Development of a glucose sensor for diabetic patients
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2 (Minimal)-invasive glucose sensors: an overview

2.1 Introduction
The concept of a glucose sensor was first introduced by Clark & Lyons in 1962. In their article dealing with continuous monitoring of blood chemistry, they suggested that a thin layer of soluble enzyme might be retained at the surface of an oxygen electrode using a dialysis membrane [77]. Glucose and oxygen would diffuse into the enzyme layer from the sample site and the consequent depletion of oxygen would provide a measurement of the glucose concentration. The first article describing an immobilised enzyme electrode was due to Updike & Hicks in 1967 [78]. They immobilised the enzyme glucose oxidase in a polyacrylamide gel at an oxygen electrode. Since this pioneer work in the 1960s, reasonable research effort has been devoted to the development of glucose sensors by a number of research groups worldwide [36, 79-81]. Today, glucose sensor research is a relatively mature and well-worked research field. The majority of sensors are based on electrochemical principles and employ enzymes as biological components for molecular recognition. Several new techniques for glucose sensing have been developed in clinical practice [82] as well as in biotechnology [83] and the food industry [84]. This has inspired the development of in vivo glucose sensing techniques other than the existing enzyme based method [85].

Improved diabetes control remains a motivation behind the research efforts being focused on development of an implantable glucose sensor. Still, the absence of a glucose sensor in clinical practice after all these years of research makes it clear that the in vivo implementation of these devices is
very difficult. Despite good in vitro sensor performance it has been observed that subcutaneous implanted glucose sensors show a significant decay in sensitivity [35, 86-90] and poor selectivity [91] over the implantation period. Several different explanations have been proposed, but in general there is no structural approach to assess the contribution of different failure mechanisms to the functional instability of implanted sensors.

In this chapter various glucose sensors and their basic detection principles are reviewed. In addition the influences of possible failure mechanisms on the in vivo performance of these sensors are discussed.

2.2 Glucose sensors
Glucose sensors can be broadly classified in three main categories depending on the number of applications under investigation:

1. The first and by far the largest category consist of the enzyme-based needle-type electrochemical glucose sensors. The detection principle of these sensors is based on the monitoring of the enzyme-catalysed oxidation of glucose. The category includes glucose sensors using amperometric or potentiometric operating principles (hydrogen-peroxide electrode based, oxygen-electrode based, mediator-based and potentiometric-electrode based).

2. The second category consists of glucose sensors based on the direct electro-oxidation of glucose on noble metal electrodes (electrocatalytic glucose sensors).

3. The third category consists of glucose sensors based on a number of different detection or glucose extraction techniques. This category includes affinity-based glucose sensors, coated wire glucose electrodes, reverse iontophoresis based glucose sensors, suction effusion fluid based glucose sensors and microdialysis based glucose sensors.
2.2.1 Enzyme-based glucose sensors

- **General principles**

  An enzyme-based glucose sensor is *de facto* a biosensor (Figure 2-1, page 20). A biosensor may be defined as:

  ‘a device that incorporates a biological sensing element either intimately connected to or integrated within a transducer. The usual aim is to produce a digital electronic signal that is proportional to the concentration of a specific chemical or set of chemicals’ [92].

  The biological component is used for molecular recognition, which contributes to the high specificity of the biosensor. The analyte is transformed by the biological component to a quantifiable property and then transformed into an electrical signal by the transducer. A major advantage that biosensors have over more conventional analytical methods is that they simplify the analysis to a great extent and make continuous detection of the analyte possible.

  The choice of biological component depends on the analyte under investigation and may involve processes such as biocatalysis, immunological coupling, and the use of micro-organisms or organelles. Important is a direct relationship between the biosensor signal and the quantity of the analyte under investigation. Besides biocatalysis, these principles are rarely used in glucose sensor designs and are not discussed here.

- **Biological component**

  Enzymes were initially used as biological recognition entity and are still widely applied. In the enzymatic reaction substrate is transformed into reaction products according the following general reaction:

  $$E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow[k_2]{\text{Product(s)}} E$$

  **Reaction 2-1.** Enzymatic reaction; E represents the enzyme, S the substrate and $k_1, k_{-1}$ and $k_2$ represent the rate constants of the reaction.
Substances liberated or substances consumed during the transformation, are
detectable by a suitable transducer. Several different enzymes can be used
depending on the substrate under investigation (Table 2-1 on page 22) Most
glucose sensors under investigation are based on the enzymatic oxidation of
glucose by the enzyme glucose oxidase (\text{god}).

\[ \text{glucose} + O_2 + H_2O \xrightarrow{\text{god}} \text{gluco-\delta-lacton} + H_2O_2 \]

**Reaction 2-2.** Enzymatic oxidation of glucose by glucose oxidase.

In this reaction glucose is oxidised to gluconic acid. Glucose oxidase acts
temporarily as an electron acceptor, which means that it is first reduced to
an inactive state and subsequently reactivated by the reduction of oxygen to
hydrogen peroxide [93].

![Figure 2-1. Schematic representation of possible biosensor construction.](image)

In the case of a glucose sensor, the enzyme glucose oxidase is used as
biological component in combination with a suitable transducer method.

