using an ultrafiltration technique.[47] However, the *in vivo* results obtained here allow the conclusion that it is feasible to use the microfluidic chip coupled to microdialysis for the continuous subcutaneous monitoring of glucose in rats. Further development and application of this system are thus warranted.

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References

1. Klonoff, D.C., Continuous glucose monitoring: roadmap for 21st century diabetes therapy. *Diabetes Care* **2005,** 28, (5), 1231-1239.
2. DCCT Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **1993,** 329, (14), 977-986.
3. UKPDS Group, Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *The Lancet* **1998,** 352, (9131), 837-853.
4. DCCT Group, Hypoglycemia in the diabetes control and complications trial. The Diabetes Control and Complications Trial Research Group. *Diabetes* **1997,** 46, (2), 271-286.
5. Davis, S.; Alonso, M.D., Hypoglycemia as a barrier to glycemic control. *J. Diabetes Complicat.* **2004,** 18, (1), 60-68.
6. Cheyne, E.; Kerr, D., Making 'sense' of diabetes: using a continuous glucose sensor in clinical practice. *Diabetes Metab. Res. Rev.* **2002,** 18, (S1), S43-S48.
7. Steil, G.M.; Panteleon, A.E.; Rebrin, K., Closed-loop insulin delivery--the path to physiological glucose control. *Adv. Drug Delivery. Rev.* **2004,** 56, (2), 125-144.
8. Heinemann, L., Continuous glucose monitoring by means of the microdialysis technique: underlying fundamental aspects. *Diabetes Technol. Ther.* **2003,** 5, (4), 545-561.
9. Wentholt, I.M.; Vollebregt, M.A.; Hart, A.A.; Hoekstra, J.B.; DeVries, J.H., Comparison of a needle-type and a microdialysis continuous glucose monitor in type 1 diabetic patients. *Diabetes Care* **2005,** 28, (12), 2871-2876.
10. Poscia, A.; Mascini, M.; Moscone, D.; Luzzana, M.; Caramenti, G.; Cremonesi, P.; Valgimigli, F.; Bongiovanni, C.; Varalli, M., A microdialysis technique for continuous subcutaneous glucose monitoring in diabetic patients (part 1). *Biosens. Bioelectron.* **2003,** 18, (7), 891-898.
11. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.
12. Hoss, U.; Kalatz, B.; Gessler, R.; Pfleiderer, H.-J.r.; Andreis, E.; Rutschmann, M.; Rinne, H.; Schoemaker, M.; Haug, C.; Fussgaenger, R.D., A novel method for

continuous online glucose monitoring in humans: the comparative microdialysis technique. *Diabetes Technol. Ther.* **2001,** 3, (2), 237-243.

13. Petrou, P.S.; Moser, I.; Jobst, G., Microdevice with integrated dialysis probe and biosensor array for continuous multi-analyte monitoring. *Biosens. Bioelectron.* **2003,** 18, (5-6), 613-619.
14. Kapitza, C.; Lodwig, V.; Obermaier, K.; Wientjes, K.J.C.; Hoogenberg, K.; Jungheim, K.; Heinemann, L., Continuous glucose monitoring: reliable measurements for up to 4 days with the SCGM1 system. *Diabetes Technol. Ther.* **2003,** 5, (4), 609-614.
15. Guihen, E.; O'Connor, W.T., Capillary and microchip electrophoresis in microdialysis: recent applications. *Electrophoresis* **2010,** 31, (1), 55-64.
16. Nandi, P.; Lunte, S.M., Recent trends in microdialysis sampling integrated with conventional and microanalytical systems for monitoring biological events: A review. *Anal. Chim. Acta* **2009,** 651, (1), 1-14.
17. Sassa, F.; Morimoto, K.; Satoh, W.; Suzuki, H., Electrochemical techniques for microfluidic applications. *Electrophoresis* **2008,** 29, (9), 1787-1800.
18. Hsieh, Y.-C.; Zahn, J.D., On-chip microdialysis system with flow-through sensing components. *Biosens. Bioelectron.* **2007,** 22, (11), 2422-2428.
19. Hsieh, Y.-C.; Zahn, J.D., On-chip microdialysis system with flow-through glucose sensing capabilities. *J. Diabetes Sci. Technol.* **2007,** 1, (3), 375-383.
20. Moon, B.-U.; Koster, S.; Wientjes, K.J.C.; Kwapiszewski, R.M.; Schoonen, A.J.M.; Westerink, B.H.C.; Verpoorte, E., An enzymatic microreactor based on chaotic micromixing for enhanced amperometric detection in a continuous glucose monitoring application. *Anal. Chem.* **2010,** 82, (16), 6756-6763.
21. Walker, E.; Wang, J.; Hamdi, N.; Monbouquette, H.G.; Maidment, N.T., Selective detection of extracellular glutamate in brain tissue using microelectrode arrays coated with over-oxidized polypyrrole. *The Analyst* **2007,** 132, (11), 1107-1111.
22. Pravda, M.; Marvin, C.A.; Sarre, S.; Michotte, Y.; Kauffmann, J.-M., A damping system for syringe pumps used for microdialysis with on-line electrochemical detection. *Anal. Chem.* **1996,** 68, (14), 2447-2450.
23. Lodwig, V.; Heinemann, L., Continuous glucose monitoring with glucose sensors: calibration and assessment criteria. *Diabetes Technol. Ther.* **2003,** 5, (4), 572-586.
24. Oldenziel, W.H.; Dijkstra, G.; Cremers, T.I.F.H.; Westerink, B.H.C., Evaluation of hydrogel-coated glutamate microsensors. *Anal. Chem.* **2006,** 78, (10), 3366-3378.
25. Feldman, B.; Brazg, R.; Schwartz, S.; Weinstein, R., A continuous glucose sensor based on wired enzyme technology - results from a 3-day trial in patients with Type 1 diabetes. *Diabetes Technol. Ther.* **2003,** 5, (5), 769-779.
26. Hamdi, N.; Wang, J.; Monbouquette, H.G., Polymer films as permselective coatings for H2O2-sensing electrodes. *J. Electroanal. Chem.* **2005,** 581, (2), 258-264.

