Design and development of a miniaturised flow-through measuring device for the in vivo monitoring of glucose and lactate

Rhemrev-Boom, Maria Martha

RIJKSUNIVERSITEIT GRONINGEN

Design and development of a miniaturised flow-through measuring device for the *in vivo* monitoring of glucose and lactate

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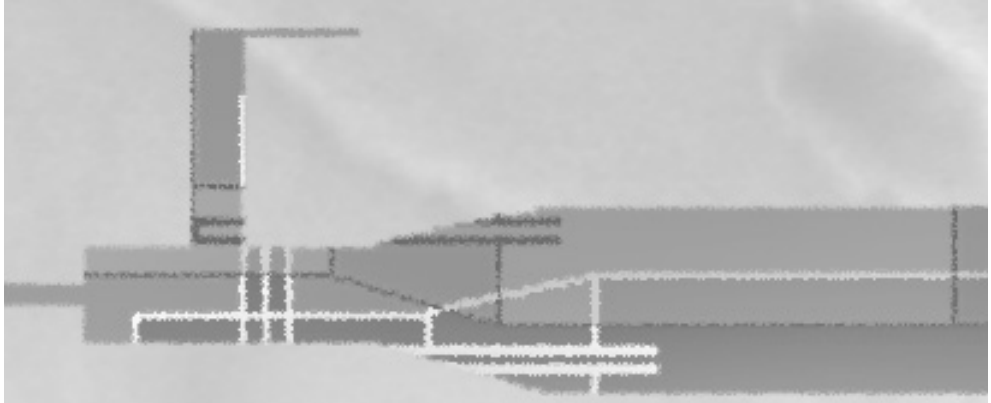

Chapter 1

Clinical motivation and scope of the thesis

1.1 Clinical motivation to monitor metabolites

The first diagnosis of a disease by measurement of a constituent in a body fluid took place in the Middle Ages. Diabetes mellitus was diagnosed by tasting the sweetness of urine[1] Glucose, one of the most important energy supplies in organisms, is normally not lost in urine, but taken up by cells and metabolized predominantly with oxygen to carbon dioxide and water (aerobic metabolism). In the absence of oxygen, glucose is metabolized to hydrogen and lactate ions (anaerobic metabolism). An interruption in the supply of energy to organs, such as the brain and the heart quickly develops life-threatening situations. Myocardial infarction and hypoglycemic coma are examples of such complications. Although complications like atherosclerosis and diabetic mellitus patients differ in many respects, both demonstrate the importance of a finely tuned uptake and release of metabolites in organisms. Apart from acute risk, this misbalance also carries long-term risks, such as the late complications from frequent high glucose levels in diabetes. Besides increased risk of heart disease or stroke, diabetes is a major cause of blindness, end stage renal disease and limb amputations. Based on results of extensive trials in lowering elevated blood glucose levels of diabetic patients, compared to standard treatment a significant reduction in microvascular complications was achieved[2-4]. However, tight blood glucose control with today's available methods is not easy to achieve. Besides intensive support from a health care team, the patient must be extremely motivated to continually manage his glucose levels by frequent finger pricking for the rest of the patient's life. While high blood glucose levels are often not felt by the patient, the consequent complications are usually years later. And although many attempts have been undertaken to monitor patients at risk, the necessary accuracy of current methods for self monitoring appears far from optimal[5,6]. Today, most parameters for the diagnosis of physiological abnormalities are measured batch-wise in discrete samples, whereas analysis takes place in the clinical laboratory. So, an important improvement for rapid clinical intervention or for a follow-up of the progression of a disease would be to apply analytical methods that can measure compounds continuously in body fluid without the need of laboratory handling procedures. For home-care monitoring of diabetic patients and/or other applications in which the mobility of the patient should be improved, the ultimate goal would be the availability of a portable real-time monitoring device, which can continuously monitor body fluid parameters. Not only is the quality of life of the patient improved, also a better control is achieved and, in addition, more information is gathered regarding the fluctuations over time.

The use of on-line continuous *in vivo* monitoring devices can also be motivated for

myocardial infarction. Due to artherosclerosis, arteries narrow and progressively diminish the blood stream and finally the supply energy to the myocardial tissue. For one-third of every heart attack victims, no chest pain is felt and electrocardiogram changes may be absent in the early hours. Routinely used markers, such as troponin and creatine kinase, are released after cell death and are present only after several hours and can therefore not be used to prevent an infarction[7]. To conclude, it is difficult to identify patients at risk for myocardial infarction with current diagnostics. However, it is well known that in the early stage of ischemia, poorly perfused tissue shifts from aerobic to anaerobic metabolism, which, among other things, is followed by an increased lactate production. Lactate production occurs within minutes between the onset and end of myocardial ischemia and a quantitative relation has been found between the extent of ischemia in the myocard and the release of lactate[8,9]. For this reason, a lactate monitoring technique could be an interesting alternative and may contribute to control patients at cardiac risk. In addition, also other critically ill patients may benefit from lactate monitoring because high lactate levels have a strong prognostic value in critical care units as it may predict organ failure[10]. Examples are trauma patients, patients with head injury or cerebral inschemia[11,12], acute intestinal ischemia[13], liver ischemia[14], transplanted organ surveillance[15] and patients with septic shock[16].

Other interesting applications for continuous *in vivo* monitoring devices include sports medicine[17-19] and hormonal diseases[20]. In the field of biotechnology, continuous monitoring devices could be used for fermentation control by following fluctuations in the nutrients and adjusting their levels when necessary[21]. In environmental technology, micro-contaminants can be analyzed and monitored more quickly and cheaper than current methods[22]. Applications in food technology can be found in the quick and cheap analysis of various nutrients to determine the quality and/or freshness of the food[23] and to determine the contamination by microorganisms[24] or the presence of prohibited compounds[25]. Additionally, continuous *in vivo* monitoring devices can also be important tool in biomedical research (see chapter 9 this thesis).

In summary, on-line continuous *in vivo* monitoring of compounds, such as glucose and lactate, in body fluids may be of great help in clinical diagnostics. Besides an improvement of the quality of life for large patients groups, these monitoring devices could be of great importance to prevent further damage. Thanks to the quick availability of data and relatively low price per analysis related to low costs, applications in other fields may benefit from this technology.

1.2 Requirements device for continuous *in vivo* monitoring

For continuous *in vivo* monitoring, the long-term reliability of the device without the necessity of frequent blood sampling for calibration is essential. For instance, the current standard for frequent self-monitoring of blood glucose (SMBG) of diabetic patients is by finger pricking. Only if the new device demonstrates a significant improvement over the current SMBG with respect to compliance (less pain, automation), reliability and frequency of the measurement and improved life quality and expectancy, it will be accepted for clinical use. In order to allow continuous monitoring of compounds *in vivo*, a constant contact between the body and the measuring device is unavoidable. For the comfort of the patient, the most less invasive technique will be chosen at all time whenever possible. Due to this and to avoid unnecessary wound reactions, the diameter and size of subcutaneous and intravenous measuring devices, should to be as small as possible, whereas inflammation, infection and the risk of release of hazardous substances should be avoided at all times. In general, a device should have the following requirements for reliable functioning[26-27].

a. The device should be selective towards the analyte of interest. The ability to recognize the analyte in a complex medium is extremely important for the accuracy of the device;
b. The sensitivity and dynamic range of the device should be such that the analyte of interest can be analyzed in a reproducible manner in clinical relevant concentrations;
c. The response time of the device should be such that, in practice, the sensor quickly responds to changes in concentration and real-time measurements are obtained;
d. The device should be biocompatable to avoid body reaction caused by materials used in the construction of the device or substances produced by the device;
e. To cause minimal discomfort to the patient the device should be of a size and shape that can be easily inserted.
f. To prevent excessive calibration, the performance characteristics of device as meant above should essentially remain the same within the period of monitoring.
g. Useful for long-term applications (e.g. several months) to prevent frequent insertion and/or calibration, the stability of the device is an essential requirement.

Besides the above-mentioned requirements, a more practical requirement is that these devices can be produced in mass in a reproducible manner[28].

1.3 Technical motivation for the set-up of the *in vivo* monitoring device

For the continuous monitoring of analytes *in vivo*, by means, (bio)sensors have the most potential[29,30]. In general, sensors can be, depending on the interaction with the patient's body, characterized as non-invasive or invasive[26]. Non-invasive sensors obtain information by using the physical and/or chemical properties of the analyte of interest without mechanical intervention. For instance the near-infrared (NIR) spectrum of glucose has been proposed for non-invasive monitoring of glucose[31]. NIR-measurements are usually performed at places on the body that are relatively well circulated with blood, such as the tips of fingers, earlobes, inner lip or oral mucosa. Although a number of commercial devices (Dream-beam®, Diasensor® and Glucocontrol®) have been introduced based on this principle, no or limited scientific *in vivo* studies have been reported. Probably due to variations in sweat, changes in the local blood circulation and/or interference from other body compounds[32], the accuracy of these techniques have found to be only good within a set of well prepared ensemble of patients. A recent overview of optical glucose sensing, including optical absorption spectroscopy, polarimetry, Raman spectroscopy and fluorescent glucose sensing, has been presented by McNicols and Cote[31a].

In contrast to non-invasive sensors, (minimal) invasive (bio)sensors use techniques that have intimate mechanical contact with the biological tissue or fluids. (Minimal)-invasive methods measure analyte concentration in the interstitial fluid of the skin (transcutaneous) or in the subcutis (subcutaneous). An example of a transcutaneous device for the continuous monitoring of glucose is the use of reverse iontophoresis as sampling technique prior to on-line analysis by an enzyme-based sensor[33] (see reaction 1 and 2, this chapter). During iontophoresis charged molecules are driven vice versa across the skin. Via this way interstitial fluid together with small amounts of glucose are transferred across the skin. A commercial device, the Glucowatch®, based upon this technique has been developed by Cygnus Inc. A major disadvantage of this device is that the measurements are intermittent and no real-time concentrations are measured. A comparable method was developed by Ito and coworkers[34]. Instead of current they used suction of glucose through the skin from which the stratum corneum is removed. This procedure is often painful and carries a high risk of infection so it is not useful for long-term monitoring. Glucose was then directly measured by a standard Ion Selective Field Effective Transistor (ISFET) glucose sensor. An ISFET sensor is a miniaturised potentiometric sensor produced by microelectronic techniques and is capable to analyze glucose by measuring the change in local pH due to

the gluconic acid, which is produced during the enzymatic conversion of glucose (see reaction 2, this chapter). Although easy to fabricate, the sensitivity of this type of sensors is limited due to the small dissociation constant of gluconic acid. Additionally, due to interaction with the produced hydrogen peroxide the sensor demonstrated only a short functional operating time.

Although measurements in the blood circulation give more direct information about the status quo of the patient without delay, the majority of the continuous *in vivo* measurements are carried out in the subcutaneous tissue. Especially due to the high risk of thrombosis, embolism and septicaemia, sensing in the vascular compartment is avoided whenever possible and only just a few sensors have been described to operate intravenously[35,36]. Although the use of an anticoagulant, such as heparin, effectively diminishes this effect, for most patients, such as those suffering from diabetic mellitus, the less invasive subcutaneous device remains the preferred option. The subcutaneous tissue is regarded as the appropriate site of implantation because implantation of the sensor is relatively easy, and can be performed without anaesthetic or complicated surgical procedures[37], whereas long-term implantation belongs to the possibilities. The first concept of a subcutaneous biosensor for the measurement of glucose was reported by Clark and Lyons[38]. Since then, an impressive amount of literature on biosensor development has appeared and extensively reviewed (see also chapter 2). Today, sensor research is a mature and well-worked research field. The majority of sensors are based on electrochemical principles and employ enzymes as biological components for molecular recognition. In the enzymatic reaction, substrate is transformed into reaction products according to the following general reaction:

$$E + S \underset{}{\overset{k_{1/-1}}{\leftrightarrow}} ES \xrightarrow{k_2} E + \text{Product(s)}, \qquad [1]$$

In which E represents the enzyme, S the substrate, k_1, k_{-1} and k_2 the rate constants of the reaction. Substances liberated or consumed during the reaction are detected by a suitable transducer (see also chapter 2). For instance, most glucose sensors are based on the enzymatic oxidation of glucose by the enzyme glucose oxidase (GOx):

$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOx} \text{gluconolacton} + H_2O_2 \qquad [2]$$

Depending on the type of transducer, the hydrogen peroxide formed or the oxygen consumed is detected amperometrically. In case of H_2O_2, a platinum work electrode is used as anode and polarized to a positive potential of +600 mV vs Ag/AgCl:

$$\text{Anode: } H_2O_2 \xrightarrow{+600\ mV} O_2 + 2H^+ + 2e^- \qquad [3]$$

If O_2 is determined, a platinum work electrode is used as cathode and polarized to a negative potential of –600 mV vs. Ag/AgCl. Both reference and working electrode are immersed in a KCl/K_2HPO_4 solution. Electrodes and electrolyte are separated from the sample via an oxygen-permeable membrane (Clark cell):

$$\text{Cathode: } O_2 + 4e^- + 4H^+ \xrightarrow{+600\ mV} 2H_2O_2 \qquad [4]$$

Amperometric electrodes have a high sensitivity, which allows detection of electroactive substances as low as 10^{-9} M and a dynamic range of three to four order of magnitude. Regarding H_2O_2 electrode based sensors, an additional advantage is the relative ease of manufacturing and the possibility to construct them in small sizes. For this reason, most *in vivo* experiments in human subjects have been performed using the so-called needle-type glucose sensor inserted in the subcutaneous tissue[26, 39-45]. A commercial needle-type biosensor CGMS®, based on the work of Mastrototaro and coworkers[46], was recently introduced by the Minimed™. In all cases, the enzyme is immobilized at the surface of the electrode with specially fabricated membranes. The main functions of the membrane are to hold the enzyme at the electrode, to restrict access of interfering substances respectively to form an interface between the body and the device as well as to act as a diffusional barrier for the analyte of interest (see also chapter 2). In general, good in vitro results have been achieved with these sensors. However, upon implantation, these devices show a progressive loss in sensor performance that can be attributed to poor biocompatibility. Frequent re-calibration *in vivo* will be necessary to obtain accurate results. Although different design approaches have been proposed to overcome these problems and some progress has been made using special membranes or sensor construction, due to these inherent characteristics this sensor design may be limited to short-term applications only.
An interesting alternative for on-line continuous glucose monitoring in subcutaneous adipose tissue using open-flow micro-perfusion was developed by Trajanoski *et al.*[47]. In this

technique a double-lumen catheter with microholes is inserted and isotonic perfusion fluid is pumped through the inner cannula and returned via the perforated outer lumen. In the outer lumen the perfusate equilibrates with the subcutaneous tissue fluid, and a peroxide-based glucose sensor continuously measures glucose for up to 24 hours.
An alternative for the hydrogen-peroxide electrode is the combination of GOx immobilized onto an oxygen electrode[48,49]. In this case, the consumption of oxygen is measured (see reaction (2), this chapter). The advantages over the hydrogen peroxide-electrode are improved sensor stability and selectivity. Thanks to co-immobilized catalase, peroxide mediated inactivation of GOx is minimized, whereas detection takes place at –600 mV vs Ag/AgCl, which avoids electrochemical interference by oxidative interfering species normally present in biological fluids. Major disadvantages are that miniaturization to the same extent as peroxide-based electrodes is difficult respectively the applicability of the oxygen electrode is largely dependent on a constant endogenous oxygen concentration.

Basically due to biocompatibility and sensor stability related problems, many researchers have focused their attention on other approaches to measure analytes *in vivo*. Among these, sampling techniques have been introduced to improve sensor stability. Of the currently proposed techniques for *in vivo* monitoring, microdialysis (MD)[50-52] and ultrafiltration (UF)[53-55] are used as a sampling interface between the analytical system and the tissue. With these techniques continuous measurements of analytes *in vivo* are possible without the direct contact of the sensor and the body[56]. Thanks to the biocompatibility and size, these devices can be easily implanted into tissue with a relatively small needle with minimum foreign body reaction. Furthermore, large molecules and cells are excluded by the membrane and a relatively clean matrix is obtained for measurement.
In classical microdialysis (MD), as introduced by Ungerstedt *et al.*[50], a fluid flows through a hollow fiber and partially equilibrates with the surrounding tissue; the sample or dialysate thus obtained is withdrawn and the content of the analyte of interest is measured outside the body. Mostly, the perfusate is pushed into the MD probe and the dialysate is batch-wise collected in an open tube. Characteristic features of MD is the stability and repeatability of the MD probe in living tissue and long operational periods, which makes this technique suitable for long-term monitoring. The term relative recovery is used for the ratio of the concentration of a substance in the dialysate and the nominal *in vivo* concentration. In general, the relative recovery is inversely dependent on the flow rate because the samples are more dilute.
Since the introduction of microdialysis[50], many researches have combined traditional MD

with continuously monitoring of the analyte in the dialysate by a sensor[57-65]. However, frequently problems were recognized concerning the accurate determination of the *in vivo* concentration due to uncertainty of the relative recovery. To improve this, several methods have been introduced to enable the calculation of the nominal *in vivo* concentration (see chapter 2). These methods are usually accompanied with complicated calibration procedures, whereas neither method accounts for changes in recovery during sampling. A more straightforward method, however, is performing MD at extremely low flow rates. As reported earlier[66-69] with current MD devices near quantitative recovery is obtained at extremely low microdialysis flow rates (< 300 nl/min) as the sample is able to equilibrate with the interstitial fluid. To prevent loss of perfusion fluid through the membrane into the tissue, Rosdahl et al[70] added a colloid to the fluid. Kaptein et al[68] used a suction pump and obtained equilibrated samples at a microdialysis flow rate of 100 nl/min but without the need of a colloid. As an alternative to microdialysis Ash *et al.*[55] introduced ultrafiltration (UF) as an alternative method to microdialysis. In their method, tissue fluid is ultrafiltered through a hollow-fibre membrane by underpressure. Because many fibers were needed to collect sufficient amount of fluid, large needles are required. A more patient friendly method was introduced by Moscone et al[71], who used a miniaturised UF probe and introduced a light-weight disposable pump to suck ultrafiltrate from the body. However, in both cases, to enable real time continuous monitoring at these low flow rates, the construction (low volume connections and flow-through cell) of the device as well as the performance (sensitivity) of the sensing part of the device needs special attention. Currently, there are no sensors available, which, besides the requirements mentioned in chapter 1.2, meet the demands for continuous real-time *in vivo* monitoring at low flow rates.

In summary, despite considerable research efforts only limited sensors are available in clinical practise. At present, any non-invasive sensor system for glucose monitoring still requires frequent blood glucose measurements for re-calibration purposes. Various implantable (invasive) glucose sensors have been developed and introduced, but in general these sensors show a significant decay in sensor performance over the implantation period and are therefore limited for short-term applications. The use of MD or UF as an interface between the body and the sensing element of the device minimizes these problems associated with the bad biocompatibility of the sensor surface. However, because with traditional MD no absolute concentrations are measured and current *in vivo* calibration techniques are insufficient, sofrequent blood sampling is still required. To avoid excessive calibration procedures, the use of ultraslow MD or UF as a sample technique is promising.

With these techniques near quantitative recoveries can be achieved. A major disadvantage of this principle is the increased delay time between the nominal *in vivo* concentration and the concentration finally measured in the dialysate or ultrafiltrate. Obviously, to be able to reduce the delay time to an acceptable level, miniaturization of the device is of extremely importance.

1.4 Aims and scope of the thesis

The aim of this thesis was to develop a miniaturised device as schematically demonstrated in figure 1 for the continuous real time *in vivo* monitoring of a variety of analytes, such as

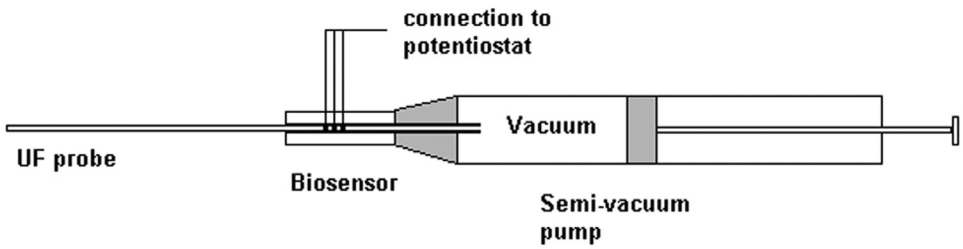

Figure 1:
Schematic demonstration of the miniaturised device for the continuous in vivo monitoring of analytes in vivo.

glucose and lactate. In general, the device should comprise a MD or UF probe, a miniaturised flow-through biosensor and a semi-vacuum pump. The use of MD or UF as a sampling technique prior to detection by a biosensor was motivated by the reported limited life time of most implanted biosensors. In this set-up it was decided that the performance of the device during *in vivo* monitoring should essentially remain the same for at least several days whereas no additional calibration steps should be necessary during that time. In order to avoid excessive calibration steps due to the application of conventional MD, ultraslow MD and UF was introduced. Accordingly near quantitative recoveries are achieved. To allow, however, real-time continuous *in vivo* monitoring at this flow perfusion flow rates, the need for a miniaturised and low dead volume biosensor was recognized. For this reason, it was decided to develop a miniaturised flow-through biosensor based upon

the amperometric detection of hydrogen peroxide, which is formed after the enzymatic conversion of the analyte of interest. Specifically, the method of enzyme immobilization is of importance in order to construct reliable biosensors of this type.

In a first attempt, several available biosensors and methods for enzyme immobilization have been tested in a Flow Injection Analysis system, for their selectivity and sensitivity (chapter 3 and 4). Based upon the results obtained and method of fabrication, their possibility for application in a miniaturised device is discussed. In chapter 4, the construction of a low volume flow through cell and low dead volume tubing connecting the probe and the biosensor is discussed. To be able to fabricate these miniature devices in the (near) future in mass in a reproducible way, several techniques of fabrication have been evaluated (chapter 4). Additionally, the semi-vacuum pump as introduced by Moscone et al[71], was tested for its performance (chapter 3 and 4).

Besides the construction of the device, good performance of the biosensor is essential to succeed in the development and applicability of this device. Although numerous methods of enzyme immobilization have been reported (see section 1.3 and chapter 2) and some of those methods have been tested (see chapter 3 and 4) to enable the introduction of the biorecognition part of the biosensor in closed micro-channels, only a few fabrication methods of enzyme immobilization are possible. One of the possibilities is electropolymerisation in the presence of a particular enzyme. The decision to apply this technique is, among other reasons, further motivated by that fact that during electropolymerization the film thickness is self-controlling. Via this way a thin and uniform membrane can be obtained, and enables the reproducible production of biosensors. Based upon this principle, miniaturised flow-through amperometric biosensors for the continuous monitoring of glucose or lactate have been developed and tested for their performance characteristics (see chapter 5). Although good performance was achieved for the glucose biosensor, the stability of the lactate biosensor was rather poor. Covalent coupling of lactate oxidase towards the electropolymerized membrane instead of inclusion within the membrane significantly improved the stability of the lactacte biosensor (see chapter 6). Comparable results were obtained when glucose oxidase was covalently coupled in a similar way. In addition, tests with these biosensors suggested a direct transfer of electrons without the need for electron transfer mediators. Glucose and lactate could be measured at –150 mV vs. Ag/AgCl.

To demonstrate the *in vivo* applicability of the miniaturised device for glucose measurements, oral glucose tolerance tests (OGTT) (chapter 7) and 24 hours profiling (chapter 8) have been carried out in healthy volunteers. During the experiments a portable

potentiostat is introduced, which enabled the volunteers to walk freely. In these experiments, the data found for the blood and subcutaneous tissue glucose values were compared. Additionally, the phenomena observed have been discussed in relation to differences in metabolism and transport of glucose between the two compartments and the consequences on the character of the tissue surrounding probe after inserting the MD probe. The performance of the lactate biosensor was evaluated when connected on-line to a novel continuous monitoring catheter placed intravascular in the coronary sinus for detection of myocardial ischemic events (see chapter 6). Finally, the turnover of brain extracellular glucose and lactate was estimated using these miniaturised biosensors (see chapter 9). Glucose and lactate were on-line continuously analyzed in dialysate during ultraslow MD after exogenously applied analytes via the probe, which was placed in the striatum of rat brain.

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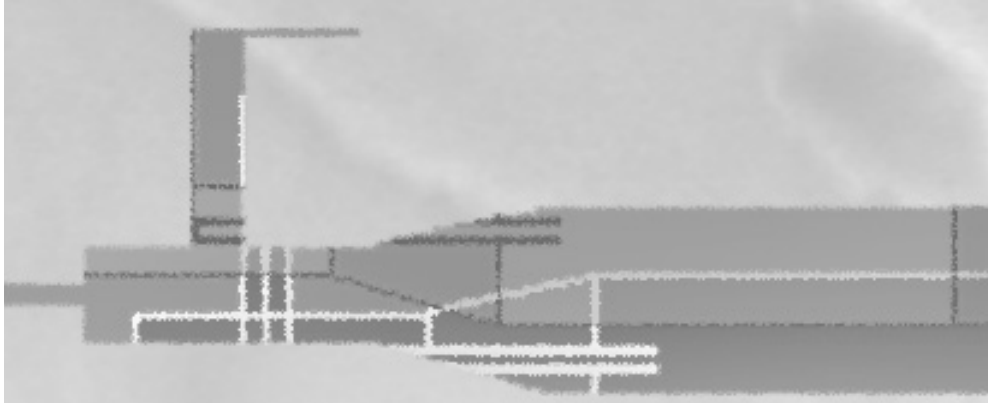

Chapter 2

Theoretical aspects

2.1 The Biosensor

2.1.1 Definitions and classification

Biosensors were first mentioned as such in the literature in 1977[1]. Before that, devices that contained a biological sensing element were simply referred to as electrodes with a description of the kind of sensing component, e.g. enzyme electrode. Today, according to the International Union of Pure and Applied Chemistry (IUPAC), an electrochemical biosensor is defined as a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is retained in direct spatial contact with an electrochemical transduction element[2]. The biological recognition system translates information from the biochemical domain, usually an analyte concentration, into a chemical or physical output signal with a defined sensitivity. The main purpose of the recognition system is to provide the sensor with a high degree of selectivity for the analyte to be measured. The transducer part, also called a detector, sensor or electrode, of the sensor serves to transfer the signal from the output domain of the recognition system to, mostly, the electrical domain.
Finally, chemical biosensors are self-contained, all parts being packaged together in the same unit, the biological recognition element in direct spatial contact with the transducing element. In figure 1, a biosensor is schematically demonstrated.
An electrochemical biosensor is a biosensor with an electrochemical transducer. It is considered to be a chemically modified electrode as a conducting material is coated with a biochemical film. Although biosensors with different transducer types, e.g. electrochemical, optical, piezoelectric or thermal type, show common features, this thesis will focus on

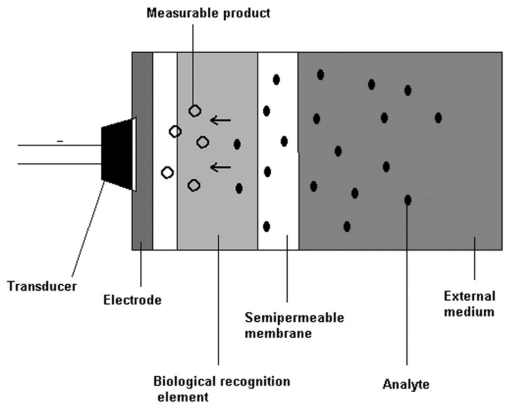

Figure 1:
Schematic demonstration of a biosensor.

electrochemical biosensors. As a biosensor is a self-contained integrated device, a biosensor should be a reagentless analytical device, although the presence of co-substrates, such as water for hydrolases or oxygen for oxidoreductases, may be required for the analyte determination. But, as part of the integrated system, some separation of amplification steps achieved by inner or outer membranes or reaction layers may be applied.

The diversity of the molecular recognition systems and of the electrochemical transducers incorporated in each biosensor is enormous. Since the development of the enzyme-based sensor for glucose, first described by Clark and Lyons in 1962, in which glucose was entrapped between two membranes[3], an impressive amount of literature on methods of immobilization and related biosensor development has appeared. These methods have been extensively reviewed elsewhere[4-11]. Biological receptors, i.e. enzymes, antibodies, cells or tissues with high biological activity, can be immobilized in a thin layer at the transducer surface by using different procedures:

a. Entrapment behind a membrane;
b. Entrapment of biological receptor within a polymeric matrix;
c. Entrapment of biological receptors within self-assembled monolayers or bilayer lipid membranes;
d. Covalent bonding of receptors on membranes or surfaces activated by means of bifunctional groups or spacers;
e. Bulk modification of entire electrode material.

Receptors are immobilized directly on the transducer surface or on a polymer membrane covering it. Covalent attachment procedures are more complicated than entrapment, but are especially useful in miniaturised devices. With these procedures more stable and reproducible activities were obtained with covalent attachment.
Besides the reacting layer, many biosensors, especially those designed for *in vivo* applications, incorporate one or several inner or outer layers. These membranes serve the following functions:

a. As a protective layer to enhance selectivity by excluding large molecules, such as proteins or cells, by preventing leakage of reacting layer components and by decreasing the influence of interfering species, such as ascorbate and urate, detected by the transducer;
b. As a diffusional outer barrier for the substrate to enlarge the dynamic range of the sensor by controlled sensor response by the substrate diffusion through the membrane

rather than by the enzyme kinetics;

c. A biocompatible and biostable surface to prevent toxic, mutagenic, carcinogenic, thrombogenic and/or immunogenic effect respectively unacceptable variations in biosensor response due to fouling by sample components.

Depending upon the sensor diameter, pre-cast membranes, such as those made of polycarbonate or cellulose acetate, deposited by dip- or spin-coating, are used. In order to prepare microsize biosensors, the enzyme is frequently immobilized by entrapping via an electropolymerization step.

Although biosensor response is an important parameter to indicate the rate limiting steps (transport or reaction) and facilitate biosensor optimization in a given matrix, other, biosensor specific, performance criteria are necessary to fully characterize the biosensor. For this reason standard protocols for evaluation of performance criteria were defined in accordance with standard IUPAC protocols[12].

Sensor calibration is generally performed by adding standard solutions of the analyte and by plotting steady-state responses possibly corrected for a blank (often called background) versus the analyte concentration. The sensitivity and linear concentration range are determined by plotting steady-state response versus the analyte concentration. Electrochemical biosensors always have an upper limit of the linear concentration range, which is directly related to the biocatalic properties of the biological receptor and can, in case of enzyme-based biosensors, be significantly extended by using a diffusion barrier to the substrate. However, this obviously will lead to a decrease in sensor sensitivity. Enzyme-based biosensors are often characterized by their apparent Michaelis-Menten constant K_M (the analyte concentration yielding a response equal to half of its maximum value). When the measured K_M is much larger than that of the soluble enzyme, it usually means that a significant substrate diffusion barrier is present between the sample and the reaction layer. The sensitivity, not to confuse with detection limits, is defined as the slope of the calibration curve.

Biosensor selectivity is expressed as the ratio of the signal output with the analyte alone to that with the interfering substance alone. Next, interfering substances can be added at their expected concentration to the sample, already containing the usual analyte concentration. The selectivity is then expressed as the percentage of variation of the biosensor response.

The term reproducibility, the drift in a series of results performed over a period of time, is

essentially the same as for any other analytical device. The reliability of biosensors for given samples depends both on their selectivity and reproducibility.
The response time is the time necessary to reach 90% of the steady-state response after adding the analyte into the measurement cell, and depends upon the analyte and product transport rate through different membrane layers. Therefore, the thickness and permeability of these layers are essential parameters.
Regarding the stability of a biosensor, it is necessary to specify whether the lifetime is a storage (shelf) or operational (use) lifetime and what the storage and operational conditions are. In addition, the mode of assessment to determine the lifetime should be specified. Actually, the lifetime is defined as the storage or operational time necessary for the sensitivity, within the linear concentration range, to decrease by a factor 10% or 50%.

2.1.2 History of the development of enzyme-based amperometric biosensors

Biosensor development began with the experience of biomolecule immobilization and stabilization on the one hand, and the miniaturization and functionalization of more sophisticated transducers, on the other[13].
Because of their specificity and catalytic properties, enzymes have found widespread use as sensing elements in biosensors. Since the development of the first enzyme-based sensor by Clark and Lyons[3], who immobilized glucose oxidase on an oxygen-sensing electrode to measure glucose, there has been an impressive proliferation of applications involving a wide variety of substrates. A variety of enzymes belonging to classes of oxido-reductases, hydrolases and lyases have been integrated with different transducers (electrochemical, optical or calorimetric transducers) to construct biosensors for applications in health care, veterinary medicine, food industry, environmental monitoring and defense[5]. Electrochemical transducers transform a biochemical reaction into an electrical potential or current. Devices developed based on such transducer are relatively easy to construct and to handle and are known for their sensitivity and possibility to miniaturize. For the construction of enzyme electrodes three types of electrochemical transducers are known: potentiometric, amperometric or conductrometric transducer. Amperometric enzyme biosensors form the majority of commercial biosensor devices available on the market today. Amperometric biosensors operate at a fixed potential with respect to a reference electrode and the current generated by the oxidation or reduction of the substrate at the surface of the working electrode is measured. Amperometric biosensors are based on redox enzymes and

a number of available oxido-reductase enzymes have been used for the construction of subsequent biosensors (see Table 1)[11].

Table 1

Selected list of oxido-reductase enzymes used for amperometric biosensors.

Enzyme	Source	Typical substrate
Glycollate oxidase	Spinach	Glycollate
	Rat liver	L-, D-Lactate
Lactate oxidase	*Pediococcus* sp.	L-Lactate
Glucose oxidase	*Aspergillus niger*	ß-D-Glucose
Xanthine oxidase	Bovine milk	Hypoxanthine
		Xanthine
		Benzaldehyde
Oxalate oxidase		Oxalate
L-Amino acid oxidase	Diamond rattle snake	L-Methionine
		L-Phenylalanine
Nitro-ethane oxidase		Nitroethane
		Aliphatic Nitrocompounds
Urate oxidase	Hog liver	Urate
Sulfite oxidase	Beef liver	Sulfite
Malate oxidase		Malate
Cholesterol oxidase	Nocardia	Cholesterol
Ascorbase oxidase	Squash	L-Ascorbate

These enzymes use molecular oxygen as an electron acceptor and produce hydrogen peroxide in the reaction with their substrates. Biosensors based on these enzymes, so-called first-generation amperometric biosensors[3], work by following either the oxygen consumption or the hydrogen peroxide production as a measure of the substrate (analyte) concentration. For instance, the traditional glucose sensor amperometrically detects the hydrogen peroxide, which is produced during the oxidation of glucose catalyzed by the enzyme glucose oxidase (GOx):

$$\text{Glucose} + O_2 \xrightarrow{GOx} \text{gluconolactone} + H_2O_2$$

This sensor has several drawbacks: denaturation of the enzyme by the hydrogen peroxide produced and the strong dependence of the electrical signal on the oxygen consumption are among the major ones. Additionally, biosensors based hydrogen peroxide measurement may suffer from interference resulting from nonspecific electrochemical oxidation of compounds, such as ascorbate, uric acid and glutathione at the 0.6 – 0.7 V (vs Ag/AgCl reference) potential required to detect H_2O_2. This has led to the development of the second-generation biosensors, in which the electrodes were chemically modified with an artificial electron carrier. In this case the electron acceptor is replaced by a so-called mediator (M), which shuttles the electrons involved in the redox process from the enzyme toward the electrode or vice versa[4]:

$$\text{Glucose} + GOx_{ox} \longrightarrow \text{gluconolactone} + GOx_{red}$$
$$GOx_{red} + M_{ox} \longrightarrow GOx_{ox} + M_{red}$$
$$M_{red} \longrightarrow M_{ox} + n\ e^-$$

Frequently reported examples of mediators are ferrocene[14,15], ferrocene derivates[16,17], tetrathiafulvalene[18,19], tetracyanoquinodimethane[20,21], and quinones[22]. Although effective in lowering the operating potential, most mediated electrodes still suffer from some ascorbic acid and uric acid interference. Hydrogen peroxide formed during the oxidase-catalyzed reactions can also be measured using peroxidase-modified electrodes. In these electrodes a reduction current, resulting from either the direct or mediated electron transfer is measured at low applied potential, thereby circumventing the interference problems encountered during electrochemical oxidation of H_2O_2[23,24]. Another way to measure H_2O_2 at low oxidation potential is by using carbon with dispersed rhodium[25,26], ruthenium[27,28] or iridium particles[29].

Direct electron transfer from an electrode to an enzyme active site simplifies biosensor design. In this case no co-substrate or mediator is required and enzyme electrochemical oxidation (reduction) determines the amperometric signal. Enzyme electrodes based on this principle are called third-generation biosensors[4]. However, some aspects have to be taken into account when considering this possibility. For instance the redox center have to approach sufficiently close to the electrode to allow rapid electron transfer. For large redox

enzymes, such as glucose oxidase, this is difficult to realize as these enzymes have thick insulating protein shells. Their catalytic centers are buried deep inside and are shielded from surroundings[30]. For these enzymes it becomes important that they are immobilized on a compatible electrode surface in a way that makes electron transfer from the catalytic center to the electrode feasible without denaturation of the enzyme[31].

Additionally, enzyme immobilization is also extremely important in terms of biosensor operational stability and long-term use. Especially due to the instability of the biological element, this is still one of the major issues in todayís biosensor research. Since this factor is to some degree a function of the strategy used, choice of immobilization technique is critical. A large number of reports, as reviewed elsewhere[5], can be found involving enzymes physically or chemically (covalently) entrapped on the transducer.

Physical methods of enzyme immobilization, such as entrapment and adsorption, have the benefit of applicability to many enzymes and may provide little or no perturbation of the native structure and function of the enzyme. Biosensors based upon direct physical adsorption of enzymes on a surface, generally show poor long-term stability due to enzyme leakage. A significant improvement, however, was demonstrated using biosensors based on the encapsulation of the enzyme[31a]. Physically retaining enzymes on the biosensor surface through the use of thin electrochemically prepared polymer films seems to be a good alternative. This is a simple procedure where a suitable monomer is oxidized in the presence of an enzyme. The resulting polymer can be conducting or nonconducting depending on the monomer employed. The film thickness can be easily controlled by adjusting the applied potential. The majority of the published work involves the use of pyrrole and derivatives thereof to entrap glucose oxidase[32-35]. It has been proposed that glucose oxidase, presenting a highly negative charge at pH 7, is electrostatically entrapped among polymer chains, which allows enzyme immobilization. The amperometric signal in these electrodes is obtained with redox mediators or with the natural co-substrate, oxygen. In the latter case, a major disadvantage results from the fact that the applied potential (+0.65 *vs.* Ag/AgCl) is susceptible to the presence of interfering compounds like ascorbic acid and uric acid. Other conducting polymers, which have been employed, are poly(thiophenes), poly(indoles) and poly(anilines)[36,37]. However, the manufacturing of these polymers presents serious disadvantages: the former requires aprotic solvents whereas the latter is performed under strongly acidic conditions (around pH 1). An interesting alternative is the use of non-conducting polymer films, such as polyphenols or

poly(phenylene-diamines)[38-41]. The resulting films are thin (nanometer range), hydrophobic and insulating and have therefore obvious advantages in terms of stability and applicability towards miniaturised devices. In addition to immobilizing the enzyme, these films allow rapid diffusion of substrate and product but also act as permselective membranes to improve selectivity and provide a barrier against electrode fouling.