To ensure maximal contact and response, the enzyme molecules are
directly or indirectly immobilised on the transducer. With the immobilised
enzyme electrode the thin enzyme layer is in close contact with the trans-
ducer surface. Preferably, the enzyme layer must be as thin as possible to
achieve rapid equilibration of concentrations. When the electrode is
immersed in the test environment, glucose is transported towards the enzy-
matic layer by convection and/or diffusion (Figure 2-2). Subsequently, glu-
Enzyme-based glucose sensors

Glucose diffuses within the enzyme layer accompanied by the enzymatic transformation into the reaction products hydrogen peroxide and gluconic acid. These reaction products migrate in all directions including backwards to the sample environment. Meanwhile oxygen, used in the enzymatic reaction, migrates towards the reaction side. Depending on the transducer method used, hydrogen peroxide or oxygen is converted at the transducer interface giving an electrical signal.

Figure 2-2. Schematic detail of an enzyme electrode. A thin enzyme layer is in close contact with the transducer surface. The substrate is transported in the enzyme layer by diffusion and/or convection. After the transformation the product(s) are transported to the transducer by diffusion.

Transducer

There are many detection techniques such as amperometry, potentiometry, thermometry or photometry, all of which can function as transducer method. The choice of method depends on the reaction type and the reaction products used or produced in the biological transformation step. Also the intended application of the biosensor is important. If a biosensor will be used in vivo the transducer should be small, should not release toxic substances, have a good biocompatibility and the interference from chemical or biological substances should be negligible [72, 94]. Unlike biological components, which have high specificity, some transducer methods are suscep-
CHAPTER 2
Enzyme-based glucose sensors

tible to interfering species [87]. Of all transducer methods, potentiometry and amperometry are mostly adopted. Both methods are comparatively simple to use and electrodes based on these principles can be miniaturised without great difficulty.

Table 2-1. Example of enzymes used in biosensors.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Transducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-amino acid oxidase</td>
<td>Amino acids</td>
<td>O₂</td>
</tr>
<tr>
<td>Cholesterol oxidase</td>
<td>Cholesterol</td>
<td>Pt</td>
</tr>
<tr>
<td>Choline oxidase</td>
<td>Choline</td>
<td>O₂</td>
</tr>
<tr>
<td>Alcohol dehydrogenase</td>
<td>Ethanol</td>
<td>Pt</td>
</tr>
<tr>
<td>Glucose oxidase</td>
<td>Glucose</td>
<td>O₂</td>
</tr>
<tr>
<td>Catalase</td>
<td>H₂O₂</td>
<td>Pt</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>Lactate</td>
<td>Pt</td>
</tr>
<tr>
<td>Glucoseamylase, Glucose oxidase</td>
<td>Maltose</td>
<td>Pt</td>
</tr>
<tr>
<td>Alcohol oxidase</td>
<td>Methanol</td>
<td>Pt</td>
</tr>
<tr>
<td>Invertase, mutarotase, Glucose oxidase</td>
<td>Sucrose</td>
<td>O₂</td>
</tr>
</tbody>
</table>

Potentiometric electrodes

Potentiometric electrodes measure the equilibrium potential between the indicating electrode and the stable reference electrode under zero current conditions. Electrodes that give selective response to certain ions in solution are known as ion-selective electrodes (ISE). These electrodes have a thin ion-sensitive glass membrane enclosing an electrolyte solution and detect potentials that arise at the glass/solution interface. The composition of the glass determines the sensitivity for certain ions in solution. The electrical potential measured is proportional to the logarithm of the activity (Nernst relationship) of the ion in solution. It is important that other species which may complex the ion of interest and lower its activity, must either be removed or masked. Best know ion-selective electrode is the pH-electrode although there are also ISEs for many other ions such as NH₄⁺, Li⁺, Na⁺ or
K⁺. The analytically useful range of these sensors is from 10⁻¹ M to 10⁻⁵ M. Potentiometric electrodes in combination with an immobilised enzyme are able to measure penicillin, urea, amino acids, DNA, RNA and glucose given a pH change. A good example of miniaturised potentiometric based systems is the pH-sensitive ion selective field effect transistor (ISFET). However, three general problems are encountered with ISFETs measurements in vivo [95-97]. First, reliable measurements require a buffered sample solution. Secondly, reducing agents such as ascorbic acid or uric acid interfere with the detection and measures should be taken to prevent them from entering the electrode space. Third, the rapid degradation of immobilised enzyme at body temperature results in an unstable sensor signal. Most potentiometric enzyme electrodes are therefore used in laboratory or industrial equipment.

### Amperometric electrodes

With amperometric electrodes, the intensity of a current crossing the electrochemical cell under an imposed potential is determined. Normally these consist of a working electrode where oxidation or reduction of the electrochemically active substances takes place, depending on the direction of the imposed potential, and a second electrode that acts as reference electrode. During electrolysis, the intensity of the current is a function of the concentration of the electro-active substance. Species that are frequently determined amperometrically are hydrogen peroxide (H₂O₂) and oxygen (O₂). In the case of H₂O₂, a platinum (Pt) working electrode is used as anode and polarised to a positive potential of +600 mV with respect to a standard calomel electrode (SCE), where a silver cathode is used as reference electrode.