27. Ramanavicius, A.; Ramanaviciene, A.; Malinauskas, A., Electrochemical sensors based on conducting polymer--polypyrrole. *Electrochim. Acta* **2006,** 51, (27), 6025-6037.

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

29. Maran, A.; Crepaldi, C.; Tiengo, A.; Grassi, G.; Vitali, E.; Pagano, G.; Bistoni, S.; Calabrese, G.; Santeusanio, F.; Leonetti, F.; Ribaudo, M.; Di Mario, U.; Annuzzi, G.; Genovese, S.; Riccardi, G.; Previti, M.; Cucinotta, D.; Giorgino, F.; Bellomo, A.; Giorgino, R.; Poscia, A.; Varalli, M., Continuous subcutaneous glucose monitoring in diabetic patients: a multicenter analysis. *Diabetes Care* **2002,** 25, (2), 347-352.

30. Zhang, Y.; Hu, Y.; Wilson, G.S.; Moatti-Sirat, D.; Poitout, V.; Reach, G., Elimination of the acetaminophen interference in an implantable glucose sensor. *Anal. Chem.* **1994,** 66, (7), 1183-1188.

31. Carelli, I.; Chiarotto, I.; Curulli, A.; Palleschi, G., Electropolymerization of hydroxybenzene and aminobenzene isomers on platinum electrodes to assemble interference-free electrochemical biosensors. *Electrochim. Acta* **1996,** 41, (11-12), 1793-1800.

32. Schoonen, A.J.M.; Wientjes, K.J.C., Glucose transport in adipose tissue. *Sens. Actuators, B* **2005,** 105, (1), 60-64.

33. Kirby, B.J., Micro- and nanoscale fluid mechanics: transport in microfluidic devices. *Cambridge University Press* **2010**.

34. Jungheim, K.; Koschinsky, T., Glucose monitoring at the arm. *Diabetes Care* **2002,** 25, (6), 956-960.

35. Aussedat, B.; Dupire-Angel, M.; Gifford, R.; Klein, J.C.; Wilson, G.S.; Reach, G., Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am. J. Physiol. Endocrinol. Metab.* **2000,** 278, (4), E716-728.

36. de Vries, M.G.; Arseneau, L.M.; Lawson, M.E.; Beverly, J.L., Extracellular glucose in rat ventromedial hypothalamus during acute and recurrent hypoglycemia. *Diabetes* **2003,** 52, (11), 2767-2773.

37. Moberg, E.; Hagström-Toft, E.; Amer, P.; Bolinder, J., Protracted glucose fall in subcutaneous adipose tissue and skeletal muscle compared with blood during insulin-induced hypoglycaemia. *Diabetologia* **1997,** 40, (11), 1320-1326.

38. Cheyne, E.H.; Cavan, D.A.; Kerr, D., Performance of a continuous glucose monitoring system during controlled hypoglycaemia in healthy volunteers. *Diabetes Technol. Ther.* **2002,** 4, (5), 607-613.

39. Wientjes, K.J.C.; Schoonen, A.J.M., Determination of time delay between blood and interstitial adipose tissue glucose concentration change by microdialysis in healthy volunteers. *Int. J. Artif. Organs* **2001,** 24, (12), 884-889.

40. Muller, M.; Schmid, R.; Nieszpaur-Los, M.; Fassolt, A.; Lonnroth, P.; Fasching, P.; Eichler, H.G., Key metabolite kinetics in human skeletal muscle during ischaemia and reperfusion: measurement by microdialysis. *Eur. J. Clin. Investig.* **1995,** 25, (8), 601-607.

41. Quinn, C.P.; Pishko, M.V.; Schmidtke, D.W.; Ishikawa, M.; Wagner, J.G.; Raskin, P.; Hubbell, J.A.; Heller, A., Kinetics of glucose delivery to subcutaneous tissue in rats measured with 0.3-mm amperometric microsensors. *Am. J. Physiol. Endocrinol. Metab.* **1995,** 269, (1), E155-161.

42. Cengiz, E.; Tamborlane, W.V., A tale of two compartments: interstitial versus blood glucose monitoring. *Diabetes Technol. Ther.* **2009,** 11, (s1), S-11-S-16.

43. Rebrin, K.; Steil, G.M.; van Antwerp, W.P.; Mastrototaro, J.J., Subcutaneous glucose predicts plasma glucose independent of insulin: implications for continuous monitoring. *Am. J. Physiol. Endocrinol. Metab.* **1999,** 277, (3), E561-571.

44. Caplin, N.J.; O'Leary, P.; Bulsara, M.; Davis, E.A.; Jones, T.W., Subcutaneous glucose sensor values closely parallel blood glucose during insulin-induced hypoglycaemia. *Diabet. Med.* **2003,** 20, (3), 238-241.

45. Kulcu, E.; Tamada, J.A.; Reach, G.; Potts, R.O.; Lesho, M.J., Physiological differences between interstitial glucose and blood glucose measured in human subjects. *Diabetes Care* **2003,** 26, (8), 2405-2409.

46. Rossetti, P.; Porcellati, F.; Fanelli, C.G.; Bolli, G.B., Evaluation of the accuracy of a microdialysis-based glucose sensor during insulin-induced hypoglycemia, its recovery, and post-hypoglycemic hyperglycemia in humans. *Diabetes Technol. Ther.* **2006,** 8, (3), 326-337.