Chemical methods of enzyme immobilization include covalent binding and crosslinking using multifunctional reagents, such as glutaraldehyde[42], reactive silanes[43] or cyanuric chloride[44]. Alternatively, by using functionalized thiols on gold electrodes, electrical communication for various layers of glucose oxidase on a self-assembled monolayer was achieved[45]. Another promising strategy is the immobilization of both enzyme and mediator using redox polymers. Via this way electron transfer between an enzyme and electrode can be established, while free diffusion of enzyme and mediator is avoided. Most of the examples involve redox organic molecules or transition metal complexes, which can be reversibly oxidized or reduced. For instance Heller and coworkers[46] reported the use of polyvinylimidazole modified with osmium complexes as redox hydrogel. The biosensor demonstrated no leachable components and a high stability. Based on these results it was decided to use this polymer for further development in the design of a subcutaneously implantable glucose electrode.

Next, three dimensional enzyme wiring was accomplished when working with redox macromolecules. The design of these macromolecules was to assure a complex with the enzyme and enable electron transfer from the buried redox site to the periphery of the enzyme. The first step involves complex formation by mixing the enzyme and redox polymer solutions. Covalent crosslinking was then achieved with diepoxide. To eliminate interference by electroactive species, a layer of immobilized peroxidase was added[47]. Biosensors based on this principle were also produced using acrylamide and vinylimidazole copolymers containing osmium redox sites[48], or a polycationic redoxy hydrogel, such as poly(allylamine), to which ferrocene was initially attached to the polymer and further crosslinked with glucose oxidase using epichlorohydrin[49].

Besides the development of new strategies to immobilize the enzyme and to eliminate interference by electroactive species, in the near future biosensor development will continue to focus on miniaturization but also multiple analyte detection. A severe drawback, however, is the instability of the biological receptor molecule, which still forms the major reason of the limited amount of commercially available biosensors today. Possibly via biotechnological processes, enzymes stable at high temperatures and produced by

thermophilic microorganisms, can circumvent this instability problem. Furthermore, as the structure of enzymes becomes better defined, it is possible to "tailor" enzymes that can function under stress conditions for a long period of time. Future research in these areas is necessary.

2.1.3 Theoretical aspects of amperometric biosensors

Electrochemical techniques

A number of electrochemical techniques have been used in this investigation, amperometry and cyclic voltammetry. These methods will be discussed briefly below.

A. Amperometry

The measurement of a current at a constant potential is called amperometry. Normally, this technique allows the detection of molecules or ions at concentrations as low as 10^{-9} M and it has a dynamic range of 3 to 4 orders of magnitude[4]. In amperometry the current is measured as a function of the fraction of converted material[50]. Thanks to the fact that in analytical measurements very little material is consumed and the bulk concentration of the electroactive specie relatively remains constant, steady state conditions can be assumed. In case of biosensor response the current is measured at various substrate concentrations and compared with the current when no substrate is present. The difference between these two situations indicates biosensor activity.

B. Cyclic voltammetry

In this technique the potential is raised from a starting point E_0 to an en potential E_1 and subsequently lowered back to E_0. Normally this is done at a constant sweep (Figure 2). In the presence of an oxidizable analyte, the potential is started at a point well below the oxidizing potential E^0 of the analyte and only non-faradaic current flows. When the potential reach E^0, oxidation starts and the current increases. At the potential E^0, the surface concentration of the oxidizable analytes is decreased. This leads to an increase in flux from the bulk solution to the electrode surface and consequently to an increase in current. Raising the potential further leads to surface concentration near zero and mass transfer to the surface reaches a maximum rate and subsequently declines due to depletion. At E_1 the

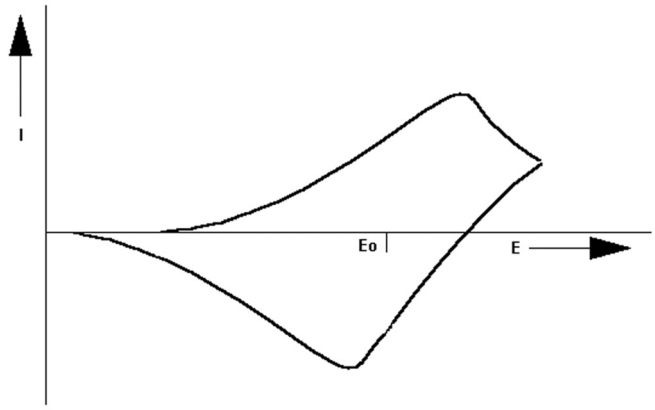

Figure 2:
Illustration of cyclic voltammetry: resulting current as a function of the potential sweep.

potential scan is reversed. At that moment a large concentration of oxidized material is present at the electrode surface. When the potential reaches E^0, reduction starts to take place and cathodic current flows. The cathodic peak has the same form as the anodic peak for basically the same reasons as mentioned above. Although it is beyond the scope of this thesis to describe this system mathematically in detail, a general rule is that the current measured is proportional to the bulk concentration of the redox analytes and to the square root of the scan rate[50]. In a reversible (Nernstian) system, the difference in potential between the oxidation and the reduction peak is about 2.3 RT/*nF*. The difference in peak potential gives information about whether or not a reaction is reversible. In case of irreversible systems, a larger potential difference between the two current peaks and possibly also a less symmetric shape is observed[50].

Description of the processes involved for amperometric biosensors

In an amperometric biosensor, the following processes are important and should be considered when designing such a sensor[7]:

A Transport of the analyte to the surface

In order to be detected, the analyte must be transported from the bulk solution to the biosensor, whereas to maintain steady-state conditions, consumed analyte must be replenished. There are three modes of transport, migration, diffusion and convection. Migration is the movement of charged species due to the presence of a gradient in electrical potential. Because biosensor measurements are generally carried out in solutions containing an excess of supporting electrolyte, which suppresses any potential gradients,

migration will not be of importance in these systems. Diffusion is the movement of species as a result of a concentration gradient, and is because it is dependent on the motion of molecules, a relatively slow process, and is described by the first and second law of Fick[51]:

$j = -D\ (dc/dx)$ [1]

$dc/dt = D(d^2c/dx^2)$, [2]

in which j is the net flux, dc/dx the concentration gradient and D the diffusion coefficient (m^2s^{-1}). If the initial condition is defined as the bulk substrate concentration c_b, and the enzymatic reaction is sufficiently fast, the surface concentration c_0 reduces to zero. The difference $c_b - c_0$ is then the concentration gradient leading to mass transport by diffusion, and equation [2] can be written as:

$c = c_\infty A\ (x/2D^{1/2}.t^{1/2})$, [3]

in which c_∞ is the concentration at an infinite distance from the electrode surface and A an error function to encounter for diffusional problems as the distance x reaches infinity. Equation [3] describes the increase in substrate concentration at increasing distance from the surface (x) respectively its decrease at increasing reaction time (t). This means that under diffusion controlled conditions a decaying biosensor response can be expected. For this reason, applying convection is of paramount importance for biosensor analysis. Not only this will lead to an increase in sensitivity, also a more stable and reproducible response is established rather than a decaying response. Convective mass transport is achieved, for instance, by forcing the solution to move past the electrode. Although convection maintains the substrate concentration at the bulk concentration level, only in a very thin layer, the so-called diffusion layer, on the surface of the electrode, convection is not possible and mass transfer is controlled by diffusion. However, most biosensors are fabricated with a membrane covering the surface of the electrode. To avoid concentration polarization as meant here, it is therefore of importance to keep the thickness of the membrane as small as possible.

B Binding of the analyte to the redox enzyme incorporated in the sensor

After transport of the substrate to the surface, an important step that takes place in a biosensor is the recognition of the substrate (S) by the biological receptor, in our case an

enzyme (E). Formation of E-S is generally very fast and limited by diffusion. In case of a redox enzyme (for instance glucose oxidase), the complex formation is followed by a redox reaction, the oxidation of glucose. This enzyme reaction can be described by a simple Michaelis-Menten model[51]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} E + \text{Product} \qquad [4]$$

$$v = (k_2[E]_0 . [S] / K_M + [S]), \qquad [5]$$

in which the Michaelis-Menten constant $K_M = (k_{-1} + k_2)/ k_1$ and E_0 is the total concentration of enzyme. A reciprocal form of the Michaelis-Menten equation (Lineweaver-Burk plot[52]):

$$1/v = [K_M/(k_2[E]_0[S])] + 1/(k_2[E]_0) \qquad [6]$$

is often used to plot the rate data in a linear way. Deviation from linearity of this plot reveals whether mass transport is important[53]. A model as described above is relatively simple and applies only to systems where the concentration of S is substantially higher that the concentration of the complex ES. In amperometric biosensors this is generally true.

C Conversion of the analyte by the redox enzyme

The steady-state response of an electrode coated with a thin film containing the immobilized glucose oxidase can be modeled using the following kinetic scheme[54]:

In the film:

$$S + E_1 \xrightarrow{K_M} [E_1S] \xrightarrow{k_{cat}} E_2 + P \qquad [7]$$

$$E_2 + O_2 \xrightarrow{k} E_1 + B \qquad [8]$$

At the electrode:

$$B \xrightarrow{k'} A \qquad [9]$$

Where S is the substrate (glucose) and P the product (gluconolactone) for the enzyme catalyzed reaction; E_1 (enzyme-FAD) and E_2 (enzyme-$FADH_2$) are the oxidized and reduced forms of the enzyme, respectively, and A (O_2) is the oxidized form of the mediator and B (H_2O_2) its reduced form. For a mathematical description of the process, it is assumed that no concentration polarization occurs. This means that the measuring layer near the electrode contains uniform concentrations of the involved species. This assumption is allowed when this layer is sufficiently thin[7,51]. According to Albery et al[55-57], the process can be described as:

$$e_\Sigma / j_S = K_M/(l.k.(s_\infty - j/k_D)) + 1/l.k + 1/l.k' \qquad [10]$$

In which e_Σ is the total enzyme concentration in the film, j_S the flux of substrate reacting within the polymer film, l the thickness of the biosensor membrane, s_∞ the analyte concentration at infinite distance from the biosensor surface and k_D the rate constant for analyte transport across the membrane. Equation [10] describes the individual possible rate limiting processes. The total process may be either controlled by enzyme kinetics, mass transfer to the biosensor surface or electron transfer. In general, mass transfer is the rate determining, and equation [10] can be rearranged as:

$$1/ j_S = K_M/(l.k.e_\Sigma.s_\infty) + 1/(k_D.s_\infty) \qquad [11]$$

In this equation enzyme kinetics and substrate transport is separated, and can be used to understand and explain biosensor response. If the biosensor behaves according to equation [11], a linear dependence of the current on the analyte concentration will be displayed. Altering the enzyme concentration e_Σ or the diffusion layer may show to what proportion this may contribute to the overall rate. However, when the enzyme loading is sufficiently high, the overall rate is determined by mass transfer. Or in other words, a properly functioning biosensor can be obtained when mass transport is not too fast in relation to the kinetics of the enzymatic reaction. When this condition is not met, a biosensor with a low dynamic range will result.

Besides the use of ultrathin polymer membranes for the production of biosensors, more recently, there has been an increasing interest to investigate reversible interfacial reactions of enzymes covalently attached at the electrode using carbodiimide immobilization procedures[58,59]. The advantage of such an approach is no desorptive losses of the enzyme as well as the possibility to 'wire' enzymes to electrodes without the need for electron transfer mediators[60]. The reversible direct electrochemistry of GOx attached to a self-assembled monolayer were first demonstrated by Jiang et al[61]. By determining the surface coverage of the enzyme, they demonstrated that the enzyme exists as a monolayer. The rate constant for direct electron transfer between the enzyme-bound flavin adenine dinucleotide (FAD) prosthetic group and the modified electrode was calculated. Based on these investigations it was demonstrated that the electron transfer reaction was a surface bound process and that this reaction could be attributed to the prosthetic group within the GOx shell and not to free FAD. In addition it was found that the reduced form of GOx was catalytically regenerated by glucose in the absence of oxygen and the enzyme was directly re-oxidized on the electrode surface. However, it was demonstrated that the data obtained with such a biosensor fitted very well to a previously developed algorithm[54], describing the kinetics of monolayers of enzymes immobilized within very thin electrochemically formed polymer matrices using the Marquardt-Levenburg algorithm:

$$\alpha/j_{obs} = n.F.A.\alpha/i = K_M/(k_{cat}.K_S.s_\infty.e_\Sigma.l) + 1/(k_{cat}.e_\Sigma.l) + 1/(k.K_A.a_\infty.e_\Sigma.l) \quad [12]$$

In which $j_{obs} = j_S / \alpha$, and α is the ratio of hydrogen peroxide detected to that lost into the bulk solution. Thus, by measuring the current for the enzyme-coated electrode as a function of the concentrations of substrate respectively mediator or enzyme loading in the film, they were able to estimate the kinetics of the reactions involved of the immobilized enzyme. It was found that the enzyme kinetic parameters were in close agreement with those previously determined for monolayers of GOx[54].

2.2 Sampling

2.2.1 Introduction

Measuring analytes in body fluids is routinely used to assist in the medical diagnosis; many clinical decisions are made based on laboratory analysis. In this case, samples are taken

batch-wise and subsequently analyzed in the clinical laboratory. The results of these measurements are usually available only after several hours and these analyses are generally rather expensive[62]. Due to these disadvantages, these methodologies can not be used in case the concentration of the analyte has to stay within a certain range or when a concentration profile of the analyte over the day is required[63]. Besides frequent sampling is avoided, continuous analysis offers the advantage of providing instantly information of the analyte concentration and facilitating the creation of time-profiles. In addition, it is expected that the quality of patient care would be improved if batch-wise sampling and analysis were replaced by on-line continuous analysis.

For continuous (bio)chemical monitoring, two methodologically different invasive approaches are generally known: measurement directly inside the body or a combination of a sampling and an analytical device. The direct measurement inside the body with biosensors has several technical limitations. Among these problems are *in vivo* calibration and instability of the sensor signal[64,65]. In addition, most biosensor probes are large enough to create artifacts in the surrounding tissue[66].
An alternative approach for measuring parameters *in vivo* can be found in combining an analytical system with a continuous sampling system, such as microdialysis or ultrafiltration sampling[67-70]. Based on the setup of our measuring device, in this chapter we will primarily focus on both of these techniques, microdialysis and ultrafiltration.

2.2.2 Continuous on-line sampling

Because all tissues are in close contact with the blood circulation and therefor changes in analyte concentration after a clinical event occur rapidly in blood, most biochemical parameters are measured in blood samples. For this reason, blood is an ideal compartment to be used for continuous undiluted sampling. However, due to serious drawbacks, among infection hazards and the risk of blood clotting, this can not be performed on a routine basis and can only be done in a hospital setting. To overcome these drawbacks, alternative body fluids in combination with alternative sampling techniques have been considered: microdialysis or ultrafiltration.

Microdialysis

Microdialysis (MD) is a dynamic sampling method based on analyte diffusion across a semi-

permeable membrane, due to a concentration gradient[71]. The membrane forms the contact area with the body fluid, and, depending on the cut-off of the semi-permeable membrane, it prevents diffusion of large molecules or cells to the analytical system and may therefore serve as a selector. Accordingly, a decrease in biosensor performance is avoided because the sensing surface remains rather clean. Because the invasive part, the probe, is relatively small and the sampling does not withdraw the scarce extracellular fluid[64], MD probes allows, besides intravenous sampling, continuous sampling in the extracellular space of virtually all tissues[72]. MD can be characterized as a minimally invasive method causing minor tissue trauma and allows to define the sampling site clearly[73].

In figure 3, a MD probe is schematically demonstrated. A fluid is pumped with a pump through the MD probe. The incoming fluid, called the perfusate, is a buffer balanced in pH and ion content with the extracellular fluid in the surrounding tissue. The outgoing fluid, the dialysate, contains body fluid constituents, which have been diffused into the fluid by passive diffusion through the membrane of the MD probe. Thanks to the semi-permeable membrane, the influx and outflux of fluid are balanced if no resistance in the outflow exits.

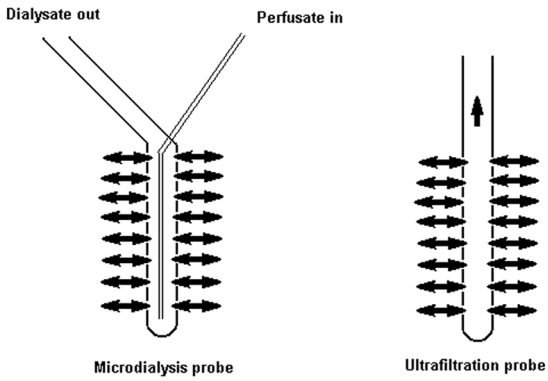

Figure 3:
Schematic demonstration of a Microdialysis probe and a Ultrafiltration probe.

A major disadvantage of using MD, however, is that no absolute concentrations of analyte are measured[74]. To be able to determine this concentration, the relative recovery has to be determined in advance. The relative recovery is defined as the percentage of the concentration found in the dialysate and of that of the original body fluid concentration. Sometimes, the absolute recovery is used to characterize the concentration of the analyte in the dialysate. The absolute recovery is the total amount of analyte in the dialysate per time interval. Although, in general, the relative recovery is independent of the concentration of analyte, there are many, both MD variables and tissue properties that may influence the relative recovery[75,76]. An overview of the most important factors are listed in Table 2.

Table 2

Factors that may influence the relative recovery

Microdialysis factors	Physiological / tissue factors
1. Perfusion flow rate	1. Temperature
2. Probe geometry and surface area	2. Tissue tortuosity
3. Molecular cutoff of the membrane	3. Extracellular space fraction
4. Diffusion characteristics of the analyte	4. Tissue/protein binding
	5. Tissue blood flow
	6. Tissue metabolism of the analyte

To determine the relative recovery, several methods enabling the calculation of the absolute concentration in the extracellular fluid have been proposed:

a. *In vivo* recovery equals *in vitro* recovery[77]
   Originally, the *in vivo* recovery was estimated by the *in vitro* recovery, and the data were not corrected for the relatively small extracellular volume and variations in viscosity and tissue tortuosity[78].
b. Changing flow rate method[78]
   In this case the relative recovery is calculated by measuring the concentration of the analyte by different flow rates and extrapolating the flow rate to zero. This method is rather time-consuming and can only be applied under steady-state conditions.
c. No-net-flux method[64,79]
   Different concentrations of analyte are added to the perfusate. If the concentration in the extracellular fluid is higher or lower than in the perfusate, the content of analyte in the dialysate will increase or decrease respectively. Interpolation of the results will yield the absolute analyte concentration. This method is not only very laborious, it is only valid if the absolute concentration does not vary during the calibration.
d. Retrodialysis method[80]
   In retrodialysis it is assumed that the diffusion of analyte through the membrane is equal in both directions. In this method, compared to the expected *in vivo* concentration, a high concentration of the analyte is added to the perfusate. The relative recovery can then be determined by calculating the fraction of retained analyte concentration in the dialysate. A major drawback of this method is that if the analyte concentration is higher

than expected, the endogenous concentration may influence the measured recovery and that tissue metabolism influences the recovery.

e. Internal reference method[81,82]
   The relative recovery is calculated by assuming that the *in vivo* and *in vitro* loss of an internal standard (or *in vivo* marker) added to the perfusate is identical. Although this method can also be used under non steady-state conditions, an *in vivo* marker with nearly identical properties of the analyte of interest has to be found and that a dual detection system is needed to detect both analyte and *in vivo* marker.
f. Low flow method[74,83,84] or stop-flow method[79]
   This concept is based upon the assumption that near quantitative recoveries are reached if perfusate and extracellular fluid are in contact with each other long enough to reach equilibrium. This equilibrium can only be reached at extreme low flow rates (< 0.5 $\mu l.min^{-1}$).
g. Independent method calibration[85]
   The MD sample is calibrated with an independent valid analytical method. Although the data will be most reliable, most sampling sites, with the exception of blood samples, have no alternative methods for sampling.

In general it can be concluded that, with the exception of the internal reference method, neither method will account for changes in recovery during sampling. These changes may occur as a result of blockage of the membrane by proteins, cell structures or to pathophysiological changes in the tissue structure[86].
Besides these problems, MD may also cause tissue artifacts due to tissue trauma as a result of insertion of the probe or to draining of the analyte present in the extracellular fluid[87]. In order to avoid this phenomenon is to minimize the absolute recovery by performing MD at extremely low flow rates and to emulate the extracellular fluid as well as possible[88]. However, continuously sampling by means of MD at these low flow rates influences in a negative way the required time resolution. In addition sensitive analytical will be required to analyze this extremely low volume samples.

Ultrafiltration

Ultrafiltration (UF) is a sampling technique in which endogenous fluid is withdrawn from the sampling site by underpressure and was introduced by Janle-Swain et al[89] as an alternative to MD. In comparison with MD, the UF probe consists of a semi-permeable

membrane that exclude large molecules whereas small analytes are extracted together with water and salts (see figure 3). When continuous sampling by UF is applied a rapid replenishment of the collected body fluid from the studied area (e.g. subcutaneous, venous) is required. So, the success of UF depends whether the withdrawn body fluid can be replenished rapidly from blood vessels. It can be used in virtually every tissue, except the brain, presumably the extracellular fluid is too small and the influx of filtrate too slow for sampling even at very low flow rates. Originally, UF was used for the continuous tissue sampling in awake, unrestrained animals, such as dogs and cats. Most experiments have been performed subcutaneously, but also intravenous measurements and the UF of saliva have been reported. Since 1987, about 25 scientific papers have appeared. In these studies, two types of UF probes have been reported. One consists of one or more loops of hollow, semi-permeable fibers that are joined to a single, non-permeable conducting tube. The tube is attached to a vacuum source that drives the UF. By using a relatively high underpressure, fluid and dissolved compounds are pulled from the capillaries to the probe. So, in this case the ultrafiltrate resembles the blood-concentration. Due to the large size these probes can not be used for application in blood. In contrast to this probe, Moscone et al[90] described the use of a very small UF probe (single hollow fiber with a diameter of 250 µm and a length of 4 cm) for the continuous sampling at extremely low flow rates (100 - 300 nl. min^{-1}). Using a low underpressure, the extracellular space is adequately replenished by the capillaries and is therefore minimally disturbed during sampling. Thus with slow UF, the ultrafiltrate resembles the extracellular space rather than blood plasma.
Typically, UF provides recoveries above 95% for low molecular weight molecules. A small correction factor due to the exclusion of restricted compounds (e.g. plasma proteins) must be included[91,92].

Microdialysis versus Ultrafiltration

Both MD and UF extract compounds by moving them across a semi-permeable membrane. In case of MD the separation is exclusively due to a concentration gradient of the analyte of interest, whereas in UF analytes can or cannot pass the membrane because of their size[93]. In contrast to UF, the use of an additional fluid in MD may lead to changes in the surrounding tissue. However, exact figures on the effect of the withdrawal of fluid on the recovery and absolute analyte concentrations in the tissue when UF sampling is performed are as yet unknown. During continuous on-line UF sampling in subcutaneous tissue in man, it was found that the probe held a resistance and the flow rate during sampling decreased[94]. As a result, it

was difficult to accurately determine the absolute concentration of the analyte investigated. To conclude, whether or not MD or UF should be used for the continuous sampling depends on for instance the sampling site of interest, the required recovery, the sensitivity of the analytical method and the conditions during the experiments (e.g. hospital setting or at home). For instance, UF holds a lower infection risk and no additional fluids needs to be carried. However, when the sampling site has limited fluid production (e.g. brain fluid), MD is a better alternative. Especially, in order to prevent drainage and excessive calibration steps, ultraslow microdialysis (uMD), MD at low flow rates (100 – 300 nl.min^{-1}), seems a good alternative. In comparison with UF, relative recoveries for uMD were found of around 100%[84] and no flow restriction over the MD probe was observed.

2.3 References

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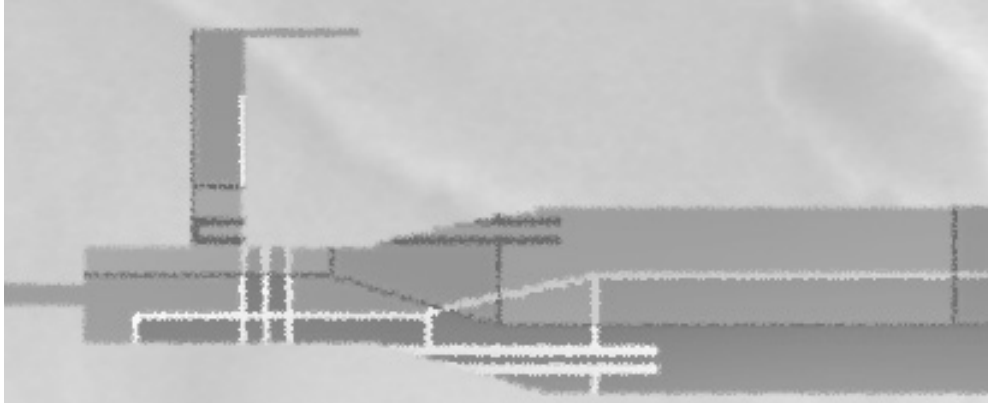

Chapter 3

Biosensor device and ultrafiltration sampling for continuous in vivo monitoring of glucose

Summary

A biosensor was tested for its applicability for the *in vivo* monitoring of glucose and sampling by ultrafiltration at submicroliter flow rate. The biosensor was integrated in a Flow Injection Analysis system and the performance characteristics of the biosensor were determined. In addition, the first results obtained with a miniaturised portable sensor device for the *in vivo* monitoring of glucose are demonstrated.

3.1 Introduction

It is well known that intravascular sensors have the disadvantage of rapid induction of blood clotting, and that most sensors suffer from serious drawback sensor performance due to fouling and contamination of the surface during operation[1]. In order to avoid this problem, microdialysis was proposed as an interface between the body and the sensor[2-4]. An alternative for *in vivo* sampling is ultrafiltration[5]. In both cases, the sample is free of large proteins and analysis can be performed nearly without further purification. An advantage of ultrafiltration over microdialysis is that the analyte of interest is nearly quantitatively recovered without dilution of the matrix sampled. However, to obtain a representative sample during monitoring, low volume samples are required. Therefore, the use of a small disposable syringe for pulse-free ultrafiltration at submicroliter flow rates have been reported[6,7]. Sampling was performed up to 24 hours and in this case on-line analysis was performed electrochemically using a bi-enzyme reactor in a flow injection system[8]. To circumvent the use of expensive equipment and to miniaturise the analytical system to improve the patient's mobility, the application of a biosensor seems justified. Besides being small, robust and easy to handle, these biosensors must be biocompatible and reliable for measurements in the complex matrix of body fluids. For instance for glucose and lactate measurements, amperometric biosensors with immobilised enzymes for the conversion of the analyte into electrochemically detectable products, are frequently applied. Since several years, various authors have described the production of biosensors utilising various methods of enzyme immobilisation on electrodes in order to improve the selectivity of the biosensor and to overcome interference from electroactive species. The use of a biosensor for off-line discontinuous monitoring is mostly described, but for continuous *in vivo* monitoring of patients little or no data have been reported. Therefore, in our laboratory we started to test several commercially available biosensors. In this article we describe the

experiments carried out and the results obtained with one commercially available biosensor. For the purpose of *in vivo* on-line monitoring of blood during ultrafiltration at low flow rate, the biosensor was tested for its sensitivity, stability and selectivity. To enhance the stability of the sensor and the linear dynamic range of the assay, the biosensor was integrated into a miniaturised Flow Injection Analysis (FIA) system.

Our ultimate goal is to define a miniaturised portable biosensor device for on-line continuous *in vivo* monitoring. Sampling should preferably be performed by means of ultrafiltration at submicroliter flow rate. Although many attempts have been reported to miniaturise biosensor devices, no hardware is yet available to perform *in vivo* sampling by ultrafiltration at 100 nl.min^{-1} and direct analysis by means of a portable biosensor device. Besides the need for a pulse free pump capable of delivering reproducibly low flow rates (100 nl.min^{-1}), extremely low internal volumes are required for these low volumes of sample. By using the disposable syringe as mentioned before[6,7], the first results of a home-made biosensor device which is capable of sampling through ultrafiltration at low flow rate and on-line analysis by means of a portable biosensor are demonstrated in this article.

3.2 Materials and Methods

3.2.1 Materials

D+-Glucose for standard solutions was obtained from Sigma Chemical Concentration. (St. Louis, MO). All other chemicals were of pro-analysis quality and purchased from Merck (Darmstadt, Germany). Double quartz distilled water was used for all aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK) to inhibit bacterial growth. The composition of the carrier solution was a Dulbecco's phosphate-buffered saline (PBS) (mmol.l^{-1}): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5), and Na_2HPO_4 (8.1). Prior to analysis, helium was purged through the carrier solution to remove air. The stock glucose solution (50 mmol.l^{-1}) was prepared by dissolving glucose in PBS and allowed to stand for 24 hours. The standard solutions were prepared from this stock solution by diluting with PBS.

3.2.2 Methods

Sampling by ultrafiltration was performed by a probe (fibers of an artificial kidney, AN69HF,

acrylonitrile and sodium methallyl sulphonate copolymer, Filtral 16, Hospal Ind., Meyzieu, France, 340 µm o.d., 240 µm i.d.) of 2 cm with a hand-made spring inside (stainless steel wire, D = 60 µm, 12 axial length windings per cm, Vogelsang, Hagen, Germany). One end of this probe was connected via a 20-30 cm long fused silica tube (i.d. 50 µm, o.d. 150 µm, Polymicron Technologies inc., Phoenix, Arizona) with a model Marathon Autosampler (Spark Holland, Emmen, the Netherlands). The probe was filled with water and the other end of the probe was closed with cyano-acrylic glue (Henkel, Nieuwegein, the Netherlands). Ultrafiltration at a flow rate of 100 nl.min-1 was carried out by the underpressure of a model 22 syringe pump (Harvard Apparatus, Kent, GB). The syringe was directly connected to the autosampler. The valve of the autosampler was automatically switched on a time-basis (load/inject 60/60 seconds). The autosampler was equipped with a 20 µL loop, which was partially filled (100 nl) with ultrafiltrate. A model LKB 2150 HPLC pump (Pharmacy Brim, Sweden) was used to pump the carrier solution at a flow rate of 0.5 ml.min-1 via the autosampler through a specially designed flow-through cell. The GOD sensor (SensLab, Leipzig, Germany) was inserted in the flow-through cell and connected to a home-made potentiostate. The potential applied was held at +250 mV vs. Ag/AgCl. The signal output was recorded with a model BD112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

3.3 Results and discussion

After testing several commercially available biosensors, promising results were obtained with the biosensors from SensLab. However, to be able to test this biosensor for our purpose, we had to construct a specific flow-through cell. The constructed flow-through cell as well as the instrumental set-up is demonstrated in figure 1. By using the instrumental set-

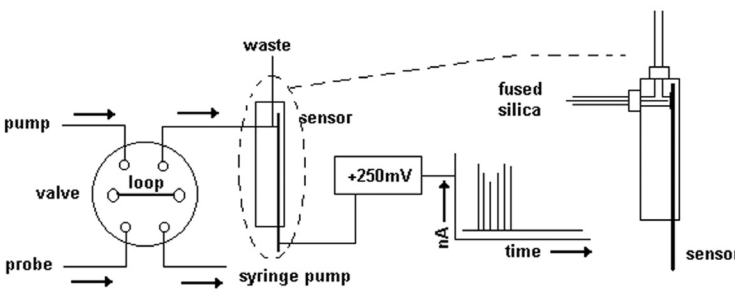

Figure 1:
Instrumental set-up for the determination of glucose.

up and the conditions mentioned in 3.2.2., the biosensor device was tested for its performance characteristics. The repeatability of the assay was determined by analysing in sixfold standard solutions of glucose of between 0.5 - 15 mmol of glucose. The repeatability, expressed as the relative standard deviation in the peak height, was found to be 1- 2 %. By analysing standard solutions of glucose in PBS, the assay was found to be at least linear up to 15 mmol (correlation coefficient of > 0.99). An example of a calibration curve is shown in figure 2. The selectivity of the biosensor was tested by analysing accordingly a standard solution of 0.15 mmol of ascorbic acid in PBS. If a diagnostic concentration of 0.04 mmol of ascorbic acid is assumed, the contribution of ascorbic acid to the peak height for glucose was calculated to be approximately 3%. The accuracy of the biosensor was tested by analysing a pooled blood sample by the hexokinase reference method (GlucoTrend, Boehringer Mannheim, Germany) as well as with the method demonstrated here. Prior to analysis, a calibration plot was calculated after analysing standard solutions of glucose in PBS. The calculated content of glucose in the sample was found to be 6.57 mmol, whereas with the reference method a glucose content of 6.67 mmol was found. With respect to the stability of the GOD sensor, standard glucose solutions in PBS as well as a pooled serum sample were monitored for up to 12 hours and no significant decline in the signal for glucose was noticed. Equilibrium after placing the biosensor in the flow-through cell was reached within several minutes, and, although a slight decrease in the signal for glucose was seen, one sensor could be used for up to 14 days. A typical chart record of monitoring by means of the method described here is presented in figure 3. From this figure the drift in the peak heights due to the pulse of the model 22 Harvard syringe pump can be easily recognised.

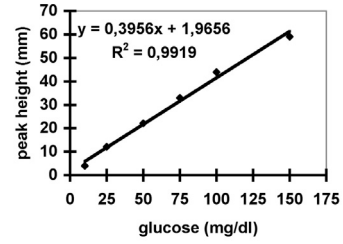

Figure 2:
Calibration curve for the determination of glucose by FIA in combination with the tested biosensor.

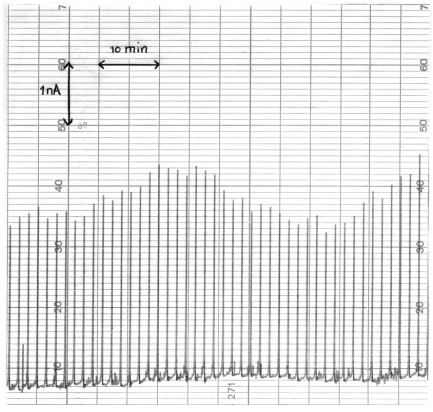

Figure 3:
Typical chart record of the determination of glucose in vivo by means of ultrafiltration and FIA.

In addition to the promising results obtained with the biosensor mentioned above, further attempts have been made regarding miniaturisation of our disposable sensor device. Therefore, the pulse-free disposable syringe pump, as reported earlier[6,7], was tested for its performance characteristics. During several days we followed the performance of the syringe pump (1.2 mL Monovette, Sarstedt, Nümbrecht, Germany, equipped with a restriction fused silica tube, 15 µm i.d., 150 µm o.d., 4 cm length, glued into the syringe) by sampling water at a flow rate of 300 nl.min^{-1} through ultrafiltration and weighing with a microbalance (Sartorius, Nieuwegein, the Netherlands) every five minutes the amount of ultrafiltrate sampled. The data were directly recorded and are presented in figure 4. The 24 hours of the recorded data presented here are typical for the performance of the syringe pump; based on these results it can be concluded that the performance characteristics of the pump are adequate for our purpose. Next, a home-made sensor was constructed by us as presented in figure 5. Ultrafiltration was carried out at a flow rate of 200 nl.min^{-1} by the underpressure of the pulse-free disposable syringe as mentioned above. Sample was introduced every minute for 30 seconds through a 25 µm i.d. fused silica tube via a home-made valve. Instead of using a diffusion controlled membrane or biocatalytic elimination as reported by many authors, in order to improve the selectivity of the biosensor we used rhodium to catalyse the electroreduction of hydrogen peroxide. In comparison with earlier results[9], electroreduction of the biochemical reaction product hydrogen peroxide could be

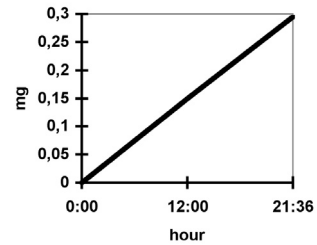

Figure 4:
Performance of the pulse-free disposable syringe pump at 300 nl.min^{-1}.

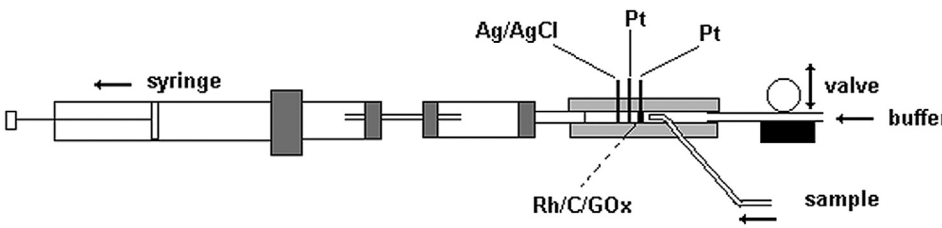

Figure 5:
Schematic construction of the home-made biosensor.

established at -150 mV. At this potential no interference of the signal by ascorbic acid was noticed by us. So far promising results have been obtained by this home-made biosensor device. In the near future, however, more work has to be done regarding the sensitivity and the dimensions of the biosensor device before we can start the experiments for *in vivo* monitoring of patients. In order to achieve in an efficient manner our ultimate goal, we will also continue with the testing of the biosensors mentioned here as well as other available biosensors.

3.4 Comments on biomedical applications

In this article we demonstrated the possibilities for the on-line and continuous *in vivo* monitoring of glucose after sampling by ultrafiltration at very low flow rates. On-line analysis can be performed with FIA or a miniaturised biosensor device. Based on the performance characteristics found for the biosensor tested here, we will proceed with some relevant biomedical applications, such as the monitoring of glucose in men and rats after subcutaneous ultrafiltration and glucose in rat after intravenous ultrafiltration. Our first measurements with a lactate sensor show that monitoring of both glucose and lactate at the same time belongs to the possibilities.

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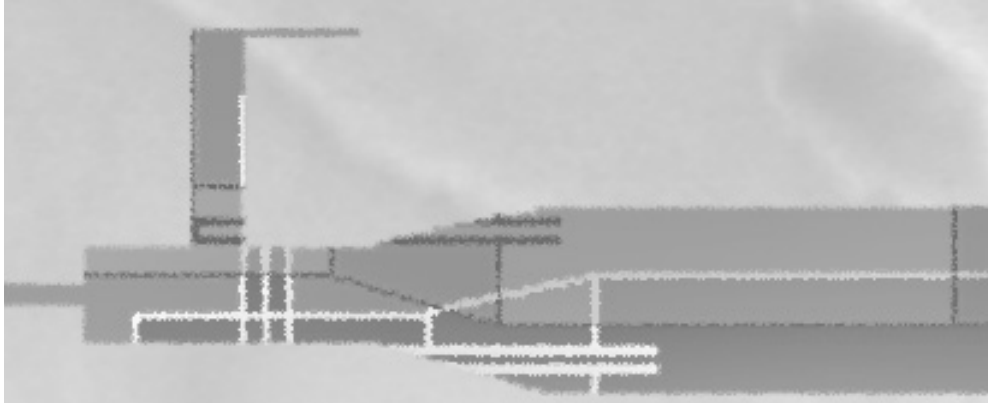

Chapter 4

A versatile biosensor device for continuous biomedical monitoring

Summary

Although biosensors are by means suitable for continuous biomedical monitoring, due to fouling and blood clotting, *in vivo* performance is far from optimal. For this reason, ultrafiltration, microdialysis or open tubular flow is frequently used as interface. To secure quantitative recoveries of the analyte of interest, sampling at submicroliter level will be necessary which in turn necessitates the development of small and versatile biosensor devices. Here, a miniaturised biosensor device, which directly can be connected to various interfaces will be presented. The biosensor device consists of a pulse free pump and a biosensor with an internal volume of 10-20 nanoliter. In this article, the production as well as the construction of the flow-through cell of the biosensor will be discussed. The advantages and disadvantages of several production processes will be demonstrated and a detailed protocol for the production of such a nanoliter flow-through cell will be presented. With respect to the bio-selector, several permselective membranes have been tested on their performance characteristics. Results obtained with these biosensors will be presented and discussed. Finally, a protocol based upon *in situ* electropolymerisation for the immobilisation of the biological component was defined and several biosensors based upon this principle have been produced and tested for the monitoring of glucose respectively lactate. To demonstrate, data obtained during a variety of *in vivo* studies at different clinical relevant applications will be presented.