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

**Reaction 2-3.** Reaction at platinum tip of peroxide electrode.

If O₂ is determined, a platinum-working electrode is used as cathode and polarised to a negative potential of -600 mV/SCE. The silver (Ag/AgCl) reference electrode (anode) and the Pt-working electrode are immersed in a
CHAPTER 2
Enzyme-based glucose sensors

0.5 M KCl/K₂HPO₄ solution. Anode, cathode and electrolyte are separated from the analyte sample by an oxygen-permeable membrane (Clark cell).

\[
\text{Cathode: } O_2 + 4e^- + 4H^+ \overset{-600 \text{ mV}}{\rightarrow} 2H_2O_2
\]

**Reaction 2-4. Reaction at platinum tip of oxygen electrode.**

Amperometric electrodes have a high sensitivity which allows detection of electro-active substances as low as \(10^{-9}\) M and with a dynamic range of three to four orders of magnitude. Among amperometric based enzyme electrodes, oxidase-catalysed reactions are most common because of the simple handling of the electrochemical O₂ and H₂O₂ detection. Besides glucose, lactate, lactose, sucrose and ethanol are other examples of substrates that can be measured with amperometric biosensors. Major difference from a potentiometric electrode is the consumption of reaction products when an amperometric electrode is used.

### Precautionary measures when using electrodes in vivo

Precautionary measures should be taken when amperometric glucose sensors are applied for on-line *in vivo* measurements [98]. Because a current crosses the electrochemical cell it is possible that charged (bio-)molecules might foul the electrode space, causing loss of sensitivity [35, 89]. Specially fabricated membranes may prevent a substantial part of electrode fouling [99-103] but the problem still remains with small bio-molecules. In addition, various reducing substances present in biological environments such as uric acid, ascorbic acid or glutathione may considerably influence the oxidation of H₂O₂ [91]. Electrode fouling does not occur with amperometric based gas electrodes, such as the Clark-type oxygen electrode, where the electrode cavity is protected by a hydrophobic membrane only permeable for gas [1, 62, 77, 104-107].

### Enzyme immobilisation

The first glucose sensor designs immobilised glucose oxidase onto the electrode by trapping the enzyme in a polyacrylamide gel that was attached to the membrane of an electrode [78]. The main functions of the membranes are to hold the enzyme at the electrode; to restrict the access of interfering
Enzyme-based glucose sensors

substances; to act as a diffusional barrier for glucose and to form an interface between the body and the device [82]. Besides physical entrapment in polyacrylamide gels or by dialysis membranes it is also possible to retain enzyme molecules by cross-linking with e.g. glutaraldehyde [108]. Chemical immobilisation may improve the long-term enzymatic stability [54, 109]. Two aspects in the use of enzymes in biosensors, made for in vivo applications, need special attention. First, if reaction products are potentially dangerous, the biosensor should not be applied in vivo without the necessary precautions. In the enzymatic conversion of glucose by GOD, hydrogen peroxide is formed which is a very reactive chemical and is known for its toxic effects. Secondly, the adequate availability of co-factors needs to be guaranteed. In the case of glucose measurements with GOD, the oxygen deficiency provokes incomplete transformation of the glucose present resulting in incorrect functioning of the biosensor. Oxygen deficiency is likely to occur in vivo because the physiological molar glucose concentrations generally exceed the molar oxygen concentrations in the body. An alternative for oxygen is the linking of biological redox reactions via a mediator to an amperometric electrode [110-116]. A mediator transfers the co-factor electrons directly to the electrode provided that the enzyme is in solution. Examples of mediators are ferrocene and its derivatives. However, ferrocene and derivatives are notoriously toxic [117] and quite soluble materials and should not be used for in vivo monitoring. Enzyme molecules can communicate directly with the electrode by organic conduction salts derived from tetracyanoquinodimethane and N-methyl phenazine immobilised by conducting polymers such as polypyrrole [111, 114, 118, 119]. In this case the presence of co-substrates or mediators is superfluous. A major disadvantage remains electrode fouling by small charged endogenous compounds when used in vivo. Alternative solutions have been developed to avoid oxygen deficiency problems. Examples are the application of hydrophobic membranes that are selective for oxygen over glucose [120] and a sensor design which include a two-dimensional cylindrical configuration in which oxygen enters the enzyme region from the end and side, while glucose enters only from the end [121].
**Hydrogen peroxide electrode-based glucose sensors**

This type of glucose sensor measures the amount of hydrogen peroxide produced in the conversion of glucose by \( \text{god} \) (see Reaction 2-2 on page 20) by an amperometric hydrogen-peroxide electrode. Evolved from the original Clark-oxygen electrode [77], the signal from the hydrogen peroxide electrode is due to the oxidation of the hydrogen peroxide at the catalytic platinum anode [122]. Clark modified his original design using a membrane where \( \text{god} \) was immobilised between a polyacrylamide and polycarbonate membrane and placed it on a platinum electrode [123].

The mass transfer of both glucose and hydrogen peroxide are the rate limiting processes. In addition, the linear range of the sensor depends on the oxygen concentration necessary in the enzymatic conversion of glucose by \( \text{god} \).

The most important advantages of the hydrogen-peroxide electrode based sensor over other types of sensors are the relative ease of manufacturing and the possibility of constructing them in small sizes. It is possible to construct them in the shape of a needle: the so-called “needle-type” glucose sensor. Due to these advantages many glucose sensors are based upon this principle. The high operating potential, which is required for the oxidation of hydrogen peroxide, can also oxidise other components present in vivo. This problem can be overcome, to some degree using, special membranes [124] although its application leads to a reducing sensitivity [91].