47. Kaptein, W.A.; Kemper, R.H.A.; Ruiters, M.H.J.; Venema, K.; Tiessen, R.G.; Korf, J., Methodological aspects of glucose monitoring with a slow continuous subcutaneous and intravenous ultrafiltration system in rats. *Biosens. Bioelectron.* **1997,** 12, (9-10), 967-976.

48. Tiessen, R.G.; Rhemrev-Boom, M.M.; Korf, J., Glucose gradient differences in subcutaneous tissue of healthy volunteers assessed with ultraslow microdialysis and a nanolitre glucose sensor. *Life Sci.* **2002,** 70, (21), 2457-2466.

49. Thome-Duret, V.; Reach, G.; Gangnerau, M.N.; Lemonnier, F.; Klein, J.C.; Zhang, Y.; Hu, Y.; Wilson, G.S., Use of a subcutaneous glucose sensor to detect decreases in glucose concentration prior to observation in blood. *Anal. Chem.* **1996,** 68, (21), 3822-3826.

50. Wientjes, K.J.C.; Grob, U.; Hattemer, A.; Hoogenberg, K.; Jungheim, K.; Kapitza, C.; Schoonen, A.J.M., Effects of microdialysis catheter insertion into the subcutaneous adipose tissue assessed by the SCGM1 system. *Diabetes Technol. Ther.* **2003,** 5, (4), 615-620.

51. Lutgers, H.L.; Hullegie, L.M.; Hoogenberg, K.; Sluiter, W.J.; Dullaart, R.P.F.; Wientjes, K.J.; Schoonen, A.J.M., Microdialysis measurement of glucose in subcutaneous adipose tissue up to three weeks in Type 1 diabetic patients. *Neth. J. Med.* **2000,** 57, (1), 7-12.

Chapter 6

Summary and Perspectives

The development and application of continuous glucose monitors (CGM) constitutes a major field in diabetes-related research. The main goal of continuous glucose monitoring is to provide better insight into the daily glucose fluctuations experienced by diabetic patients to help them achieve better management of their glucose levels. The existing conventional ways of discontinuous glucose monitoring, such as finger-prick and HbA_{1c} level tests, are insufficient for detection of hyperglycemic and, especially important, hypoglycemic episodes.[1] Various techniques have been investigated for the development of CGMs for diabetic patients, involving both invasive and noninvasive methods. Even though noninvasive approaches based on optical or transdermal sensing mechanisms are appealing because of their user-friendliness and convenience, they are still underdeveloped in terms of accuracy and exhibit insufficient precision for clinical use.[2] Invasive subcutaneous glucose monitoring methods using needle-type or microdialysis-based CGMs to determine glucose levels in the interstitial fluid have obtained promising results in clinical settings.[3, 4]

Chapter 1 presents a general introduction to glucose biosensing, continuous glucose monitoring and microfluidics, and describes the scope of this thesis. The aim of this research is to demonstrate the development of a continuous glucose monitor combining microdialysis sampling and microfluidics to achieve the further miniaturization of existing systems. Our approach to continuous glucose monitoring distinguishes itself in three ways. First, we have adopted microdialysis for sampling glucose in the interstitial fluid, a technique which has proven to be biocompatible and safe for use in both animals and humans.[5] Glucose diffuses over the microdialysis membrane and is transported to an extracorporeal sensor, where glucose oxidase facilitates the conversion of glucose to gluconic acid and H_2O_2. Second, we have taken a cue from an existing subcutaneous continuous glucose monitoring system (SCGM), in which glucose detection is based on the reaction in solution of glucose oxidase (GOx) and glucose to produce H_2O_2.[6] The main advantage of a solution-based enzymatic reaction is that fresh enzyme can be provided for each determination, circumventing the slow loss of reactivity over time to which immobilized GOx is prone. An added benefit is therefore the reduced requirement for calibration of the solution-based system compared to approaches employing (micro)electrodes with immobilized GOx, since there is no need to compensate for signal drift. However, a system based on microdialysis and solution-based assay requires additional components such as a pump and tubing, in addition to a detector. Therefore, the third distinguishing feature of the system presented in this thesis is that it uses microfluidics technology to reduce system bulk and

complexity resulting from the necessity of having flow. The microfluidic system allows for a substantially reduced consumption of chemical reagents and short reaction times. This system represents a step forward towards a more fully miniaturized, portable monitoring system.

In order to better understand the issues involved in continuous glucose monitoring, we review in **Chapter 2** the technical requirements that a CGM has to satisfy and the physiological influences affecting accuracy, selectivity, sensitivity and durability. The review focuses on invasive glucose monitors based on either implantable needle-type sensors or microdialysis. It describes the physiological factors affecting the performance of these CGMs, including the sources for differences between interstitial fluid and blood glucose concentrations, implantation effects and location of the CGM. Though the studies of currently available CGMs showed clinically acceptable results, their performance is still sub-optimal and awaiting technical improvements. In this chapter, we also present our approach using a microfluidic enzymatic reactor with integrated electrochemical detector coupled to microdialysis sampling, which we envision could contribute to the further development of microCGM.

