Design and development of a miniaturised flow-through measuring device for the in vivo monitoring of glucose and lactate
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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\textsuperscript{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\textsuperscript{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.](image-url)
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 \textit{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\textsuperscript{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. 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, 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. 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 conductometric 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
Chapter 2

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

Table 1
Selected list of oxido-reductase enzymes used for amperometric biosensors.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Typical substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycollate oxidase</td>
<td>Spinach</td>
<td>Glycollate</td>
</tr>
<tr>
<td>Lactate oxidase</td>
<td>Rat liver</td>
<td>L-, D-Lactate</td>
</tr>
<tr>
<td>Lactate oxidase</td>
<td><em>Pediococcus</em> sp.</td>
<td>L-Lactate</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td><em>Aspergillus</em> niger</td>
<td>β-D-Glucose</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Bovine milk</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td></td>
<td>Xanthine</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
<td><em>Diamond</em> rattle snake</td>
<td>L-Methionine</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
<td></td>
<td>L-Phenylalanine</td>
</tr>
<tr>
<td>Nitro-ethane oxidase</td>
<td></td>
<td>Nitroethane</td>
</tr>
<tr>
<td>Nitro-ethane oxidase</td>
<td></td>
<td>Aliphatic Nitrocompounds</td>
</tr>
<tr>
<td>Oxalate oxidase</td>
<td>Oxalate</td>
<td></td>
</tr>
<tr>
<td>Sulfite oxidase</td>
<td>Beef liver</td>
<td>Sulfite</td>
</tr>
<tr>
<td>Malate oxidase</td>
<td></td>
<td>Malate</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td><em>Nocardia</em></td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td>Squash</td>
<td>L-Ascorbate</td>
</tr>
</tbody>
</table>

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, 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):
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 H₂O₂. 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:

\[
\text{Glucose} + \text{GOx} \rightarrow \text{gluconolactone} + \text{H}_2\text{O}_2
\]

\[
\text{GOx}_{\text{red}} + \text{M}_{\text{ox}} \rightarrow \text{GOx}_{\text{ox}} + \text{M}_{\text{red}}
\]

\[
\text{M}_{\text{red}} \rightarrow \text{M}_{\text{ox}} + n\text{ e}^-
\]

Frequently reported examples of mediators are ferrocene, ferrocene derivatives, tetrathiafulvalene, tetracyanoquinodimethane, and quinones. 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 H₂O₂. Another way to measure H₂O₂ at low oxidation potential is by using carbon with dispersed rhodium, ruthenium or iridium particles.

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. 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. 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.

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 today’s 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, 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. 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. 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). 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). 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, reactive silanes or cyanuric chloride. Alternatively, by using functionalized thiols on gold electrodes, electrical communication for various layers of glucose oxidase on a self-assembled monolayer was achieved. 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 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. Biosensors based on this principle were also produced using acrylamide and vinylimidazole copolymers containing osmium redox sites, or a polycationic redox hydrogel, such as poly(allylamine), to which ferrocene was initially attached to the polymer and further crosslinked with glucose oxidase using epichlorohydrin.

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. In amperometry the current is measured as a function of the fraction of converted material. 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
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. In a reversible (Nernstian) system, the difference in potential between the oxidation and the reduction peak is about \( 2.3 \frac{RT}{nF} \). 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.

**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:

**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:

\[ j = -D \frac{dc}{dx} \]  
\[ \frac{dc}{dt} = D\frac{d^2c}{dx^2} \]

in which \( j \) is the net flux, \( \frac{dc}{dx} \) the concentration gradient and \( D \) the diffusion coefficient (m²s⁻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:

\[ c = c_\infty A \left( \frac{x}{2D^{1/2}t^{1/2}} \right) \]

in which \( c_\infty \) 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.

**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:\[51:\]

\[
\begin{align*}
\text{E} + \text{S} & \xrightleftharpoons[k_1]{k_2} \text{ES} \\
\text{ES} & \rightarrow \text{E} + \text{Product}
\end{align*}
\]

\[v = \left(\frac{k_2[E_0]_0}{K_M + [S]}\right)\]

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

\[
\frac{1}{v} = \frac{K_M}{k_2[E_0][S]} + \frac{1}{k_2[E_0]} \quad [6]
\]

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:

\[
\begin{align*}
\text{S} + \text{E}_1 & \rightarrow [\text{E}_1\text{S}] \\
[\text{E}_1\text{S}] & \rightarrow \text{E}_2 + \text{P} \\
\text{E}_2 + \text{O}_2 & \rightarrow \text{E}_1 + \text{B}
\end{align*}
\]

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At the electrode:

\[
\begin{array}{c}
B \\
\rightarrow
\end{array}
\quad \begin{array}{c}
k' \\
\quad A
\end{array}
\]

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\(_2\)O\(_2\)) 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\(^7\). According to Albery et al\(^{55-57}\), the process can be described as:

\[
e_j^S = \frac{K_M}{(l.k.s_\infty - j/k_D)} + \frac{1}{l.k} + \frac{1}{l.k'}
\]

In which \( e_j^S \) 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, \( s_\infty \) 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:

\[
1/j_S = K_M/(l.k.e_j^S.s_\infty) + 1/(k_D.s_\infty)
\]

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 \( e_j^S \) 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. 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. The reversible direct electrochemistry of GOx attached to a self-assembled monolayer were first demonstrated by Jiang et al. 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, describing the kinetics of monolayers of enzymes immobilized within very thin electrochemically formed polymer matrices using the Marquardt-Levenburg algorithm:

\[
\frac{\alpha}{j_{\text{obs}}} = n \cdot F \cdot A \cdot \frac{\alpha}{i} = K_{K_M}/(k_{-cat} \cdot \text{s}_{S\text{x}} \cdot \text{e}_{S\text{y}} \cdot i) + 1/(k_{-cat} \cdot \text{e}_{S\text{y}} \cdot i) + 1/(k \cdot K_a \cdot \text{a}_{S\text{z}} \cdot \text{e}_{S\text{y}} \cdot i) \] [12]

In which \(j_{\text{obs}} = j_S / \alpha\), and \(\alpha\) 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.

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\textsuperscript{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\textsuperscript{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\textsuperscript{64,65}. In addition, most biosensor probes are large enough to create artifacts in the surrounding tissue\textsuperscript{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\textsuperscript{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 therefore 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. 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, MD probes allows, besides intravenous sampling, continuous sampling in the extracellular space of virtually all tissues. MD can be characterized as a minimally invasive method causing minor tissue trauma and allows to define the sampling site clearly.

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.

A major disadvantage of using MD, however, is that no absolute concentrations of analyte are measured. 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. An overview of the most important factors are listed in Table 2.
### Table 2
Factors that may influence the relative recovery

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

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 µl.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 al90 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 included91,92.

**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 size93. 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 decreased94. 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% and no flow restriction over the MD probe was observed.

2.3 References

Theoretical aspects


Chapter 2