4.1 Introduction

In general, a biosensor consists of two elements, the bioselector and the transducer. The selector recognises the analyte of interest and reacts whereas the transducer monitors the biochemical reaction initiated by the sample[1]. Most biosensors described in the literature as in our set-up use an enzyme as selecting element. For instance, the enzyme glucose oxidase (GOx) converts the analyte of interest glucose into hydrogen peroxide, which in turn can electrochemically be detected with the electrochemical transducer[2]. Preferably, both elements should be located close to each other, and for this reason the enzymes are mostly immobilised on the working electrode. Although biosensors are devices that have the potential to continuously monitor analytes *in vivo*, till date, only a relatively small number of biosensors realised any commercial success. Especially due to fouling and contamination

of the electrode surface, frequently the performance of a (bio)sensor diminishes during *in vivo* monitoring. For this reason, several approaches for invasive, such as the open flow microperfusion needle enzyme electrode[3], and noninvasive biosensors, such as the GlucoWatch Biographer[4], have been proposed to improve sensor stability. More frequently, microdialysis (MD) or ultrafiltration (UF) has been reported as an interface[5,6]. In this case a semi-permeable membrane is implanted and filtrate or perfusate containing analytes are transported by pumping and analysed outside the body. Because the membrane excludes cells and large molecules, a clean matrix for measurement with the biosensor is acquired. In addition, wound reactions respectively inflammation is avoided and strict regulations regarding sterility and/or leakage of biosensor components into the body are circumvented. To obtain, however, quantitative recoveries, ultraslow MD or UF at low flow-rates (50 – 100 $nl.min^{-1}$) by means of underpressure is inevitable. For this reason, our laboratory developed a disposable pump, which produces a stable low flow rate for several days[7]. To prevent, however, unacceptable delay times and/or in case real time monitoring is required, miniaturisation of the measuring device will clearly be necessary. In this article, a miniaturised flow-through cell integrated between the interface and the disposable pump will be presented. During the development of this miniaturised flow-through cell, different aspects which may influence the performance of the final measuring device, such as maximum cell volume, use of materials, low dead volume connections and low back-pressure, were specially taken into account. Based upon the criteria set for such a device, different available production processes were evaluated on their possibilities (merits) and will be discussed here.

Besides the hardware, the availability of a selective and sensitive bioselector is of major importance for the applicability of biosensors for continuous monitoring purposes. Throughout the years, numerous authors have described the immobilisation of enzymes for the development and production of all sorts of biosensors[8]. To investigate the possibilities in our set-up, different permselective membranes from several available biosensors have been tested on their performance. Although these biosensors, which have been described into more detail earlier[3,9], were not suitable in our instrumental set-up due to the unacceptable high cell volume, conclusions could be drawn regarding their applicability. An overview of the results obtained will be presented and discussed in this article. To establish, however, a miniaturised flow-through biosensor as needed in our approach, it was decided to immobilise the bioselective component via *in situ* encapsulation by electropolymerisation. Because of their reported advantages[10,11], much attention has recently been given to the use of electropolymerised conducting or non-conducting

permselective membranes, such as poly(1,3-phenylenediamine). Thanks to the self-controlling film thickness of the non-conducting polymer during electropolymerisation and reported electrocatalytic action of poly(1,3-phenylenediamine), a sensitive biosensor is obtained which can thus reproducibly be produced. Based upon this approach, several biosensors have been produced for the determination of glucose and lactate during various experiments involving continuous monitoring. Data obtained during several *in vivo* monitoring studies will be presented to demonstrate the suitability and applicability of the instrumental set-up.

4.2 Experimental section

4.2.1 Apparatus

For the testing of several permselective membranes a Flow Injection Analysis (FIA) system, as schematically demonstrated in figure 1, consisting of a model Marathon autosampler, equipped with a 20 µl loop (Spark Holland, Emmen, the Netherlands), a model LKB HPLC pump (Pharmacia, Bromma, Sweden) and a home-made potentiostat, is used. By automatically switching the injection valve on a time-basis (load/inject 60/120 seconds), aliquots of sample (100 nl) are injected and analysed every two minutes. A specially designed flow-through cell, in which the biosensor to be tested is inserted, is directly connected to the injection valve of the autosampler.

For the production of the miniaturised biosensors a model DECADE electrochemical detector (Antec Leyden, the Netherlands) and a model 22 (Harvard Apparatus, Kent, GB)

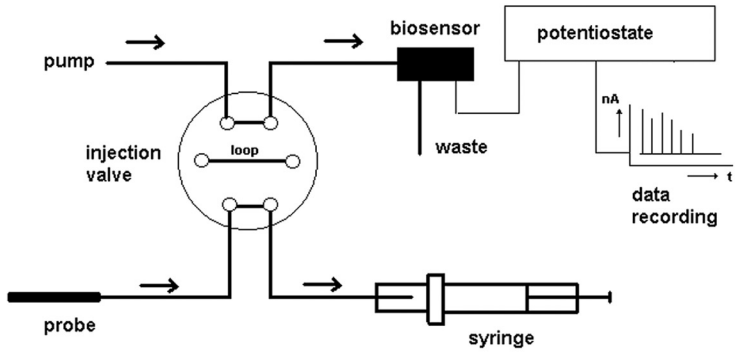

Figure 1:
Schematic demonstration of the FIA instrumental set-up

syringe pump is used to pump the monomer solution through the flow-through cell during electropolymerisation. Testing of the miniaturised biosensor is performed by FIA as well as in the continuous mode. The FIA consisted of a model DECADE electrochemical detector (Antec Leyden, the Netherlands) equipped with a VICI Cheminert C4 valve with a 100 nl internal loop (Valco Instruments, Houston, USA) for sample injection and a model 22 (Harvard Apparatus, Kent, GB) syringe pump to transport at a flow-rate of 5 $\mu l.min^{-1}$ the carrier solution and sample to the miniaturised biosensor. The valve is automatically switched on a time-basis (load/inject 120/60). Measurements are carried out at +0.5 V vs. Ag/AgCl.

Sampling in the FIA mode is performed by ultraslow MD at a flow rate of 100 $nl.min^{-1}$ by the underpressure of a homemade semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as described previously[12]. Connections between the various parts of the FIA are made with fused silica tubing (i.d. 50 µm, o.d. 150 µm) to avoid high dead volumes.

For measurements in the continuous mode, the UF or MD probe is directly connected to the miniaturised biosensor via fused silica tubing (i.d. 50 µm, o.d. 150 µm). Ultraslow MD or UF is carried by the underpressure of the home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany). The pump is directly connected to the miniaturised biosensor, and ultraslow MD or UF is carried out at a flow-rate of 100 $nl.min^{-1}$. Measurements are carried out at +0.5 V vs. Ag/AgCl by means of a home-made potentiostat.

Signal output is recorded with a model BD112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

4.2.2 Materials

The enzyme glucose oxidase from *Aspergillus niger* (EC 1.1.3.4., grade I) and lactate oxidase from *Pediococcus species* (grade I) are obtained from Boehringer Mannheim (Germany). D(+)-glucose and L(+)-lactate for standard solutions and 1,3-phenylenediamine for the permselective membrane is purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals are of pro-analysis quality and are purchased from E. Merck (Germany). Double quartz distilled water is used for all aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon UK) to inhibit bacterial growth. The composition of the carrier solution during MD is a Dulbecco's buffer phosphate-buffered saline (PBS) ($mMol.l^{-1}$): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5), Na_2HPO_4 (8.1)

and EDTA (2). The pH is adjusted to pH 7.4 and purged with helium before use. Standard solutions of glucose or lactate are prepared by diluting the stock solution of glucose (50 $mMol.l^{-1}$) respectively lactate (25 $mMol.l^{-1}$) in PBS and are allowed to reach mutarotational equilibrium before use (24 h).
For the construction of the biosensor, tygon tubing (ID 0.005 inch) is purchased from Skalar Analytical (Breda, the Netherlands). The auxiliary and work electrode is made from platinum wire (0.10 mm diameter) whereas the reference electrode is made from silver wire coated with melted AgCl (0.125 mm diameter). All these materials are purchased from Drijfhout (Amsterdam, the Netherlands). To connect the biosensor to the probes, fused silica (150 µm OD, 50 µm ID) is used (Aurora Borealis Control, the Netherlands) which is glued with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands).
For *in vivo* measurements by means of ultraslow MD, a CMA 60 microdialysis catheter (Aurora Borealis Control, the Netherlands) is used. Sampling by UF is performed by a 2 cm UF probe placed in a catheter as developed and provided by Cordis Europe N.V. (Roden, the Netherlands).
To test several permselective membranes, biosensors were provided by SensLab (Leipzig, Germany), the University of Manchester (Hope Hospital, England) and the University of Freiburg (IMTEK, Freiburg, Germany).

4.2.3 Procedures

With a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) two platinum wires and a Ag/AgCl wire are placed carefully close to each other into a 0.005 inch ID tygon tubing as demonstrated in figure 2. By a multimeter the correct position of the electrodes in the tubing is controlled. Possible leakages formed in the tygon tubing are eliminated with cyanoacrylic glue. The flow-through cell prepared in this way is washed with methanol, 10 v/v% hydrogen peroxide solution in water and 0.1 M phosphate buffer pH 7.0 by means of a 1 mL syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun, Melsungen, Germany). The syringe is filled with a solution containing 2 $mg.ml^{-1}$ of enzyme and 10 $mg.ml^{-1}$ of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 7.0. The syringe is placed in the model 22 (Harvard Apparatus) syringe pump and the wires outside the tubing are connected with the potentiostate by means of crocodile clips. Electropolymerisation is performed at +0.8 V vs Ag/AgCl for one hour at a flow rate of 500 $nl.min^{-1}$ followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Before storage and/or use, the

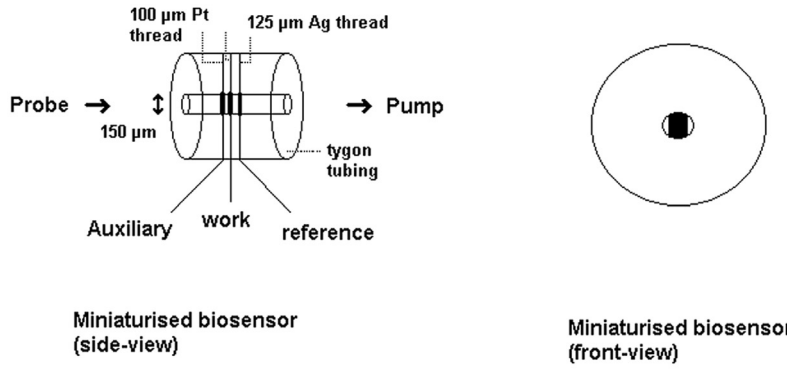

Figure 2:
Schematic demonstration of the miniaturised biosensor

biosensors are rinsed with 0.1 M phosphate buffer pH 7.0 for 30 minutes. In between, the biosensors are stored in the refrigerator at 4-8 °C.

4.3 Results and discussion

4.3.1 Testing of the permselective membranes

Several permselective membranes were investigated for their applicability by testing commercial available biosensors as well as biosensors provided by colleague research institutes. An overview of the results obtained is given in table 1. Although promising results, which have been described in more detail elsewhere[7], were obtained with the glucose biosensors from SensLab (Leipzig, Germany), miniaturisation was attended with an unacceptable decrease in sensitivity and a large deviation in the performance of each biosensor produced (data not shown). Another biosensor, obtained from the University of Manchester and described in detail by Rigby et al[3], comprises of a needle electrode system for *in vivo* monitoring of in this case lactate based upon open flow microperfusion. Due to the fact that this sensor is actually developed for monitoring in the continuous mode, the sensitivity of the biosensor when tested in the FIA was limited. Damaging the tip of the needle electrode when introduced in a specially designed flow-through cell probably caused the limited stability observed. The biosensor provided by the University of Freiburg is produced by thin film technology, whereas the flow-through cell is produced by means of

printed circuit board technology as described in detail by Jobst et al[9]. Based upon the promising results (see table 1) with this type of biosensor, more work was carried out. However, unfortunately during measurements in the continuous mode as in our set-up, a major drawback of this biosensor was observed. Especially due to the construction of the flow-through cell and connections, air bubbles easily retained in the flow-through cell and frequently disturbed the signal. In addition, by pulling the dialysate or filtrate through the flow-through cell instead of pushing, possible air bubbles were enlarged and difficult to remove. Because this biosensor could not be produced in an alternative way at that time and other tested biosensors did not fulfil our criteria, it was decided to construct a new biosensor.

Table 1:

Testing of different biosensors.

Characteristics	**Biosensor 1[1]**	**Biosensor 2[2]**	**Biosensor 3[3]**
Mode	FIA	FIA	FIA
Internal volume	± 10 µl	± 10 µl	± 1 - 2 µl
Repeatability (as %SD in peak height)	1-2%	Not determined	2-4%
Linearity	Up to 15 mM	Not determined	Up to 25 mM
Limit of Detection	0.1 mM of glucose	22 mM of lactate	0.3 mM of glucose
Stability	14 days	2-4 hours	21 days
Selectivity (interference by ascorbic acid and/or uric acid)	No interference	Not determined	No interference

1] Biosensor obtained from SensLab, tested in the FIA mode at a flow rate of 0.5 ml.min^{-1} at +0.25 V vs. Ag/AgCl;

2] Biosensor obtained from Manchester, tested in the FIA mode at a flow rate of 100 µl.min^{-1} at +0.65 V vs. Ag/AgCl;

3] Biosensor obtained from Freiburg, tested in the FIA mode at a flow rate of 10 µl.min^{-1} at +0.35 V vs. Ag/AgCl.

4.3.2 Construction of the miniaturised biosensor

Our ultimate goal was to define a miniaturised biosensor device connected directly to a UF or MD probe for on-line continuous *in vivo* monitoring. Ideally, for continuous monitoring the total device comprises of, besides a potentiostat and data recording, a sampling probe (UF or MD) directly connected to the biosensor and the semi-vacuum syringe pump. To our knowledge no hardware is yet available to perform *in vivo* sampling by UF or MD at 100 $nl.min^{-1}$ and continuous analysis by means of a portable biosensor device. In order to succeed, several criteria have to be met for this miniaturised flow-through cell. To enable continuous monitoring on a real-time basis, unacceptable delay times should be avoided. For this reason, the internal cell volume should not exceed 25 nanoliter whereas dead volumes due to inefficient connections between the different parts of the device should be minimised. Next, to prevent the formation and/or retaining of air bubbles in the device and/or bad functioning of the pump, restrictions in the flow should be avoided. In addition, the flow-through cell should be made of biocompatible material and should contain three electrodes for amperometric measurements. Based upon these criteria set, several fabrication technologies have been reviewed to allow the reproducible (mass)-production of these devices in the near future. However, most of these techniques could not offer a total solution. For instance, micro-injection moulding[13] can be used for the mass-production of the flow-through cell, but the integration of the electrodes must take place afterwards and additional techniques are needed to provide for the connections. Because the production of a mould is extremely expensive and success could not be guaranteed,

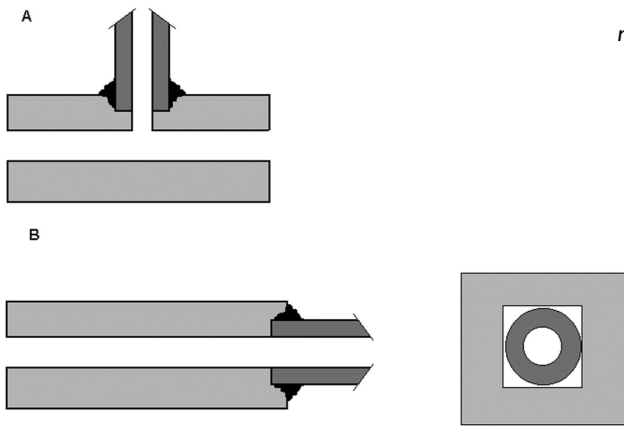

Figure 3: *Connections with microchannels produced via lithography; (a) connections perpendicular on the microchannel, (b) connections in line with the microchannel.*

other alternatives were investigated. Although lithography is frequently used for the production of microproducts[14,15], again the main problem lies in the connection with other parts of the device. Because most channels are produced via anisotropic etching[16], rectangular channels are formed. As a consequence, most connections are made perpendicular on the channel (see figure 3a)[17]. In our set-up, however, connections direct in line with the channel are preferred (see figure 3b). As can be seen in figure 3, it is obvious that difficulties might be expected by connection the other parts of the measuring device to the flow-through cell in such a way. Another interesting technology is Rapid Micro Product Development (RMPD) by means of stereo lithography[18]. With this technology, a three-dimensional microproduct can be rapidly developed and produced, and other components can be easily integrated. A main disadvantage is the relatively long production time (several hours) per product and the high costs involved if other components have to be integrated. As with lithographic techniques, the possibilities with thin film technology[19] did not convince us to start the production of a prototype. Difficulties were expected with the connections and the production of a reproducible multi-layer model. Additional techniques, like micromilling and the application of laser for the production of microproducts, can merely be seen as additional techniques. In summary, due to the high costs involved for the production of the first prototypes and the fact that success was not guaranteed due to the difficulties expected by connecting the different parts of the device, it was decided to produce and test the first prototypes in our laboratory. By using tygon tubing with a defined internal diameter, a flow-through cell with a defined volume can be easily constructed, as can be schematically seen in figure 2. With an internal diameter of 0.005 inch (0.127 mm), the tubing has a specified volume of 127 nl.cm^{-1}. In this case the electrodes are positioned within 1-2 mm of each other, and a flow-through cell with a total volume of approximately 10-20 nl can thus be obtained. *By using* 4 cm of 150 µm OD x 50 µm ID fused silica capillaries (with a specified volume of 20 nl.min^{-1}) to connect the biosensor with the probe respectively pump, the total internal volume will be 100 nl or less. Via this way, continuously monitoring at 100 – 300 nl.min^{-1} on a real-time basis with an acceptable delay time (less than a minute) is possible. Additionally, by using this type of construction it is not likely that air bubbles will retain in this linear type flow-through system and disturb the analysis, whereas restrictions in the wet part of the system (probe, flow-through cell, pump and connections) which may affect the performance of the semi-vacuum syringe pump are avoided as much as possible. Regarding the *in vitro* and/or *in vivo* testing of the biosensor, the low costs involved and easy fabrication in the laboratory without excessive investments can be remarked as an additional advantage.

4.3.3 Introduction of the permselective membrane

Immobilisation of the bioselective component via *in situ* encapsulation by electropolymerisation of a non-conductive polymer, poly(1,3-phenylenediamine), enabled us the production of reliable and sensitive biosensors. Thanks to the self-controlling film thickness of the non-conducting polymer poly(m-phenylenediamine) during electro-polymerisation, a thin and uniform film thickness can thus reproducibly be obtained. In addition, it has to be emphasised that the introduction of the biorecognition part of the biosensor on submicron levels and/or immobilisation of biomolecules in these closed micro-channels can only be performed via this way. As reported earlier into more detail[20], the reproducibility of the production of the biosensors in our laboratory was investigated by testing 11 produced biosensors in the FIA mode. The reproducibility was found to be approximately 15% (see table 2).

Table 2:

Some performance characteristics of the miniaturised biosensor.

Characteristics studied	**Performance of the biosensor**	
Precision[1]	**Test with one sensor**	**Test with 11 sensors**
	Peak height (nA)	Peak height (nA)
Injection no.:		
X1	0.28	0.30
X2	0.27	0.32
X3	0.28	0.28
X4	0.28	0.25
X5	0.26	0.25
X6	0.28	0.34
X7	0.26	0.35
X8	0.28	0.28
X9	0.28	0.37
X10	0.28	0.25
X11	-	0.28
X_{mean}	0.273	0.297
S_x	0.008	0.042
% S_x	3%	14.3%

Selectivity[2]	Recorder output[3] (nA)
Sample:	
Blank (Dulbeccoís buffer)	0
0 mM glucose + 0.1 mM ascorbic acid	0
0 mM glucose + 0.25 mM Uric acid	0
5 mM glucose	1.000
5 mM glucose + 0.1 mM ascorbic acid	0.975 (2.5%)
5 mM glucose + 0.25 mM Uric acid	1.025 (2.5%)
25 mM glucose	5.45
25 mM glucose + 0.1 mM ascorbic acid	5.55 (1.8%)
25 mM glucose + 0.25 mM Uric acid	5.50 (0.9%)

1] The repeatability of the FIA system in combination with the miniaturised biosensor was tested by injecting several times (n = 10) a standard solution of 5 mM of glucose in Dulbecco's buffer. The repeatability of the production of this biosensor was tested in the FIA mode. A standard solution of 5 mM glucose in Dulbecco's buffer was injected six times; the data presented in the table are mean values of the peak heights measured.

2] The selectivity of a three week old biosensor was tested in the continuous mode.

3] From the data displayed, the recorder out-put from the blank (off-set) is subtracted.

The repeatability of the measurements by means of FIA in combination with the biosensor, determined after ten consecutive injections of a standard glucose solution, was found to be 3%. Linearity of the biosensor was observed up to 25 mM of glucose as tested in the FIA mode as well as the continuous mode (see figure 4), whereas a limit of detection of 0.5 mM glucose could be calculated. By analysing in the continuous mode standard solutions, containing 0.1 mM ascorbic acid or 0.25 mM uric acid in combination with 0, 5 respectively 25 mM of glucose, the selectivity of the biosensor could be demonstrated (see table 2). In addition, the selectivity and accuracy of the biosensor was proven by analysing approximately sixty serum samples and comparing the results with those obtained from the clinical laboratory thereby using validated methods (see figure 5). The accuracy was found to be well in line with the criteria set for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus[21]. The stability of the biosensor was extensively tested, and based upon these results, as demonstrated in figure 6, the performance of the biosensors

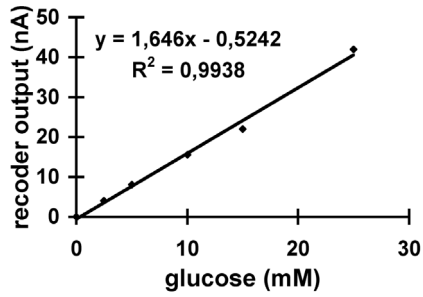

Figure 4:
Calibration curve for the determination of glucose by means of the miniaturised biosensor, determined in the continuous mode.

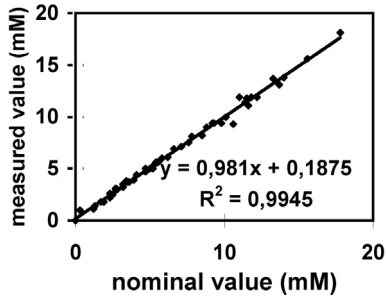

Figure 5:
Accuracy of the miniaturised biosensor: Correlation between glucose in dialysate measured with the biosensor and glucose content in serum determined by the clinical laboratory. The Y-axis represents the individual values determined in 60 serum samples by the biosensor; the Y-axis represents the individual values determined in 60 serum samples by a validated clinical method.

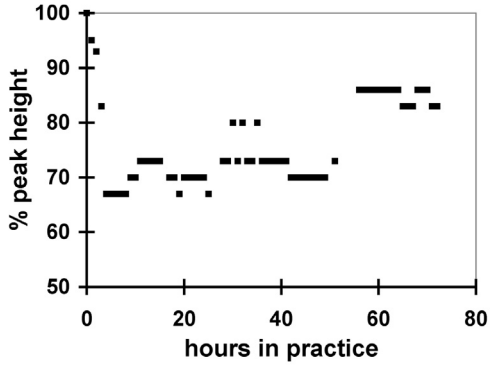

Figure 6:
Stability of the glucose biosensor, determined by continuously monitoring dialysate of a serum sample containing approximately 6 mM of glucose.

produced in this way could be guaranteed for almost a week, whereas frequently one biosensor could be used in the continuous mode for up to 3 weeks (data not shown).

4.3.4 Performance characteristics of the syringe pump

Besides the availability of a miniaturised biosensor, a pulse-free pump is required for delivering reproducibly low flow rates (100 – 300 nl.min^{-1}). By using a disposable syringe as

mentioned before[12], ultraslow MD or UF by means of underpressure can reproducibly be performed. The performance of the disposable syringe pump is clearly demonstrated in figure 7. From this figure, the drift in the peak heights due to the pulse of the model 22 Harvard syringe pump can easily be recognised, whereas hardly no drift is seen when the disposable syringe pump is used. Data reported earlier[7], when water was ultrafiltrated at a flow rate of 300 nl.min^{-1} and collected filtrate weighed every 5 minutes for more than 3 consecutive days, demonstrated the reliability of this syringe pump. Based upon these results, the disposable syringe pump is routinely used in our laboratory during *in vivo* monitoring experiments for a variety of biomedical applications as presented below.

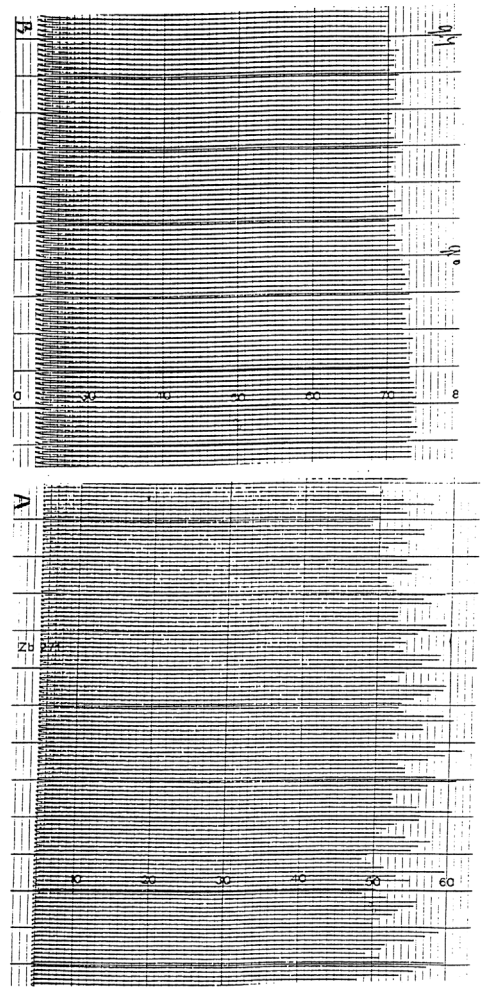

Figure 7:
Performance of the syringe pumps used for sampling at a flow rate of 300 nl/min. The content of glucose in dialysate is determined by FIA by switching on a time base (60/120 load/inject) the partly filled 20 µl loop on-line with the detection system; (a) Performance of the model 22 Harvard syringe pump, (b) Performance of the disposable syringe pump.

4.3.5 Biomedical applications

To demonstrate the applicability of the measuring device developed, results of some ongoing *in vivo* studies will be presented here. In figure 8, typical data obtained during glucose tolerance tests on healthy volunteers are demonstrated. Subcutaneous sampling was performed at a flow rate of approximately 100 nl.min^{-1} by means of ultraslow MD. The dialysate was continuously analysed by connecting the biosensor directly to the MD probe (CMA 60 probe). During the glucose tolerance test, blood samples were obtained and analysed in the clinical laboratory. The results show a remarkably good correlation between the blood glucose concentrations and the subcutaneous glucose concentration measured with the biosensor. Another biomedical application, which clearly demonstrated the importance of continuous monitoring, is the measurement of lactate in blood during myocardial ischemia in anaesthetised pigs. In contrary to other markers of heart ischemia, the monitoring of lactate may offer an opportunity to detect tissue oxygen deficiency more directly and therefore may prevent irreversible heart damage. To examine this, an UF catheter was placed in the coronary sinus, allowing cardiac monitoring without heart surgery. The content of lactate in the ultrafiltrate thus obtained was continuously monitored for several hours during which ischemia was induced for various periods of time by inflating a balloon catheter in the left anterior descending coronary artery. During this study, which will be described in more detail by Tiessen et[22], the first *in vivo* observations of prompt myocardial lactate changes have been found as can be seen in figure 9.

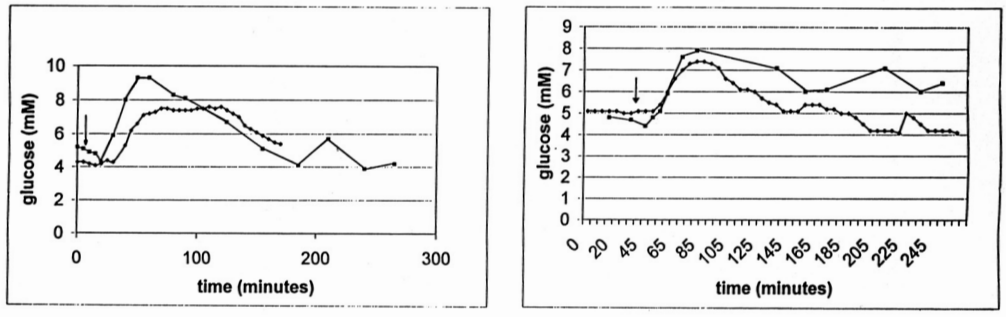

Figure 8:
Typical graphs obtained for in vivo monitoring of glucose during glucose tolerance tests on respectively two healthy volunteers (a) and (b); blood glucose concentrations in time (■), and subcutaneous glucose concentrations in time measured by the biosensor after ultraslow microdialysis (◆). The arrow in the figures indicates the oral administration of glucose.

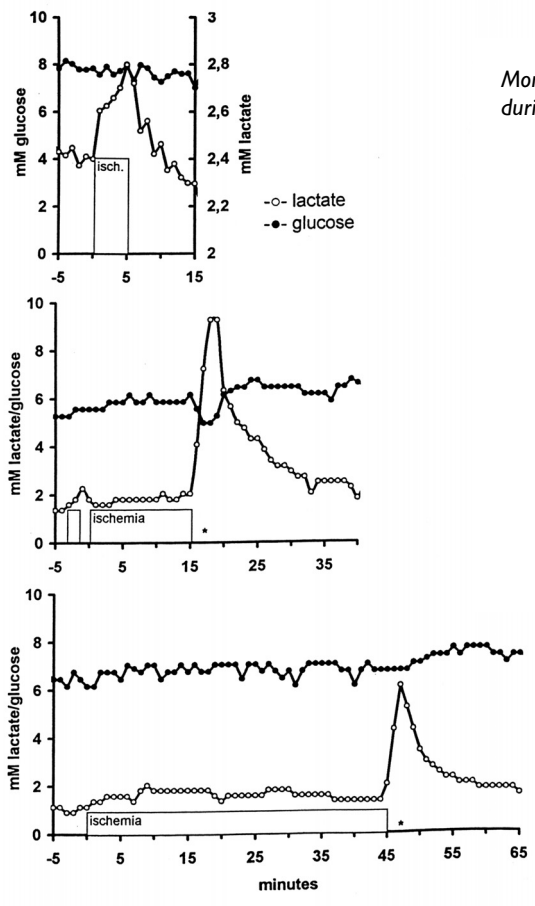

Figure 9:
Monitoring of lactate in blood during myocardial ischemia in anaesthetised pigs.

4.4 Conclusions

A miniaturised device, comprising a sampling unit (UF or MD probe), a biosensor and a semi-vacuum syringe pump, has been developed and tested for the on-line monitoring of glucose or lactate in biological matrices at very low perfusion flow. The permselective membrane of the bio-selector, based on an electropolymerised m-phenylenediamine provided good performance characteristics for in *vivo* studies. The good selectivity as well as enhanced sensitivity and quick response (several seconds) enabled us to develop a miniaturised flow-through nanoliter biosensor. Based upon the chosen production process, a cost-effective and reliable biosensor can reproducibly be produced. The results obtained so far from *in vivo* studies demonstrate the potential of the measuring device presented here.

4.5 References

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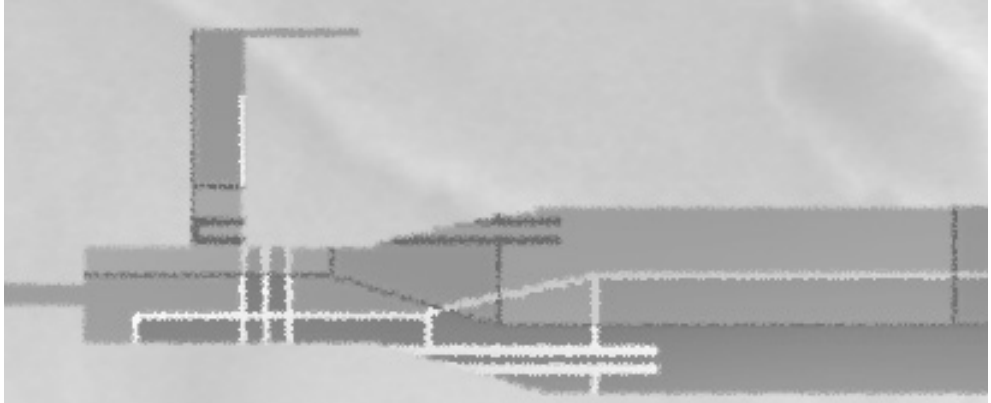

Chapter 5

On-line continuous monitoring of glucose and lactate by ultraslow microdialysis combined with a flow-through nanoliter biosensor based on poly(m-phenylenediamine) ultra-thin polymer membrane as enzyme electrode

Summary

A miniaturised flow-through biosensor with a cell volume of only a few nanoliters was developed in our laboratory. The biosensor can be directly coupled to a microdialysis or ultrafiltration probe. Sampling and continuous on-line monitoring can be thus carried out at submicroliter levels and as a consequence quantitative recoveries of the analyte of interest are achieved. Via this way excessive calibration procedures as with conventional microdialysis are avoided. Here, the construction and the performance of such a biosensor for the continuous on-line monitoring of glucose and lactate will be presented. The biosensor is based on the amperometric detection of hydrogen peroxide after conversion of the analyte of interest by an immobilised oxidoreductase enzyme. Immobilisation of the enzyme is performed through electropolymerisation of m-phenylenediamine. Strategies to improve the performance (e.g. linearity, selectivity and stability) of the miniaturised biosensor are discussed and ex vivo and *in vivo* experiments carried out thus far demonstrates the potential of this miniaturised flow-through biosensor.

5.1 Introduction

Biosensors have by means the potential to monitor analytes continuously *in vivo*. An ideal biosensor can be used in the ambulant patient to monitor metabolism and warn in case of life-threatening emergency. Major problems associated with biofouling such as the rapid loss of biosensor performance, limited the number of biosensors currently used in clinical applications. To circumvent these problems, microdialysis (MD) and – more recently also – ultrafiltration (UF) has been introduced as an interface[1,2] between the biological matrix and the measuring device. UF and MD use an implanted semi-permeable membrane to filtrate or dialyse analytes from the biological matrix. The filtrate or perfusate containing analytes is being transported by pumping and analysed outside the body. Because the membrane excludes cells and large molecules, MD and UF are able to deliver a clean matrix for measurement with a biosensor. A major drawback of conventional MD is that no quantitative recoveries of the analyte *in vivo* is obtained and excessive (*in vivo*) calibration procedures are required for accurate monitoring. Unlike MD, UF-sample concentrations are independent on probe diffusion characteristics and analytes with low molecular weight are quantitatively recovered. Quantitative recoveries are also obtained with ultraslow MD. In

this case dialysate is collected by means of underpressure at extremely low flow rates (less than 300 nl.min^{-1}) instead of the conventional way of pushing the carrier solution through the probe. To achieve this a disposable pump capable of producing a stable low flow rate (50 – 300 nl.min^{-1}) for several days has been developed in our laboratory[3]. To enable, however, real time continuous monitoring at these very low flow rates, the construction as well as the performance of the biosensor needs special attention. Critical parameters, such as cell volume (≤ 25 nanoliter), low dead volume connections, biocompatibility of the materials and low backpressure is of importance. In addition, to allow the reproducible production of these measuring devices, the final construction of the biosensor will more or less depend on today's available manufacturing procedures. Based upon these criteria, a miniaturised flow-through cell with a maximum volume of 20 nanoliter has been developed in our laboratory. Thanks to the construction, this flow-through cell, as schematically demonstrated in figure 1, can be directly coupled to an UF or MD probe.

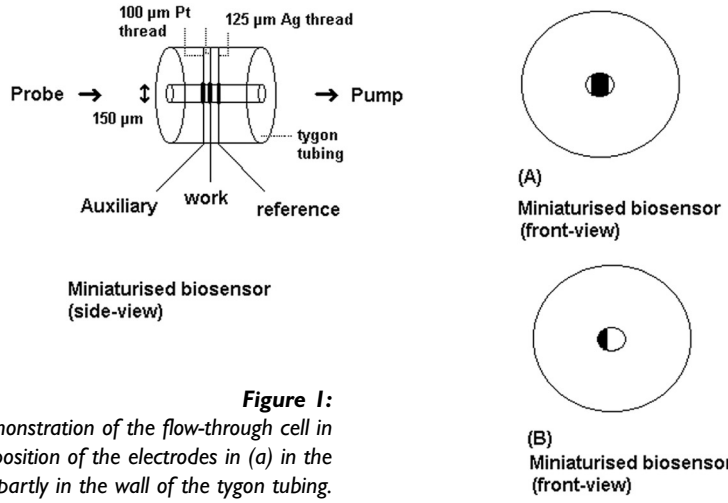

Figure 1:
Schematic demonstration of the flow-through cell in detail; position of the electrodes in (a) in the centre, or (b) partly in the wall of the tygon tubing.