The development of a microfluidic chip is described in Chapters 3 and 4. In **Chapter 3**, we demonstrate an enzymatic glucose reactor based on chaotic mixing in a microfluidic channel network for continuous glucose monitoring. An on-chip amperometric detector consisting of a thin-film Pt electrode was located at the end of the microreactor channel to detect the reaction product, H_2O_2. Chaotic mixing was achieved through the integration of an array of slanted microgrooves in the upper surface of the microchannel. The mixing characteristics of this device were compared to microchannels containing no microgroove arrays. A linear calibration curve for glucose in the clinical range of interest was obtained in both microchannels with slanted grooves and no grooves, using a solution-based enzymatic reaction. However, higher sensitivity was obtained in slanted-grooved micromixers compared to no-grooved micromixers, due to enhanced mixing in the former device. The flow rates used were selected to reduce solution consumption and because of their compatibility with microdialysis .

A microfluidic system incorporating chemiluminescence (CL) detection as an alternative approach for glucose sensing is presented in **Chapter 4**. With a two-reactor system, glucose is converted into a CL output signal detected by a silicon photodiode embedded in the chip. The fabricated CL microfluidic chip was characterized and reaction conditions optimized e.g. flow rate, pH, and concentrations of luminol and catalyst. The main advantage of this optical system is

that the silicon photodiode is not directly exposed to the sample or chemical reagents. This is in contrast to systems using subcutaneously implanted microelectrodes in direct contact with interstitial fluid, which are subject to biofouling, and as a result, signal drift. The system exhibited a linear calibration curve for glucose concentrations between 2 mM and 10 mM. With these results, it could be concluded that this new CL-based microfluidic system is promising for sensing glucose. Further investigation of how compounds in complex biological samples could adversely affect CL detection is required. It would also be of interest to use this alternative approach for *in vivo* glucose monitoring. Furthermore, it would be interesting to integrate both electrochemical and CL detection onto a single device to improve analytical function. Amperometric detection of H_2O_2 is simple and robust from an instrumental point of view, but is subject to signal drift over time. Because the photodiode is insensitive to drift, periodic CL analysis of a g lucose standard could be used to calibrate microelectrode response to correct for drift, thus extending the period of time between required replacements of the microfluidic/amperometric detection component.

Chapter 5 demonstrated an *in vivo* application of the enzymatic microreactor (EMR) with integrated amperometric detection for continuous glucose monitoring. Introduced in Chapter 3, the EMR was further developed and coupled with a microdialysis probe which was inserted into the subcutaneous tissue of anesthetized rats. A polypyrrole (PPy)-coated microelectrode was studied with respect to its ability to withstand interaction with biologically interfering substances. The recovery of the selected microdialysis probe was also evaluated *in vitro*. The EMR coupled with microdialysis exhibited a linear calibration curve for measured current as a function of glucose concentration in the clinical range of interest when the probe was immersed in glucose solutions of different concentrations. The *in vivo* performance of the EMR was assessed by implanting the microdialysis probe in an anesthetized rat and modulating blood glucose concentrations with saline, glucose and insulin injections. Recorded glucose levels increased and decreased as expected with the intravenous injection of glucose and insulin, respectively. Furthermore, values measured with the microCGM correlated well with blood glucose values measured concurrently. With this newly-designed glucose sensing system, we concluded that it is feasible to use the microfluidic EMR chip coupled to microdialysis for continuous subcutaneous measurement of glucose in rats.

In the research leading up to this thesis, a new sensing system for continuous glucose monitoring based on an alternative microfluidic approach for glucose sensing has been developed. Two different types of sensing systems were realized

based on amperometric and CL detection of the hydrogen peroxide produced in the GOx-catalyzed reaction of glucose with oxygen. Both systems employed microreactors incorporating chaotic mixing channels. One future R & D goal should focus on the further miniaturization of the microfluidic system to achieve a true μ-SCGM. The microfluidic EMR is a continuous flow system characterized by low fluid consumption, offering a promising alternative for glucose sensing. On-going research is required to scale down the system and integration of the system parts. An important consideration in this regard is the compatibility of all the system components with respect to materials and operating parameters. In our enzymatic reactor, we successfully developed an on-chip detector as a first step. The new compact design and realization of pumps, control valves, fluidic reservoirs, and electronics will also be important aspects to consider when pursuing the goal of business-card size devices.[7]

Another interesting bioanalytical aspect to explore might be to use the same principle of solution-based enzyme assay on the microscale for the analysis of other analytes of interest, such as lactate, ethanol, and urea. The switch to another enzyme is easily accomplished, since the enzyme is simply introduced to the EMR as a solution via one the device inlets. This is in contrast to approaches utilizing surface-immobilized enzymes, which require an extra immobilization step that generally needs to be modified for each new enzyme applied. The adjustment of microfluidic channel length allows the mixing/reaction time (analyte sensitivity) to be easily tuned for different applications. Multiple reaction channels are also possible to detect different substrates simultaneously.

To gain more insight in physiological and pharmacological studies of glucose concentration fluctuations under different challenges, it will be important to be able to monitor glucose in freely moving rats instead of anesthetized rats. Animals that are awake provide a better representation of physiological glucose delivery than anesthetized rats, which should ultimately also lead to a better understanding of the complexities associated with glucose metabolism. Anesthesia influences metabolic conditions and glucose consumption in the tissue/cells during pharmacological challenges. Studies with freely moving rats will also provide a better means of investigating “the push-pull theory” as a possible explanation for observed differences in blood and ISF glucose levels, and rates of change in these levels, during periods of increasing and decreasing blood glucose levels.[8]

Ultimately, it is very desirable to link continuous glucose monitoring to an insulin delivery system to achieve the so-called “artificial pancreas”. Insulin delivery in such a system would be controlled by feed-back information delivered by a

continuous glucose monitor. Alongside further development of highly accurate and reliable CGM, other components such as an insulin pump and control algorithms are required.[9] An insulin pump plays an important role in the delivery of fast-acting insulin. The control algorithms ensure the adequate delivery of insulin based on information from the CGM. To complete the ultimate goal of a fully automated system, the challenges of variable insulin absorption after administration and system configuration need to be addressed, and adequate control algorithms developed.