Shichiri et al. were the first to report success in miniaturising a glucose sensor by introducing a needle-type glucose sensor, which had an outer diameter of 1 mm [44]. The sensor (Figure 2-3, page 27) consisted of a fine glass isolated platinum wire with at the end a non-isolated bulbous tip (anode). On this tip a layer of glucose oxidase was immobilised using a cellulose-diacetate membrane. An outer steel tube stained with silver, serves as the cathode. The platinum anode converted hydrogen peroxide produced in the enzymatic conversion of glucose at 600 mV. The bulb-end of the electrode was further coated with a polyurethane membrane to overcome oxygen limitation. Glucose sensors inserted in the subcutaneous tissue of seven dogs demonstrated a directly proportional relation between the blood glucose concentration and current of the sensor [45]. The sensor sensitivity,
however, gradually decreased to 81% of the initial level during 3 days of continuous monitoring. The authors imputed this loss of sensitivity to the fixation of albumin and other proteins on the inserted sensor. This needle-type sensor could be implemented in a closed-loop glycaemic control system together with a microcomputer system and an insulin pump. They also developed a telemetry unit for integration with their glucose sensor equipment. This device was used for monitoring and control of insulin delivery [125]. With this closed-loop system it was possible to establish glycaemic control in diabetic patients for several days [46, 47, 126], although a reduction to 57% of the initial signal level was noticed after 4 days of in vivo measurements [127].

Figure 2-3. The Shichiri glucose electrode. Pfeiffer *et al.* constructed a similar needle-type glucose sensor. They immobilised glucose oxidase on a standard stainless needle, which functioned as cathode [128]. A second generation of this sensor used a centrally placed platinum wire (0.3 mm) surrounded by a stainless steel tubing. By successive dipcoating procedures, layers of cellulose acetate, glucose oxidase (crosslinked with glutaraldehyde) and polyurethane were placed on its sur-
Enzyme-based glucose sensors

In vitro these electrodes were stable for at least 6 days and had a linear range extending to 28 mM glucose with response times less than 100 sec. [54]. The sensor was implanted in the subcutaneous tissue of a sheep and it was found that the sensor signal and the delay response did not exceed 5 minutes [129]. Sternberg et al. developed a needle-type glucose sensor where glucose oxidase was covalently coupled to a cellulose acetate layer, using bovine serum albumin, and deposited it on a platinum tip [130, 131]. Due to the multi-layer structure and composition, small anions such as ascorbate were partially discriminated. When implanted subcutaneously in anaesthetised rats, sensor responses correlated correctly with blood glucose concentration but presented sensitivity coefficients significantly different to those determined in vitro. Several improvements have been made to this sensor design to enhance in vivo stability and reduce the effect of interfering substances [58, 132-134].

Vehlo et al. investigated the ability of several cathode-needle materials to behave as a reference electrode in a two-electrode glucose sensor to present a stable auxiliary electrode potential in order to improve in vivo stability [135]. They concluded that improvements in sensor analytical characteristics could be obtained with silver/silver-chloride-coated cathodes. Vadgama et al. constructed a glucose sensor similar to the design of Shichiri [136-139]. To overcome surface fouling of the electrode they designed a needle enzyme electrode incorporating an open micro-flow technique, in which the sensor surface is subjected to a flow of fluid. Implantation of these sensors in rats indicated that there was little or no surface fouling avoiding the requirement for repeated in vivo calibrations at least over the initial implantation period [140]. Updike et al. constructed a total implantable glucose sensor [56, 141-143]. Their sensor-transmitter system was implanted subcutaneous into non-diabetic dogs for 20 to 114 days [141]. The implanted devices operated only during intermittent measuring periods of a few days. The sensor signal decayed continuously over the implant period and required re-calibration before each recording session. Sensor units eventually failed because of electronic problems or because of bio-fouling of the electrode.
Several other needle-type glucose sensors have been developed based on the principle of amperometric detection of $\text{H}_2\text{O}_2$ produced by the enzymatic conversion of glucose by $\text{god}$ [48-50, 144-158]. These sensors mainly differ in size, shape, type of membrane used and way of glucose oxidase immobilisation.

A number of studies have been published, describing the negative influence of endogenous proteins on the functioning of amperometric peroxide detecting electrodes [49, 98, 139, 159, 160]. It is suggested that the foreign body reaction at the implantation side eventually induced by the bio-incompatibility of sensor materials may be a reason for observed reduction or loss of sensitivity. Moreover, the electrode oxidises any species present at the electrode surface that is oxidisable at the applied potential, contributing to regularly observed poor in vivo performance. Well-known bio-chemicals that interfere at the electrode, either by oxidation or by the reaction with $\text{H}_2\text{O}_2$, include ascorbic acid, uric acid and urea [35, 100-103, 161-163]. A strategy to overcome the problem of interferants is the application of several types of membranes such as hydrophilic polyurethane, polyhema and Nafion® , to make the sensor more biocompatible [56, 98, 100-103, 120, 158, 163-165]. In addition, there must be a means of counteracting the relatively low ratio of oxygen to glucose (oxygen deficit) in the body [166]. Membranes with relatively high oxygen solubility may be helpful to minimise the steady-state oxygen deficit [141, 167] but this sensor design provides no means to account for the effects of local oxygen variations on the signal.