Besides the construction of the measuring device, the performance of the biosensor is mainly dependent on the bio-selector. Because of the high substrate specificity and high turnover rate, immobilised oxidoreductase enzymes, such as glucose oxidase (GOx) or lactate oxidase (LOx), are well known for their use in biosensors. The measurement is based upon the conversion of the analyte of interest into the electrochemically detectable product, hydrogen peroxide. Due to the fact that the anodic response of the liberated

peroxide species is accompanied by large contributions from coexisting oxidizable constituents such as ascorbic acid and uric acid, the specificity of the bio-selector is compromised by the partial selectivity of the electrochemical transducer[4]. To minimise this effect, the enzyme is immobilised on the working electrode preferably by the use of a permselective membrane. An ideal permselective membrane does not only adequately immobilise the enzyme of interest, but also act as a substrate diffusion-limiting barrier and is capable of reducing surface fouling. Via this way the biosensor becomes more membrane diffusion controlled instead of following the Michaelis-Menten kinetics, and as a consequence extends the linear range of the biosensor. To avoid a combination of membranes, which inevitably extend the response time and lower the response, these functions are preferably combined in a single membrane. To achieve high solute fluxes and maintain good chemical selectivity, ultra-thin polymer-films are used for this reason. Much attention has been given to the application of electropolymerised conducting films of polypyrrole[5-7], poly(N-methylpyrrole)[8] and polyaniline[9,10] and recently of the non-conducting polymers, such as polyphenol[11], poly(2-naphthol)[12] and poly(o-phenylene-diamine)[13-23]. Biosensors using these polymers exhibit several advantages, such as they are effective in preventing the biosensor from fouling and eliminating the interference from electroactive species, whereas an enhanced sensitivity and electrocatalytic action for poly(1,3-phenylenediamine) permselective films was observed, which was attributed to the high efficiency of transformation of enzymatically generated H_2O_2 to an electrical signal[21,22]. Thanks to the self-controlling film thickness of the non-conducting polymer during electropolymerisation, a thin and uniform film can thus reproducibly be obtained. In addition, it has to be emphasised that the introduction of the biorecognition part of the biosensor on submicron levels and/or immobilisation of biomolecules in these closed micro-channels can only be performed via this way. Based on these advantages, the applicability of poly(1,3-phenylenediamine) as a permselective membrane in our miniaturised flow-through biosensors has been investigated. The present paper reports the studies conducted and results obtained with these miniaturised flow-through biosensors. To investigate and improve the performance of the biosensor, the conditions during electropolymerisation have been investigated and optimised. The linearity, precision and sensitivity as well as the selectivity and stability of various biosensors thus produced has been investigated and will be described in detail. Finally, to demonstrate the potential respectively possibilities of this instrumental set-up, results of some ongoing studies will be shown.

5.2 Experimental

5.2.1 Materials and solutions

The enzyme glucose oxidase (GOx) from *Aspergillus niger* (EC 1.1.3.4., grade I, 25,000 units/84.5 mg solid) and lactate oxidase (LOx) from *Pediococcus species* (grade I, 200 units/6.74 solid) are obtained from Boehringer Mannheim (Almere, the Netherlands). D(+)-glucose and L(+)-lactate for standard solutions and 1,3-phenylenediamine for the permselective membrane is purchased from Sigma Chemical Co. (Amstelveen, the Netherlands). All other chemicals are of pro-analysis quality and are purchased from E. Merck (Amsterdam, the Netherlands). Double quartz distilled water is used for all aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK) to inhibit bacterial growth. The composition of the carrier solution during MD is a Dulbecco's buffer phosphate-buffered saline (PBS) (mMol.l-1): NaCl (136.9), KCl (2.7), KH2PO4 (1.5), CaCl2 (0.9), MgCl2 (0.5), Na2HPO4 (8.1) and EDTA (2). The pH is adjusted to 7.4 and purged with helium before use. Standard solutions of glucose or lactate are prepared by diluting the stock solution of glucose (50 mMol.l-1) in PBS and are allowed to reach mutarotational equilibrium before use (24 h).

5.2.2 The flow-through cell

With a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) two platinum wires (0.10 mm diameter) and a Ag/AgCl wire (0.125 mm diameter) (Drijfhout, Amsterdam, the Netherlands) are placed carefully close to each other into a 0.005 inch ID tygon tubing (Skalar Analytical, Breda, the Netherlands) as demonstrated in figure 1. By means of a multimeter the correct position of the electrodes in the tubing is controlled. Possible leakage formed in the tygon tubing is eliminated with cyanoacrylic glue. Low dead volume connections with the biosensor are made with fused silica (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands).

5.2.3 The permselective membrane

The flow-through cell is washed with methanol, 10 v/v% hydrogen peroxide solution in water and 0.1 M phosphate buffer pH 7.0 by means of a 1 ml syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun,

Melsungen, Germany). The syringe is filled with a solution containing 2 $mg.ml^{-1}$ (approximately 500 units per ml) of enzyme and 10 $mg.ml^{-1}$ (90 $mmol.l^{-1}$) of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9. The syringe is placed in the model 22 syringe pump (Harvard Apparatus, Kent, UK) and the wires outside the tubing are connected with model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by means of crocodile clips. Electropolymerisation is performed at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 500 $nl.min^{-1}$ followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Before storage and/or use, the biosensors are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes. In between, the biosensors are stored in the refrigerator at 4-8 °C.

5.2.4. Measurements by means of Flow Injection Analysis (FIA)

The biosensors thus obtained have been used for measurements in the discontinuous mode (by means of FIA) and the continuous mode. For measurements in the discontinuous mode, a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) equipped with a VICI Cheminert C4 valve with a 100 nl internal loop is used. The biosensor is connected to the VICI Cheminert C4 valve for sample injection. With the model 22 syringe pump (Harvard Apparatus, Kent, UK) carrier solution and sample is transported to the biosensor at a flow rate of 5 µl/min. The internal loop of the valve was continuously filled with dialysate by connecting a CMA 60 MD catheter (Aurora Borealis Control, Assen, the Netherlands) and a home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[24] each at a side of the VICI Cheminert valve. Ultraslow MD is carried out at a flow rate of 300 $nl.min^{-1}$. To avoid high dead volumes, connections between the various parts of the FIA were made with fused silica (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Injection and analyses of the sample was carried out every three minutes. Measurements were carried out at +0.5 V vs. Ag/AgCl. Data is recorded with a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

5.2.5 Measurements in the continuous mode

For measurements in the continuous mode, one side of the biosensor is connected to a CMA 60 MD catheter (Aurora Borealis Control, Assen, the Netherlands) whereas the other side of the biosensor is directly connected to a home-made semi-vacuum syringe

pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[24]. Dialysate is continuously analysed at a flow rate of 300 nl.min^{-1}. Accordingly, measurements were carried out at +0.5 V vs. Ag/AgCl. Data is recorded with a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands). Before measurements, the biosensor was allowed to settle a stable current over a period of approximately 30 minutes.

5.2.6 Electron microscopy

Electron micrographs were taken using field emission scanning electron microscopy by a model JEOL 6301 F electron microscope (Jeol, Japan). Samples were sputter coated with an Au-Pd layer of 2 nm.

5.2.7. Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR)

Infrared spectra were recorded on a Mattson Galaxy 6020 FTIR spectrophotometer equipped with a single reflection diamond ATR (Specac Golden Gate) and a narrow bandpass mercury-cadmium-telluride (MCT) detector at a resolution of 4 cm^{-1}. Coaddition of 50 scans was used.

5.3 Results

5.3.1 Construction of the miniaturised flow-through cell

By using tygon tubing with a defined internal diameter, a flow-through cell with a defined volume can be easily constructed. With an internal diameter of 0.005 inch (0.127 mm), the tubing has a specified volume of 127 nl.cm^{-1}. If the electrodes are positioned within 1-2 mm of each other, a cell with a total volume of approximately 10-20 nl is obtained. Low dead volume connections between the probe and the semi-vacuum syringe pump are obtained with 150 µm OD x 50 µm ID fused silica capillary. For each centimetre of fused silica used, an additional dead volume of 20 nl is introduced. In practice the biosensor has been connected with approximately 2 cm of fused silica tubing. An instrumental set-up for monitoring in the continuous mode as thus obtained is demonstrated in figure 1. Although for every device the exact delay is determined during calibration prior to *in vivo* measurements, compared to the relatively high dead volume of the CMA 60 MD probe

(approximately 3 µl), the dead volume of the measuring device is negligible. Delay times in combination with the CMA 60 MD probes are found to be 2-3 minutes, whereas the response time of the biosensor without the MD probe is only in the order of seconds. These delay times are likely to be acceptable for the purpose of clinical monitoring.

5.3.2 Production of the miniaturised flow-through cell

The reproducibility of the production of these miniaturised flow-through cells is investigated by producing in our laboratory at 4 consecutive days a total of 11 biosensors. After storage at 4 - 8 °C during 3 days, each biosensor has been tested by analysing a standard solution of 5 mM of glucose in six-fold by means of FIA. The repeatability of the method, expressed as the relative standard deviation in the peak height after six consecutive injections was found to be 2 - 4% depending on the biosensor tested. For all biosensors tested, an average peak height for a glucose was calculated and was found to be 0.66 nA. The reproducibility of the production of these biosensors, expressed as the relative standard deviation in the mean peak height of 11 tested biosensors, was found to be 15%. Although the advantage of fabricating enzyme layers with highly reproducible thickness by controlling the amount of charge during electropolymerisation is recognised, only limited data is available to demonstrate the reproducible fabrication of biosensors in such a way. Gooding *et al.*[25] reported for their thin-film biosensors a deviation of between 7% and 15% between four biosensors produced at different days but prepared with the same batch of reagents. Although these data are comparable with our data, we believe that the deviation found by us is mainly caused by the fact that the electrodes are positioned in the flow-through cell by hand and as a consequence the surface of the working electrode will differ per biosensor (see also figure 1). And for this reason it is likely that through automation an improvement in the reproducibility of the manufacturing of these biosensors will be achieved.

5.3.3 Polymer formation of poly(phenylenediamine) films for use in biosensors

Although in this case m-phenylenediamine is used, even the formation of the frequently used poly(o-phenylenediamine) films has never been fully understood. To our knowledge, a polymerisation mechanism of o-phenylenediamine has only been proposed by Jang *et al.*[26]. They suggested that during electropolymerisation, at least two different polymeric

components which are responsible for electron mediation are produced: an active component containing phenazine, formed mainly in the early stage of polymerisation, and an inactive component containing noncyclically coupled species. Chiba *et al.*[27] investigated the structure of a 2.5 µm thick electropolymerised poly(o-phenylenediamine) films by means of infrared (IR) spectroscopy. They proposed a 'ladder' polymer with an asymmetrical 'quinoid' structure, which is partially ring-opened and involves moieties of oxidised forms of the quinone-imine type. To investigate the properties of the poly(m-phenylenediamine) films as described here, a previously washed 1 mm thick platinum electrode with a total surface area of 40 mm^2 was coated with electropolymerised m-phenylenediamine. For this reason, the electrode was dipped in a 0.1 M phosphate buffer pH 6.9 solution containing 10 $mg.ml^{-1}$ 1,3-phenylenediamine, a platinum counter electrode and a Ag/AgCl reference electrode, and electropolymerised at +0.8 V for 60 minutes. A brownish coloured electrode was obtained, which was completely coated with polymer with a thickness of approximately 1 µm, as investigated with electron microscopy. Structural information about the polymer was obtained by means of Total Reflectance Fourier Transform Infrared Spectroscopy. Although limited information can be obtained from the fingerprint area (1200 - 650 cm^{-1}), the stretching vibration of the N-H bonds (3300 – 3500 cm^{-1}), C-H bonds (2930 cm^{-1}) and C-C bonds (1500 – 1600 cm^{-1}) are clearly distinguished, whereas absorption peaks observed at ~ 1650 cm^{-1} and around 1600 cm^{-1} can be ascribed to C=O stretching vibrations of the quinone respectively C=C stretching vibrations (see figure 2). Although no full qualitative analyses of the polymer is possible with these thin polymer films, the spectra thus obtained are comparable with those found by Chiba *et al.*[27]. This means that the formation of m-phenylenediamine based polymer films is comparable with o–phenylenediamine based polymer films.

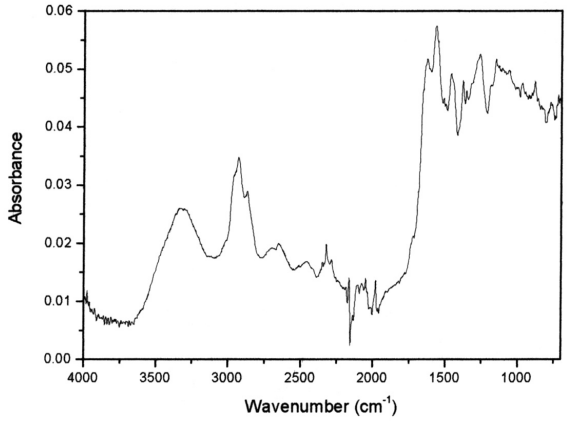

Figure 2:
Infrared spectrum of an electropolymerised poly(m-phenylenediamine) film on a platinum disk electrode (film thickness of ~ 1 µm).

5.3.4 Dynamic range of the poly(m-phenylenediamine) based biosensor

The polymerisation time on the performance of the biosensor in our set-up was investigated. Different biosensors were made in which GOx was immobilised at +0.8V for 15, 30, 60, 90 respectively 120 minutes of polymerisation. After an equilibration time of 15 minutes, each biosensor was tested by analysing a standard solution containing 5 mM glucose in Dulbecco's buffer by means of FIA, and the mean peak height (nA) was determined for each individual biosensor. Based on the results (see table 1) it was decided to immobilise the enzyme by electropolymerisation at +0.8 V for 1 hour.

Table 1:

Effect of polymerisation time on the performance of the biosensor

Polymerisation time (min)	***Peak height (nA)***
15	0.23
30	0.48
60	1.83
90	0.82
120	0.58

Several biosensors produced accordingly were tested *in vitro* in the continuous mode. A typical calibration plot as shown in figure 3 was obtained, and linearity was found up to approximately 5 mM of glucose. However, for continuous monitoring of for instance patients suffering from diabetes mellitus, glucose concentrations of up to 30 mM can be expected, which makes this biosensor less suitable for clinical applications. So, in order to

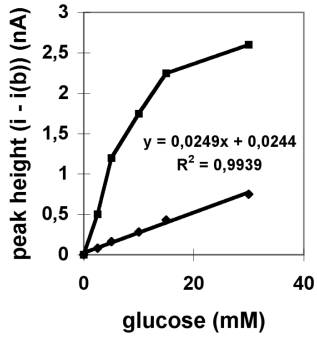

Figure 3:
Calibration curves for (a) 'one layer' of poly(m-phenylenediamine) (■), and (b) an 'additional' layer of poly(m-phenylendiamine) (◆).

avoid Michaelis-Menten kinetics, and to improve the substrate diffusion-limiting barrier respectively, several options were investigated. By extending the polymerisation time, a 'densification' of the polymer layer in combination with a slight thickness increase of the poly(o-phenylenediamine) film was observed by Jobst[29]. In our set-up this was investigated by dipping 0.1 mm previously washed platinum wires in a 10 mg/ml 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9 containing a platinum counter electrode and a Ag/AgCl reference electrode, and electropolymerise at +0.8 V for 60 respectively 90 minutes. Although a 'densification' cannot be clearly seen with electron microscopy (see figure 4), a slight increase of 200 up to 240 nm of the thickness of the permselective membrane is observed. These layers are clearly thicker than found by others[13-14,21], who reported a thickness of only 10 – 35 nm. Because polymerisation is performed at comparable pH (pH 6.9 versus pH 5.2 - 7.4), the contradicting thickness of the layer cannot be explained by the

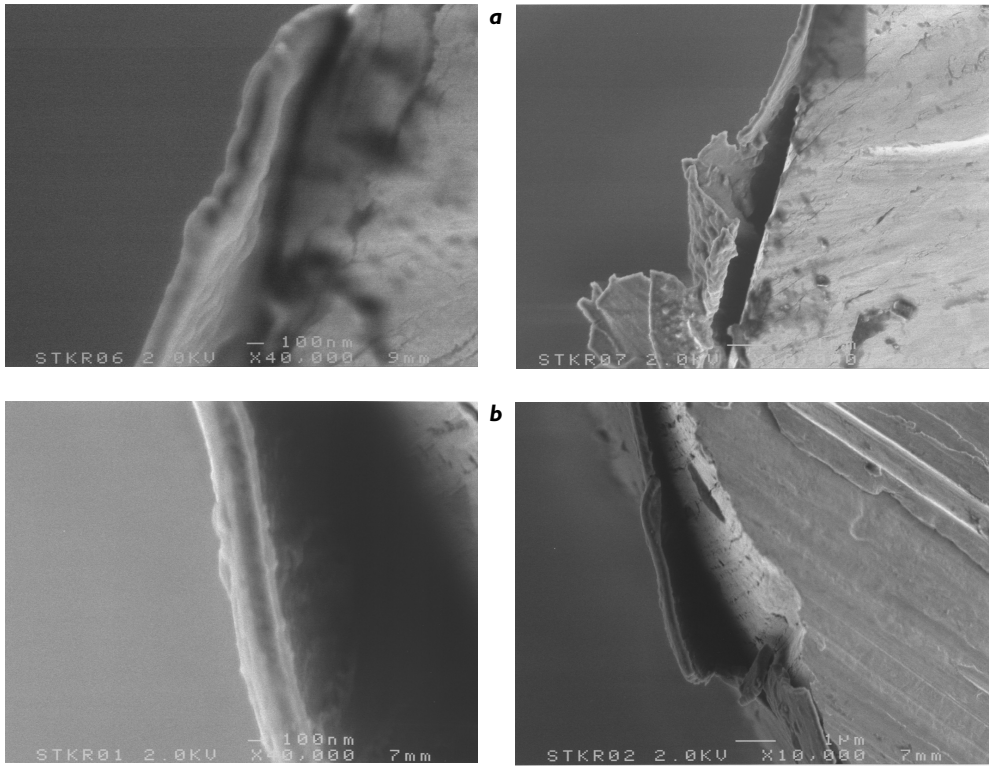

Figure 4:
Electron Microscopy pictures of an electropolymerised poly(m-phenylenediamine) film on a platinum wire for (a) 60 minutes, and (b) 90 minutes.

pH of the polymerisation solution. More likely, other parameters such as the polymerisation time seems to be of more importance. For instance our polymerisation time is considerably longer (1.5 hours compared to 10-15 minutes). However due to the diversity of protocols and reported contradicting data, no full explanation can be given for this phenomenon. More interesting is, however, whether or not the characteristics of the diffusion-limiting barrier of the permselective membrane can be affected via this way. For this reason, biosensors were produced by electropolymerisation of an additional 30 minutes with a solution containing the monomer only. After testing the biosensors *in vitro* by means of analysing standard solutions of glucose in the continuous mode, a typical calibration curve, as shown in figure 3, was obtained. Obviously, the linear range (up to 30 mM glucose) is now satisfactory. Although the sensitivity also decreased as expected, with a calculated limit of detection of 0.05 mM of glucose, the sensitivity was found to be adequate enough for our purpose. Also, no significant increase in the response time (only several seconds) was observed. An additional advantage of producing biosensors this way is that the electropolymerisation time is less critical whereas broad dynamic range biosensors can be obtained without the use of complex and/or a diversity of procedures for the fabrication of (multilayers) of permselective membranes.

5.3.5 Enzyme immobilisation

Approximately 3 – 10 U of GOx per cm^2 of electrode surface can be incorporated into poly(o-phenylenediamine) films[13-14,21]. In our set-up, this means that, with a maximum electrode surface area of 0.0571 mm^2, 0.14 – 0.50 µU of GOx is available, which theoretically can convert 0.0035 – 0.0120 pMol/s of glucose at pH 7.0 at 25 °C. Based upon the measurements performed, a maximum conversion of approximately 0.0150 $pMol.s^{-1}$ of hydrogen peroxide has been calculated according to Faraday's law. The transformation coefficient in poly(o-aminophenol) films, defined as the efficiency of the transformation of H_2O_2 produced by the enzymatic reaction to an electronic signal, varies between 15 – 77%[21]. Although not completely comparable, a maximum experimental conversion of 0.020 – 0.100 $pMol.s^{-1}$ of hydrogen peroxide can be estimated. Obviously, the amount of active enzyme entrapped in the polymer film is 1 order of magnitude higher than assumed.

5.3.6 Selectivity of the biosensor

It is hypothesised that a high electron flux may lead to the protonation of the amino-groups

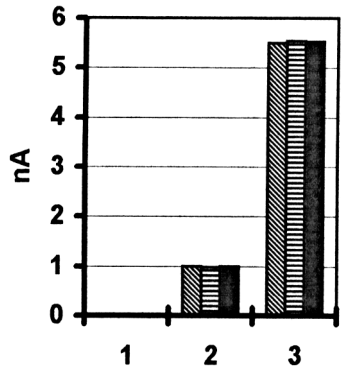

Figure 5A:
Contribution by interferences on the glucose signal (the data presented are corrected for the baseline current and the Y-axis represents (i – i(b)) (nA)): (1) 0 mM glucose, 0 mM glucose + 0.1 mM ascorbic acid, 0 mM glucose + 0.25 mM uric acid; (2) 5 mM glucose, 5 mM glucose + 0.1 mM ascorbic acid, 5 mM glucose + 0.25 mM uric acid; (3) 25 mM glucose, 25 mM glucose + 0.1 mM ascorbic acid, 25 mM glucose + 0.25 mM uric acid.

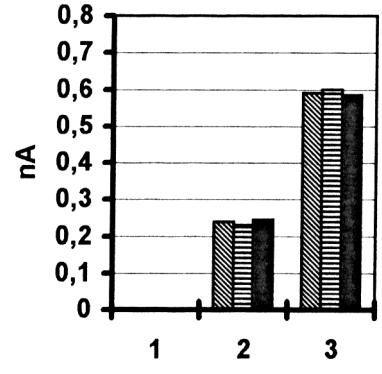

Figure 5B:
Contribution by interference on the lactate signal (the data presented are corrected for the baseline current and the Y-axis represents (i – i(b)) (nA)): (1) 0 mM lactate, 0 mM lactate + 0.1 mM ascorbic acid, 0 mM lactate + 0.25 mM uric acid; (2) 2 mM lactate, 2 mM lactate + 0.1 mM ascorbic acid, 2 mM lactate + 0.25 mM uric acid; (3) 5 mM lactate, 5 mM lactate + 0.1 mM ascorbic acid, 5 mM lactate + 0.25 mM uric acid.

of the poly(m-phenylenediamine) film. As consequence the permselective membrane might swell allowing interferents to gain access to the working electrode and which may affect the selectivity of the biosensor. For this reason it is worthwhile to investigate the selectivity of the permselective membrane at high substrate flux. For this reason, the selectivity of the poly(m-phenylenediamine) based biosensor as in our set-up is investigated by analysing in the continuous mode standard solutions, containing 0.1 mM ascorbic acid or 0.25 mM uric acid in combination with 0, 5 respectively 25 mM of glucose. Based on the results shown in figure 5, no significant contribution to the signal is observed from the possible inferences tested. This means that even at a high substrate flux within the dynamic range of the biosensor, the permselectivity of the poly(m-phenylenediamine) film is not altered.

5.3.7 The stability of the glucose biosensor

To examine the stability of the biosensor as presented here, several experiments have been conducted. Biosensors with only 'one layer' of poly(m-phenylenediamine) (60 minutes of

electropolymerisation) and stored in the refrigerator at 4 °C. The biosensors were tested after respectively 0, 3, 5 and 7 days of storage by analysing a standard solution containing 5 mM glucose in Dulbecco's buffer by means of FIA and measuring the mean peak height (nA). In contrast, the biosensor tested at t = 0, was kept at room temperature and retested after respectively 1, 2, 3, 7 and 8 days by means of FIA. The results of these tests are shown in table 2.

Table 2:

Stability of glucose biosensor under different storage conditions, in use and effect of preservative

Age of sensor (days)	**Storage**[1]		
	4 °C no preservative	Room temperature no preservative	Room temperature with preservative
0	100	100	100
1	-	22	-
2	-	19	80
3	51	17	-
5	20	-	58
6	-	-	-
7	16	5	58
8	-	0	50

[1] Results are expressed as percentage of original peak height at day 0.

Remarkably, although storage at 4 °C does prolong the lifetime of the biosensor, an unacceptable decrease in the performance after 7 days of storage is observed. After 15 days of storage, the performance had grown worse and no signal for a standard solution 5 mM glucose could be measured (data not shown). Because the biosensors tested after respectively 3, 5 and 7 days of storage at 4 °C were not tested before, leaching out of enzyme is probably not the only explanation for stability problems with these biosensors. Due to the fact that the biosensors were not completely stored dry, dissolution of the film into the solution could be an explanation for this phenomenon. However, positive experience with preservatives regarding the stability of enzymes in instrumental set-ups,

leaded to the next experiment, in which a biosensor with 'one layer' of poly(m–phenylenediamine) (60 minutes of electropolymerisation) was kept at room temperature and retested accordingly after 2, 5, 7 and 8 days by means of FIA. However, in this case, 0,1% of Kathon CG (a preservative) was added to the carrier solution. From the results shown in table 2, a significant increase in the stability can be observed. The first drop from 100% (t = 0) to approximately 60% (t = 5 days) of signal is probably due to leaching out of enzyme and/or partly dissolution of the polymer film. After five days, equilibrium is seen whereas the performance of the biosensor gradually decreased and after approximately 30 days (data not shown) no signal could be detected anymore. Although no clear explanation can be given for these observations, it is questionable whether or not the ingredients within the preservative used may affect in a positive way the stability of the biosensor. In order to be able to answer these questions, more research regarding the stability of biosensors during (different) storage conditions will be necessary. Equally important, however, is the stability of the biosensor in practice. During the measurements, gluconic acid as well as hydrogen peroxide is generated. Especially hydrogen peroxide may affect the stability of the enzyme glucose oxidase. To examine these two biosensors, with 'one layer' of poly(m-phenylenediamine) (60 minutes of electropolymerisation) and another with an 'additional layer' of poly(m-phenylenediamine) (90 minutes of electro-polymerisation), were made and tested accordingly by continuously monitoring of a standard solution of 5 mM glucose. The results are shown in figure 6. Again, for both sensors an initial decrease in sensitivity from 100% to approximately 70% was seen within one day of practice, and is probably caused by leaching out of not fully immobilised enzyme and/or polymer film[13,21]. However, as demonstrated in figure 6, the biosensor with the 'additional layer' retained its activity whereas the performance of the other biosensor gradually decreased within 70 hours. These results suggest that the stability of the biosensor is merely dependent on the denaturation of the enzyme by hydrogen peroxide[28]. Others[30] attributed this decline in sensitivity on electrode fouling by small endogenous proteins. To investigate this in our set-up, a serum sample containing approximately 5 mM of glucose was monitored for several days in the continuous mode. The data obtained, as demonstrated in figure 6, are typical for biosensors thus produced. After an initial decrease in sensitivity, the biosensor was stable for at least three days whereas no significant additional decrease in the performance of the biosensor was observed. Based on these ex-vivo results, it might be concluded that the biosensor can be used for up to three days for continuous *in vivo* monitoring of glucose. Although more stability studies will be conducted in the near future, these results clearly indicate the performance of this type of biosensor.

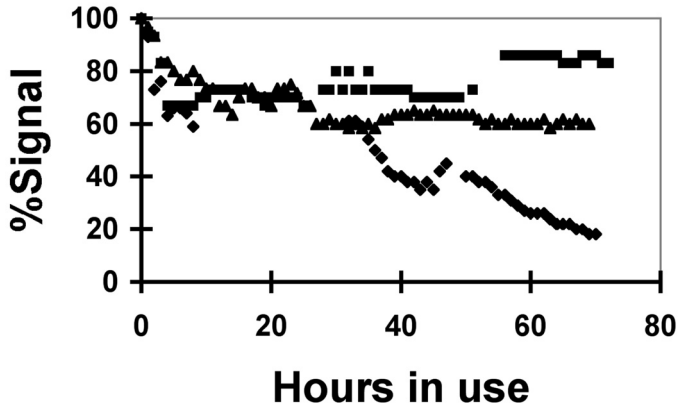

Figure 6:
Stability of the glucose biosensor:
(a) Continuous monitoring of a 5 mM glucose solution with a '60 minutes' poly(m-phenylenediamine) film based biosensor (◆), and
(b) Continuous monitoring of a 5 mM glucose solution with a '90 minutes' poly(m-phenylendiamine) film based biosensor (■), and
(c) Continuous monitoring of a serum sample with a '90 minutes' poly(m-phenylenediamine) film based biosensor (▲).

Obviously, the initial decrease in sensitivity is determined by leaching out of enzyme and/or dissolution of the polymer film followed by biodegradation during storage and/or in practice and denaturation of the enzyme by hydrogen peroxide in practice.

5.3.8 Performance characteristics of the lactate biosensor

Calibration curves of the lactate biosensor were performed by analysing standard solutions of lactate by means of FIA, and linearity was found up to approximately 5 mM of lactate. Reference values of lactate in blood range from 0.5 till 2.2 mM, which means that the lactate biosensors produced here are sufficiently linear for this purpose. Although similar characteristics were observed regarding the selectivity (see figure 5), response time (in the order of seconds) and reproducibility (4% as determined by FIA and expressed as the relative standard deviation in the peak height after six consecutive injections), the stability of the lactate biosensor was of our most concern. In figure 7, the stability data are demonstrated which were obtained after analysing by means of FIA a standard solution of 2 mM of lactate in Dulbecco's buffer. From this figure a 60% decrease in the performance of the biosensor can be seen within 5 hours of practice. Experiments with these biosensors

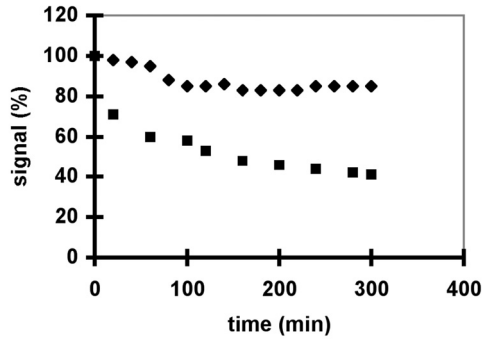

Figure 7:
Effect of electropolymerisation by cyclic voltammetry on the stability of a lactate biosensor: (a) electropolymerisation at +0.8 V(■); (b) electropolymerisation by cyclic voltammetry (◆).

in the continuous mode showed a rapid decrease in signal to almost zero within one hour of practice. Based upon these results it is evident that both the stability towards denaturation as well as immobilisation within a poly(m-phenylenediamine) polymer film is significantly poorer for LOx compared to GOx. From work by others it is known that the potential scan range for polymerisation can affect the film morphology, and thus the performance of the biosensor. Zhang *et al.*[21] demonstrated that electropolymerisation by cyclic voltammetry generates a more uniform and compact insulating film. For this reason, a lactate biosensor was produced by sequentially potentially cycling from –0.2 to +1.0 V vs. Ag/AgCl at a scan rate of 50 $mV.s^{-1}$ for at least 20 potential sweeps in a 0.1 M phosphate buffer solution pH 7.0, containing 2 $mg.ml^{-1}$ of enzyme and 10 $mg.ml^{-1}$ of 1,3-phenylenediamine. Although, as can be seen from figure 7, the stability of the lactate biosensor produced in this way is significantly increased, during continuous monitoring however, these biosensors still show an unacceptable decline in the performance of the biosensor. For *in vivo* studies by means of continuous monitoring of lactate, more work needs to be done to improve the stability of these biosensors.

5.3.9 Applications

To demonstrate the applicability of the biosensors described in this article, some results of our ongoing *in vivo* studies will be presented here. Only a few studies reported the use of these devices *in vivo* neurochemical studies. Besides the fact that the extracellular fluid (ECF) contains several potential electrocatalysts (e.g. glutathione and ascorbic acid), which might affect the performance of the biosensor, also the restriction of mass-transport to the electrode surface by brain tissue, reflects the difficulties associated with performing direct neurochemical measurements in this complex environment. J.P. Lowry *et al.*[17-19] and Cass

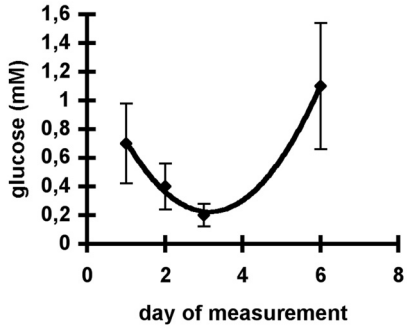

Figure 8:
Monitoring of brain extracellular glucose in rat striatum of anaesthetised rats (n = 6, day 1 directly after implantation of the probe) and freely moving rats (day 2, 3 and 6 after implantation of the probe).

et al.[31] demonstrated the use of such a biosensor, for the monitoring of brain extracellular glucose in rat striatum in awake freely-moving rats. As the reported ECF glucose contents differed significantly (0.5 – 2 mM), we decided to investigate these ECF glucose concentrations in rat striatum. Small I-shaped home-made MD probes, as described earlier in detail[32], were implanted in rat striatum of anaesthetised rats. Immediately after implantation, the biosensor was coupled to the probe and glucose was monitored continuously at a flow rate of 100 nl.min^{-1} in dialysate. One day respectively 3, 5 and 6 days after implantation, glucose was monitored similarly in rat striatum of freely moving rats. Although the study is still ongoing, tentative results (see figure 8) have been obtained which are in accordance with reported preliminary results. In our set-up, directly after implantation of the probe, a concentration of approximately 0.7 mM of glucose is found; this concentration decreases to about 0.2 mM within 3 days followed by an increase to above 1 mM 6 days after implantation. These levels are among the lowest reported levels and illustrate both sensitivity and selectivity of the biosensor.

5.4 Conclusion

A miniaturised device, comprising a sampling unit (UF or MD probe), a biosensor and a semi-vacuum syringe pump, has been developed and tested for the continuous on-line monitoring of glucose or lactate in biological matrices at very low perfusion flow. The permselective membrane of the bio-selector, based on a 'double layer' of electropolymerised m-phenylenediamine provided good performance characteristics for *in vivo* studies. The good selectivity, sensitivity and quick response (several seconds) enabled

us to develop a miniaturised flow-through nanoliter biosensor. Although still made by hand in the laboratory, a cheap and reliable biosensor can thus reproducibly be fabricated. The linearity as well as stability was extended by increasing the time of electropolymerisation with m-phenylenediamine. The stability of lactate based biosensors, however, is still cumbersome. Although good performance is demonstrated in the FIA mode, in order to apply these lactate biosensors in the continuous mode more work needs to be done to improve the stability of the lactate biosensor. The results obtained so far with the glucose biosensors from *in vivo* studies demonstrate the potential of the measuring device presented here.

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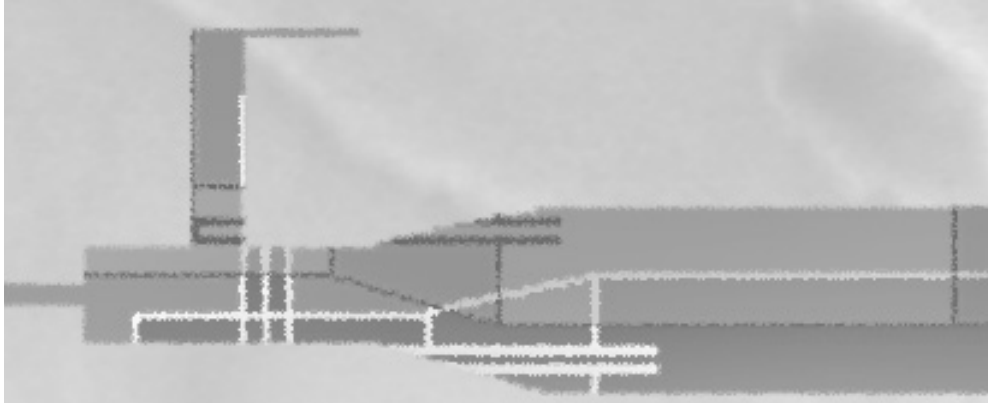

Chapter 6

Covalent immobilization of enzymes throughout a poly(m-phenylenediamine) ultra-thin polymer membrane for the production of stable flow-through nanoliter biosensors

Summary

The fabrication of a stable flow-through nanoliter lactate and glucose biosensor is reported. The fabrication is based on the EDC mediated covalent immobilization of the enzymes lactate oxidase respectively glucose oxidase onto an *in situ* electropolymerized poly(m–phenylenediamine) polymer film. Without the additional use of a mediator, detection of the subsequent analyte was found to be possible at –150 mV vs Ag/AgCl. The dynamic range as well as the sensitivity of the lactate biosensor was sufficient for the analysis of lactate in clinical relevant levels. *In vitro* experiments with ascorbic acid and uric acid demonstrated the selectivity of the lactate biosensor. The stability of the biosensor was found to be sufficient for continuous *in vivo* monitoring of lactate in blood ultrafiltrate. Intermittent stability studies demonstrated the stability: no significant decline in biosensor sensitivity was seen within 15 days of practical use.

6.1 Introduction

The coupling of a biological-sensing element (such as en enzyme, cell or antibody) with a transducer, either electrochemical, optical or piezoelectric, is the basis of a biosensor[1]. These two components are usually integrated by the immobilization of the biorecognition molecule onto the surface of the transducer. Ideally, the biorecognition molecule is immobilized close to the transducer surface and retaining its biological activity in a reproducible manner. In addition, the desired immobilization layer should possess over good permselective properties, act as a substrate diffusion-limiting barrier in order to avoid Michaelis-Menten kinetics and is capable of reducing surface fouling. Furthermore, it is desirable that the immobilization layer gives the biological molecule enhanced stability, applicable for the immobilization of many different biological molecules, robust and chemically resistant to the reactants and products of the biochemical reaction.

Based on the criteria mentioned above, the reproducible deposition of a defined enzyme immobilization matrix continuous to present a challenge to biosensor development. Studies[2,3] demonstrated that the response of enzyme electrodes is highly sensitive to the thickness of the enzyme layer. As a consequence to establish the reproducible production of biosensors, a precise method of depositing enzyme layers is required. Electrochemical deposition of enzyme layers provides a simple immobilization procedure for controlling the

spatial distribution of the polymer[4-6]. In addition, it has to be emphasized that the introduction of the biorecognition part of the biosensor on submicron levels and/or immobilization of biomolecules in closed micro-channels, as in our set-up[7,8], can only be performed via this way. For this purpose, much attention has been given to the application of ultra-thin polymer films obtained by the electropolymerization of a wide variety of different monomers (such as pyrrole, indole, aniline, phenol, aminophenol, diaminobenzene)[9-16]. Biosensors using these polymers exhibit several advantages, such as they are effective in preventing the biosensor from fouling and eliminating the interference from electrocatalytic species. Due to the self-controlling film thickness during electropolymerization of especially non-conducting polymers (such as polyphenol- and polyphenylenediamine-based films), a thin and uniform film can thus reproducibly be obtained. Besides the above-mentioned advantages, poly(1,3-phenylenediamine) based films exhibit additional advantages. Probably thanks to the high efficiency of transformation of enzymatically generated H_2O_2 to an electrical signal, an enhanced sensitivity and electrocatalytic action is observed for biosensors produced this way[15]. For this reason, flow-through nanoliter biosensors, in which enzyme is immobilized in a poly(m-phenylenediamine) ultra-thin polymer membrane, have been investigated in our miniaturised flow-through measuring device, as described in detail elsewhere[7,8]. Although sufficient stability was observed for the glucose biosensors, lactate biosensors demonstrated unacceptable loss in activity when used in the continuous mode.