References

1. Davis, S.; Alonso, M.D., Hypoglycemia as a barrier to glycemic control. *J. Diabetes Complicat.* **2004,** 18, (1), 60-68.
2. Wentholt, I.M.E.; Hoekstra, J.B.L.; Zwart, A.; DeVries, J.H., Pendra goes Dutch: lessons for the CE mark in Europe. *Diabetologia* **2005,** 48, (6), 1055-1058.
3. Cox, M., An overview of continuous glucose monitoring systems. *J. Pediatr. Health Care* **2009,** 23, (5), 344-347.
4. Oliver, N.S.; Toumazou, C.; Cass, A.E.G.; Johnston, D.G., Glucose sensors: a review of current and emerging technology. *Diabet. Med.* **2009,** 26, (3), 197-210.
5. Kapitza, C.; Lodwig, V.; Obermaier, K.; Wientjes, K.J.C.; Hoogenberg, K.; Jungheim, K.; Heinemann, L., Continuous glucose monitoring: reliable measurements for up to 4 days with the SCGM1 System. *Diabetes Technol. Ther.* **2003,** 5, (4), 609-614.
6. Schoemaker, M.; Andreis, E.; Roper, J.; Kotulla, R.; Lodwig, V.; Obermaier, K.; Stephan, P.; Reuschling, W.; Rutschmann, M.; Schwaninger, R.; Wittmann, U.; Rinne, H.; Kontschieder, H.; Strohmeier, W., The SCGM1 System: subcutaneous continuous glucose monitoring based on microdialysis technique. *Diabetes Technol. Ther.* **2003,** 5, (4), 599-608.
7. Kinoshita, H.; Aoki, K.; Yanagisawa, I.; Fujii, T., Miniaturization of integrated microfludic systems. *Proceedings of microTAS 2010* **2010**, 1397-1399.
8. Aussedat, B.; Dupire-Angel, M.; Gifford, R.; Klein, J.C.; Wilson, G.S.; Reach, G., Interstitial glucose concentration and glycemia: implications for continuous subcutaneous glucose monitoring. *Am. J. Physiol. Endocrinol. Metab.* **2000,** 278, (4), E716-728.
9. Hovorka, R., Closed-loop insulin delivery: from bench to clinical practice. *Nat. Rev. Endocrinol.* **2011,** 7, (7), 385-395.

Appendix

Samenvatting

De ontwikkeling en toepassing van continue metende glucosesensoren zijn een belangrijke tak in diabetes gerelateerd onderzoek. Het voornaamste doel van het continue meten van glucose is om betere informatie te genereren en meer inzicht te verkrijgen in de dagelijkse fluctuaties van de glucosegehaltes bij diabetespatiënten, en op deze manier te helpen om gezonde glucosegehaltes te kunnen handhaven. De bestaande methods van niet-continue glucosemetingen zoals de vingerprik en HbA1c-gehalte test zijn onvoldoende om bij patiënten voorvallen van hypoglykemie en hyperglykemie afdoende te detecteren. Om doorlopende glucose meetapparaten te ontwikkelen voor diabetespatiënten zijn in het recente verleden verschillende methods uitgeprobeerd, zowel (zo min mogelijk) invasief als volledig niet-invasief. De niet-invasieve methoden zijn echter nog lang niet ver genoeg ontwikkeld om alle problemen aan te kunnen. Onderhuidse glucosesensoren zijn de meest veelbelovende methode voor toekomstige klinische toepassingen. De mogelijkheid van mobiel gebruik, stabiliteit op lange termijn, en verhoogde nauwkeurigheid zijn de belangrijkste succesfactoren bij de verdere ontwikkeling van onderhuidse glucosesensoren.

In **Hoofdstuk 1** wordt het bereik van dit proefschrift beschreven samen met een algemene inleiding tot biosensoren en microvloeistoffenleer. Het doel van dit onderzoek is om een nieuw type te ontwikkelen van een continue metende glucosesensor gebasseerd op een alternatieve methode dat gebruik maakt van de microvloeistoffenleer. Er zijn drie factoren in het bijzonder waarmee onze aanpak zich onderscheidt van bestaande systemen voor continue glucosesensoren. Ten eerste hebben wij een aangepaste microdialysetechniek gebruikt dat in een andere context reeds als veilig wordt erkend. Glucose dat vanuit het lichaam door de porien van een microdialysemembraan diffundeert wordt daarna getransporteerd naar het sensorische gedeelte van het meetapparaat alwaar de analytische reactie plaatsvindt. In tegenstelling tot gangbare in vivo methoden wordt door deze opzet het agressieve reactieproduct waterstofperoxide buiten het lichaam geproduceerd en ook volledig buiten het lichaam gehouden. Ten tweede hebben wij gebruik gemaakt van een onderhuidse continue glucosesensor (SCGM) waarin de analytische reactie wordt gekatalyseerd door glucose oxidase (GOx) dat zich vrij in oplossing bevindt. Dit in tegenstelling tot bestaande systemen waarbij GOx op een vaste drager wordt geïmmobiliseerd. Het grootste voordeel van een continue aanvoer van katalyserend enzym in oplossing is dat enzymactiviteit veel hoger is. Bovendien wordt voordurend vers GOx aangevoerd. Eén van de positieve gevolgen hiervan is dat er

minder calibraties nodig zijn vergeleken met een electrode waarop GOx is geïmmobiliseerd. Echter, het gebruik van microdialyse en een op opgelost GOx gebasseerde aanpak vereisen allebei aanvullende componenten zoals een minipomp, slangetjessysteem en een detector. Om deze nadelen te verhelpen gebruiken wij de alternatieve aanpak uit de microvloeistoffenleer. Het microfluidische system maakt het mogelijk om minder chemische stoffen te gebruiken en een snellere reactietijd te verkrijgen in een heel klein microfluidisch kanaaltje. Op deze manier zou het in de nabije toekomst mogelijk kunnen zijn om een geminiaturiseerde, compacte configuratie te bouwen met volledig geïntegreerde componenten.

