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 sepsicaemia, 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 \( \leq 300 \text{ nl.min}^{-1} \)), 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 \( \leq 300 \text{ nl.min}^{-1} \)) 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 \( \mu \text{m} \) 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 electroactive 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

Summary and final comments

interfering species is extremely important.
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 10