To solve this problem, direct covalent attachment has, thanks to the stability of the resultant bond, the greatest potential for the development of commercial enzyme sensors. For this reason, some investigators[17-20] studied the effect of the covalent attachment of the enzyme to the polymer, and demonstrated improved enzyme stability in addition to a reduction of leaching. For instance, the immobilization of lactate oxidase (LOx) on a polytyramine membrane by using glutaraldehyde to cross-link the enzyme to the polymer, as described by Palmisano et al[21], is easy and a straightforward approach for the covalent coupling of biomolecules. However, cross-linking the enzyme with glutaraldehyde is, due to its homobifunctional nature, a relatively uncontrolled method of immobilization and actually negates the precise spatial control of the electropolymerization. Cosnier and co-workers[22] applied the avidin-biotin interaction for biomolecule immobilization. Although this technique seemed promising, the observed performance characteristics were disappointing. Linearity was only up to 2 mM, whereas the stability was cumbersome; a loss of 50% in sensitivity was seen within 10 days of storage and of 15% within 190 minutes when in operation.

A more popular method was described by Situmorang et al[23,24]. They presented the covalent coupling of Glucose oxidase (GOx) to an electrodeposited polytyramine film

through the use of the coupling reagents 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide. Highly reproducible and stable devices could thus be prepared: no loss in electrode response was seen after four months of dry storage and exhibited only minor loss in response after 20 days of repeated use. However, besides the limited dynamic range reported and the considerable influence on the signal by ascorbic acid (5-10%), a major limitation of the polytyramine membrane is that the polymerization conditions are incompatible with maintaining the activity of most biomolecules. Especially if relatively unstable enzymes are applied, such as LOx, this permselective membrane is not an option. Another approach was reviewed by Gooding et al[25]; they described the use of Self Assembled Monolayers (SAM) for the fabrication of immobilized enzyme layers. Alkanethiols adsorb spontaneously from solution onto particularly a gold surface, and a well ordered monolayer is formed and enzymes are immobilized close to the electrode surface. Thanks to the control over the orientation and distribution of the enzyme, the application of SAM can potentially provide a reproducible and robust method of fabricating immobilized enzyme layers. Unfortunately, gold is not a suitable metal for the oxidation of H_2O_2. For this reason, the biorecognition events in enzyme electrodes constructed using SAMs have most commonly been transduced using redox mediators. In general, a variety of mediators have been used, which are free to diffuse in solution[26-28], adsorbed[29-46] or covalently bound to the immobilization matrix[47-51] and/or the enzyme[52,53]. For instance, Gooding et al[54] described the preparation of GOx-SAM electrodes, assembled on gold and then platinized electrochemically by hexochloroplatinate. The subsequent Pt-GOx-SAM electrode was able to monitor the enzyme reaction via the oxidation of the hydrogen peroxide, and demonstrated that the platinum particles effectively mediated the conversion of H_2O_2 at the gold surface.

Another way to improve the efficiency of the mediator-enzyme reaction is to 'wire' the enzyme to the electrode by bonding a mediator to the enzyme cofactor. Willner and co-workers[53] described a novel method to electrically wire flavoenzymes on the electrode surface by reconstitution of the apo-flavoenzyme with a ferrocene-thethered FAD diad. Next, a pyrroloquinoline quinon-FAD diad monolayer was assembled on a gold electrode. Apo-glucose oxidase was reconstituted on the surface with the FAD-cofactor site to yield the aligned biocatalyst on the electrode. The PQQ redox unit acts as an electron relay that electrically contacts the FAD redox-site of the enzyme with the electrode. The surface reconstituted enzyme exhibits direct electrical communication with the electrode and acts as bioelectrocatalyst for the oxidation of glucose. One particular advantage of this approach is that it provides a method of controlling the orientation of the enzyme onto the surface. By

attaching the FAD active center to the electrode, structural alignment of the enzyme redox-center with respect to the electrode and the positioning of the redox relay component for efficient electrical contact is ensured. Jiang et al[55] also suggested a direct electron transfer between native GOx and an electrode as long as GOx is immobilized with a favorable orientation. According to Marcus theory, the rate of electron transfer is mainly dependent on the potential difference, the reorganisation energy and the distance between the involved redox centers, in this case the active site of the enzyme and the electrode. So far, this theory has been used to evaluate the results obtained with SAMs. Csoregi et al[56] already demonstrated the electrocatalytic reduction of H_2O_2 by immobilizing Horse Radish Peroxidase (HRP) through covalent coupling using a carbodiimide on a heat-pretreated graphite fiber. Based on these results an apparent direct electron transfer between the electrode and the active center of the immobilized peroxide was suggested. Via this way a H_2O_2-selective amperometric microbiosensor could be constructed, whereas detection of H_2O_2 was possible at –200 mV vs SCE.

For this reason and to establish our goal, the fabrication of stable and selective miniature flow-through biosensors as in our approach[7,8], our laboratory investigated the covalent coupling of GOx and LOx onto a preformed poly(m-phenylenediamine) ultra-thin polymer membrane by using a water soluble carbodiimide. The fabrication as well as the *in vitro* performance characteristics and the long-term stability in practice of these biosensors will be presented in this paper. The applicability of the lactate biosensor is demonstrated by the presentation of some results of an *in vivo* study conducted in our laboratory, in which lactate is continuously monitored in blood of anaesthetized pigs.

6.2 Experimental

6.2.1 Materials and solutions

The enzyme glucose oxidase (GOx) from *Aspergillus niger* (EC 1.1.3.4., grade I, 25,000 units/84.5 mg solid) and lactate oxidase (LOx) from *Pediococcus species* (grade I, 200 units/6.74 solid) are obtained from Boehringer Mannheim (Almere, the Netherlands). D(+)-glucose, L(+)-lactate, 1,3-phenylenediamine and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is purchased from Sigma Chemical Co. (Amstelveen, the Netherlands). All other chemicals are of pro-analysis quality and are purchased from E. Merck (Amsterdam, the Netherlands). Double quartz distilled water is used for all aqueous

solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK) to inhibit bacterial growth. The composition of the carrier solution during MD is a Dulbecco's buffer phosphate-buffered saline (PBS) (mMol/l): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5), Na_2HPO_4 (8.1) and EDTA (2). The pH is adjusted to 7.4 and purged with helium before use. Standard solutions of glucose or lactate are prepared by diluting the stock solution of glucose (50 mMol/l) in PBS and are allowed to reach mutarotational equilibrium before use (24 h).

6.2.2 The flow-through cell

The fabrication of the flow-through cell is essentially carried out as described in detail elsewhere[7]. With a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) two platinum wires (0.10 mm diameter) and a Ag/AgCl wire (0.125 mm diameter) (Drijfhout, Amsterdam, the Netherlands) are placed carefully close to each other into a 0.005 inch ID tygon tubing (Skalar Analytical, Breda, the Netherlands). By means of a multimeter the correct position of the electrodes in the tubing is controlled. Possible leakage formed in the tygon tubing is eliminated with cyanoacrylic glue. Low dead volume connections with the biosensor are made with fused silica (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands).

6.2.3 The permselective membrane

The flow-through cell is washed with methanol, 10 v/v% hydrogen peroxide solution in water and 0.1 M phosphate buffer pH 7.4 by means of a 1 ml syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun, Melsungen, Germany). The syringe is filled with a solution containing 10 mg/ml (90 mM/L) of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 7.4. The syringe is placed in the model 22 syringe pump (Harvard Apparatus, Kent, UK) and the wires outside the tubing are connected with model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by means of crocodile clips. Electropolymerization is performed at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 500 nl/min. Before introducing the bioselector, the flow-through cell is washed for 30 min with 0.1 M phosphate buffer pH 4.65 at a flow rate of 500 nl/min. The syringe is then filled with a solution containing 2 mg/ml of LOx respectively GOx and 30 mg/ml of EDC in 0.1 M phosphate buffer pH 4.65. Covalent coupling of the enzyme is then performed at room temperature for 4 hours at a flow rate

of 100 nl/min. Before storage and/or use, the biosensors are rinsed with 0.1 M phosphate buffer pH 7.4 for 30 minutes at a flow rate of 500 nl/min. In between, the biosensors are stored in the refrigerator at 4-8 °C.

6.2.4 Measurements in the continuous mode

For measurements in the continuous mode, one side of the biosensor is connected to a CMA 60 MD catheter (Aurora Borealis Control, Assen, the Netherlands) or a ultrafiltration catheter (Cordis Europe N.V., Roden, The Netherlands) whereas the other side of the biosensor is directly connected to a home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[57]. Dialysate or ultrafiltrate is continuously analysed at a flow rate of 100 nl/min. Accordingly, measurements were carried out at –150 mV vs. Ag/AgCl by means of a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands). Data is recorded with a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands). Before measurements, the biosensor was allowed to settle a stable current over a period of approximately 30 minutes.

6.2.5 Measurements by Flow Injection Analysis (FIA)

For measurements in the discontinuous mode, a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) equipped with a VICI Cheminart C4 valve with a 100 nl internal loop is used. The biosensor is connected to the valve for sample injection. With a model 22 syringe pump (Harvard Apparatus, Kent, UK) carrier solution and sample is transported to the biosensor at a flow rate of 5 $\mu l.min^{-1}$. The internal loop was continuously filled with sample at a flow rate of 300 $nl.min^{-1}$ by a home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[57]. Connections between the parts of the FIA were made with fused silica (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Injection and analysis was carried out every three minutes. Measurements were carried out at –0.15 V vs. Ag/AgCl. Data is recorded with a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

6.2.6 Attenuared Total Reflection Fourier Transform Infrared (ATR-FTIR)

Infrared spectra were recorded on a Mattson Galaxy 6020 FTIR spectrophotometer equipped with a single reflection diamond ATR (Specac Golden Gate) and a narrow

bandpass mercury-cadmium-telluride (MCT) detector at a resolution of 4 cm^{-1}. Coaddition of 50 scans was used.

6.2.7 Ultrafiltration catheter

The ultrafiltration catheters used during the *in vivo* experiments are essentially the same as described by Tiessen et al[58]. A 5F (1.78 mm o.d.) Nylon blend double lumen catheter of 120 cm length (Modified Cordis Multipurpose, Cordis Europe N.V., Roden, The Netherlands) was designed to simultaneously withdraw blood and blood ultrafiltrate. The large lumen, 0.98 mm i.d., was used as guide wire/flush/blood sample lumen. The proximal end of the large lumen was provided with a luer connector. Five side holes in the large lumen (i.d. 0.635 mm) were made in the last 4 cm of the catheter tip. The small lumen, oval with id 0.83 mm x 0.35 mm, was provided with two closely adjoining fenestration of 2 times 2 cm length at 2 mm from the distal tip, to place a hollow fiber membrane AN69HF (Hospal, Meyzieu, France; 340 µm o.d.; 240 µm i.d.) with a MWCO of 20 kD. Two marker bands, gold 18 Kt, width 1.0 mm, were placed at either end of the fenestration to locate the probe on the X-ray. A helical platinum wire was placed inside the hollow fiber to support the wall of the probe. The fiber was closed and fixed with two components polyurethane adhesive (E.V. Roberts, Culver City, CA, USA) in the distal end of the catheter window. At the proximal end, the fiber was glued to the biosensor.

6.2.8 *In vivo* experiments

All procedures were reviewed and approved by the Animal Experiments Committee of the Groningen University. Experiments were performed on two Yorkshire swines, weighing approximately 35 kg. The swines were anaesthetised using a combination of ketamine (15 mg/kg), acepromazine (0.2 mg/kg) and atropine (0.05 mg/kg) intramuscularly. An endotracheal tube was inserted for O_2/NO_2 ventilation. Ventilation with 2% isoflurane (Forene®, Abbott, USA) was used to ensure adequate anaesthesia throughout the experiments. Levodromaron (2 mg subcutaneously) was given as analgesic at the beginning of the procedure and supplemented if necessary. Lidocaine (50 mg intravenously) was given prophylactically. Throughout the experiments, the condition of the animals was carefully monitored by means of continuous transcutaneous oximetry, intra-arterial blood pressure measurement and electrocardiographic registration (ECG) of the limb leads and one modified precordial lead.

By means of a 9F introducer sheath (AvantiTM, Cordis Europe, Roden, The Netherlands), the ultrafiltation catheter was introduced in the left jugular vein. To protect the tip of the membrane from damage while introducing, a tube was temporally placed in the valve of the introducer. From the left jugular vein, the ultrafiltration catheter was advanced and positioned at least 5 cm within the coronary sinus. The position of the catheter was checked by contrast injection. After successfully placing the ultrafiltration catheter, the semi-vacuum pump, located at the other side of the lactate biosensor, was set in action allowing to continuously monitoring the lactate content in the ultrafiltrate. The lag-time between sampling and the actual measurement was determined during the calibration of the biosensor. Calibration was performed *in vitro* prior to and after each *in vivo* measurements by placing the ultrafiltration catheter in a standard solution containing 2.5 mM of L(+)lactate. The sensitivity *in vitro* of the lactate sensor did not significantly decrease before and after each experiment. The lag-time thus determined was found to be approximately 25 min.

Forty-five minutes after placing the probe, the response was measured *in vivo* by injecting 0.1 g/kg of lactate 20% solution in 0.9% saline solution intravenously in 2 minutes time. Whole blood samples (2 ml) were withdrawn every 2, 5 or 10 minutes from the catheter in the coronary sinus vein. The test tubes contained sodium fluoride and potassium oxalate to arrest coagulation and metabolism. The tubes were kept on ice until analysis. The concentration of lactate was measured in blood plasma after centrifugation with the Vitros 750 analyser (Ortho-Clinical Diagnostics, Illkirch Cedex, France) in the hospital routine laboratory.

6.3 Results and discussion

Based upon the data obtained during earlier studies[7], the need to improve the stability of the lactate biosensors was recognized. Because even under storage conditions, a rapid and unacceptable decline in sensor performance was observed (data not shown), this could not be fully explained by leaching out of enzyme. Presumably both rapid degradation as well as leaching out of enzyme accounts for these observations. It is well known that biomolecules, and specifically enzymes, maintain their activity for a longer periods of time when immobilized through covalent coupling[59]. Also for the fabrication of stable biosensors, several investigators[17-20] have successfully demonstrated this phenomenon. For a successful covalent coupling, however, several criteria have to be met. Not only should the surface

and/or (permselective) polymer contain the appropriate moieties to allow the coupling reaction to proceed, the conditions during immobilization should be such that the enzyme of interest remains its biological activity. Mainly thanks to its performance characteristics determined in earlier studies[7] and its applicability in these miniaturised flow-through devices, coupling onto a poly(m-phenylenediamine) membrane is preferred in our case. Although only limited structural information is available about this type of polymers, Jang and coworkers[60] proposed a mechanism of the electropolymerization of o–phenylene-diamine. According to them, the suggested polymer contained both primary and secondary amine groups. The presence of primary amines was confirmed by us in earlier studies[7], where AT-FTIR measurements were performed on electropolymerized poly(m-phenylene-diamine) membranes. According to Hermanson et al[59], coupling of biomolecules, in this case an enzyme, onto an amine-containing support can for instance easily be performed without further activation steps using a water-soluble carbodiimide. Especially EDC has been widely used and facilitates particularly the formation of stable amide bonds between a carboxylate group and an amine. EDC is a so-called 'zero-length' cross-linker, since it mediates in the formation of amide linkages without leaving a spacer molecule (see figure 1). N-substituted carbodiimides react with a carboxylic group to form a highly reactive and short live O–acylisourea derivative. With proteins, this complex is formed from C-terminal and Glu or Asp side-chain carboxyl groups. This activated species can react with a primary amine to form an amide bond. Based upon the protocols suggested by Hermanson et al[59], several lactate biosensors were manufactured as described in the experimental section. During the first tests with these biosensors a remarkable phenomenon was observed. In contrary to the biosensors reported earlier[7], detection with these new biosensors was possible at -150 mV vs Ag/AgCl. These observations are comparable with the observations reported by Csoregi et al[56], who explained this phenomenon by an apparent direct electron transfer between the electrode and the active center of the immobilized enzyme. However, they used immobilized HRP for the enzymatic reduction of H_2O_2, whereas the oxidized form of HRP is believed to convert back via a direct electron-transfer process or a mediated one. At +600 mV a small anodic current, attributed to the oxidation of H_2O_2, was reported, whereas a cathodic current starts at +400 mV and increases rapidly as the potential is made more negative. In our case, H_2O_2 is formed during the reaction between lactate and lactate oxidase, leaving the enzyme in the oxidized form. When measurements were carried out in the FIA mode at +500 mV vs Ag/AgCl, compared to the biosensors reported earlier[7], a relatively small anodic current, owing to the oxidation of H_2O_2, was observed. Similar to the results of Csoregi *et al.*[56], when the potential was lowered to +150 mV vs Ag/AgCl, a

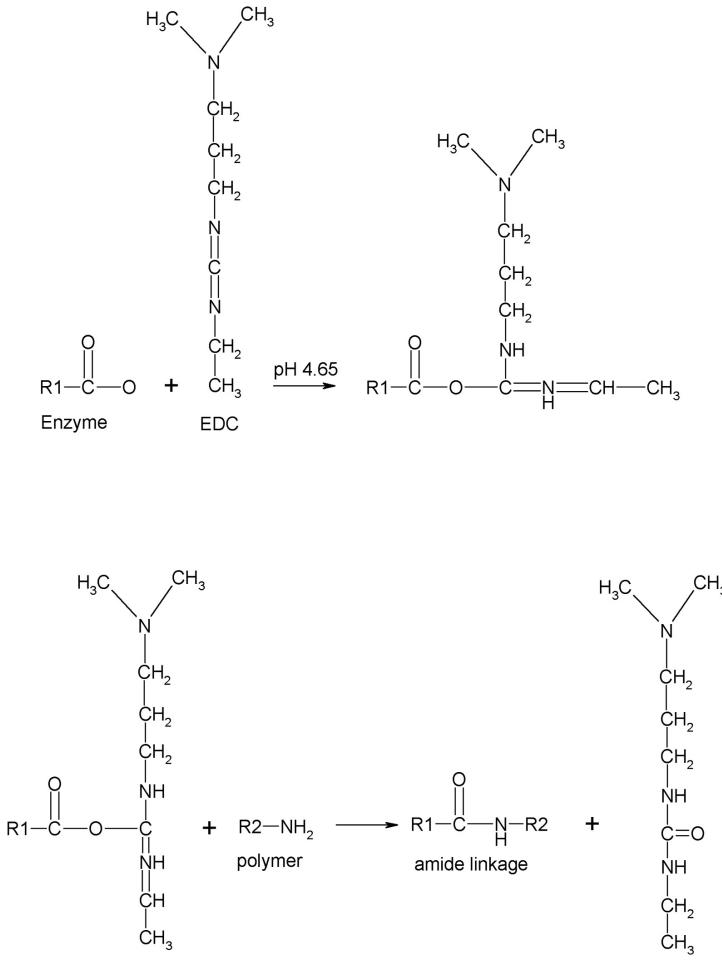

Figure 1:
EDC mediated formation of amide linkages.

cathodic current was observed, which significantly increased at –150 mV vs Ag/AgCl. Although these results are in accordance with the data reported by Csoregi et al[56], in our case this phenomenon can not be explained by a direct electron transfer between the electrode and the immobilized enzyme. Due to the fact that a response dependent on the concentration of the substrate was observed, instead of a cathodic current, an anodic current should then be expected. Because in this case a cathodic current was measured, a more plausible explanation for this phenomenon might be the electrocatalytic reduction of the H_2O_2, according to:

[1]	Substrate + Enzyme$^{(ox)}$	<=>	Product + Enzyme$^{(red)}$
[2]	Enzyme$^{(red)}$ + O_2	=>	Enzyme$^{(ox)}$ + H_2O_2
[3]	$2H_2O_2 + 4^{e-}$	=>	$2H_2O + O_2$

Normally, due to a considerable interference from oxygen, the reduction of H_2O_2 as a method for detection is not the method of choice for these type of measuring systems. However, Ravishankaran *et al.*[61], among others, described the fabrication of a metal dispersed composite electrode and demonstrated the electrocatalytic reduction of H_2O_2 at a reduced overpotential of – 50 mV. In this case, the presence of silver in the composite exerted an electrocatalytic effect for the reduction of H_2O_2 resulting in a significant reduction of the potential.

As in our case no signal could be detected below +300 mV vs Ag/AgCl with the biosensors reported earlier[7], altering of the permselective membrane during the covalent coupling of the enzyme mediated with EDC has been hypothesized. To investigate this, two poly(m-phenylenediamine) coated electrodes were made by dipping an platinum electrode in a 0.1 M phosphate buffer pH 7.4 containing 10 mg.ml^{-1} 1,3-phenylenediamine, a platinum counter electrode and a Ag/AgCl reference electrode, and electropolymerised at +0.8 V for 60 minutes. One of the coated electrodes was dipped in a solution containing 30 mg.ml^{-1} EDC in 0.1 M phosphate buffer pH 4.65 for an additional 4 hours. The polymer films thus

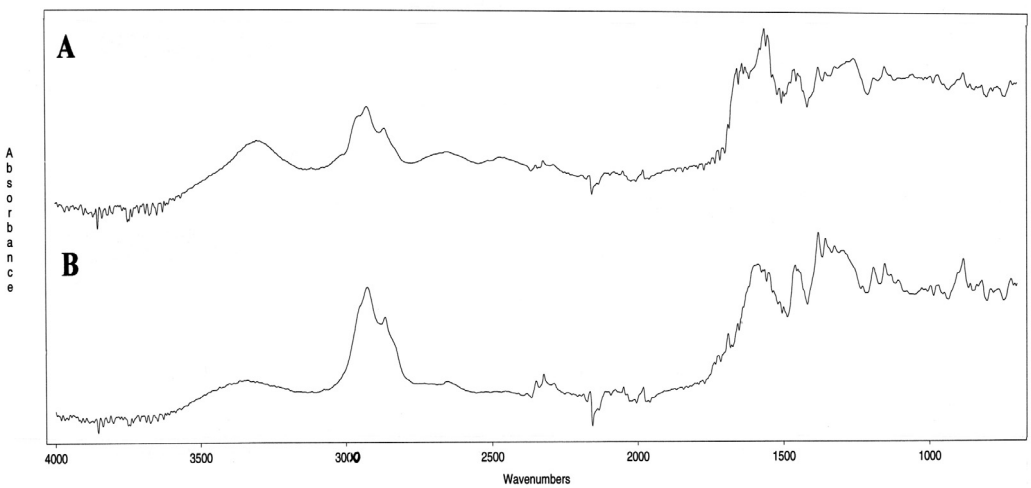

Figure 2:
Infrared spectra of (a) electropolymerised poly(m-phenylenediamine) film on a platinum electrode, and (b) electropolymerised poly(m-phenylenediamine) film on a platinum electrode with EDC treatment.

obtained were investigated by means of Total Reflectance Fourier Transform Infrared Spectroscopy. As shown in figure 2, no significant spectral differences between both polymer films can be seen. Although no altering in the permselective membrane could be detected via this way, it has to be remarked that a full identification of the molecular structure of these ultrathin polymer films remains extremely difficult. Although more work needs to be done to fully prove our hypothesis, we continue to believe that the observed phenomenon might be attributed to the electrocatalytic reduction of H_2O_2. Glucose biosensors produced accordingly clearly demonstrated an enzyme-independent effect. As these glucose biosensors exhibited the same characteristics, a direct electron transfer between the electrode and the active center of the immobilized enzyme due to a favorable orientation of any enzyme offered onto the surface seems to be unlikely to occur.

To investigate, however, the applicability of these biosensors, the performance characteristics were studied further. At first, the reproducibility of the production of these biosensors was investigated by producing at 3 consecutive days a total of 6 biosensors, and analysing with each biosensor a standard solution of 2.5 mM of lactate in six-fold by means of FIA. The repeatability of the method, expressed as the relative standard deviation in the peak height of six consecutive injections was found to be 4%. The reproducibility of the production of the biosensors, expressed as the relative standard deviation in the mean peak height of six tested biosensors, was found to be 13%. These figures are well in line with the earlier reported data[7].

The dynamic range of the lactate biosensor was tested in the continuous mode at a flow rate of 100 nl/min. The linearity was found to be up to 10 mM for lactate (correlation coefficient R^2 of 0.998) with a limit of detection of 0.05 mM. A typical calibration curve is demonstrated in figure 3. As can be seen from this figure, the dynamic range of the lactate biosensor is found to be sufficient for the analysis of lactate on clinical relevant levels.

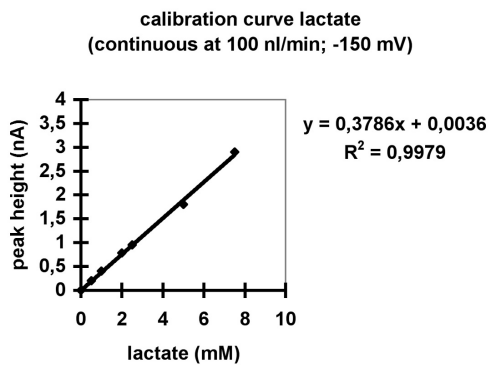

Figure 3:
Calibration curve of lactate biosensor.

The selectivity of the lactate biosensor was tested by analyzing in the continuous mode standard solutions containing 0.1 mM ascorbic acid or 0.25 mM uric acid with or without in combination with 2.5 mM of L(+)-lactate. As can be seen in figure 4, in neither case, a significant contribution to the signal was observed from the possible interferences tested. More interestingly, however, is the stability of the lactate biosensor. At first the stability was investigated by analyzing in the continuous mode a standard solution containing 1 mM of L(+)-lactate overnight. The first results were promising; no decline in sensor sensitivity was observed for at least a day.

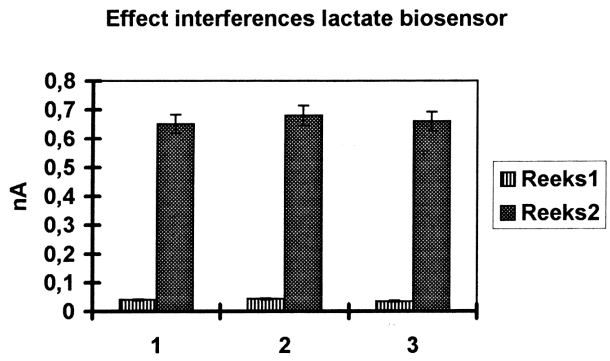

Figure 4:
Contribution by interferences on the lactate signal (Reeks 1 = 0 mM L(+)-Lactate; Reeks 2 = 2.5 mM L(+)-Lactate in PBS buffer): (1) 2.5 mM L(+)-Lactate in PBS buffer; (2) 2.5 mM L(+)-Lactate + 0.1 mM ascorbic acid in PBS buffer; (3) 2.5 mM L(+)-Lactate + 0.25 mM uric acid in PBS buffer.

Based upon this significant improvement in stability compared to the biosensors presented in earlier studies[7], a limited *in vivo* study was started with these biosensors. To demonstrate, a typical result of the lactate content monitored in blood ultrafiltrate of anaesthetized pigs before and after an intravenous injection of L(+)-lactate, is shown in figure 5. As the lactate content in the blood ultrafiltrate, determined by the biosensor, closely follows the lactate content in the plasma, determined by the clinical lab, the applicability of the lactate biosensors is clearly demonstrated.

During the *in vivo* experiments two lactate biosensors have been used, and were found to remain both stable throughout the experiments whereas no significant decline in activity

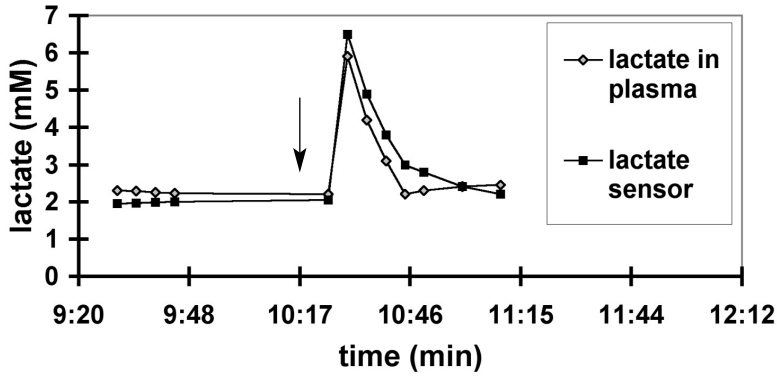

Figure 5:
Typical graph obtained during the in vivo monitoring of lactate in blood ultrafiltrate of anaesthetized pigs before (start) and after (arrow) an intravenous injection of lactate.

was seen over a period of four days of *in vivo* testing. Additionally, the same biosensors were further tested for a period of more than two weeks. Intermittent tests were performed in the continuous mode, analyzing a standard solution containing 2.5 mM of L(+)-lactate. In between the biosensors were stored at 4-8 °C. As can be seen from figure 6, no significant decline in sensor performance over the tested period of 15 days for both lactate biosensors was observed. Based on our knowledge, these types of lactate biosensors are among the most stable lactate biosensors reported.

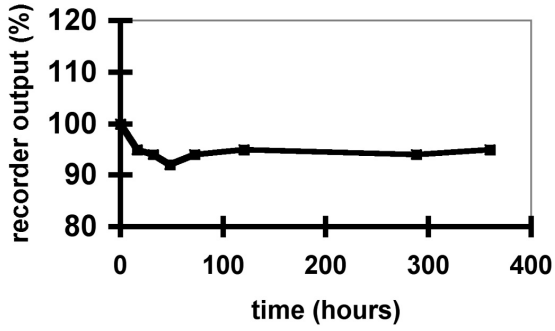

Figure 6:
Typical stability curves for lactate biosensors during intermittent in vivo and in vitro experiments

6.4 Conclusion

Stable flow-through nanoliter lactate and glucose biosensors can be produced by the covalent immobilization of the enzymes LOx respectively GOx using EDC as mediating coupling reagent onto an *in situ* electropolymerized poly(m-phenylenediamine) polymer film. As detection of the subsequent analyte was possible at –150 mV vs Ag/AgCl measuring a cathodic current, electrocatalytic reduction of hydrogen peroxide was suggested for this phenomenon. Both dynamic range and sensitivity were sufficient for the analysis of lactate in clinical relevant levels, whereas *in vitro* experiments with ascorbic acid and uric acid demonstrated no significant contribution to the signal from these electroactive species. The stability, determined during intermittent experiments, was found to be excellent; no significant decline in sensitivity was seen during 15 days of practical use. Especially for lactate biosensors as in this concept, these biosensors are among the most stable ever reported.

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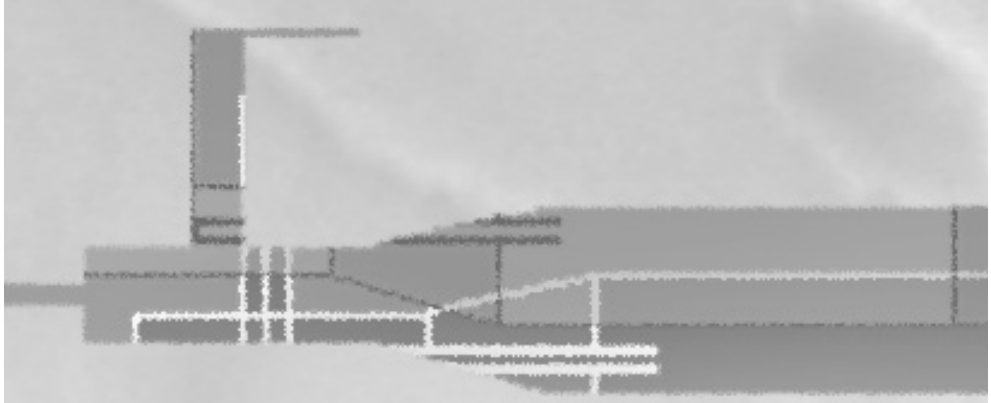


Chapter 7

A lightweight measuring device for the continuous in vivo monitoring of glucose by means of ultraslow microdialysis in combination with a miniaturised flow-through biosensor

Summary

A lightweight measuring device for continuous *in vivo* monitoring of glucose in biological compartments is presented. The measuring device consists of a miniaturised flow-through biosensor, connected to a microdialysis probe and a semi-vacuum pump. The biosensor is based on the amperometric detection of hydrogen peroxide after conversion of glucose by immobilised glucose oxidase. A portable potentiostat equipped with data logging is used for detection and registration. The device is validated for its accuracy, precision, linearity, selectivity and stability during ex vivo and *in vivo* experiments. The accuracy was found to be well in accordance with the criteria set for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus. *In vivo* monitoring of glucose in dialysate of subcutaneous sampled tissue during glucose tolerance tests in healthy volunteers demonstrates the potential of this measuring device.

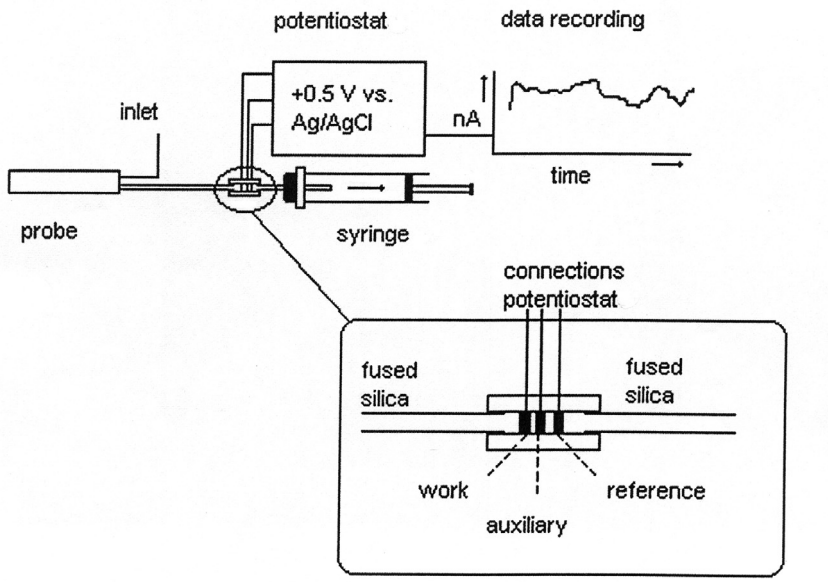
7.1 Introduction

Diabetes mellitus is a chronic disorder characterised by insulin deficiency, hyperglycaemia and a high risk of the development of irreversible damage to eyes, kidneys, peripheral nerves, heart and blood vessels[1,2]. A long-term study performed by the Diabetes Control and Complications Trial Research Group[3] has conclusively demonstrated that if glucose levels are tightly regulated diabetic complications are reduced. In addition, hypoglycaemic events can be prevented. In practice, blood glucose levels are controlled with the finger-prick method. The measurements thus obtained however are limited and inform only about the blood glucose levels at intermittent moments. Continuous *in vivo* glucose monitoring may therefore be a significant improvement. For this reason, throughout the years elaborate research efforts have been devoted to the development of a glucose sensor[4]. Biosensors may not only offer a viable route for continuous real-time glucose monitoring carried out by clinical laboratories at the patient's bedside or during operations but also for self monitoring at home[5]. An ideal biosensor has many advantages over conventional analysis such as reagentless analysis and independence of laboratory facilities. Several investigators reported the use of a peroxide based needle-type amperometric biosensor, that can be easily manufactured and miniaturised. To avoid thromboembolism and complications arising

from surface fouling of the electrode by proteins and coagulation composites (e.g. fibrinogen/fibrin, platelets, leukocytes and red cells), the needle-type glucose sensor was most often inserted in the subcutaneous tissue[6-12]. As an alternative to implanted needle glucose biosensors, microdialysis (MD) and/or ultrafiltration (UF)[13-24] as a sampling interface between the body and the biosensor have been proposed. These techniques are regarded as minimally invasive because the needles required for the insertion of the MD or UF probe are relatively small[25]. And although the effect of probe implantation on the glucose measurements is still a matter of concern, these sampling techniques have demonstrated an improvement of the stability and reliability of the biosensors tested thus far[26,27]. The sampling principle of MD is based on diffusion down the concentration gradient existing between the perfusion fluid (dialysate) and the outside environment of the semi-permeable membrane (interstitial fluid). Small analytes, such as glucose, are perfused whereas the semi-permeable membrane excludes cells and/or large molecules. A relatively clean matrix is obtained which is transported by pumping and analysed outside the body with a glucose biosensor. A major drawback of conventional MD is that the concentration of glucose is always lower in the dialysate than in the sampled interstitial fluid, and excessive (*in vivo*) calibration procedures are required for accurate monitoring[28]. The *in vivo* recovery, the dialysate/interstitial concentration ratio, depends on variables such as probe dimensions and perfusion flow rate as well as on tissue characteristics as tissue tortuosity, extracellular volume and metabolism[29-31]. A generally accepted method for the quantitative analysis of the interstitial concentration is based on the principle that diffusion of substances from the interstitial fluids tends to reach equilibrium between dialysate and interstitial concentrations at extremely low perfusion flow rates[32,33]. In our laboratory, (near) quantitative *in vivo* recoveries have been observed at flow rates of less than 300 $nl.min^{-1}$ (ultraslow MD)[28]. To allow, however, continuous *in vivo* monitoring at these low perfusion flow rates, the need for a small and low dead volume biosensor was recognised. Because, to our knowledge, no hardware is yet available to perform *in vivo* sampling by ultraslow MD followed by on-line continuous analysis, a portable lightweight measuring device was developed by us as demonstrated schematically in figure 1. The measuring device comprises a MD probe, a flow-through biosensor with an internal volume of 10 – 20 nanoliter and a semi-vacuum pump. The semi-vacuum pump is capable of producing a stable low flow rate for several days[34] and does not need (additional) batteries. A home-made portable potentiostat equipped with rechargeable battery and a data-logger is used for detection and registration of the analytical results during monitoring.

In this paper the construction and production of the lightweight portable measuring device

Figure 1:
Schematic demonstration of the instrumental set-up.



are presented. The performance characteristics (precision, selectivity, linearity, accuracy and stability) of the measuring device, which have been tested *in vitro* and ex vivo, will be described. The performance of the measuring device *in vivo* was assessed with glucose tolerance tests (OGTT).

7.2 Materials and methods

7.2.1 Materials

The enzyme glucose oxidase from *Aspergillus niger* (EC 1.1.3.4., grade I) is obtained from Boehringer Mannheim (Almere, the Netherlands). D(+)-glucose for standard solutions and 1,3-phenylenediamine for the permselective membrane is purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals are of pro-analysis quality and are purchased from E. Merck (Amsterdam, the Netherlands). Double quartz distilled water is used for all aqueous solutions containing 0.1% (by volume) Kathon CG (Rhom and Haas, Croydon, UK)

to inhibit bacterial growth. The composition of the carrier solution during microdialysis is a Dulbecco's buffer phosphate-buffered saline (PBS) (mmol.l^{-1}): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5), Na_2PO_4 (8.1) and EDTA (2). The pH is adjusted to pH 7.4 and purged with helium before use. Standard solutions of glucose are prepared by diluting the stock solution of glucose (50 mmol.l^{-1}) in PBS and are allowed to reach mutarotational equilibrium before use (24 hr).

For the construction of the flow-through biosensor, tygon tubing (ID 0.005 inch) is purchased from Skalar Analytical (Breda, the Netherlands). The auxiliary and work electrode is made from platinum wire (0.10 mm diameter) whereas the reference electrode is made from silver wire coated with AgCl (0.125 mm). All these materials are purchased from Drijfhout (Amsterdam, the Netherlands). Connections with the flow-through biosensor are made by using fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Connections are glued with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands).