Om de klinische relevantie van continue glucosesensoren te beschouwen en daarmee de richting van dit onderzoek vast te stellen hebben we in **Hoofdstuk 2** de recente vooruitgangen besproken op het gebied van continue glucosesensoren (CGMs) en de technische uitdagingen die hierbij nog overwonnen moeten worden. Met name (zo min mogelijk) invasieve glucosesensoren, waaronder naaldgelijkende glucosesensoren en op microdialyse gebasseerde systemen worden in dit hoofdstuk besproken. Bovendien hebben wij uitgebreid literatuuronderzoek gedaan naar een aantal factoren die wellicht zorgen voor verschillen tussen de glucosegehaltes in de interstitiële weefselvloeistof (ISF) en die in het bloed. Deze factoren zijn onder andere: implantatie effecten; bioverontreiniging; bloedglucose versus weefselvloeistof gehaltes en de positie van geimplanteerde stoffen.

Hoewel uit het literatuuronderzoek is gebleken dat de recente studies naar verkrijgbare CGMs resultaten lieten zien die in de kliniek aanvaardbaar zijn, zijn hun prestaties nog sub-optimaal en vereisen de huidige beperkingen dat ze in technisch opzicht nog aanzienlijk worden verbeterd. Wij stelden een veelbelovende aanpak voor die gebruik maakt van zowel een enzymatische minireactor en een electrochemische detector, beide geïntegreerd op e en microfluidische chip welke zijn aanvoer krijgt door middel van microdialyse.

De ontwikkeling van een microfluidische chip wordt besproken in **Hoofdstuk 3-4**. In **Hoofstuk 3** hebben we de werking van een enzymatische glucose reactor aangetoond dat gebasseerd is op chaotische menging in een microfluidisch kanalennetwerk al s essentieel onderdeel van een continue glucosesensor. Om het reactieproduct waterstofperoxide te meten werd een electrode (gemaakt van een dun laagje platina) aan het einde van het microfluidische kanaaltje gebruikt als amperometrische detector op de chip. In het voor klinische doeleinden interessante bereik werd een lineaire calibratiecurve voor glucose verkregen voor zowel het microkanaaltje met groeven als het kanaaltje met gladde wanden. Dit werd gedaan door gebruik te maken van een colloidale enzymatische reactie. Micromixers

voorzien van een schuin groevenpatroon hadden een hogere gevoeligheid vergeleken met micromixers zonder groeven, vanwege de betere dooreenmenging van de vloeistofstroom. Een aanzienlijke reductie van de hoeveelheid gebruikte reagentia is mogelijk dankzij de combinatie van optimale doorstroomsnelheid, efficiente menging, en monsteraanvoer door middel van microdialyse. In **Hoofdstuk 4** hebben we een alternatieve analytische techniek ontwikkeld waarmee glucosegehaltes gemeten kunnen worden, namelijk een microfluidisch systeem met detectie door middel vanchemoluminescentie (CL). Ten opzichte van de gangbare electrochemische detectie hopen we hiermee de specificiteit van het glucosesignaal te vergroten en de interferentie door andere stoffen in het lichaam te verminderen. In een systeem met twee reactoren wordt glucose door een silicium photodiode omgezet in een CL output signaal.. We hebben zelf microfluidische chips gebouwd voor CL detectie. Deze werden gekarakteriseerd en de reactieparameters, zoals doorstroomsnelheid, pH en concentraties van luminol en katalysator werden geoptimaliseerd. Het aldus aangepaste systeem genereerde een lineaire calibratiecurve voor glucose concentraties tussen 2 en 10 mM. Het grootste voordeel van dit optische systeem is dat de detector niet direct aan de chemische reactanten wordt blootgesteld. Het is een robust systeem vergeleken met de veelgebruikte electrochemische detectie waarin de naaldgelijkende electrode erg gehinderd wordt door bioverontreiniging en signaaldrift. Uit onze resultaten hebben wij geconcludeerd dat dit nieuwe CL microfluidisch systeem geschikt te maken is als toekomstige glucosesensor. Als verdere onderzoek is het wellicht interessant om een studie te doen naar verstoringen veroorzaakt door complexe biologische monsters en de mogelijkheid tot eleminatie van die verstoringen in diverse biomedische toepassingen. Met het oog op de twee onderzochte meetmethodes (hoofdstuk 3 en 4) zou het daarnaast interessant zijn om de beste eigenschappen van beide meettechnieken in één system te combineren om zowel goede nauwkeurigheid als lange levensduur te verkrijgen, aangezien de electrochemisch methode heel gevoelig is en optische detectie meer geschikt is voor stabiliteit op de langere termijn.