A fundamental shortcoming in design of all hydrogen peroxide-based enzyme electrode sensors is the inevitable peroxide-mediated enzyme inactivation [149, 168, 169]. Hydrogen peroxide, produced in the enzymatic conversion of glucose, deactivates the glucose oxidase molecules. The application of special membranes may prevent electrode fouling and electro-chemical interference substantially, and it contributes to an improved bio-performance. Even so frequent re-calibration in vivo may be necessary to account for enzyme inactivation [150, 170]. Considering these inherent
characteristics, this sensor design may be limited to short-term applications at best.

**Oxygen electrode-based glucose sensors**

An alternative for the hydrogen-peroxide electrode is the combination of glucose oxidase immobilised onto an oxygen electrode. In this case, oxygen that is consumed during the enzymatic conversion of glucose can be measured (see reaction 2.2, page 20). The signal output of the electrode is the difference between the base oxygen level and the level attained as a result of oxygen depletion by the enzymatic reaction. Oxygen electrode-based glucose sensors are composed of a Clark-type oxygen sensor (Figure 2-4), which is covered with a membrane containing the immobilised enzymes glucose oxidase and catalase [78, 166]. The common Clark-type amperometric oxygen sensor consists of a two-electrode system; a centrally placed platinum wire (cathode) enclosed in a silver/silver chloride case (anode). The cavity between the cathode and anode is filled with electrolyte and the whole face-end of the sensor is covered with an oxygen-permeable membrane. By applying a constant potential of -600 mV between the platinum cathode and silver anode, oxygen is electrochemically reduced resulting in an amperometric signal. The advantages of amperometric oxygen detection over H₂O₂ are twofold [171] and manifest themselves in sensor stability and selectivity, especially in vivo. First, catalase is co-immobilised in excess to forestall peroxide-mediated inactivation. Second, a nonporous hydrophobic membrane, which is only permeable for gases, protects the electrode cavity. This vastly reduces electrochemical interference compared with peroxide-based sensors and because the hydrophobic membrane retains the current within the oxygen sensor, electrode fouling with polar molecules is not likely to occur. The nominal disadvantage is that miniaturisation of an oxygen electrode to the same extent as a peroxide-based electrode is difficult; oxygen electrodes have more components and are therefore more difficult to make. Initially most of the developed oxygen electrode-based glucose sensors are intended to be used intravascularly [62, 166, 171, 172]. In all these designs together with catalase was immobilised on top of a Clark-type oxygen electrode and covered with hydrophobic membranes.
A disadvantage of this approach is the dependency on a constant environmental oxygen concentration i.e. the oxygen concentration in the tissue surrounding the sensor. Gough et al. sensor design included a two dimensional cylindrical configuration to overcome the oxygen deficit problem [104, 121, 168, 173]. In this sensor oxygen enters the enzyme region from the end and side, while glucose enters only from the end, allowing adequate oxygen availability even at substantial concentration mismatches [121]. Amour et al. used sensors that were based on the sensor geometry developed by Gough et al.[171]. They implanted glucose sensors in the superior vena cava of six dogs. The results demonstrated that their sensor could remain operable on demand, during a period of 333 days. The sensor response to glucose showed little change over the implant period. Factors as biocompatibility, enzyme lifetime, $O_2$ availability, $O_2$ sensor stability, and biochemical interference were not limitations.

Measuring in the blood stream has the advantage of providing direct information about the blood glucose concentration. On the other hand, it has
CHAPTER 2

Enzyme-based glucose sensors

de the disadvantage of possible problems with blood compatibility. To lower the risk of thrombosis, oxygen electrode-based glucose sensors that can be inserted subcutaneously were developed by Ertefai et al. [174] and Atanasov et al. [106, 107, 175]. To study the response of glucose sensors that were chronically implanted in subcutaneous tissues, Ertefai et al. developed a physiological preparation employing a chamber which was permanently mounted on the back of a rat. The chamber supported the growth of vascularised subcutaneous tissue around the sensors and it was used in conjunction with chronic implanted intravascular catheters for blood sampling and fluid infusion. They found that the Glucose sensors responded to glucose infusions with a lag-time of 10 to 15 minutes. Using an in vitro calibration, the sensors indicated at best only relative rather than absolute values of blood glucose concentrations [174]. Atanasov et al. developed a rechargeable oxygen electrode-based glucose sensor that makes it possible to extend the sensor lifetime by in situ sensor refilling [106, 107, 176-178]. Replacing spent immobilised \textit{god} with fresh enzyme facilitates recharging of the implanted sensor without surgical removal from the patient. The glucose sensor has been implanted and tested in vivo in sheep [179] and dogs [180]. A good correlation was found between glucose serum levels measured by routine technique and those measured using the glucose sensor.

- Mediator-based glucose sensor

The use of an artificial electron acceptor or mediator to replace the natural acceptor oxygen in the oxidation of glucose by glucose oxidase is another approach that has been explored to overcome the tissue oxygen dependence [181]. In addition, the oxidation of the reduced mediator occurs at a low potential thus reducing the sensitivity of the sensor to interfering substances.