During *in vitro* and *in vivo* measurements, sampling is performed by means of ultraslow microdialysis thereby using a CMA 60 microdialysis probe (Aurora Borealis Control, Assen, the Netherlands).

7.2.2 Production of the flow-through biosensor

By pushing a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) perpendicularly through a 0.005 inch ID tygon tubing, consecutively two platinum wires and a Ag/AgCl wire are placed close to each other into the 0.005 inch ID tygon tubing (see figure 1). By means of a multi-meter the correct position of the electrodes in the tubing is checked. Possible leakage formed in the tygon tubing is eliminated with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands). The flow-through cell thus prepared is washed prior to electropolymerisation by connected the cell with a 1 ml syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun, Melsungen, Germany). The flow-through cell as well as the electrodes are washed by filling the syringe and pushing the following solutions consecutively through the flow-through cell: methanol (pro analysis, E. Merck, Amsterdam, the Netherlands), 10 %v/v hydrogen peroxide solution in water (pro analysis, E. Merck, Amsterdam, the Netherlands) and finally 0.1 M phosphate buffer pH 6.9. After washing the syringe is filled with a solution containing 2 mg.ml^{-1} of enzyme and 10 mg.ml^{-1} of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9. The syringe is placed in a model 22 (Harvard Apparatus, Kent, United

Kingdom) syringe pump. The flow-through cell is connected to the syringe and the wires outside the tubing of the flow-through cell are connected to a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by means of crocodile clips. Electropolymerisation is performed at +0.8 V vs Ag/AgCl for one hour at a flow rate of 0.5 µl.min^{-1} followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Before storage and/or use, the biosensors are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes at a flow rate of 0.5 µl.min^{-1}. In between, the flow-through biosensors thus produced are stored in the refrigerator at 4–8 °C.

7.2.3 Testing of the flow-through biosensor by means of Flow Injection Analysis

For the *in vitro* testing of the flow-through biosensor, measurements are carried out by means of Flow Injection Analysis (FIA). The flow-through biosensor is connected to a VICI Cheminert C4 valve (Valco Instruments, Houston, USA) and equipped with a 100 nanoliter internal loop for sample injection. With the model 22 (Harvard Apparatus, Kent, GB) syringe pump, carrier solution and sample is transported to the biosensor at a flow rate of 5 µl/min. The internal loop of the valve is continuously filled with dialysate by connecting the microdialysis probe and a home-made semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[34] each at a side of the VICI Cheminert C4 valve. Dialysis is carried out at a flow rate of 300 nl.min^{-1} by means of under-pressure. To avoid high dead volumes, connections between the various parts of the flow injection analysis system are made with 150 µm OD x 50 µm ID fused silica capillary (Aurora Borealis Control, Assen, the Netherlands). Injection and analysis of the sample is carried out every two minutes. Detection is carried out at +0.5 V vs. Ag/AgCl by connecting the wires outside the tubing of the flow-through cell to a model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by crocodile clips. Data is recorded on a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands).

7.2.4 A lightweight measuring device for the continuous monitoring of glucose

For the continuous monitoring of glucose during *in vitro* and *in vivo* experiments, a lightweight measuring device (see figure 1) was constructed. One side of the flow-through

biosensor is connected with the microdialysis probe whereas the other side is connected with the semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[34]. Low dead volume connections between the different parts of the device are made with fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Dialysate is sampled by means of under-pressure and is continuously analysed by the flow-through biosensor at a flow rate of 300 nl.min^{-1}. Detection is carried out at +0.5 V vs. Ag/AgCl and data is collected with a model Dextralert^{TM} (Analytic Devices, Zeist, the Netherlands) portable potentiostat.

7.2.5 Oral glucose tolerance test (OGTT)

OGTTs were performed with healthy young female subjects. All subjects gave their informed consent and the study was approved by the Ethical Committee of the University of Groningen. On day 1, a microdialysis probe is placed in one side of the umbilicus in the subcutaneous fat tissue by means of a 16G catheter. At day 2, following an overnight fast, subjects are monitored from 8.30 a.m. until 1 p.m. At 8 a.m. the implanted probe is washed and de-aerated with sterile 0.9% NaCl solution. One end of the probe is connected to the lightweight measuring device as described, whereas the other end of the probe is connected to a buffer reservoir containing sterile PBS buffer (without Kathon CG). By pulling the plunger of the semi-vacuum syringe pump a vacuum is created and sampling is started. The dialysate thus obtained is continuously analysed for 5 hours at a flow rate of 300 nl.min^{-1}. After reaching a steady state (mostly within 30 minutes), the subjects received 100 g of glucose dissolved in 200 ml of water via an oral administration. During the OGTTs, blood samples were taken from a forearm vein cannula and analysed by the clinical laboratory with an ECA 180 glucoanalyser (Medingen GmbH, Dresden, Germany). To correct for the difference between glucose molarity (mol/l blood) of haemolysed whole blood and molality (mol/kg water), the data obtained from the blood samples are multiplied by 1.176 as prescribed by the manufacturer's report. In this way a correct comparison can be made between the content of glucose in blood versus dialysate. At the end of the study, the subject is disconnected and the flow-through biosensor is coupled to the microdialysis probe, which is placed in PBS buffer containing 5 mmol.l^{-1} of glucose for one-point calibration.

7.3 Results and discussion

7.3.1 Performance characteristics

The determination of glucose is based upon the conversion of glucose by the immobilised glucose oxidase (GOx) into the electrochemically detectable product, hydrogen peroxide. To enable the efficient immobilisation of the enzyme and to improve the performance characteristics of the final biosensor, we preferred a permselective membrane based upon the electropolymerisation of the monomer 1,3-phenylenediamine in the presence of the enzyme GOx. Accordingly, a substrate diffusion-limiting barrier is introduced which prevents the reaction from following the intrinsic enzyme kinetics and as a consequence extends the linear range. The linearity is investigated by analysing standard solutions of glucose in the FIA and the continuous mode. For both modes a linearity of up to 30 mmol.l^{-1} of glucose is found. The signal/noise (S/N = 3) ratio indicates a limit of detection of 0.5 mmol.l^{-1} in the FIA mode and 0.05 mmol.l^{-1} in the continuous mode. This means that the linear range as well as the sensitivity of the biosensor is sufficient for the determination of glucose levels in diabetic patients. The precision of several biosensors (n = 10) was tested by analysing a standard solution of 5 mmol.l^{-1} of glucose in six-fold in the FIA mode. The repeatability, expressed as the relative standard deviation in the peak height after six consecutive injections was found to be 2-4% depending on the biosensor tested. To investigate the selectivity of the biosensor regarding interference from electroactive species, standard solutions, containing 0.1 mmol.l^{-1} ascorbic acid or 0.25 mmol.l^{-1} uric acid in the presence of 0, 5 or 25 mmol.l^{-1} of glucose, are analysed in the continuous mode with several (fresh and one week old) biosensors. No significant contribution to the signal is observed and demonstrated the selectivity and stability of the permselective membrane.
Regarding the stability of the measuring device, it has to be emphasised that due to electrode fouling by small endogenous proteins and the hydrogen peroxide mediated enzyme inactivation, theoretically, *in vivo* measurements will not take longer than a couple of days without re-calibration of the device. For this reason, it was decided to test the stability of several flow-through biosensors by monitoring for only up to three days in the continuous mode respectively a standard solution of 5 mmol.l^{-1} glucose and a dialysate of serum sample containing approximately 6 mmol.l^{-1} glucose. It was observed that after an initial decrease in sensitivity from 100% to approximately 70% within several hours of practice, the biosensors remained their activity during the period tested. This is in accordance with data reported by others[35], who attributed this initial decline to leaching

out of not fully immobilised enzyme and/or polymer film. Although more studies are performed to evaluate the storage conditions and to increase the stability of the biosensors in use and during storage, the biosensors are sufficiently stable for the purpose of this study. To examine the accuracy of the measuring device, ex vivo studies have been conducted by analysing serum samples obtained from the clinical laboratory for their content of glucose in the FIA mode. By comparing the results with those obtained from the clinical laboratory which have been analysed with validated methods, the accuracy of the measuring device is determined. The results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus. For this reason the method presented by Clarke et al[36] was used. They described the error grid analysis, where the x-axis represents the reference blood values and the y-axis the value generated by the measuring device tested. The diagonal represents perfect agreements whereas data points above and below the diagonal represent overestimates and underestimates. Based on the assumption that the target blood glucose level ranges from 70 – 180 $mg.dl^{-1}$, the grid is divided into five regions of varying degrees of accuracy. In short, values found in zone A and B are clinically acceptable, whereas values in zone C, D and E are potentially dangerous for patients and are therefore clinically significant errors. As can be seen in figure 2, the results are well in line with those obtained by the clinical laboratory; a correlation of $Y = 0.98X + 0.19$ with a correlation coefficient of 0.99 for $n = 54$ was calculated. No results were found which deviated more than 20% from the reference value. Based on these results, it was decided to proceed with *in vivo* monitoring studies.

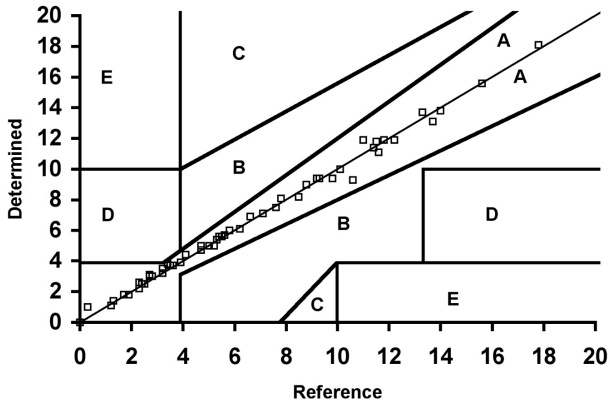


Figure 2:
Error grid analysis for the evaluation of clinical implications of patient-generated blood glucose values. The Y-axis represents the values determined in the serum samples by means of the biosensor; the X-axis represents the values determined in the serum samples by means of validated clinical methods.

7.3.2 The lightweight measuring device

Although numerous parameters determine the performance of the sampling by MD, in general (near) quantitative recoveries are obtained at very low flow rates (less than 0.5 $\mu l.min^{-1}$)[28]. To enable, however, real time on-line monitoring of the dialysate, the internal volume of the measuring device has to be extremely small. By using tygon tubing with a defined diameter, a flow-through cell with a defined volume can be easily constructed. With an internal diameter of 0.005 inch (0.127 mm), the tubing has a specified volume of 127 $nl.cm^{-1}$. If the electrodes are positioned within 1-2 mm of each other, a cell with a total internal volume of 10-20 nanoliter is obtained. By connecting the flow-through cell with, for instance, 4 cm of 50 µm ID x 150 µm OD fused silica tubing (with a specified volume of 20 $nl.cm^{-1}$), the total internal volume of the measuring device is 100 nanoliter or less. This means that at a flow rate of 300 $nl.min^{-1}$, the delay time between sampling and analysis is less than a minute. Because of the relatively large internal volume of the CMA 60 MD probe however, a total delay time of approximately 2-3 minutes was found. Although this delay time is one of the smallest reported for a MD based measuring device, *in vivo* correlation studies will have to prove whether or not this delay time will be sufficiently low for adequate and practical use of diabetic patients.

By using a semi-vacuum syringe pump, as described earlier in detail[34], a stable flow rate of 300 $nl.min^{-1}$ can be produced for up to several days[3] without the need of (additional) batteries. This means that the wet part of the measuring device outside the body (biosensor, pump, fused silica and electrical connections and housing) weighs less then 5 gram, which is negligible, compared to the insulin pumps worn by many diabetic patients.

For detection and data collection, a portable potentiostat provided with internal data-logging and rechargeable battery is used. The Dextralert™ collects continuously data and every minute the mean value of the sampled minute is filed in its internal memory. The results can directly be recorded by connecting the Dextralert™ to a flatbed recorder or via the Dextralert™ software program installed on a Windows95/98/NT PC. This first prototype of the Dextralert™, with a weight of approximately 250 gram can be easily worn by attaching the instrument to a belt with the provided clip.

7.3.3 Oral glucose tolerance test (OGTT)

To demonstrate the applicability of the lightweight measuring device described in this article, the first set of results of our ongoing *in vivo* studies are presented. On the first day,

a MD probe is placed in one side of the umbilicus in the subcutaneous fat tissue by means of a 16G catheter. On the second day a fresh biosensor is connected to the implanted MD probe after allowing to reach an equilibrium for 30 minutes. Dialysate is then continuously analysed at a flow rate of 300 nl.min^{-1} as schematically demonstrated in figure 1. During the OGTT blood samples are collected and analysed in the clinical laboratory on their content of glucose. In figure 3, some typical data obtained during the OGTT on healthy volunteers are demonstrated. The data presented are corrected for the delay time, which is found to be several minutes. As can be seen in figure 3a, in one case the content of glucose in the dialysate reasonably follows the blood glucose content. However, frequently we have observed a lower glucose content in the dialysate (see figure 3a) compared to blood and a relative low correlation was found ($Y = 0.82$, $R^2 = 0.56$) (see figure 4a). Based upon the *in vitro* and *ex vivo* performance characteristics of the measuring device, this phenomenon can not (only) be attributed to the measuring device. We believe, as already suggested by others[37,38], that the values found in the interstitial fluid are influenced by the characters of the tissue surrounding the probe. The present results are comparable with those obtained during similar studies[24] applying other (validated) analytical methods. For instance Wientjes[37] demonstrated increasing glucose recoveries in interstitial fluid until a plateau is reached 6-9 days after placing the probe. Especially the (individual) change in recovery during the first day was significant. This effect was explained by him that insertion of the probe can cause trauma to cells and capillaries. As a consequence, no (fully) functional layer of tissue surrounds the probe and limits diffusional glucose transport. The increase of glucose recoveries during the days after implantation follows from repair of the tissue microstructure around the probe. Tiessen et al[24] also observed relatively low interstitial glucose contents compared to blood glucose levels in fat tissue. He attributed this effect to that, depending on the location of the MD probe, the diffusion path of glucose as well as possible glucose uptake by the cells in the subcutaneous interstitium may differ. Although the amount of data are in this stage too limited to draw conclusions concerning this phenomenon, it seems worthwhile to investigate whether or not a better correlation is obtained if the measuring device is calibrated by means of the glucose levels found in blood. Especially during home-care, diabetic patients must be able to calibrate and control in a simple way their measuring device. It has to be emphasised however that, in order to improve the quality of life of diabetic patients excessive calibration should be avoided. For this reason, the data from the sampled interstitium are recalculated on the first blood glucose value only. The results are demonstrated in figure 3b and 4b, and as can be seen from both figures a better correlation is found between the values in blood and dialysate

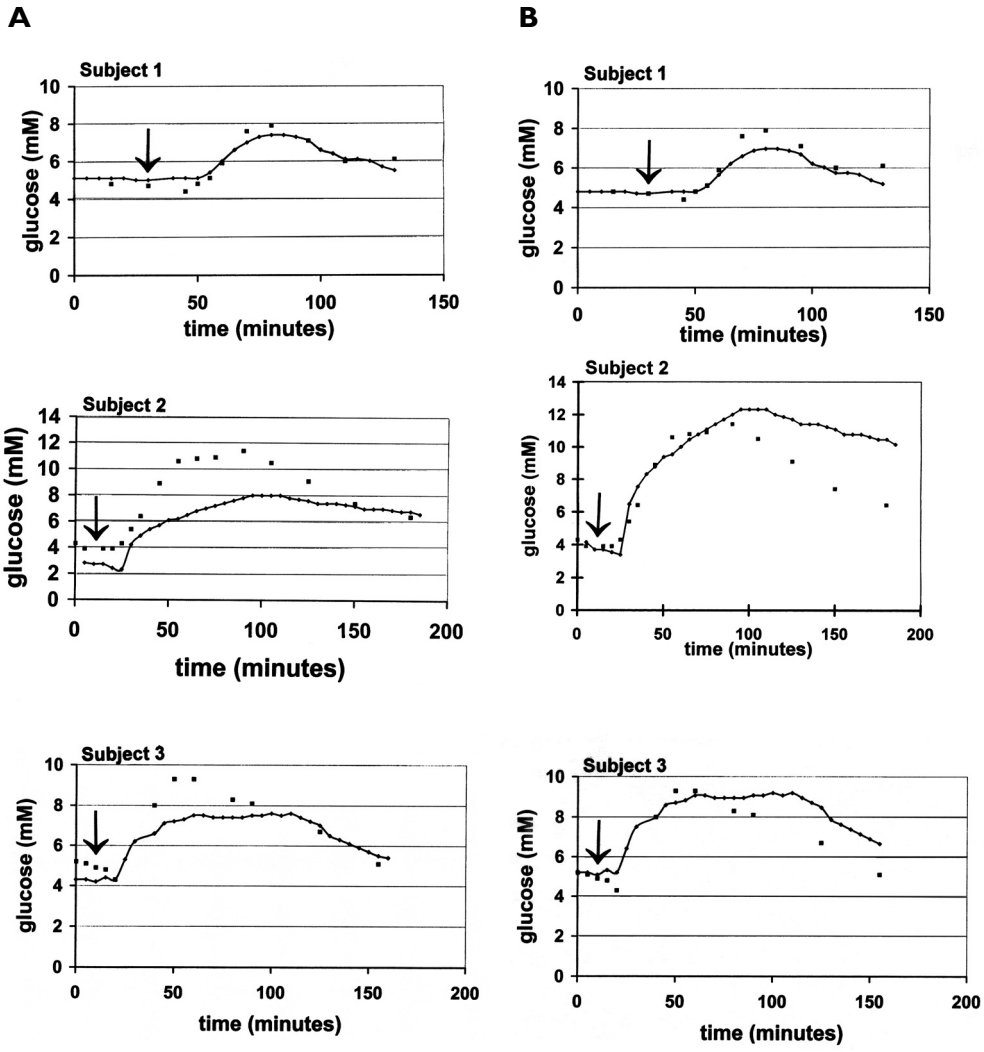


Figure 3a and 3b:

Typical graphs obtained for in vivo monitoring of glucose during glucose tolerance tests on respectively three healthy volunteers (a) calibration of biosensor in vitro and (b) calibration of biosensor on the first blood glucose value measured. Blood glucose concentrations in time (■), and subcutaneous glucose concentrations in time measured by the biosensor after ultraslow microdialysis (◆).The arrow in the figures indicates the oral administration of glucose.

(Y = 1.06, R^2 = 0.80). However, for several subjects a significant delay time is found in the decrease of the dialysate glucose content compared to the decrease in the blood glucose values (see figure 3b), and may lead to a potentially dangerous situation for the diabetic patient. Although these typical data have been observed in earlier studies[24], no explanation has been given so far.

In order to investigate this phenomenon more thoroughly, more clinical studies will therefore be necessary. For this reason and to investigate the possibilities of the measuring device for the monitoring of glucose for a longer period of time, more clinical studies will be carried out in the near future. These studies will be extended by the monitoring of both healthy volunteers and diabetic patients.

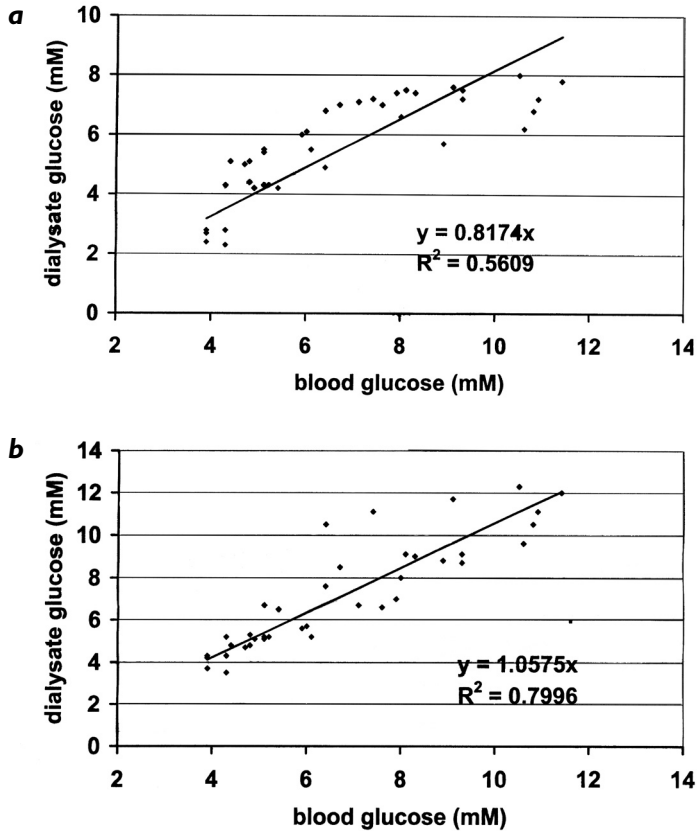


Figure 4a and 4b:
Correlation between glucose in dialysate measured with biosensor and blood glucose measured in the clinical laboratory: (a) calibration of biosensor in vitro, and (b) calibration of biosensor on the first blood glucose value measured.

7.4 Conclusions

Our ultimate goal is to develop a measuring device, which reliably measures glucose for at least several days without excessive and difficult calibration steps. In addition, if only (disposable) parts of the measuring device outside the body have to be replaced after defined moments without the need of hospital personnel, the quality of life of diabetic patients can thus be improved. Here, a portable lightweight measuring device is presented, which comprises a sampling unit (MD probe), a miniaturised flow-through biosensor and a semi-vacuum pump. Thanks to the low perfusion rate, (near) quantitative *in vivo* recoveries are established which circumvents excessive calibration normally used for microdialysis based measuring devices. The performance characteristics (linearity, precision, accuracy, selectivity and stability) which have been determined during *in vitro* and *ex vivo* studies, justifies the application of this measuring device for the *in vivo* monitoring of glucose in subcutaneous sampled interstitium of diabetic patients.

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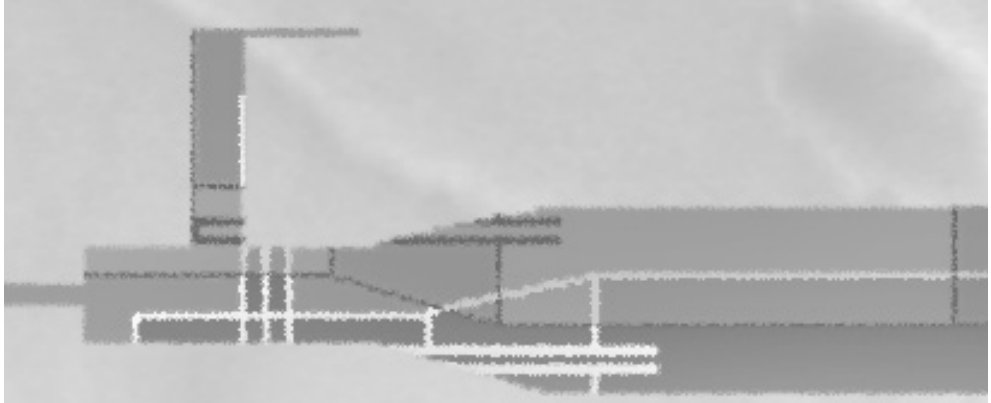


Chapter 8

Twenty-four hours profiling of glucose in the subcutaneous tissue of healthy volunteers by means of a lightweight portable measuring device

Summary

A portable lightweight measuring device for continuous long-term *in vivo* monitoring of glucose in biological compartments is described. The measuring device consists of a flow-through glucose oxidase based biosensor of only a few nanoliters internal volume, a microdialysis probe and a disposable vacuum pump. A portable potentiostat equipped with data logging is used for detection and registration. Sampling and continuous on-line monitoring is carried out at submicroliter levels and as a consequence quantitative recoveries of glucose are achieved. Accordingly, excessive calibration procedures, as are necessary with conventional microdialysis, are avoided. The clean matrix obtained for measurements, results in an improved stability and reliability of the biosensor, thereby creating the possibility to use the measuring device for long-term *in vivo* monitoring. The sensor is based on the amperometric detection of hydrogen peroxide after conversion of glucose by immobilised glucose oxidase. The clinical potential of this device was examined in freely moving healthy volunteers subcutaneously monitored for 24 hours. The data obtained were found to correlate well with the blood glucose levels determined throughout the day.

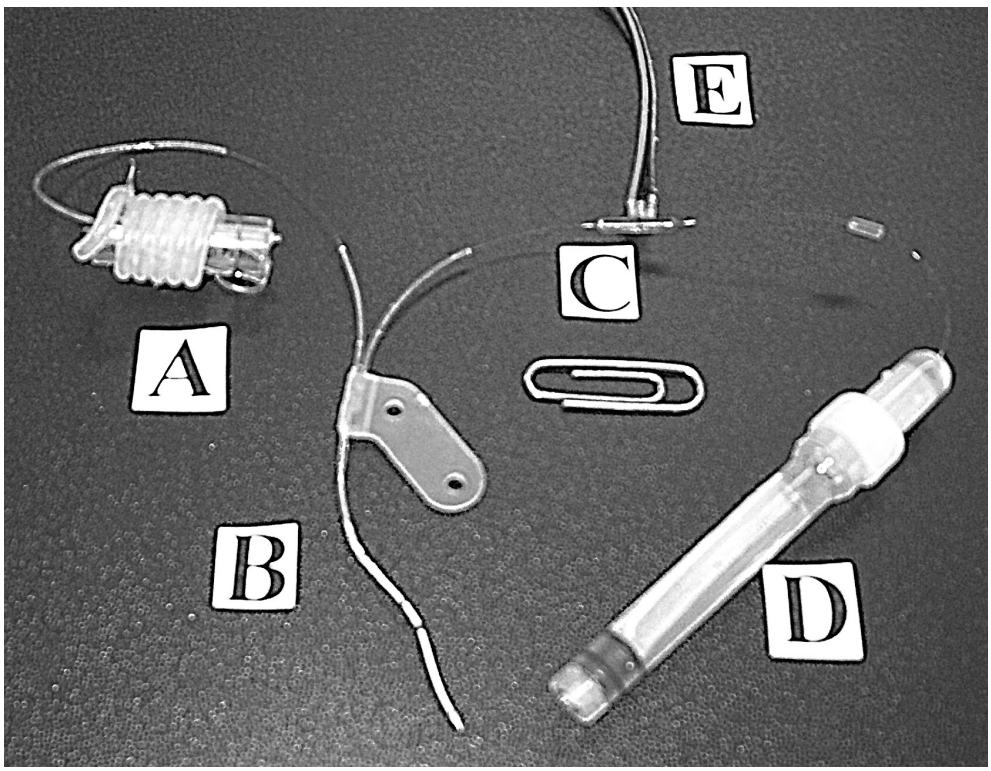
8.1 Introduction

Today, most biochemical parameters for the diagnosis of physiological abnormalities are measured batch-wise in discrete samples, whereas analysis takes place in the clinical laboratory. To allow rapid therapeutic intervention or to follow-up the progression of a disease, the need for methods that can measure continuously components in body fluids without the need of laboratory facilities is recognised. For instance for the home-care monitoring of diabetic patients, the ultimate goal would be the availability of a portable real-time device, which can continuously monitor glucose for long time (a week or more). Although biosensors are devices that have the potential to continuously monitor analytes *in vivo*, due to fouling and contamination of the electrode surface, the performance of a biosensor frequently diminishes during *in vivo* monitoring[1]. For that reason, several approaches, non-invasive as well as invasive, have been proposed to overcome this problem. For instance, near-infrared detection of glucose has been proposed for non-invasive

monitoring of glucose[2]. However, due to variations in sweat, changes in local blood circulation and/or interference from other body components, this technique is generally characterised as indicative. The GlucoWatch Biographer[3], a minimally invasive transcutaneous device using reverse iontophoresis as a sampling technique and an enzyme-based biosensor for analysis, can only be used for intermittent measurements. For measurements in the subcutaneous tissue, needle type biosensors with improved stability, such as the open flow microperfusion needle enzyme electrode[4] and the commercial needle type biosensor CGMS[5] have been presented for short-term applications. As an alternative to implanted needle glucose biosensors, microdialysis (MD) and/or ultrafiltration (UF)[6-9] as a sampling interface between the body and the biosensor have been proposed. In both cases large molecules and cells are excluded by the semi-permeable membrane of the MD or UF probe and a relatively clean matrix is obtained for measurement leading to a subsequent improvement of the stability and reliability of the biosensor[10,11]. Although the effect of probe implantation on the glucose measurements is still a matter of concern, characteristic features of these minimally invasive techniques is the stability and repeatability of the probe in living tissue and long operational period, which makes these techniques suitable for long-term monitoring. A major drawback of conventional MD is that the concentration of the analyte of interest is always lower in the dialysate than in the sampled interstitial fluid. To determine the nominal *in vivo* concentration of the analyte of interest, complicated calibration methods have to be applied[12]. A more straightforward method is, however, performing MD at extremely low flow rates[13,14]. With current MD devices a near quantitative recovery is obtained at extremely low microdialysis flow rates (< 300 nl.min^{-1}) as the sample is able to equilibrate with the interstitial fluid. However, data obtained during a four hours oral glucose tolerance test in healthy volunteers, frequently showed a lower glucose content in the dialysate compared to blood. Although the effect of probe implantation cannot be fully neglected[15], data obtained during similar studies in our group clearly demonstrated the importance of the location of the MD probe[16]. During these studies it was demonstrated that subcutaneous glucose measurements were shown to correlate better with blood levels provided that the probe was placed in subcutaneous loose connective tissue instead of adipose tissue. They found that glucose levels measured in the deep subcutaneous layer of loose connective tissue were very close to arterial levels, whereas glucose contents in adipose tissue were lower than in blood. Due to higher insulin levels they saw an increased glucose uptake by adipose cells at higher glucose levels. These insulin levels are mediated by GLUT4 glucose transporters, and are known to be more present in adipose than in connective tissue.

To allow, however, continuous *in vivo* monitoring at these low perfusion flow rates, the need for a small and low dead volume biosensor was recognised. For this reason, a flow-through biosensor with an internal volume of 10 – 20 nanoliter, as described elsewhere in detail[17], was developed by us for the continuous *in vivo* monitoring of glucose. The biosensor is based upon the amperometric detection of hydrogen peroxide after conversion of glucose by the immobilised enzyme glucose oxidase. Immobilisation of the enzyme was achieved by electropolymerisation of m-phenylenediamine, basically because immobilisation of biomolecules in these closed micro-channels can only be performed via this way. An additional advantage is that thanks to the self-controlling film thickness of this non-conducting polymer during electropolymerisation, biosensors can reproducibly be

Figure 1:
The measuring device comprised of a buffer reservoir (A), a MD probe (B), the biosensor (C) and a semi-vacuum pump (D). The biosensor is connected (E) to a home-made portable potentiostat. The dimensions of the miniature measuring device can be derived from the paperclip in the picture.



manufactured. With this biosensor, a lightweight device is composed as demonstrated in figure 1. The measuring device comprises a commercial available MD probe, the biosensor and a semi-vacuum pump as reported earlier[18] and weighs less than 5 gram. The home-made portable potentiostat used for detection and registration of the analytical results is equipped with rechargeable battery and a data-logger and can be easily worn by attaching the instrument to a belt with the provided clip.
Previous, this device has been validated during *ex vivo* experiments and some limited short-term *in vivo* tests[19]. However, in order to investigate the potential of the lightweight portable measuring device more thoroughly, *in vivo* monitoring of glucose in subcutaneous tissue of freely moving healthy volunteers was performed for a more extended period of time (24 hours). In this paper, the results of these *in vivo* experiments will be presented.

8.2 Materials and methods

8.2.1 Apparatus

For the production of the miniaturised biosensors a model DECADE electrochemical detector (Antec Leyden, Leiden, The Netherlands) and a model 22 syringe pump (Harvard Apparatus, Kent, United Kingdom) is used to pump the monomer solution through the flow-through cell during electropolymerisation. Signal output during electropolymerisation is recorded with a model BD112 flatbed recorder (Kipp & Zonen, Delft, The Netherlands). During the *in vivo* studies, detection is carried out at +0.5 V vs. Ag/AgCl and data is collected with a model Dextralert™ (Analytic Devices, Zeist, The Netherlands) portable potentiostat.

8.2.2 Materials

The enzyme glucose oxidase from *Aspergillus niger* (EC 1.1.3.4., grade I) is obtained from Boehringer Mannheim (Almere, the Netherlands). D(+)-glucose for standard solutions and 1,3-phenylenediamine for the permselective membrane is purchased from Sigma Chemical Co. (St. Louis, MO). The composition of the carrier solution during microdialysis is a sterile 0.9% saline solution. Standard solutions of glucose are prepared by diluting the stock solution of glucose (50 mmol.l^{-1}) in sterile 0.9% saline solution and are allowed to reach mutarotational equilibrium before use (24 hr). All other chemicals are of pro-analysis quality

and are purchased from E. Merck (Amsterdam, The Netherlands). Double quartz distilled water is used for all other aqueous solutions.
For the construction of the flow-through biosensor, tygon tubing (ID 0.005 inch) is purchased from Skalar Analytical (Breda, the Netherlands). The auxiliary and work electrode is made from platinum wire (0.10 mm diameter) whereas the reference electrode is made from silver wire coated with AgCl (0.125 mm). All these materials are purchased from Drijfhout (Amsterdam, the Netherlands). Low dead volume connections are made with fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands). Connections are glued with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands).
Sampling is performed by means of ultraslow microdialysis thereby using a CMA 60 microdialysis probe (Aurora Borealis Control, Assen, the Netherlands).

8.2.3 Production of the measuring device

Production of the measuring device is essentially carried out as described in detail elsewhere[17]. By pushing a 0.50 x 16 mm Luer Lock needle (B.Braun, Melsungen, Germany) perpendicularly through a 0.005 inch ID tygon tubing, consecutively two platinum wires and a Ag/AgCl wire are placed within 1-2 mm to each other into the 0.005 inch ID tygon tubing. A multi-meter was used to check the correct position of the electrodes in the tubing. Leakages in the tygon tubing are eliminated with cyanoacrylic glue (Henkel, Nieuwegein, the Netherlands) and the flow-through cell thus obtained is washed with respectively methanol (pro analysis, E. Merck, Amsterdam, The Netherlands), 10 %v/v hydrogen peroxide solution in water (pro analysis, E. Merck, Amsterdam, The Netherlands) and 0.1 M phosphate buffer pH 6.9. Electropolymerisation is performed at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 0.5 µl.min^{-1} using a solution containing 2 mg.ml^{-1} of enzyme and 10 mg.ml^{-1} of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9, followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Afterwards, the biosensors thus produced are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes at a flow rate of 0.5 µl.min^{-1} and stored in the refrigerator at 4-8 °C. The measuring device was constructed by connecting one side of the flow-through biosensor with the microdialysis probe and the other side with the semi-vacuum syringe pump (1.2 ml monovette, Sarstedt, Nümbrecht, Germany) as previously described[18]. Low dead volume connections between the different parts of the device are made with 4 cm fused silica tubing (150 µm OD, 50 µm ID) (Aurora Borealis Control, Assen, the Netherlands).

8.2.4 Performance characteristics of the measuring device

The device had been validated earlier in detail for its accuracy, precision, linearity, sensitivity, selectivity and stability during *ex vivo* experiments[19]. The linearity was found to be up to 30 mmol.l^{-1} with a detection limit of 0.05 mmol.l^{-1}. The precision was found to be 2-4%, whereas no contribution to the signal could be observed from electroactive species, such as ascorbic acid and uric acid. The accuracy of the device had been investigated by analysing 50 serum samples for their content of glucose. The results were compared with those obtained from the clinical laboratory, which have been analysed with validated methods, and demonstrated that the accuracy of the device was found to be well in accordance with the criteria set for methods of Self Monitoring of Blood Glucose. After an initial decrease from 100% to 70% in sensor response within several hours of practice, no further loss of the signal for three consecutive days was demonstrated during continuous monitoring of a dialysate of a standard solution of 5 mmol/l of glucose or a serum sample respectively. The semi-vacuum syringe pump, as reported earlier[20], produces a stable flow rate of 300 nl.min^{-1} for almost a week and does not need additional batteries. The portable potentiostat, the Dextralert^{TM} detects and collects data and files every minute the mean value of the sampled minute in its internal value.

8.2.5 Twenty-four hours profiling of glucose in subcutaneous tissue

In vivo tests were performed with healthy young male and female subjects. All subjects gave their informed consent and the study was approved by the Ethical Committee of the University of Groningen. On day 1, a microdialysis probe was placed in one side of the umbilicus in the subcutaneous loose connective tissue by means of a 16G catheter. At day 2, subjects were monitored from 9.00 a.m. up to 24 hours until the next day 9.00 a.m. At 8.00 a.m. the implanted probe was washed and de-aerated with sterile 0.9% NaCl solution. One end of the probe was connected to the lightweight measuring device, as described and provided with a fresh biosensor, whereas the other end to a 1 ml buffer reservoir containing sterile 0.9% saline solution. By pulling the plunger of the semi-vacuum syringe pump a vacuum was created and sampling was started. The dialysate thus obtained was continuously analysed for at least 24 hours at a flow rate of 300 nl.min^{-1}. After reaching a steady state (mostly within 30 minutes) the blood glucose content was determined every hour until 6.00 p.m. Blood samples were taken by finger pricking and the content of glucose was analysed with the Accutrend method (Boehringer Mannheim, Almere, The

Netherlands). After 6.00 p.m. the subject was free to go. At day 3, following an overnight monitoring, the last blood sample was taken at 9.00 a.m. and the subject was disconnected from the measuring device. After that, the measurements obtained were recorded via the Dextralert^{TM} software program installed on a Windows 95/98/NT PC.
To stabilise the biosensor and to examine the performance, the measuring device was calibrated at day one and at day 3, prior after the study, by means of a standard solution containing 5 mmol.l^{-1} of glucose.

8.2.6 Presentation of the data

The results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus[21]. This method describes an error grid analysis where the x-axis represents the reference blood values and the y-axis the value generated by the measuring device tested, whereas the diagonal represents the perfect agreement. Based upon the assumption that the target blood glucose levels ranges from 70 – 180 mg/dl, the grid is divided into five regions of varying degrees of accuracy. In short, values found in zone A and B are clinically acceptable, whereas values in zone C, D and E are potentially dangerous for patients and are therefore clinically significant errors.

8.3 Results and discussion

To enable real time continuous on-line monitoring at very low flow rates (less than 0.5 µl/min), the internal volume of the measuring device has to be extremely small. In this case a device is presented which is equipped with a flow-through biosensor with an internal volume ≤ 20 nl. Connections between the microdialysis probe and biosensor are such that the internal volume of the measuring device is calculated to be less than 100 nl. In combination with the CMA 60 MD probe, which has a relative large internal volume, a total delay time of approximately 3 minutes was found at a dialysis flow rate of 300 nl/min. The device has been validated earlier in detail for its performance characteristics[19]. Based upon the results obtained during these *ex vivo* tests and some limited short-term *in vivo* tests, the potential of this lightweight portable measuring device was further investigated. Additionally, to avoid deviations between the blood and subcutaneous glucose levels due to glucose gradient differences in subcutaneous tissue, as mentioned by others[16], it was decided to place the probe in the subcutaneous loose connective tissue of the subject. For

practical reasons and to comfort the volunteer, the MD probe was placed only one day before the measurements. The next day, continuous *in vivo* measurements were carried out up to twenty-four hours whereas during these measurements the volunteers were entirely free to move.