Als concrete toepassing van een microfluidisch glucose meetsysteemlaten we in **Hoofdstuk 5** zien dat een continue en nauwkeurige registratie van glucose mogelijk is met een enzymatische microreactor (EMR) in een in vivo opstelling. Met Hoofdstuk 3 als uitgangspunt werd de EMR verder ontwikkeld en gekoppeld aan een microdialysesensor die in het onderhuidse weefsel van een rat (onder anesthesie) werd geplaatst. Een detector met een laagje polypyrrole (PPy) werd gebruikt voor het weren van interfererende biologisch stoffen. De EMR gekoppeld met microdialyse vertoonde een goede correlatie tussen het continue meetsignaal en de

concentraties van glucose in het bloed in het klinisch interessante bereik. De in vivo prestatie van de EMR werd uitgevoerd door bloed glucosegehaltes te moduleren met intraveneuze injecties van glucose (glucose omhoog), insuline (glucose omlaag) en een fysiologische zoutoplossing (controle; glucose blijft onveranderd). De snelle en nauwkeurige registratie van dynamische veranderingen van glucose gehaltes door de EMR waren veelbelovend. Wij zijn van mening dat het zeer de moeite waard is om verdere onderzoek te doen naar continue glucosesensoren door gebruik te maken van microfluidische systemen. Met deze studie naar de haalbaarheid van een dergelijke systeem hebben we aangetoond dat het mogelijk is om veranderingen in onderhuidse glucosegehaltes nauwkeurig waar te nemen onder praktische in vivo omstandigheden. Met een pas-ontworpen microfluidisch glucose sensor hebben wij geconcludeerd dat het haalbaar is om op een microfluidische chip microdialyse te koppelen aan het doorlopende metingen van onderhuidse glucose in ratten.

Als alternatieve microfluidisch methode hebben we een nieuw sensorisch systeem ontwikkeld voor de doorlopende glucosesensor. Twee verschillende meetsysteemtypen werden gerealiseerd, één met electrochemische en één met een chemoluminiscentiemethode. Beide maken gebruik van chaotisch mengende microkanaaltjes. De prestaties van het uiteindelijke systeem waren uitstekend voor in vitro doeleinden. Voor de in vivo toepassing werd het microfluisdisch systeem gekoppeld met microdialyse en meette continue de onderhuidse glucoseconcentraties in een rat. Een voor de hand liggende uitbreiding voor dit onderzoek is verdere verkleining van het microfluidisch systeem naar micro SCGM. Aangezien het gebruik van een microfluidisch systeem ons in staat stelt een continue doorstroming te meten met slechts een klein vloeistofverbruik biedt een dergelijk systeem veel mogelijkheden. Aanvullend onderzoek zal er op gericht zijn om dit syteem te verkleinen en de verschillende onderdelen nog beter met elkaar te integreren. Dit moet opgevolgd worden met vooruitgang in zowel de technische aspecten van het systeem als de compatibiliteit van alle onderdelen. Hiertoe hebben we als eerste stap, en met succes, met onze enzymatische reactor een detector op een chip ontwikkeld. Het nieuwe compacte ontwerp en het realiseren van van een minipomp, controle ventiel, microfluidische reservoirs, werkbare accu en electronica vormt een verdere uitdaging in het streven naar een apparaat op bankpasformaat.

Een andere interessante onderzoeksvraag gebasseerd op een microfluisdisch systeem is het gebruik van andere enzymen dan glucose oxidase in een oplossing van interessante analieten. Het is mogelijk om, naast glucosegehaltes, de concentraties van andere stoffen te analyseren door simpelweg andere oxidsase-enzymen in een inlaatkanaaltje in te brengen. Het gebruik van opgeloste enzymen

zorgt voor snelle omzetting van reactieproducten vergeleken met geïmmobiliseerde enzymen, en een hogere gevoeligheid en snelle reactie kunnen behaald worden in een klein mengkanaaltje. Door de lengte van het microfluidisch kanaaltje aan te passen kan men de meng/reactie tijd (analietgevoeligheid) veranderen voor andere toepassingen. Meerdere reactiekanalen zouden verschillende substraten tegelijkertijd kunnen meten.

Om meer inzicht te verkrijgen in de fysiologische en farmacologische aspecten van het systeem en haar mogelijke toepassingen is het belangrijk om verder onderzoek te doen in vrijbewegende ratten, en uiteindelijk ook in mensen (zowel gezonde proefpersonen als patiënten met diabetes). Vrijbewegende proefdieren zijn een beter model voor de mens omdat anethesie het glucosemetabolisme op een complexe manier beïnvloedt. Onderzoek in vrijbewegende ratten zal ook meer inzicht opleveren in de gepostuleerde push-pull theorie over de dynamische verschillen in glucose concentraties tussen het bloed en de interstitiële weefselvloeistof.

De ultieme geavanceerde technische toepassing is een 'kunstmatige alvleesklier' dat zelf voortdurend de glucoseconcentraties meet en zonodig precies de juiste hoeveelheid insuline toedient. Een dergelijke toepassing maakt doorlopende metingen van glucose noodzakelijk, en voor het hierbovengenoemd gesloten systeem is de ontwikkeling van CGM dan ook een vereiste eerste stap. Naast verdere ontwikkeling van zeer nauwkeurige en betrouwbare CGMs moeten andere onderdelen zoals een insulinepomp en controlealgoritmes nog verder ontwikkeld worden. Een insulinepomp speelt een belangrijke rol om gemakkelijk en geleidelijk insuline toe te dienen, op een manier die heel erg lijkt op een gezonde pancreas. De controlealgoritmen zorgen ervoor dat een passende hoeveelheid insuline toegedient wordt afhankelijk van de informatie verkregen uit de CGM. Daarbij spelen ook patiëntgebonden, en nog altijd slecht begrepen, factoren een belangrijke rol. De combinatie van zowel continue glucosemetingen als een goede toediening van insuline is belangrijk voor de verbetering van een goede glucosehuishouding bij patiënten. De uitdaging van een volledig geautomatiseerde systeem, de aanpak van variabele insuline absorptie na toediening, verbeterde systeem configuratie en goede algorithmen vormen belangrijke onderdelen voor een vervolgonderzoek. De uitdagingen van een volledig geautomatiseerd systeem, waarbij nauwkeurige en continue glucosemetingen de input vormen voor een adaptief regulerend systeem dat ook nog eens op e en prettige manier door patiënten te gebruiken is, vormen een mooie richtlijn voor een volgend promotieonderzoek.