In the design of Cass et al. ferrocene was deposited on the surface of a graphite electrode [182]. Glucose oxidase was covalently attached to the surface of the electrode and covered by a polycarbonate membrane. Claremont et al. were the first who reported an implantable amperometric ferrocene-modified glucose sensor [183]. They implanted the sensor into the subcutaneous tissue of anaesthetised, non-diabetic pigs. Subcutaneous tissue glucose concentrations, as measured by the sensor, were about 20% of blood glucose values, measured by a conventional glucose detection
method. After an intravenous bolus glucose injection, electrode responses increased with almost no time lag, but the subsequent rates of rise and fall of electrode-measured tissue glucose concentrations were slower than that of the blood values. The authors implanted the sensor also in the subcutaneous tissue of normal and insulin-dependent diabetic patients [184]. The subcutaneous tissue glucose concentration mirrored simultaneously measured changes in blood glucose after an glucose oral load and after short-acting insulin injections, though increases and decreases in the sensor signal output were slower than the glycemic changes. The authors noted that the construction of the sensor configuration was difficult and virtually impossible to manufacture for general clinical use. A number of amperometric glucose sensors designs are reported in literature incorporating different types of mediators to establish electron transfer between the enzyme and electrode [34, 113, 116, 185-187].

However, the initial promise exhibited by mediator based glucose sensors for in vivo applications, has failed to materialise. The main problem remains the limited long-time-use stability of mediated glucose sensors, which has been attributed to the leaching of the mediator [188]. In addition, the loss of mediator is a particular important issue for implantable sensors because of the inherent toxic effect of the mediators used [71, 117].

Another approach is the development of amperometric glucose sensor based on the principle of direct electron transfer between the enzyme glucose oxidase [114, 115, 189-191]. Conducting organic salts or polymers have been used in the construction of these electrodes. In this case, no co-substrates such as oxygen or mediators are required. These glucose sensors are less vulnerable to electrochemical interference, although substances such as ascorbate still have a strong effect on the sensor signal. Even so, no in vivo studies have been published using sensors based on this principle.
2.2.2 Potentiometric enzyme based glucose sensors

Another approach is the possibility to measure the change in local pH due to the gluconic acid produced in the GOD reaction (see reaction 2.2, page 20) at a potentiometric sensor, usually a coated wire pH-electrode or an ion selective field effective transistor (ISFET) [96, 97, 108, 192]. Coated wire sensors are easy to fabricate and suitable for miniaturisation. Several coated wire potentiometric glucose sensors have been proposed for in vivo use [193]. However, the numerous interfering processes caused by components other than glucose have limited the applicability of these potentiometric glucose sensors greatly. Main disadvantage is the low sensitivity of the sensor due to the small dissociation constant of the produced gluconic acid. The introduction of microelectronic techniques in the sensor development has made it possible to miniaturise the sensor (ISFET). It is difficult to apply ISFET based glucose sensors directly in vivo. In particular the corrosion of semiconductor materials by physiological-salt solutions is a problem. In addition, being potentiometric measuring devices, ISFETs have the same disadvantages as the coated wire sensors: dependence on the buffer capacity of the measurement solution, sensor instability due to interference of produce hydrogen peroxide in the enzyme layer and oxygen deficit problems limiting the dynamic range of the sensor.
2.2.3 Electrocatalytic glucose sensor

Direct glucose oxidation at the surface of a platinum electrode makes it possible to measure glucose without the use of an enzyme [59, 194-201]. The lack of specificity in biologic fluids is one of the major drawbacks of electrocatalytic glucose sensors. The current that can be attributed to the oxidation of glucose is only a small fraction of the total current; there are interfering effects of other species [202], in particular urea and amino acids [198]. This leads to the relatively poor signal-to-noise ratio and low sensitivity for glucose. Interference of species can be decreased by the application of selective membranes but not prevented. In addition, a gradual decline of electrode activity is seen due to the absorption of reaction products on the electrode, reducing the “active” surface area. Gebhardt et al. suggested a “potential jump” technique to reduce the poisoning of the electrodes and additional membranes to avoid the interference of other substances than glucose [197]. Lerner et al. reported a method to reduce the poisoning of the electrode surface by varying the voltage in a trapezoidally shaped cyclic waveform [203]. Preidel et al. developed an electrocatalytic glucose sensor for long-term implantation [59, 196, 199-201]. They implanted the sensor as a flow-through cell in sheep’s to test the in vivo functioning over a longer period [59]. The sensor was inserted into the carotid artery and driven by a portable electronic unit worn by the animal. They were able to determine the glucose concentration in sheep for more than 71 days and with an improved version it was even possible to measure 130 days with tolerable deviations from glucose reference measurements [199, 201]. Although these results look very promising, only intermittent glucose measurements were conducted. Every time a measurement started, the sensor needed approximately a two hours running-in period followed by a rather complicated calibration procedure. Also a number of sensors had problems with red gelatinous clots reducing the active surface area of the electrode.

Given this lack of specificity in vivo, it is questionable whether the electrocatalytic glucose sensor ever will be used in clinical practice.


2.2.4 Other glucose sensor concepts

A variety of other glucose sensor concepts have been explored. Wilkins and Wilkins applied the principle of the liquid membrane coated wire electrode in a coated wire glucose electrode [204]. The electrode utilises a quaternary ammonium salt with a sparingly soluble metallic salt of glucose, in a matrix of polyvinyl chloride. The concept is to convert the non-ionic glucose to an ionic substance by using the associative-dissociation mechanism of glucose salts. The dissociation or the association of the glucose salt in equilibrium with a glucose solution can be measured by polarographic, potentiometric or amperometric methods. The study has indicated that only small interference effects are noted from uric acid and ascorbic acid. A major problem is the leaching of barium salt from the electrode, which should be avoided when in vivo measurements take place. By covering the electrode with a hydrogel the leaching can only be partially prevented and this is not a definitive solution for this problem.