Some typical data obtained during this study are presented in Figure 2 (A till F). To mimic the practical use of this measuring device as much as possible, calibration of the biosensor was carried out on the first blood glucose value only. The data presented are corrected for the delay time, which was approximately 3 minutes. As can be seen in Figure 2, in most cases the content of in the dialysate reasonably follows the blood glucose content. However, probably due to bad connections between the electrical parts of the measuring device, a relatively large noise in the baseline was observed, whereas in some cases even a default value was given corresponding to an overload of the potentiostat.

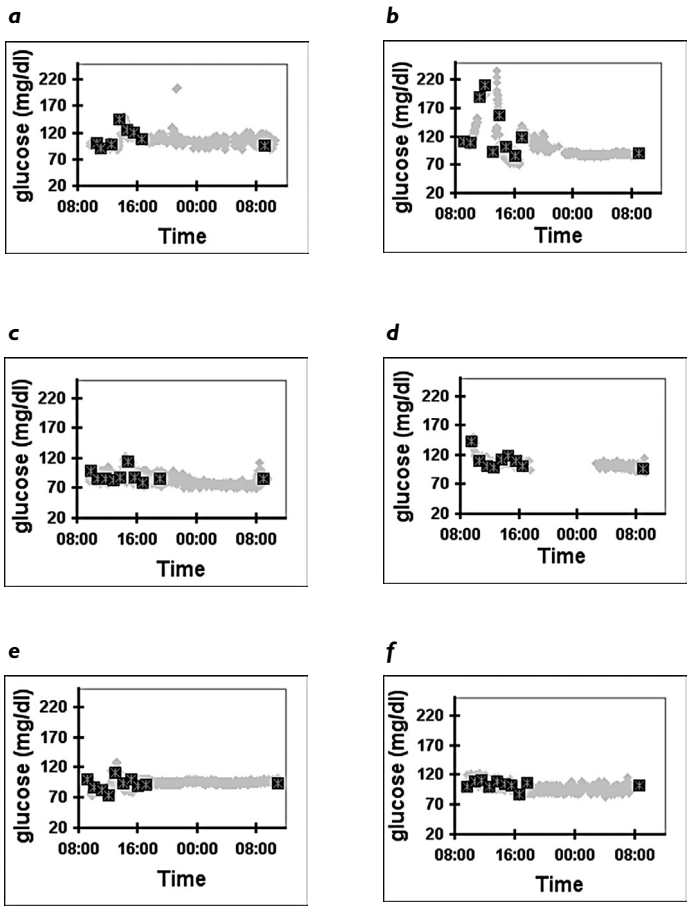


Figure 2:
Typical graphs obtained during the in vivo monitoring of glucose in subcutaneous tissue by ultraslow MD for twenty-four hours in freely moving healthy volunteers: (a) till (f). Blood glucose concentrations in time (■), and subcutaneous glucose concentrations in time (◆). Calibration of the biosensor was performed on the first blood glucose value measured.

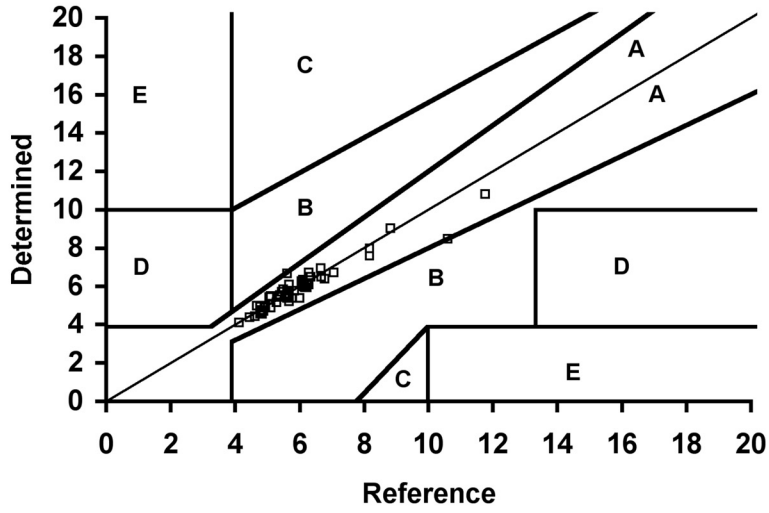


Figure 3:
Error grid analysis for the evaluation of clinical implications of patient-generated blood glucose values. The Y-axis represents the values in the subcutaneous sampled interstitium by means of ultraslow MD; the X-axis represents the values determined in the blood samples by means of a validated method.

Nevertheless, if the results are presented as prescribed for methods of Self Monitoring of Blood Glucose for patients with diabetes mellitus, a good correlation was observed. As can be seen in figure 3, the results are well in line with the criteria set, and no results were found in zone C, D and E.

Regarding the performance of the measuring device after a period of 24 hours monitoring, a mean loss in of 3% in signal was found for all biosensors tested; this loss is in accordance with earlier observations and was found to be negligible and felt within the deviation of the output of the measuring device.

Based upon the present results, the studies will be extended by the monitoring of both healthy volunteers and diabetic patients for a longer period of time (a week). Before we are capable to do so, the robustness of the device needs to be improved. Additionally, extended stability studies will have to be performed to proof the performance of these biosensors necessary during these long-term monitoring studies. As an alternative or in addition, the connection between the measuring device and the MD probe needs to adjusted such, that the (disposable) parts of the measuring device (biosensor and pump) outside the body can be easily replaced by any person without the need of trained personnel.

8.4 Conclusions

A portable lightweight measuring device of less than 5 gram is presented, which comprises a sampling unit (MD probe), a miniaturised flow-through biosensor and a semi-vacuum pump. Owing to the low perfusion rate, (near) quantitative *in vivo* recoveries are established which circumvents excessive calibration normally used for microdialysis based measuring devices. Provided that the MD probe was placed in the subcutaneous loose connective tissue, a good correlation was found with blood glucose levels during a twenty-four hours profiling of glucose in the subcutaneous tissue of freely moving healthy volunteers. Our next goal is to test this measuring device for the monitoring of glucose for up to a week without excessive and difficult calibration steps in both volunteers and patients suffering from diabetes mellitus.

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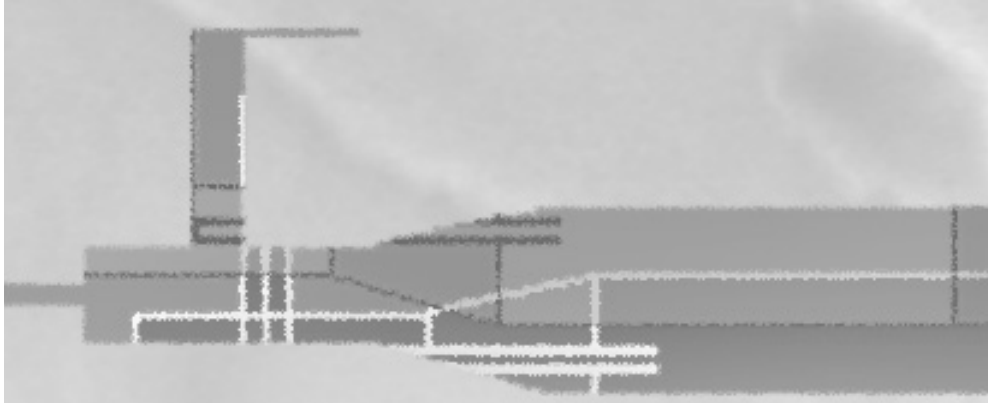

Chapter 9

Turnover of extracellular glucose and lactate in the rat striatum estimated by equilibrium microdialysis and sensor technology

Summary

Intercellular trafficking of energy substrates is essential to meet cerebral energy demand. It is as yet not known which proportion of total energy metabolism is derived from the intercellular compartment. The aim of the present study was to quantify glucose and lactate trafficking *in vivo* through the intercellular space of the striatum of the conscious rat.

Ultraslow (equilibrium) microdialysis of the striatum of freely moving rats was used to estimate intercellular levels and the turnover rates of glucose or lactate. No or increasing amounts of glucose or lactate were infused until steady state. From the difference of the concentration between the in- and out-flowing perfusate the turnover rate constants of glucose and lactate in the intercellular space is calculated. From these constants, the steady state levels and the size of the intercellular space turnover rates per g tissue were derived. Steady state levels (± SD) of glucose and lactate 2-4 days after probe implantation were 0.23± 0.12 and 0.66± 0.36 mM, respectively. The turnover rate of lactate was 0.089 ± 0.016 µMol/g/min and that of glucose 0.024 ± 0.003 µMol/g/min. These figures show that less than 10% of rat striatal energy substrates is transferred via the extracellular space and was mostly attributed to lactate trafficking. The values are close to those of the glucose-lactate shuttle estimated from a total energy balance.

9.1 Introduction

Trafficking of substrates between cells is considered essential to meet energy demand of the brain. Accordingly, it has been hypothesized that neuronal energy consumption depends, at least in part, on the release of glucose and lactate from astroglia cells and on subsequent diffusion to neurons via the brain intercellular space (ICS)[1-10]. The importance of an estimation of the concentration of glucose and lactate in the ICS has been recognized by several authors[11-21]. Concentrations ranging from 0.35 – 3.3 mM glucose[11] and 0.35 – 1.1 mM lactate[18-21] have been reported. There are, however, no reports on either estimates of the turnover rate of glucose and lactate of the ICS or of the proportion of energy substrates that is transferred via this compartment.

Here, we describe estimates of the steady state levels and the amounts of lactate and glucose that diffuse through the ICS of the striatum of the conscious and freely moving, rats. Our approach is based on the application of equilibrium microdialysis (eMD). With the conventional microdialysis (MD) perfusion rates over 1 $\mu L.min^{-1}$ are most often used. Such flow rates result in considerable concentration gradients of the analytes over the MD probe membrane ranging from ratio's between 5 up to 20, depending, among other, on the size of the dialysis probe and the perfusion rate applied[22,23]. Therefore, rather complicated methods have to be proposed to derive the real ICS levels from dialysate measurements. A relatively simple approach is based on the assumption that the recovery *in vivo* is the same as that *in vitro*. In most cases, including brain investigations, such an assumption is not allowed, because *in vivo* diffusion is limited as the result of the tortuosity of the tissue and, moreover, the probe may – at least in part – become occluded with tissue[24,25]. We[26] and others[27,28] have proposed eMD for the quantitative estimation of concentrations of substrates in the ICS. Using perfusion rates of 100 nanoliter per minute (10^{-9} $L.min^{-1}$ = $nL.min^{-1}$) or less and conventional microdialysis probes, complete equilibrium between ICS and the perfusate is reached, even if more than 80% of the surface of the probe becomes covered with tissue[26]. Moreover, we demonstrated that ultraslow ultrafiltration (collecting interstitial fluid directly through a semi-permeable membrane) and eMD resulted in identical levels of glucose and lactate[26]. A major advantage of eMD is that the real *in vivo* concentrations in the ICS are continuously measured[29].

eMD does not only allow to monitor the real levels of glucose and lactate in the ICS of a conscious rat brain striatum, but to estimate their turnover rates in that compartment as well. We propose here a simple approach to estimate these turnover rates based on the loss of glucose or lactate added to the perfusate at steady state levels. In practice, the decrease in the concentrations of lactate or glucose in the influx and efflux were measured at a constant flow rate of 100 $nLmin^{-1}$. This approach is justified, because it has been shown that acute hyperglycemia does not affect glucose cerebral consumption in conscious rats[30,31]. During low rate perfusion rapidly new steady state levels are achieved. The difference between the amounts infused and collected per time unit reflects the sum of diffusion and consumption of the substrates in the ICS surrounding the MD-probe. The cellular transporters of both lactate and glucose are bi-directional, so the rate of disappearance is particularly due to metabolism at steady state perfusion, rather than to transport over the cellular membrane. Because passive diffusion may also contribute to disappearance the present approach reveals maximal turnover rates of glucose or lactate in the ICS. Assuming a normal ICS compartment of 18% of tissue[12], we calculated the

maximal ICS turnover of glucose and lactate per g wet weight tissue. On-line eMD of glucose and lactate was possible because of the application of adapted micro-sensor technology, as described previously by us[31].

9.2 Materials and methods

9.2.1 Materials

Glucose oxidase from *Aspergillus niger* (EC 1.1.3.4, grade I, 25,000 units per 84.5 mg solid) and lactate oxidase from *Pediococcus species* (grade I, 200 units per 6.74 mg solid) were obtained from Boehringer Mannheim (Almere, Netherlands). D(+) glucose and L(+)-lactate for standard solutions and 1,3-phenylenediamine for the permselective sensor membrane was from Sigma Chemical Co. (Amstelveen, Netherlands). All other chemicals were of pro-analysis quality and were purchased from E. Merck (Amsterdam, The Netherlands). Double quartz-distilled water was used for all aqueous solutions. The composition of the carrier solution during eMD is a phosphate-buffered saline (PBS) solution (mM): NaCl (136.9), KCl (2.7), KH_2PO_4 (1.5), $CaCl_2$ (0.9), $MgCl_2$ (0.5) and Na_2HPO_4 (8.1). The pH was adjusted to 7.4 and the solution gassed with He before use. Standard solutions of glucose or lactate were prepared by diluting the stock solutions of glucose (50 mM) in PBS and were allowed to reach mutarotational equilibrium before use (24 h).

9.2.2 Probe construction

Microdialysis probes were constructed by inserting fused silica (150 µm od, 50 µm id) (Aurora Borealis Control, Assen, Netherlands) into a fiber of an artificial kidney (AN69HF, acrylonitrile and sodium methallyl sulfonate copolymer, Filtral 16; Hospal Ind., Meyzieu, France, 290 µm od, 240 µm id, molecular mass cut off 20 kDa). The tip was sealed with cyanoacrylic glue. A second fused silica tube is inserted into the fiber and served as the outlet. The fiber (total length 6 mm) as well as the inlet (total length of 8 mm) and outlet fused silica tubing (total length of 5 mm) were glued into a stainless steal cannula with cyanoacrylic glue, leaving an active length of 4 mm.

9.2.3. Surgery

Male Wistar rats (250 - 400 g, Harlan Nederland, Horst, Netherlands) were housed individually in a light-controlled room (light/dark: 0700/1900 h). Food and drink were provided *ad libitum*. The rats were anaesthetized by an intramuscular (i.m.) injection of 0.4 mg/kg body wt Hypnorm (Janssen, Beerse, Belgium) and an i.p. injection of 0.24 mg/kg body wt pentobarbital sodium (Sanofi, Maassluis, Netherlands) and placed in a stereotaxic frame. Using a rectal temperature probe and an infant heat pad (IGB, Germany), the body temperature was maintained between 36.5 and 37.5 °C during surgery. The microdialysis probe was implanted into the left striatum (from bregma, A +1.0 mm, L 2.5 mm; -6.0 mm for the surface of the skull) and secured with skull screws and dental acrylate. Additionally, a home-made aluminum screw was placed around the inlet and outlet of the probe and glued on the skull with dental acrylate. A home-made aluminum cap (diameter about 2 cm) could be easily screwed around the inlet and outlet of the probe for protection, that can easily be removed. Before measurements the animals were allowed to recover for 24 h. The Committee on Animal Bio-Ethics of the University of Groningen approved the here described procedures.

9.2.4 Preparation of glucose and lactate biosensor

Flow-through nanoliter biosensors were used as described earlier in detail[32]. Briefly, the flow-through cell was constructed by placing close to each other two platinum wires (0.10 mm diameter) and a Ag/AgCl wire (0.125 mm diameter; Drijfhout, Amsterdam, Netherlands) in a 0.005 inch id Tygon tubing (Skalar Analytical, Breda, Netherlands) by means of a 0.50 x 16 mm Luer Lock needle (B. Braun, Melsungen, Germany). Possible leakage from in the Tygon tubing was prevented with cyanoacrylic glue. Prior to immobilization of the enzyme, the flow-through cell is washed with methanol, 10% v/v hydrogen peroxide solution in water and 0.1 M phosphate buffer pH 7.0 by means of 1 ml syringe (Becton Dickinson, Etten-Leur, Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B. Braun, Melsungen, Germany). Biosensors were produced by filling the syringe with a solution containing 2 $mg.ml^{-1}$ of enzyme (approximately 500 units per ml of glucose oxidase respectively 50 units per ml of lactate oxidase) and 10 $mg.ml^{-1}$ (90 $mM.l^{-1}$) of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9. The syringe was placed in a model 22 syringe pump (Harvard Apparatus, Kent, UK) and the wires outside the tubing were connected with a model DECADE electrochemical detector (Antec Leyden, Leiden,

Netherlands) by means of crocodile clips. Glucose biosensors were produced by electropolymerization at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 500 nl.min^{-1} followed by an additional 30 minutes using the monomer solutions without the enzyme. Lactate biosensors were produced by electro-polymerization by sequentially potentially cycling from –0.2 to +0.1 V vs. Ag/AgCl at a scan rate of 50 mV.s^{-1} for at least 20 potential sweeps. Before storage and/or use, the biosensors were rinsed with 0.1 M phosphate buffer pH 6.9 for 30 min. In between, the biosensors were stored in the refrigerator at 4-8 °C. Lactate or glucose were measured in a discontinuous and a continuous mode, respectively.

9.2.5. Experimental conditions

Following surgery, the rats were individually housed in plastic animal's cages (20x30cm) with free access to food and water. Experiments were carefully carried out to maintain sterility. On experimental days, the probe was washed with sterile physiological saline solution (0.9% NaCl) and the inlet of the probe was connected to a 1 ml syringe (Becton Dickinson, Etten-Leur, Netherlands) via polyethylene tubing (id 400 µm, od 750 µm, Skalar, Breda, Netherlands) with a length of approximately 40 cm, whereas the outlet of the probe was connected to the measuring device via fused silica tubing (150 µm od, 50 µm id) (Aurora Borealis Control, Assen, Netherlands) also with a length of approximately 40 cm, allowing the animals free movement. Microdialysis was performed with PBS buffer at a flow rate 100 nl.min^{-1} by placing the syringe in a model 22 syringe pump (Harvard Apparatus, Kent, UD). Measurements were performed on day 1, 2, 3, 4, 5 and 6 after implantation of the probe. On every day steady state concentrations of respectively glucose and lactate were measured approximately 30 minutes after connecting the rat, allowing stabilization of the signal. In this case both syringe and inlet tubing was filled with PBS buffer. Next, the turnover rate of glucose or lactate was measured by filling the syringe and inlet tubing with standard solutions in PBS buffer containing increasing amounts of glucose or lactate. For every measurement, new steady state levels of both substrates were achieved and recorded within 45 minutes of perfusion. After the experiments, the rat was disconnected, the probe was washed again with physiological salt solution and the rat was brought back to the light-controlled room.

9.2.6 Histology

On completion of testing, rats were killed by overdose of sodium pentobarbital. Brains

were removed and placed in 4% formalin buffered solution, pH 7.4 for a minimum of 3 days. Before sectioning, brains were dehydrated for approximately 18 hours in a 30% sucrose saline buffer pH 7.4, frozen and sliced with a Reichert-Jung cryostat at a temperature of –13 °C. Sections of 40 µm were taken through the brain areas of cannula placement. Animals showing signs of tissue damage other than cannula track, or where the probe was placed outside the striatum were discarded.

9.2.7 Calculations

The calculations are based on the model as defined in Figure 1. It is assumed that the cerebral release of lactate into the blood circulation is negligible[33]. We assume that the properties of the tissue surrounding the probe are identical to those of the undisturbed ICS, thus of brain tissue without an implanted MD-probe. Under steady-state conditions with a probe that is perfused at low rates (= eMD) the following equation defines the

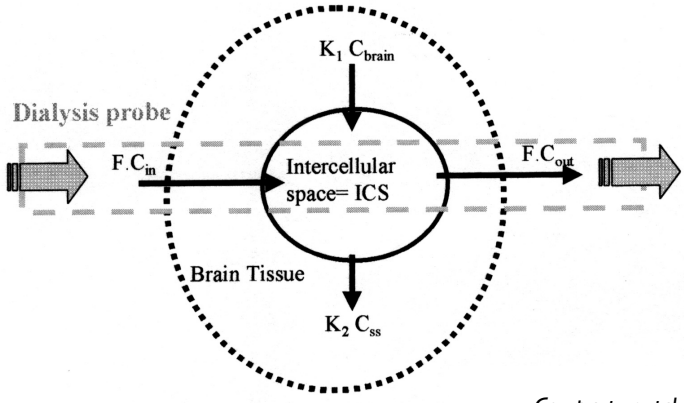

Figure 1:
Compartmental model used for the calculations.
For definitions of the used parameters see section methods.

amounts of lactate or glucose that is per time unit transported towards and outwards the dialysis probe. At such low rates we assume that the concentrations of glucose and lactate in the (out-flowing) dialysate is the same as that in the ICS (and at 0-rate perfusion, meaning that the diffusion over the probe membrane is not limiting), so the following equation can be written:

$K_1 . C_{brain} = k_2 . C_{ss} + F . C_{out} = (k_2 + F) C_{ss}$,, [equation 1]

in which,

K_1 = a transport constant (or composition of various constants; arbitrarily defined) of brain glucose or lactate towards the probe;

C_{brain} = apparent (both cellular and intercellular) concentration of the substrates in the brain;

So the term $K_1 . C_{brain}$ equals the amount of lactate or glucose that is released from brain tissue per time unit reaching the probe.

k_2 = transport constant from the probe as a part of the ICS into the brain tissue;

C_{ss} = measured concentration under steady state conditions (without added glucose or lactate to the perfusate).

So the product $k_2 . C_{ss}$ represents the amount of lactate or glucose that diffuses per time unit from the probe into brain tissue.

F = flow rate (in the present experiments, set at 100 nl/min);

C_{out} = C_{ss} concentration glucose or lactate measured in the dialysate (only when no glucose or lactate was added to the perfusate).

When glucose or lactate are added to the perfusate (C_{in}) and assuming that the rates of inflow and the outflow are identical and (new) steady states are reached, the amounts of the substrates transported or diffused per time unit can described as:

$K_1 . C_{brain} + F . C_{in} = (k_2 + F)$. Cout, [equation 2]

C_{in} = concentration glucose or lactate added to the dialysis solution;

Substituting the term $K_1 . C_{brain}$ by combining equation [1] and [2] results in:

$(k_2 + F) C_{ss} + F. C_{in} = F . C_{out} + k_2 . C_{out}$

that can be rewritten as:

$k_2 = F (C_{in} - C_{out.} + C_{ss}) / (C_{out} - C_{ss})$. [equation 3]

The constant k_2 (possibly a combination of rates of transport constants and catabolism, see discussion below) as defined here is related to the volume of the probe. The volume of the probe (V_{prob}) in the striatum is 0.48 µl.; the ICS volume is 180 µl/g of brain tissue[34]. In the calculations it is assumed that the turnover of lactate equals $^1/_2$ that of glucose. Based upon these assumptions, the flux of glucose or lactate per gram brain tissue (in µM/ g/ min) can thus be calculated as:

Flux = (180/0.48) C_{ss} (k_2 +F).

The percentage of metabolic trafficking via the ICS (R %) is calculated as the ratio between the calculated Flux and the known Cerebral Metabolic Rate (CMR) for glucose in striatum (0.82 µM/g/min; the mean value of the values given by Barbelivien *et al.*[35], Duelli *et al.*[36] and Horinaka *et al.*[37]) x100% as follows:

R (%) = [(Flux)/(CMR)]. 100% [equation 4]

9.2.8 Presentation of the results

Values are given ± SEM. Significance of the differences was calculated with Student t test. P–values < 0.05 are considered significant. ANOVA was used to test possible significant trends of steady state levels over time following implantation and of concentration dependency of calculated ICS turnover rate.

9.3 Results

Examples of recordings are shown in Figure 2. Rat striatal steady state concentrations of ICS (C_{ss}) glucose (n = 10) and lactate (n= 6) were measured up to 6 days following the

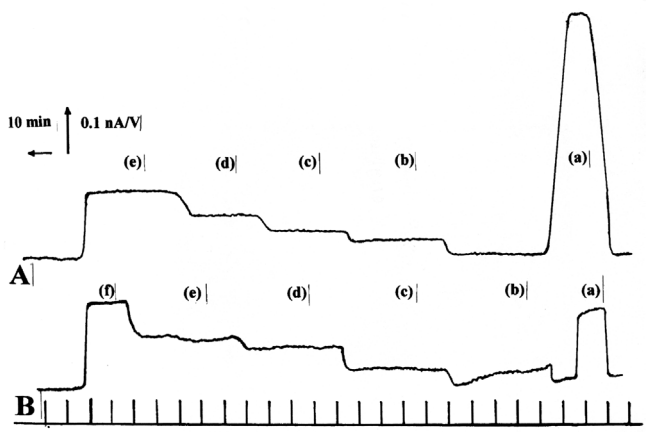

Figure 2:
Typical recorder outputs during the continuous monitoring of glucose (a) and lactate (B): In vitro calibration of the biosensor with 5 mM glucose respectively 2.5 mM lactate (a), directly followed by in vivo measurements: (b) steady state concentration of glucose respectively lactate, and new steady state concentrations after the addition of 0.5 mM glucose respectively 1.5 mM lactate (c); 1 mM glucose respectively 3 mM lactate (d); 2,5 mM glucose respectively 4 mM lactate (e); and 5 mM lactate (f).

implantation of a MD probe. The time course of C_{ss} is given in figure 3. The lowest levels (+ SEM) of glucose were measured at days 2, 3 and 4 and were 0.35 ± 0.07, 0.18 ± 0.04 and 0.31 ± 0.05 mM, respectively. The lactate levels (± SEM) did not significantly vary over 4 days; these levels were at day 2, 3 and 4, 0.64 ± 0.03, 0.68 ± 0.03 and 0.58 ± 0.02 mM, respectively.

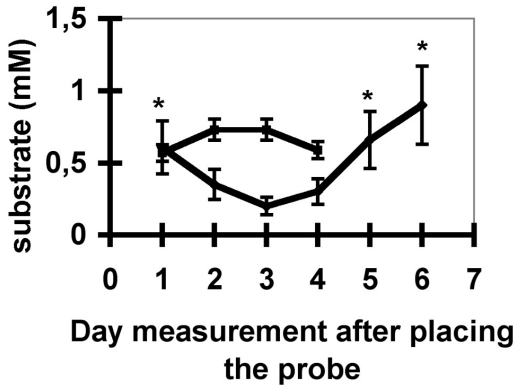

Figure 3:
*Measured steady state concentration of respectively glucose (◆) (n = 10) and lactate (■) (n = 6) in the striatum of freely moving rats up to six days following probe implantation. * indicates different from levels at day 3 ($p < 0.05$).*

At day 3 and 4 the turnover rates of glucose (n = 6) and lactate (n = 6) were estimated by infusing glucose (0.5, 1.0, 2.0, 2.5 or 5.0 mM) or lactate (1.5, 2.5, 3.0, 4.0 or 5.0 mM). On day 3 six rats were studied either with lactate (3 rats) or with glucose (3 rats); the same rats were studied on day 4, but now applying the other substrate. Based upon these measurements, the transport constant k_2 and R (%) were calculated, using equations [3] and [4]. We calculated also the correlation between the amounts of added glucose or lactate and k_2, as an index of utilized substrate. There was no significant correlation between added substrate and calculated turnover rate or R% of glucose and lactate (Figure 4). From these results, the mean ratio R (%) per added amount of glucose and lactate as well as the overall mean ratio R (%) were estimated to be less than 10% of the total energy consumption (Figure 4 and Table 1). It appeared that less than 3% of glucose and 7% of lactate is transported via the ICS, so less than 10% of the striatal energy metabolism can be attributed to diffusion of substrates via the intercellular compartment.

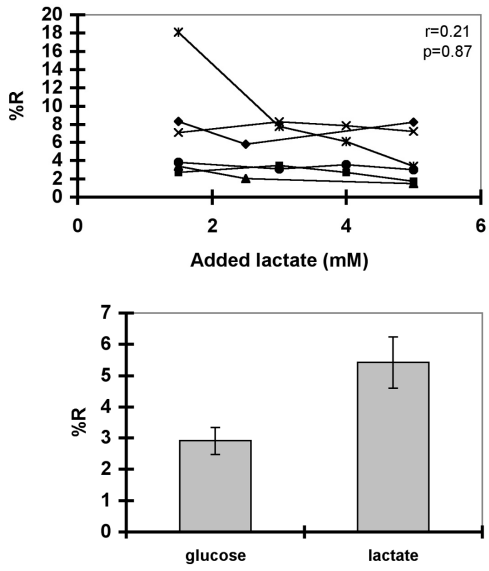

Figure 4:
Mean estimated proportion (as % + SEM) of extracellular glucose and lactate turnover in the conscious rat as related to the total glucose consumption. Upper panels show that the estimates were independent on added glucose and lactate. Lower figure is composed from the data of individual rats at days 3 and 4 following probe implantation. Number of rats: 6.

Table 1:

Steady state levels, absolute and relative turnover rate of intercellular glucose and lactate measured in the striatum of freely moving rats (6 rats studied 3/4 days after implantation of the dialysis probe; M ± SD, N= 6).

Glucose	
Steady state levels Css.	0.23 + 0.12 mM
Turnover ICS	0.024 ± 0.003 µMol/g/min
Relative Turnover	2.9 ± 0.4 %
Lactate	
Steady state levels Css.	0.66 + 0.36 mM
Turnover ICS	0.089 ± 0.016 mMol/g/min
Relative turnover	5.4 ± 0.8 %
Total in Glucose equivalents	
Turnover ICS	0.068 ± 0.01 mMol/g/min
Relative turnover	8.3 ± 0.7 %

9.4 Discussion

9.4.1 ICF glucose and lactate levels

The combination eMD with a flow-through nanoliter biosensor allowed us to monitor on a real time basis quantitatively and accurately ICS glucose or lactate levels in freely moving rats at various time intervals following probe implantation without complicated calibration procedures. Accordingly, concentrations ranging from 0.20 – 0.90 mM for glucose and 0.57 – 0.73 mM for lactate in the striatum of freely moving rats were obtained. These values are among the lowest reported (see introduction). The ICS levels of glucose decreases up to 3 days followed by an increase 4 to 6 days after implantation of the probe. These observations are comparable with those found by Fellows *et al.*[14], who reported a reduction of 75% of the ICS glucose content over 72 h following implantation of the probe and were attributed to a disturbances in glucose metabolism immediately after the implantation of the probe. According to them, the blood-brain-barrier is resealed within 2 h, whereas the local cerebral blood flow and glucose utilization in tissue surrounding the probe becomes normal within 24 h. Groothuis *et al.*[38] reported, however, that the blood-brain-barrier permeability is affected biphasically: a prompt increase shortly after insertion, followed by a second increase several days following placement of the probe. They indicate that blood-brain-barrier dysfunction persists for at least 28 days after probe implantation. If this is true, the ICS glucose content may even be lower than observed here. Measurements at longer time intervals are not recommended because of gliosis, that starts in the vicinity of the probe already within 2 days after implantation[39,40]. Although the blood-ICS-barrier may fluctuate over time, we never observed glucose values approaching blood levels, demonstrating that the barrier remained largely intact throughout the experiments. In contrast to glucose, no significant deviations in lactate content were observed over the 4 days after probe implantation. This observation may indicate that lactate levels are determined by properties of the bi-directional membrane transporters, thereby expanding the volume of distribution of lactate and thus suppressing local and short lasting fluctuations. Brain tissue levels of lactate may also remain relatively constant, because even under normoxic conditions lactate leaves the brain, particularly when local levels eg. in glia adjacent to the vascular bed, tend to become higher than blood plasma levels[32].

9.4.2 About the method of turnover rate estimations

It is argued here that the turn-over values presented here are probably maximum values and may therefore be considered as over-estimations of the turnover rates at physiological steady state conditions. Basic in our model is the assumption that the rate constants of disappearance of glucose or lactate from the dialysis probe at eMD and the ICS were similar and that they were not affected by the concentration of the substrates. The concentrations of glucose and lactate applied are well below the K_m values of their transporters, so it is unlikely that the rates of disappearance become limited at high concentrations. Indeed, the calculated k_2 values were concentration-independent, thereby suggesting that this value was also valid at steady state perfusion, thus without addition of substrates to the perfusate. With the present experimental design it is impossible to distinguish between – passive – diffusion or – active – metabolism of substrates added to the perfusate. Such a distinction would be possible, when high and carrier-saturating concentrations of lactate or glucose were applied. If diffusion is the most prominent route of disappearance of the substrate out of the probe (and ICS) the calculated k_2 values should be concentration-independent, even when very high amounts of glucose or lactate (e.g. at 25mM glucose or 10 mM lactate) were infused. Conversely, if glucose and lactate loss is due to carrier-mediated transport, the k_2 should approach a maximum value at such high substrate exposures. Direct diffusion from the probe to blood is highly unlikely, considering the large concentration gradient of glucose that has to be overcome at any of the presently applied concentrations of glucose.

According to the classical model, ICS glucose in the brain is derived directly from the bloodstream and changes in neuronal energy requirements are met by changes in the cerebral blood flow[41,42]. A direct relationship between blood and ICS glucose (and lactate) is unlikely, considering the energy buffering potential of endogenous glycogen[10,43] and the role of intracerebral lactate for energy metabolism. Glucose utilization within the brain is believed to be limited by phosphorylation, rather than by transport[12,44]. So exposure of brain tissue to high glucose does not necessarily affect energy metabolism. Indeed, Duckrow and Bryan[30] and Orzi *et al.*[31] showed that acute hyperglycemia does not lead to altered glucose consumption. Several studies[13-15] have shown that transient changes in extracellular glucose concentration occur following local neuronal activity, which is compatible with the idea that metabolism plays an important role in determining the ICS levels.

Although the applied ICS-turn-over rates may be maximum values as argued, it is as yet

uncertain whether in addition to the studied routes, alternatives and here unnoticed transfer of substrates is possible. Such alternative route could, for instance, be a direct exchange of substrates between glia and neurons in close apposition to each other, so little substrate could reach the MD probe. Alternatively, glucose and lactate may diffuse in brain tissue through close junctions, that have been observed between astroglia cells[45]. Such close junctions, however, have never been seen between neurons and glia. Moreover, there is the possibility that lactate leaves brain tissue via a venous affluent[33] and that lactate may accumulate in brain tissue downstream. In addition to lactate and glucose other energy-related substrates, such as pyruvate and in particular alanine, may also be transported via the ICS and these substrates may even leave the brain. If the latter is the case, the present turnover values lead to an underestimation of the ICS turn-over rates. Nevertheless some uncertainties, the present investigation has to be considered as a first approximation to assess the ICS turn-overrates of energy substrates in the conscious rat.

9.4.3 Implications of the extracellular turnover rates

The consumption of energy of the rat striatum is estimated to be 0.82 µmol/g/minute[35-37]. The present results suggest that maximally 10% of the energy metabolites traffic through the ICS, implying that the majority of energy substrates do not traffic through this compartment. A compartment model of brain glucose suggests a reservoir behind the blood-brain-barrier[1] and recently it has been suggested that glial glycogen is such a reservoir[43]. One consequence of this model is that extracellular glucose is not derived directly from the bloodstream, but is delivered by astrocytes[10,17,43,46]. Lactate is, besides through increased glycolysis under normoxic conditions, often thought to be produced following anaerobic glycolysis of astrocytes[47,48]. These and other findings indicate that astrocyte glycolysis plays an important role in regional energy homeostasis in brain in response to increases in local neuronal activity[3,5,6,49].

According to the glutamate-recyling-shuttle hypothesis of Magistretti and coworkers[49] only lactate coupled to glutamate neurotransmission should traffic through the ICS. Indeed, in the present investigation the relative amount of glucose diffusing through the ICS is far smaller than that of lactate (<1% versus > 7% of total energy consumption). Attwell and Laughlin[50] came to the conclusion that the glutamate-recycling shuttle may account for 3% of the total energy consumption, whereas far the most energy (80% or more) was spend in action potentials and postsynaptic effects evoked by e.g. glutamate. For general maintenance and resting potentials about 15% of the total energy spenditure was required.

Although our data do not support the suggestion that the majority of brain energy need is due to the glutamate-recycling shuttle[49], they emphasize that lactate, rather than glucose, is the major trafficking energy substrate.

The present results suggest that under these conditions less than 10% of the glucose and lactate consumed by the brain is transported via the ICS. It can be argued that the present experiments are done at low activity of e.g. the glutaminergic neurotransmission, thus underestimating the contribution of ICS glucose and lactate in the activated brain. Rat striatal ICS-lactate is increased during severe physical exercise to a (maximum) increase of only 30%[51]. This increase was mediated by glutaminergic innervation (presumably from the cerebral cortex as it appears to be sensitive to N-methyl-D-aspartate receptor blockade[42,52]. These observations suggest that the lactate response is due to both released glutamate and glutamate receptor mediated signaling. Because the k_2-values were concentration-independent (see our previous discussion), the relative contribution is also not more than 30% (i.e. still less than 13% of the total energy expenditure). Moreover, during enhanced energy demand a large proportion of the glucose and lactate consumption will be utilized for (postsynaptic) signaling, rather than in the glutamate-lactate-cycle[50]. So the low figure of 10% estimated with the present approach is not necessarily substantial higher during neuronal activation.

9.5 Conclusions

The present study provides estimates of extracellular turnover rates of glucose and lactate in the rat striatum. The turnover rates of lactate 0.089 ± 0.016 µMol/g/min and of glucose 0.024 ± 0.003 µMol/g/min were obtained. Together these figures show that less than 10% of rat striatal energy substrates is transferred via the ICS. This amount is compatible with the relative contribution of the glucose-lactate shuttle as estimated from a total energy balance.

9.6 References

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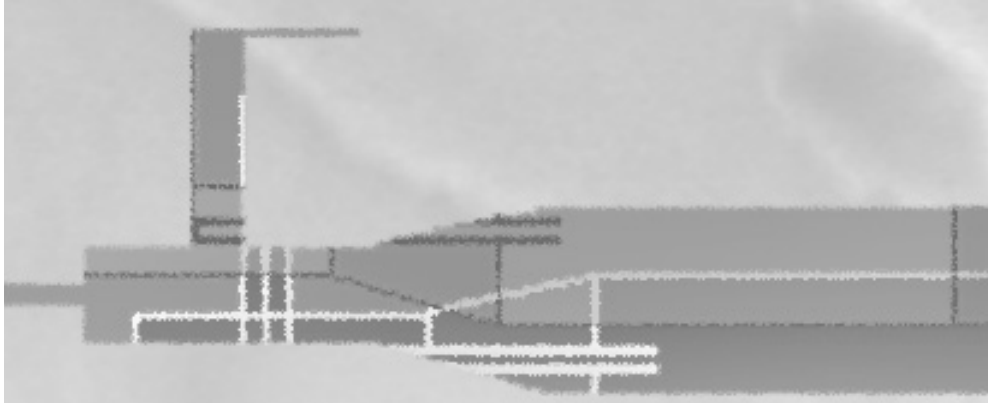


Chapter 10

Summary and final comments

General introduction

The aim of this thesis was to develop a (portable) miniaturized device for long-term continuous real time *in vivo* monitoring of analytes, such as glucose and lactate. Both glucose and lactate are markers for energy metabolism, as glucose is the major energy substrate for the body and lactate is released during oxygen deficiency. Glucose and lactate can be monitored in a wide variety of settings, such as during athletic performance and pathological situations like brain trauma, diabetes and heart failure. Because an interruption in the energy supply to organs, such as the brain and the heart, can quickly lead to life-threatening situations, the need and potential of these devices have been long recognized in clinical diagnostics. By means of real time continuous *in vivo* monitoring rapid clinical intervention can be established and, as a consequence, may prevent further damage. Additionally, for large patient groups, such as patients suffering from diabetes mellitus, the quality of life will be improved when frequent finger pricking to control their blood glucose level can be significantly reduced.