List of abbreviations

BG: Blood glucose
CAD: Computer-aided design
CCD: Charge coupled device
CE: Counter electrode
CG-EGA: Continuous glucose-error grid analysis
CGM: Continuous glucose monitor
CGMS: Continuous Glucose Monitoring System
CL: Chemiluminescence
EGA: Error Grid Analysis
EMR: Enzymatic microreactor
FDA: Food and drug administration
FIA: Flow injection system
FRET: Fluorescence resonance energy transfer
GOx: Glucose oxidase
HbA_{1c}: Glycated hemoglobin
HMDS: Hexamethyldisilazane
HRP: Horseradish peroxidase
IACUC: Institutional Animal Care and Use Committee
IDDM: Insulin-dependent diabetes mellitus
ISE: Ion-selective electrodes
ISF: Interstitial fluid
ISFET: Ion-selective field-effect transistor
KI: Potassium iodide
L/I: Luminol and 4-iodophenol
LADA: Latent autoimmune diabetes in adults
LIF: Laser-induced fluorescence
MCE: Microchip capillary electrophoresis
MEMS: Microelectromechanical systems
μAPD: Microavalanche photodiode
μTAS: Miniaturized total analysis systems
MIR: Mid-infrared
MODY: Maturity onset diabetes of the young
MWCO: Molecular weight cut-off
NIDDM: Non-insulin-dependent diabetes mellitus
NIR: Near infrared

PBS: Phosphate-buffered saline solution
PDMS: Polydimethylsiloxane
PMT: Photomultiplier tube
PPD: Polyphenylenediamine
PPy: Polypyrrole
R: Recovery
Re: Reynolds number
RE: Reference electrode
SCGM: Subcutaneous glucose monitoring system
SD: Standard deviation
SMBG: Self-monitoring blood glucose
SPR: Surface plasmon resonance
UV: Ultraviolet
WE: Working electrode
WHO: World health organization

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List of publications

Full papers

B.-U. Moon, S. Koster, K.J.C. Wientjes, R.M. Kwapiszewski, A.J.M. Schoonen, B.H.C. Westerink, E. V erpoorte, "An enzymatic microreactor based on chaotic micromixing for enhanced amperometric detection in a continuous glucose monitoring application" *Analytical Chemistry* **2010**, *82*, 6756-6763.

B.-U. Moon, J. Droge, E. Verpoorte, B.H.C. Westerink, M.G. de Vries, "Physiological processes influence continuous monitoring of subcutaneous glucose: A review" in preparation.

B.-U. Moon, M.G. de Vries, B.H.C. Westerink, E. Verpoorte, "Development and characterization of a microfluidic glucose sensing system based on a n enzymatic microreactor and chemiluminescence detection" in preparation.

B.-U. Moon, M.G. de Vries, C. A. Cordeiro, B.H.C. Westerink, E. Verpoorte, "In vivo application of an enzymatic microreactor coupled with microdialysis for continuous monitoring of subcutaneous glucose in rats" in preparation.

B.-U. Moon, J.-M. Lee, C.-H. Shim, M.-B. Lee, J.-H. Lee, D.-D. Lee, J.-H. Lee, "Silicon bridge type micro-gas sensor array" *Sensors and Actuators B: Chemical*, **2005**, 108, 271-277.

J.-M. Lee, **B.-U. Moon**, C.-H. Shim, B.-C. Kim, M.-B. Lee, D.-D. Lee, J.-H. Lee, "H_2S microgas sensor fabricated by thermal oxidation of Cu/Sn double layer" *Sensors and Actuators B: Chemical*, **2005**, 108, 84-88.

Oral presentations

B.-U. Moon, M.G. de Vries, C.A. Cordeiro, A.J.M. Schoonen, B.H.C. Westerink, E. Verpoorte, "Application of an enzymatic microreactor coupled with microdialysis for continuous monitoring of subcutaneous glucose in rats" *MicroTAS 2010 conference*, October 4-7, **2010**, Groningen, The Netherlands.

B.-U. Moon, A.J.M. Schoonen, B.H.C. Westerink, E. V erpoorte, "Development of microreactors for continuous glucose monitoring" *Annual meeting of NWO/CW*

studiegroep Analytische Scheikunde, November 2-3, **2009**, Lunteren, The Netherlands.

B.-U. Moon, K.J.C.Wientjes, A.J.M.Schoonen, "Microdialysis glucose sensor system compared with needle type glucose sensor in vivo during OGTT and physical exercise**"** The *5th IEEE Conference* on *Sensors,* October 22-25, **2006**, Daegu, Korea.

B.-U. Moon, J.-M. Lee, C.-H. Shim, M.-B. Lee, J.-H. Lee, D.-D. Lee, J.-H. Lee, "Silicon bridge type micro-gas sensor array" The *10th International Meeting on Chemical Sensors***,** July 11-14, **2004**, Tsukuba, Japan.

Poster presentations

B.-U. Moon, A.J.M. Schoonen, B.H.C. Westerink, E. Verpoorte, "Development of microreactors for continuous glucose monitoring" *The 5th International Conference on Microtechnologies in Medicine and Biology*, April 1-9, **2009**, Quebec City, Canada.

B.-U. Moon, A. J. M. Schoonen, B. H. C. Westerink, and E. Verpoorte, "An enzymatic microreactor for continuous glucose monitoring" *MicroTAS 2008 conference*, October 12-16, **2008**, San Diego, USA.

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