Another technique to measure glucose is the use of an affinity glucose sensor. Schultz et al. introduced a miniature optical sensor based on affinity binding [205, 206]. The principle of detection is based on the competitive binding of a particular metabolite and a fluorescein-labeled analogue with receptor sites specific for the metabolite and the labelled ligand. The sensor consisted of an optical fibre in a hollow dialysis fiber where on the inside surface concanavalin A was immobilised. Fluorescein-labelled dextran was selected as the competitive labelled ligand. During in vitro experiments a linear response to glucose in the physiologic range could be obtained although no in vivo experiments have been conducted so far.

A rather new approach is the development of a transcutaneous glucose monitoring technique using reverse iontophoresis [207-209]. Iontophoresis makes it possible to drive charged molecules across the skin using an electric current. Electrodes (Ag/AgCl) positioned in electrode chambers are placed on the skin. The passage of current across the skin drives ions into the tissue, from the electrode chambers positioned on the skin surface, and simultaneously pulls ions from the body in the opposite direction. A conventional enzyme-based glucose sensor is used to measure the glucose concentration in the electrode chambers. A major disadvantage of the sensor technique is
that glucose measurements are intermittent; the content of an electrode chamber can be analysed after two hours of current passage across the skin. On the basis of this technique the Cygnus Inc. USA is developing “the GlucoWatch®”, a commercial glucose sensor in the form of a wristwatch [208, 210].

A similar method for the transcutaneous monitoring of glucose has been developed by Ito et al. [211, 212]. Instead of a current they use suction to get glucose across the skin. They combine a suction effusion fluid collecting technique with a standard isfet glucose sensor. The effusion fluid is directly collected by a weak evacuation through skin from which the stratum corneum is removed. The isfet glucose sensor has been tested on human subjects for the monitoring of blood glucose levels during a 4-hour effusion fluid sample measurement. During these in vivo tests, glucose level changes in the effusion fluid followed actual blood glucose level changes with a time delay of 5 to 10 minutes. Disadvantage of the system is the need of an external pump system to supply for the vacuum and the short functional operating time.

Trajanoski et al. developed an interesting technique for on-line continuous glucose measurement in subcutaneous adipose tissue using open-flow micro-perfusion [213]. The method combined an open flow micro-perfusion of subcutaneous adipose tissue using a perforated double lumen catheter and an extracorporeal peroxide-based glucose sensor. An isotonic ion-free solution was perfused through the inner lumen of the catheter and returned via the perforated outer lumen. In the outer lumen the perfusate could equilibrate with the subcutaneous tissue fluid. Glucose concentration was calculated on-line from the measured glucose in the sampled fluid and the measured recovery in healthy volunteers during hyperglycaemic glucose loads and hypoglycaemic hyperinsulinemic clamps. They concluded that by combining open-flow micro-perfusion and a glucose sensor it was possible to monitor glucose concentration in the subcutaneous adipose tissue on-line for at least 24 hours and during hyper- or hypoglycaemic events.

Sensor designs based on the combination of existing needle-type glucose sensors and a microdialysis system have received growing attention lately. The microdialysis technique can be used to get dialysates of the subcutaneous tissue, which can be continuously measured by an ex vivo glucose sen-
Microdialysis based glucose sensors will be discussed in greater detail later in this chapter (see “Microdialysis based glucose sensors” on page 39).

2.3 In vivo performance of needle-type glucose sensors in human subjects

Most in vivo experiments in human subjects have been performed using the peroxide-based needle-type glucose sensor inserted in the subcutaneous tissue [47, 51, 125, 167, 214, 215]. In these experiments the sensors typically remained in place for several days. Sensors were placed in the subcutaneous tissue without anaesthesia, connected by percutaneous leads to wearable instrumentation whereas output signals were recorded in response to blood glucose challenges. Statistical correlations has been reported between groups of sensors and blood glucose concentrations determined by a standard-lab method. In vitro characteristics of the sensors before implantation usually showed a fast response to concentration changes and were linear over clinically useful ranges. In most studies it was noted, however, that the subcutaneous implantation of the sensor resulted in a gradual decrease in sensitivity, and eventually in the complete loss of sensor function within hours.

The observed decay in sensor sensitivity can be related to the basic design of this type of sensor. As mentioned before, problems are associated with oxygen limitation, bio-fouling, electrochemical interference and peroxide-mediated GOD inactivation. This has lead to the retrospective calibration in which the sensor sensitivity is adjusted after the experiment to match the independently determined blood glucose values. It is questionable whether the in vivo determined calibration factors can be useful in real time monitoring unless there is a highly reproducible decay pattern. Frequently, an in vitro calibration is made prior to implantation and after explantation to interpret the sensor signal during implantation. It is then assumed that the basal current and sensitivity of the sensor are identical under both in vivo and in vitro conditions, which is not realistic because after implantation sensor performance has changed [89, 141]. To cope with the sensitivity loss during implantation, in vivo calibration procedures are introduced [187, 216, 217]. In the one-point in vivo calibration procedure, the sensor output
current at a certain time after implantation (running-in period) is related to the simultaneously measured blood glucose concentration resulting in an in vivo calibration factor. In the two-point calibration method the sensor output signal is related to two different steady state levels of blood glucose as a result of an insulin or glucose infusion. The two-point calibration method is considered most reliable [170] but the method is only feasible in a clinical setting.