For continuous monitoring of analytes *in vivo*, by means, (bio)sensors have the most potential. Biosensors are defined as self-contained integrated devices, which provide specific (semi)-quantitative information thereby using a selective biological recognition element or bioselector. Because the preferred non-invasive sensors, which are available today, lack the necessary accuracy, many researchers have focussed on the development of a (minimal) invasive biosensor. Due to the high risk of thrombosis, embolism and septicaemia, only a few intravascular sensors have been described remaining the less invasive subcutaneous device as the preferred option. Implantation of these devices are relatively easy, while long-term monitoring belongs to the possibilities. However, most subcutaneous devices show a progressive loss in sensor performance within a relatively short time after implantation, which excludes them from applications for long-term monitoring. To avoid these biocompatibility and sensor stability related problems, sampling techniques, such as microdialysis (MD) and ultrafiltration (UF), have been introduced as a sampling interface between the device and the tissue. These sampling devices are small, biocompatible and exclude large molecules and cells to obtain a relatively clean matrix for measurement, which makes these techniques suitable for long-term monitoring. In traditional MD, however, the relative recovery is highly uncertain and complicated calibration procedures are necessary to accurately determine the *in vivo* concentration. But, if sampling is performed at an extremely low flow rate, the sample is able to equilibrate with the interstitial fluid. As a consequence a near quantitative recovery is obtained and excessive

calibration steps can be avoided. To enable real time continuous *in vivo* monitoring at this low flow rate, however, a low volume and sensitive measuring device is required.
This thesis describes the development of a (portable) miniaturised flow-through biosensor for the continuous *in vivo* monitoring of glucose and lactate. These biosensors have been incorporated into a (portable) miniaturised measuring device, comprising a MD or UF probe, the biosensor and a semi-vacuum pump. These devices have been tested for their performance characteristics and have been used during several *in vivo* applications.

Microdialysis and ultrafiltration as an interface

To avoid problems like fouling, MD and UF have been frequently used as an interface between the biological matrix and the measuring device. Both MD and UF probes can be placed subcutaneously and intravenously. Although intravenous probes may become clotted with blood clots soon after being inserted in the blood stream, it does not necessarily affect the sampling of the analytes of interest. The membranes used for MD and UF probes are often made of polyacrylonitrile, which is also used in haemodialysis and haemofiltration and is well tested for biocompatibility.
Both techniques separate chemicals by moving them across a semi-permeable membrane. The molecular cut-off value of the semi-permeable filtration membrane determines the maximum size of particles that can pass through the membrane. Via this way cells and large molecules are excluded and a relatively clean matrix is obtained for analysis. *In vivo* UF collects a filtrate of body fluids by applying a negative pressure as a driving force. MD is based on the use of a carrier solution, a concentration gradient and diffusion of analytes across the semi-permeable membrane. The analyte concentration in the UF sample directly reflects the tissue concentration, whereas the recovery of the analytes in the MD sample is influenced by many parameters, such as diffusion characteristics of the membrane, flow rate, composition of the carrier solution and tortuosity of the tissue. As a consequence, in order to calculate the actual analyte concentration when using MD as a sampling technique, many calibration methods have been proposed. In **chapter 2** a detailed description of these methods are discussed. Due to the fact that most of these methods are complicated and time consuming, a more straightforward method, performing MD at extremely low flow rates (flow rate ≤ 300 $nl.min^{-1}$), was chosen by us. An additional advantage is that the amount of matrix withdrawn is minimal, thereby minimizing the local effects of measurement.

Design of the miniaturised flow-through measuring device

In this thesis, the development of a miniaturised flow-through biosensor is described. The biosensor is based upon the amperometric detection of hydrogen peroxide, which is formed after the enzymatic conversion of the analyte of interest, e.g. glucose or lactate. In general, amperometric electrodes demonstrate a high sensitivity and a dynamic range of three to four orders of magnitude. Although these electrodes are relatively easy to manufacture in small sizes, to allow, however, real time measurements at extremely low flow rates (flow rate ≤ 300 nl.min-1) in a flow-through set-up, specifically the design and fabrication of such a measuring device is of importance and is discussed in **chapter 3** (partly) and **chapter 4**. At first, due to the limited dynamic range and stability of most biosensors, several biosensors were tested for their performance characteristics when integrated into a Flow Injection Analysis system (FIA). Although some promising results were obtained, the reproducible fabrication of a miniaturised portable FIA system with minimum dead volume connections could not be guaranteed. Although still aspects such as maximum cell volume, low dead volume connections and low backpressure have to be taken into account, measurements in the continuous mode seemed more feasible. The measuring device comprises then of a MD of UF probe, a pump and a flow-through biosensor connected between the probe and the pump. Despite all available fabrication techniques, a homemade flow-through biosensor was fabricated by using tubing with an internal diameter of 0.127 mm, in which electrodes are positioned within 1-2 mm of each other. Via this way a flow-through biosensor with a total volume of 10 – 20 nanoliter is fabricated. By using 50 µm internal diameter fused silica tubing to connect the biosensor with the probe and the pump, a measuring device with a total internal volume of 100 nl or less is obtained. At a flow rate of 100 – 300 nl.min^{-1}, continuous real time monitoring with an acceptable delay time of less than one minute is possible. By using a disposable syringe, ultraslow MD or UF by means of underpressure can reproducibly be performed. Introducing a restriction in the flow path according to the Poiseuille law controls the flow rate. Via this way a pulse free stable flow rate for almost a week is obtained without the need of batteries. This pump can be easily integrated in the device and is by means suitable for long-term monitoring with a portable device. Compared to biosensors integrated in a FIA system, when used in the continuous mode, however, difficulties may specifically arise with respect to the stability, dynamic range and selectivity of the biosensor. So, in order to guarantee a stable and selective biosensor with an adequate dynamic range, the choice of immobilization of the bioselector and/or membranes to control diffusion and to exclude

interfering species is extremely important.

The permselective membrane and bioselector immobilization

Permselective membranes as meant in biosensors serve many functions. Immobilization of the bioselector (in this case the enzyme), protection against interfering species, leakage of bioselector and diffusional control for the substrate in order to enhance the selectivity, stability respectively dynamic range are important characteristics of the permselective membrane. Today, an impressive amount of research has been conducted and reported for the development of amperometric biosensors in general and their permselective membranes in particular (see **chapter 2**). The immobilization of the bioselector in closed micro-channels, as in our approach, however, can only be performed through *in-situ* formation of the permselective membrane. A relative simple procedure is the electrochemical oxidation of a monomer in the presence of the bioselector. Via this way the bioselector is encapsulated within an *in-situ* formed polymer film. In **chapter 5** the manufacturing and performance of a flow-through miniaturised glucose biosensor based on poly(m-phenylenediamine) membrane as enzyme electrode is described. This non-conducting polymer film exhibits several advantages. Thanks to the self-controlling film thickness of the non-conducting polymer during electropolymerization, the resulting polymer film is thin and can reproducibly be obtained and demonstrates therefore obvious advantages in terms of stability and applicability towards miniaturised devices, as in our approach.

In addition, the prevention of fouling and eliminating interference of electro-active species is achieved, whereas enhanced sensitivity and electroactive action due to high enzymatic transformation of hydrogen peroxide to an electrical signal is induced. Glucose biosensors thus obtained demonstrated a sufficient sensitivity (a calculated limit of detection of 0.05 $mMol.l^{-1}$ of glucose) and dynamic range (linearity of up to 30 $mMol.l^{-1}$ of glucose) and no significant interference from elecroactive species like ascorbic acid and uric acid. The accuracy of these biosensors was proven by analyzing approximately 60 serum samples and comparing the results with those obtained from the clinical laboratory thereby using validated methods. The stability of the glucose biosensor was found to be sufficient to be able to monitor glucose continuously *in vivo* up to three days. In contrast, the lactate biosensor fabricated as such demonstrated a 60% decline in sensor performance within 5 hours of practice and was therefore not applicable for experiments involving continuous *in*

vivo monitoring. Possibly due to a combined effect of enzyme leakage and denaturation of the enzyme by the hydrogen peroxide produced, compared to encapsulation, immobilization of the bioselector through covalent coupling seemed a better approach, as explained in **chapter 2** and **chapter 6**. Biosensors, which are fabricated through the covalent coupling of glucose oxidase or lactate oxidase onto a preformed poly(m-phenylenediamine) polymer membrane by using a water-soluble carbodiimide, demonstrated a significant increase in the stability. The lactate biosensor, for instance, could be used for up to 15 days of intermittent continuous *in vivo* monitoring experiments without significant decline in biosensor performance. A remarkable phenomenon with both the glucose and lactate biosensor was, however, that detection was possible at –150 mV vs Ag/AgCl instead of the conventional +500 mV vs Ag/AgCl. Although not fully understood, the electrocatalytic reduction of hydrogen peroxide was suggested as a possible explanation for this phenomenon (see **chapter 6**). The performance of these biosensors as related to the sensitivity, selectivity and dynamic range was found to be comparable with that of the bioselector encapsulated based biosensors. Obviously, the covalent coupling based biosensors demonstrate an excellent stability over the encapsulating based biosensors. Based upon the stability data obtained so far these biosensors have demonstrated a great potential for long-term continuous *in vivo* monitoring.

Subcutaneous monitoring

Diabetes mellitus is a chronic disorder characterized by insulin deficiency, hyperglycaemia and a high risk of the development of irreversible damage to eyes, kidneys, peripheral nerves, heart and blood vessels. If glucose levels are tightly regulated diabetic complications are reduced, and hypoglycaemic events can be prevented. Self-control, based on fingerprick blood glucose measurements is painful, a burden on the patient and is often performed less than necessary, whereas nightly hypo- or hyperglycemia can be missed. The automatic and continuous monitoring of subcutaneous glucose levels with biosensors could be a significant improvement. Because the relation between subcutaneous and intravenous glucose levels was unclear, the possible use of the subcutaneous space for monitoring glucose was explored. As described in detail in **chapter 7** and **chapter 8**, *in vivo* studies pointed out that the subcutaneous glucose concentration did not rapidly follow changes in blood, and the relation between these compartments emphasized that the subcutaneous glucose is not directly and exclusively linked to the blood compartment. In most cases, a

lower glucose content in the dialysate compared to blood was found, and at first, this phenomenon was attributed by characteristics of the tissue-surrounding probe. By investigating the subcutaneous tissue characteristics more closely, it was found that the subcutaneous fat and underlying loose connective tissue differ in metabolism and interstitial structure. In contrary to the results in the subcutaneous fat, when the probe is placed in the loose connective tissue, subcutaneous glucose levels match blood glucose levels. These observations represent differences in tissue physiology and point to the preferential use of the loose connective tissue for an accurate representation of blood glucose levels. These studies encourage proceeding with diabetic patients.

Intravenous and intercerebral monitoring

An important advantage of biosensors is that they can be applied directly bedside of the patient and can give information about the patient's condition continuously. In the intensive care unit, biosensors can become important tools in continuous monitoring of the patient, for example, to observe if blood flow or aerobic metabolism is under threat. As lactate is produced when poorly perfused tissue shifts from aerobic to anaerobic metabolism, the monitoring of lactate allows detecting tissue oxygen deficits in time for therapeutic intervention. Nowadays, information on glucose and lactate metabolism is not only dependent on the time and frequency of blood-withdrawal, but also on the availability of laboratory facilities. With biosensors for intravenous measurements, changes in blood and lactate levels can be detected allowing timely intervention. One biosensor technology application encompassed the venous lactate concentration around myocardial ischemia in pigs (see **chapter 4** and **chapter 6**). During these studies the existence of a tight link between cardiac pathological events and metabolic shifts in the venous efflux, expressed as a sharp increase in the blood lactate concentration, was demonstrated. As the early detection of myocardial ischemia is of major importance, monitoring changes in lactate levels in the cardial venous efflux can be clinically useful.

In brain research, quantification of glucose and lactate in the brain intercellular space to calculate intercellular trafficking of energy to meet cerebral energy demand has been regarded as an important tool. By using ultraslow (equilibrium) MD in combination with the miniaturised device as presented in this thesis, intercellular levels and turnover rates of glucose and lactate could be estimated (see **chapter 9**). Thanks to this low flow rate, local effects around the in rat striatum implanted probe due to undesirable concentration

gradients of the analytes are minimized. Via this way, intracellular concentrations ranging from 0.20 – 0.90 mM for glucose and 0.57 – 0.73 mM for lactate in the striatum of freely moving rats were observed. These values were comparable with the lowest estimates reported. By adding a defined amount of substrate (glucose or lactate) via the probe and measuring the new steady state levels, the maximal turnover rate of glucose or lactate in the brain intercellular space could be estimated. Based upon the results it was suggested that a maximum of only 10% of energy metabolites are trafficking through the brain intercellular space, implying that the majority of energy substrates do not traffic through this compartment. Although this amount is compatible with the relative contribution of the glucose-lactate shuttle as estimated from a total energy balance, some uncertainties remain. Nevertheless, the studies presented in **chapter 9** represent a first approximation to determine brain intercellular space turnover rates of energy substrates in conscious rats and clearly demonstrate the applicability of this measuring device.

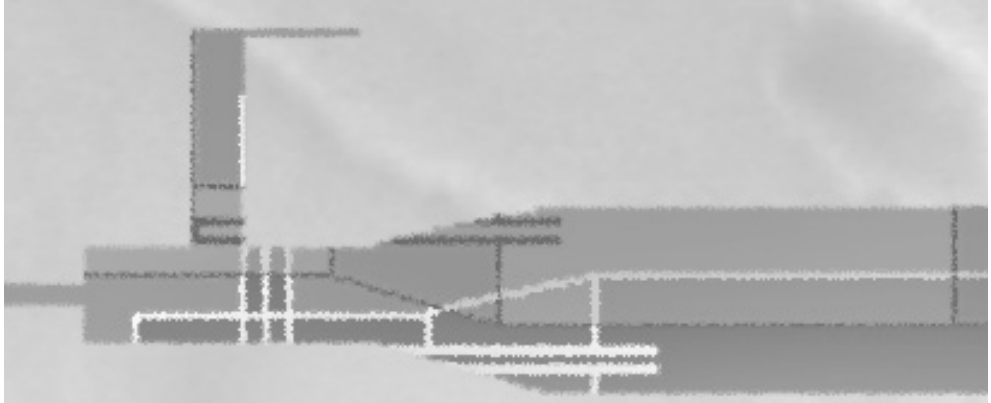
Conclusions

By means of a miniaturised measuring device, comprising a sampling unit (UF or MD probe), a flow-through biosensor and a semi-vacuum syringe pump, continuous real time *in vivo* monitoring of glucose or lactate are possible. The biosensor, in which the bioselector is incorporated through the use of *in-situ* electropolymerized m-phenylenediamine, provided good performance characteristics during both *ex vivo* and *in vivo* studies. The disposable semi-vacuum pump delivers a pulse free stable flow rate in the sub-microliter range for almost a week without the need of additional batteries. Both components can be easily integrated in a miniaturised portable device and coupled to an implanted UF or MD probe. The results obtained from several *in vivo* studies demonstrated the potential of the measuring device for long-term *in vivo* measurements, as desired for patients suffering from diabetes mellitus. Additionally, by means of this device it was shown that monitoring lactate levels in cardial venous efflux could be of use for early detection of myocardial ischemia. The study conducted in brains of freely moving rats is one of the examples of the possibilities of this measuring device for (biomedical) research.

Future

Despite considerable research efforts, only a few biosensors have become available today and are only limited applied in clinical practice. Although the potential of biosensors is recognized, this restraint is frequently caused by the observed deviations between the subcutaneous and blood values respectively the stability of these biosensors for long-term application. To overcome these problems, frequent calibration by measuring the blood values thereby using validated methods are necessary. As a consequence, possible advantages to decide in favor for the biosensor will thereby be diminished.

And, although, new strategies to improve the permselective membrane and the immobilization of the bioselector, in order to obtain selective and stable biosensors, have successfully been proposed, more effort still needs to be undertaken to investigate the appropriate place of (subcutaneous) sampling and/or measuring. Furthermore, to improve the stability of enzyme-based biosensors, in the near future it may be possible via new biotechnological processes to have access to better defined and stable enzymes that can function under stress conditions for a longer period of time. To obtain minimal invasive devices for long-term real-time *in vivo* measurements, biosensor development will continue to focus on miniaturization and multi-analyte detection.



Chapter 11

Samenvatting

Samenvatting

Het doel van het in dit proefschrift beschreven onderzoek is het ontwikkelen van een draagbaar lichtgewicht meetsysteem dat in staat is continu en 'real-time' gedurende een lange periode stoffen, zoals glucose en lactaat, in het lichaam te meten. De prestatiekenmerken van dit meetsysteem dienen dusdanig te zijn dat het in staat is patiënten constant te bewaken op bovenstaande parameters.
Zowel glucose als lactaat kunnen worden gezien als biomarkers voor het volgen van de energiebalans in het lichaam. Terwijl glucose voor het lichaam de voornaamste bron van energie vormt, zal juist lactaat worden gevormd bij een tekort aan zuurstof. Het meten van glucose en lactaat in het lichaam is niet alleen van groot belang bij patiënten die lijden aan suikerziekte, hart- en vaatziekte of in geval van een hersentrauma, maar ook bijvoorbeeld bij sporters ter voorkoming van de verzuring van de spieren. Indien bijvoorbeeld de toevoer van energie naar het hart of de hersenen wordt onderbroken, dan kunnen levensbedreigende situaties ontstaan. Bijvoorbeeld bij een verslechterde doorbloeding van de hartspier zal bij gebrek aan zuurstof glucose worden omgezet in lactaat en zal, als de doorbloeding langer slecht blijft, het weefsel door energiegebrek afsterven. Bij diabeten worden vaak zeer wisselende glucose concentraties in het bloed aangetroffen. Te weinig glucose in het bloed kan leiden tot bewusteloosheid, terwijl te veel glucose op termijn schade veroorzaakt aan ogen, nieren, bloedvaten en zenuwen.
Tot op heden vindt bewaking plaats door af en toe bloed te prikken, waarna de uiteindelijke analyse plaatsvindt in het klinische laboratorium. Behoudens het feit dat deze metingen te weinig informatie leveren omtrent de schommelingen in de concentraties gedurende de dag, is de tijdspanne tussen de bemonstering en het uiteindelijke analyseresultaat te groot om snel te kunnen ingrijpen. Daarentegen zal bij constante bewaking mogelijke schade kunnen worden beperkt respectievelijk voorkomen. Er is dan ook vraag naar een dergelijke techniek voor constante bewaking die daarbij kan helpen.

Het continu meten van stoffen is mogelijk met behulp van zogenaamde biosensoren. Biosensoren zijn geïntegreerde meetsystemen waarmee, door gebruik te maken van een biologisch stof, (semi)-kwantitatieve analyses kunnen worden uitgevoerd. In **hoofdstuk 2** wordt nader ingegaan op de theoretische achtergrond van biosensoren.
Ideaal voor de patiënt zijn de niet-invasieve meetsystemen; echter de meeste van deze biosensoren worden veelal gekenmerkt door onvoldoende nauwkeurigheid en zijn daardoor niet geschikt. Omdat het plaatsen van een biosensor in een bloedvat de nodige

risico met zich meebrengt, zoals trombose en embolie, is een meetsysteem ontwikkeld waarbij de stoffen subcutaan worden gemeten. Door de geringe biocompatibiliteit van de meeste biosensoren, echter, kan nadat de biosensoren onderhuids zijn aangebracht slechts gedurende een beperkte tijd worden gemeten. Dit probleem kan worden voorkomen door vooraleerst extracellulair vocht in het subcutane weefsel te bemonsteren, waarna het buiten het lichaam kan worden geanalyseerd. Bekende technieken die hiervoor gebruikt kunnen worden zijn microdialyse (MD) en ultrafiltratie (UF). Bij MD of UF wordt gebruik gemaakt van een semi-permeabele membraan met een gedefinieerde poriegrootte en vindt scheiding plaats op basis van het molecuul gewicht. Terwijl UF lichaamsvloeistoffen filtreert door een onderdruk over het membraan aan te brengen, is MD gebaseerd op een concentratie gradiënt tussen de dialyse vloeistof en de te bemonsteren matrix. Zogenaamde MD of UF sondes kunnen relatief eenvoudig onderhuids worden aangebracht, zijn klein en biocompatibel en laten, afhankelijk van het gebruikte membraan, slechts kleine moleculen, zoals glucose en lactaat, door waardoor een relatief schone matrix wordt verkregen die de biosensor nauwelijks vervuilt. Op deze wijze kan een meetsysteem worden verkregen waarbij stoffen gedurende een lange tijd continu in het lichaam kunnen worden gemeten.

In tegenstelling tot MD, reflecteert de concentratie in het ultrafiltraat direct de actuele concentratie in de bemonsterde matrix. Bij MD is echter de relatieve terugwinst in het dialysaat afhankelijk van diverse factoren, zoals de diffusie kenmerken van de te meten stof, het debiet, de samenstelling van de dialyse vloeistof en die van de matrix, en zijn voor de berekening van de actuele *in vivo* concentratie uitgebreide kalibratie procedures nodig. Indien nu echter wordt bemonsterd bij een extreem lage debiet (< 300 nl.min^{-1}) zal er een evenwicht optreden tussen de dialyse en de interstitiële vloeistof en kunnen semi-kwantitatieve terugwinsten worden bereikt. Alhoewel op deze wijze arbeidsintensieve kalibratie procedures worden vermeden, zullen hoge eisen moeten worden gesteld aan het interne volume en de gevoeligheid van het meetsysteem.

Dit proefschrift beschrijft de ontwikkeling en toepassing van een (draagbaar) meetsysteem waarmee continu glucose of lactaat in het lichaam kan worden gemeten. Het uiteindelijke meetsysteem bestaat uit een MD of UF sonde met daaraan gekoppeld de biosensor en een semi-vacuüm pomp.

De in dit proefschrift beschreven biosensoren zijn gebaseerd op de amperometrische detectie van waterstof peroxide, dat wordt gevormd na enzymatische omzetting van glucose of lactaat door het overeenkomstige enzym glucose oxidase respectievelijk lactaat

oxidase. Het voordeel van het gebruik van enzymen is de specificiteit voor de reactie die zij katalyseren. Door gebruik te maken van enzymen kunnen meetsystemen worden gecreëerd met een hoge selectiviteit. In het algemeen zijn amperometrische systemen gevoelige meetsystemen en beschikken over een voldoende dynamisch bereik voor de in dit proefschrift beschreven applicaties. Een additioneel voordeel is dat ten opzichte van andere detectietechnieken, systemen gebaseerd op amperometrische detectie relatief eenvoudig te miniaturiseren zijn. In **hoofdstuk 3** en **hoofdstuk 4** wordt nader ingegaan op de eisen en prestatiekenmerken die aan het meetsysteem worden gesteld, het ontwerp en uiteindelijke fabricage. Hiertoe zijn een aantal reeds beschikbare biosensoren getest met behulp van Flow Injectie Analyse (FIA). Op deze wijze wordt zowel de verzadiging van het enzym (enzym - substraat complex) als de afbraak van het enzym bij continue blootstelling aan de gevormde waterstof peroxide beperkt, waardoor een beter dynamisch bereik alsmede een langere gebruiksduur van de biosensor kan worden bewerkstelligd.
Ondanks hoopgevende resultaten met een aantal biosensoren is uiteindelijk geen fabricage techniek gevonden voor de productie van een FIA systeem met een intern volume van minder dan 100 nanoliter, en is overgestapt naar een systeem gebaseerd op continue meting. Schematisch bestaat het systeem vervolgens uit een MD of UF sonde, een wegwerp pompje met daartussen een doorstroom biosensor. Doorstroom detectorcelletjes met een intern volume van 10 – 20 nanoliter zijn handmatig gemaakt door twee platina draadjes en één Ag/AgCl draadje binnen een afstand van 1-2 mm ten opzichte van elkaar aan te brengen in een tygon slangetje met een interne diameter van 0.127 mm. De pomp bestaat uit een wegwerp spuitje waarbij, volgens de wet van Poiseuille, door het aanbrengen van een restrictie in de vloeistofstroom het debiet kan worden geregeld. Dit pompje kan relatief eenvoudig worden gekoppeld aan het meetsysteem en levert gedurende een week een continu pulsvrije debiet zonder dat daarvoor additionele batterijen nodig zijn. Met behulp van fused silica capillair met een interne diameter van 50 µm kunnen laag dood volume connecties worden gemaakt, zodat uiteindelijk een meetsysteem wordt verkregen met een intern volume van minder dan 100 nanoliter. Met een dergelijk systeem kan bij een maximaal debiet van 100 – 300 nl.min^{-1} continu en 'real-time' worden gemeten.

In vergelijking met FIA systemen zal extra aandacht besteed moeten worden aan het dynamisch bereik, de stabiliteit en selectiviteit van meetsystemen indien gebaseerd op continue meting. Met name de wijze waarop het enzym wordt geïmmobiliseerd kan invloed uitoefenen op bovenstaande prestatiekenmerken. Bij de fabricage van biosensoren wordt, in dit geval, het enzym geïmmobiliseerd rond de werkelektrode waarbij veelal gebruik

gemaakt wordt van een of meerdere lagen zogenaamde permselectieve membranen. Deze membranen dienen meerdere functies, waaronder de immobilisatie van het enzym, bescherming van de elektrode tegen storende componenten en gereguleerde diffusie van substraat vanuit de matrix naar het enzym, waardoor zowel de stabiliteit alsmede de selectiviteit en het dynamisch bereik van de biosensor kan worden verbeterd. In **hoofstuk 2** wordt een, ongetwijfeld onvolledig, overzicht gegeven van in de loop der jaren beschreven biosensoren en de daarbij behorende diverse toegepaste permselectieve membranen. Indien gebruik gemaakt wordt van een gesloten microkanaal, zoals in deze opzet, wordt de keuze echter beperkt tot het *in situ* formeren van het permselectieve membraan. Een relatief eenvoudige wijze gebaseerd op dit principe is de *in situ* electropolymerisatie van een monomeer in aanwezigheid van het te immobiliseren enzym, waardoor het enzym als het ware rond de werkelektrode wordt ingekapseld. In **hoofdstuk 5** wordt de fabricage van een geminiaturiseerde doorstroom biosensor beschreven alsmede de prestatiekenmerken daarvan, waarbij het enzym rond de werkelektrode is geïmmobiliseerd met behulp van een *in situ* gevormd poly(m-phenylenediamine) membraan. Het blijkt dat deze niet-geleidende polymeer diverse voordelen ten opzichte van andere membranen biedt. Het uiteindelijk gevormde polymeer is relatief dun en kan, door zijn niet-geleidende karakter, zeer reproduceerbaar op werkelektrodes met een zeer kleine oppervlakte worden aangebracht. De aldus geproduceerde glucose sensoren zijn voldoende gevoelig (detectielimiet van 0.05 $mMol.l^{-1}$ glucose) en beschikken over een goed dynamisch bereik (lineaire relatie tot 30 $mMol.l^{-1}$ glucose), terwijl geen noemenswaardige bijdrage in het signaal door elektroactieve verbindingen, zoals ascorbine zuur en urinezuur, is waargenomen. Door circa 60 serum monsters met behulp van deze sensoren te analyseren op het gehalte aan glucose en de resultaten daarvan te vergelijken met die van het klinisch lab is de nauwkeurigheid van de glucosesensor aangetoond. Terwijl de glucosesensor voldoende stabiel is om gedurende drie dagen glucose continu *in vivo* te meten, laten de lactaatsensoren echter een onacceptabele afname in activiteit reeds binnen 5 uur zien. Denaturatie van het enzym en/of weglekken van het enzym uit het membraan zouden een verklaring hiervoor kunnen zijn.
In **hoofdstuk 2** en **hoofstuk 6** wordt, in navolging hiervan, de immobilisatie van lactaat oxidase en glucose oxidase door covalente koppeling met behulp van een wateroplosbaar carbodiimide aan een op de werkelektrode vooraf aangebrachte poly(m-phenylene)diamine membraan beschreven. Op deze wijze kunnen biosensoren worden geproduceerd waarmee minimaal 15 dagen *in vivo* kan worden gemeten. Een additioneel voordeel van deze biosensoren is dat in plaats van bij +500 mV vs Ag/AgCl, nu gemeten kan worden bij –150 mV vs. Ag/AgCl, waardoor eventuele bijdrage door elektroactieve verbindingen wordt

geminimaliseerd. Alhoewel dit fenomeen niet eenduidig kan worden verklaard, duiden de gedane observaties op een mogelijke directe elektronenoverdracht tussen het geïmmobiliseerde enzym en de elektrode. In vergelijking met de hiervoor beschreven biosensoren, vertonen de biosensoren, met uitzondering van de stabiliteit, vergelijkbare prestatiekenmerken. Ten opzichte van diverse beschreven biosensoren is de stabiliteit van deze biosensoren echter uitzonderlijk goed te noemen, en zullen deze biosensoren mede hierdoor uitermate geschikt zijn voor toepassingen waar glucose of lactaat gedurende een lange tijd continue *in vivo* gemeten moet worden.

Eén van die toepassingen wordt beschreven in **hoofdstuk 7** en **hoofdstuk 8**, waarbij glucose continu *in vivo* wordt gemeten in het dialysaat van subcutaan bemonsterd interstitiële vloeistof. Onderzoeken hebben aangetoond dat indien het glucose gehalte in het bloed van diabeten beter wordt gecontroleerd en gereguleerd, complicaties die pas na lange termijn optreden, zoals irreversibele schade aan ogen, nieren, haarvaten en bloedvaten, kunnen worden uitgesteld respectievelijk voorkomen. De huidige methode, bloedprikjes in de vinger, wordt door diabeten als lastig en pijnlijk ervaren, en levert geen duidelijk beeld in de bloedglucose fluctuaties gedurende het etmaal. Alhoewel ten behoeve hiervan diverse onderzoeken zijn beschreven met uiteenlopende glucose biosensoren geplaatst in het subcutane vetweefsel, is tot op heden niet gekeken in hoeverre de actuele glucose concentratie in het subcutaan vetweefsel overeenkomt met de actuele glucose concentratie in het bloed. Met behulp van de in dit proefschrift beschreven biosensoren is aangetoond dat de subcutane glucose concentratie minder snel de bloedglucose concentratie volgt, en ten opzichte van de bloedglucose waarde veelal een lagere glucose concentratie in het dialysaat wordt aangetroffen. Door de MD sonde zowel in het subcutane vetweefsel als in het onderliggende losse bindweefsel te plaatsen, is een verschil in metabolisme aangetoond. In tegenstelling tot de resultaten waarbij de MD sonde in het subcutane vetweefsel is geplaatst, wordt een goede correlatie gevonden tussen de bloedglucose waarde en de glucose concentratie in het dialysaat indien de sonde is geplaatst in het losse bindweefsel. Gebaseerd op deze resultaten wordt bij verder onderzoek aan diabeten aanbevolen de MD sonde te plaatsen in het losse bindweefsel.

Een andere mogelijke interessante toepassing van het in dit proefschrift beschreven meetsysteem betreft de continue meting van lactaat in bloed bij hartpatiënten op intensive care. Ten behoeve hiervan is de lactaat concentratie in het bloed van varkens gemeten waarbij de bloedtoevoer naar het hart gedurende een bepaalde tijd middels een ballon

katheter is stopgezet (zie **hoofdstuk 6**). Door de gemeten scherpe toename in de lactaat concentratie in het bloed is een duidelijk verband aangetoond tussen een, in dit geval geïnitieerde, pathologische verandering en de daarmee gepaard gaande verandering van aërobe naar anaërobe omzetting. Gebaseerd op deze resultaten kan worden geconcludeerd dat het monitoren van de lactaat concentratie in het bloed klinisch relevant kan zijn bij de bewaking van hartpatiënten.
Naast klinische toepassingen kan het meetsysteem tevens worden gebruikt bij relevante R&D toepassingen. In **hoofdstuk 9** wordt de meting van glucose en lactaat in het striatum van vrij bewegende ratten beschreven met behulp van dit meetsysteem. Door toepassing van microdialyse bij een extreem laag debiet, is het mogelijk de intercellulaire concentratie van het gehalte aan glucose en lactaat in de hersenen te meten. Voor glucose en lactaat is een intercellulaire concentratie van 0.20 – 0.90 mM respectievelijk 0.57 – 0.73 mM gevonden. Deze concentraties komen overeen met de laagste in de literatuur gerapporteerde waarden, waarmee deze resultaten duidelijk wijzen op de goede selectiviteit van het toegepaste meetsysteem. Door nu een gedefinieerde hoeveelheid glucose of lactaat via de sonde toe te dienen totdat een nieuw evenwicht in de concentratie wordt bereikt, kan het maximale intercellulaire verbruik van glucose of lactaat in de hersenen worden berekend. Via deze wijze is aangetoond dat binnen de hersenen slechts 10% van de energie metabolieten worden getransporteerd via de intercellulaire ruimte en dat het grootste deel hoogstwaarschijnlijk via een ander compartiment wordt getransporteerd. Alhoewel nog vele vragen rond de cerebrale energie behoefte onbeantwoord zijn, is het mogelijk gebleken om op deze wijze voor het eerst schattingen te maken omtrent het intercellulaire verbruik van energie substraten in de hersenen.

De afgelopen decennia zijn wereldwijd door diverse onderzoekers een groot aantal biosensoren beschreven. Desalniettemin zijn slechts een beperkt aantal hiervan commercieel verkrijgbaar, die pas op kleine schaal op het klinisch chemisch laboratorium worden ingezet voor kortdurende metingen. De reden hiervoor is veelal de beperkte stabiliteit van de biosensor enerzijds en de gemeten deviaties tussen de bloed glucose waarde en de glucose concentratie in het subcutane vetweefsel anderzijds. Alhoewel deze tekortkomingen kunnen worden omzeild door frequenter te kalibreren, wordt daarmee tevens het voordeel van het gebruik van een biosensor voor deze toepassing tenietgedaan. Hopelijk zal, door de beschikbaarheid van nieuwe protocollen binnen de biotechnologie, het op termijn mogelijk zijn de beschikking te krijgen over ruim beschikbare en goed gedefinieerde biologische stoffen met een verbeterde stabiliteit. Ondanks het feit dat de

nieuwe generatie biosensoren verbeterde eigenschappen zullen vertonen, zal in de toekomst eveneens meer onderzoek moeten plaatsvinden om de juiste plaats van bemonstering te kunnen definiëren. Voor het verbeteren van het comfort van de patiënt zal de verdere ontwikkeling van de toekomstige meetsystemen zich focussen op miniaturisering en de simultane kwantificering van meerdere stoffen.

List of Publications

1. Rhemrev-Boom, R.M., Tiessen, R.G., Venema, K., Korf, J.
   Biosensor device and ultrafiltration sampling for continuous in vivo monitoring of glucose.
   Biocybernetics & Biomedical Engineering, 1999, **19**, 97-104.

2. Rhemrev-Boom, R.M., Jonker, M.A., Venema, K., Jobst, G., Tiessen, R.G., Korf, J.
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   Analyst, 2001, **126**, 1073-1079.

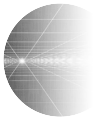
3. Rhemrev-Boom, M.M., Korf, J., Venema, K., Urban, G., Vadgama, P.
   A versatile biosensor device for continuous biomedical monitoring.
   Biosens. Bioelectron., 2001, **16**, 839-847.

4. Rhemrev-Boom, R.M., Tiessen, R.G., Jonker, A.A., Venema, K., Vadgama, P., Korf, J.
   A lightweight measuring device for the continuous in vivo monitoring of glucose by means of ultraslow microdialysis in combination with a miniaturised flow-through biosensor.
   Clin. Chim. Acta, 2002, **316**, 1-10.

5. 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***, 2457-2466.

6. Korf, J., Tiessen, R.G., Venema, K., Rhemrev, M.M.
   Glucose and melkzuur biosensoren voor continue bewaking
   Ned. T. Geneesk., accepted: 2003.

7. Rhemrev-Boom, M.M., Venema, K., Korf, J.
   Covalent immobilization of enzymes throughout a poly(m-phenylenediamine) ultra-



thin polymer membrane for the production of stable flow-through nanoliter biosensors.
Submitted for publication

8. Rhemrev-Boom, M.M., Venema, K., Korf, J.
Twenty-four hours profiling of glucose in the subcutaneous tissue of healthy volunteers by means of a lightweight portable measuring device.
Submitted for publication

9. Rhemrev-Boom, R.M., Leegsma-Vogt, G.H., Venema, K., Korf, J.
Turnover of extracellular glucose and lactate in the rat striatum estimated by equilibrium microdialysis and sensor technology.
Submitted for publication

10. Rhemrev, R., Korf, J., Venema, K., Urban, U., Vadgama, P.
A versatile biosensor device for continuous biomedical monitoring.
Presentation held during the Sixth World Congress on Biosensors, San Diego, 24-26 May 2000.

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Curriculum vitae

De auteur van dit proefschrift werd op 28 maart 1960 geboren te Gouda. In 1976 behaalde zij haar MAVO diploma aan de Maarten Luther school te Gouda, waarna in hetzelfde jaar, via een brugjaar, werd aangevangen met de studie HTS-chemie aan het Van 't Hoff Instituut te Rotterdam. Het examen werd, na een jaar stage te hebben gelopen bij TNO Hoofdgroep Maatschappelijke Technologie te Delft, afgelegd in juni 1981. In augustus 1981 werd zij aangenomen als hoofdanalist, en later als hoofd, van de afdeling Verwerkingsbaden van de firma Chemco Inc. te Soestduinen. In hetzelfde jaar startte zij met de avondopleiding MO–A Natuur- en Scheikunde, gevolgd door de studie Scheikunde, beide aan de Rijksuniversiteit van Utrecht. In 1983 werd het kandidaatsexamen afgelegd, waarna vervolgens het doctoraalexamen, met als hoofdvak analytische chemie (Prof. dr. de Ligny), werd afgelegd in 1986. Inmiddels was zij van werkgever veranderd en werkte zij van 1985 tot 1993 als werkgroepleider bij TNO Hoofdgroep Maatschappelijke Technologie (later: Instituut voor Milieu Wetenschappen), afdeling Analytische Chemie te Delft. In 1993 richtte zij, in samenwerking met Spark Holland bv, Lauer Labs bv, LC Service bv en de Hogeschool Drenthe, het applicatie laboratorium CASE Lab te Emmen op, alwaar zij tot 1996 werkte. Vanaf 1996 is zij thans werkzaam bij haar eigen bedrijf ResQ Lab bv te Nijeveen. Samenwerking tussen de vakgroep Psychiatrie van de Rijksuniversiteit Groningen en ResQ Lab bv startte in 1998 en mondde uit tot de in dit proefschrift beschreven onderzoek.