Another point of concern, which applies to all implantation studies, is that sensor or microdialysis probe implantation will lead to a tissue response. Trauma associated with sensor/probe placement leads to tissue inflammation and the wound-healing process may interfere with the stabilisation of the sensor signal. The inflammatory phase starts after infliction of the wound and takes about 3 days. The implantation of a sensor damages cells, blood vessels and connective tissue and initiates a number of healing mechanisms [218]. As a result biological molecules from the exudate that forms around the sensor, mainly proteins, are attracted to the sensor-tissue interface. The damage, inflicted on the subcutaneous tissue upon the implantation, can result in a limitation of the blood supply to the sensor surrounding and can cause a decrease in sensor output through poor availability of glucose and oxygen. Given these considerations, it is unlikely that the sensor can be inserted and used shortly thereafter for glycaemic control.

In general, good in vitro results have been achieved with these sensors. Upon implantation, however, these devices show a progressive loss in sensor performance that can be attributed to poor biocompatibility. Questions concerning the influences of wound healing, tissue response to the sensor and blood supply to the surrounding tissue on the sensor performance need to be addressed systematically.

### 2.4 Microdialysis based glucose sensors

Biocompatibility and sensor stability related problems have focused attention on other approaches to measure glucose continuously in vivo. Microdialysis is such a method; it allows glucose measurements in blood or the subcutaneous tissue without the direct interaction of a glucose sensor and
Microdialysis based glucose sensors

tissue or blood [70]. It can be seen as a special means of transport to deliver glucose from body compartments to the glucose sensor.

Microdialysis was introduced by Ungerstedt et al. to monitor neurotransmitter release in the brain [219]. In addition, microdialysis has also become a frequently used research tool for study in many other tissues or body compartments [220–227].

Basically the technique comprises the implantation in tissue or the blood compartment of a small hollow semipermeable dialysis-membrane construction (termed probe) that is perfused with a water-based solution (termed perfusate). The dialysis membrane provides a barrier between the tissue or blood compartment and the perfusion fluid. The driving force of molecular movement is diffusion down the concentration gradient existing between the perfusion fluid and the outside environment of the dialysis membrane (or vice versa). Water-soluble molecules diffuse across the membrane and enter the perfusate when this flows along the dialysis membrane. The term relative recovery is used for the ratio of the concentration of a substance in the perfusion fluid leaving the dialysis tube and outside environment of the dialysis tube (equation 2.5, page 40)

\[
\text{Relative recovery} = \frac{C_{\text{out}}}{C_{\infty}}
\]

\[\text{Equation 2-5. Relative recovery, } C_{\text{out}} \text{ is the glucose concentration in the outgoing perfusion fluid, } C_{\infty} \text{ is the glucose concentration outside the dialysis tube.}\]

The relative recovery is inversely dependent on the flow rate because the samples are more dilute. The absolute recovery is defined as the total mass removed per time interval; it depends directly on the flow rate until a plateau value is reached at maximal diffusion flux.

The kind of molecules that diffuse through the membrane depends on the molecular weight cut off (MW CO) or pore size of the dialysis membrane. Generally the MW CO of a membrane is carefully chosen so that high-molecular weight compounds such as proteins can't penetrate the mem-
brane. This results in a dialysate that is relatively pure and the amount of the low molecular weight glucose molecules present in the perfusate can be measured outside the body with an ex vivo glucose sensor. The disadvantages of microdialysis based glucose sensors such as increased lag-time and bulkiness of system may be well balanced by the improved biocompatibility and stability of the sensors when used in vivo. Hydrogen peroxide based enzyme electrode sensors may benefit from the cleaner environment because of the absence of large molecules resulting in a lower rate of electrode fouling. Although the in vivo performance may be improved, the combination of electrode fouling by small endogenous proteins and the H$_2$O$_2$ mediated enzyme inactivation prevents in vivo measurements longer than a couple of days without re-calibration of the system.

Microdialysis improves the usability of oxygen-based enzyme electrodes for in vivo glucose measurements. The size of the oxygen electrode is less important when applied in an ex vivo glucose sensor-system. In general oxygen deficit problems seen with in vivo measurements of glucose are not encountered when microdialysis based glucose sensing systems are used. The glucose concentration found in the dialysate is, depending on the flow rate used (2.5-10.0 µL/min), lower than in vivo and the oxygen concentration found in the dialysate solutions is usually higher than in tissues. This, together with the proportional stability and sensitivity preservation of oxygen electrodes, and the improved bio-compatibility and stability of the microdialysis technique, makes a glucose sensor system based on these techniques potentially successful when applied in vivo.

The difficulties associated with in vivo glucose monitoring with needle-type glucose sensors have turned researchers, including some of the leading groups in implantable glucose sensor development, to this technique [1, 63, 64, 67-69, 228-230].

Pfeiffer et al. combined the microdialysis technique with a measuring flow chamber incorporating their previous developed needle-type glucose sensor [228, 229]. They used a commercially available needle-type dialysis probe together with a micro-perfusion pump and the glucose sensor to obtain a device for continuous glucose measurement in dialysate. This device was tested in thirteen healthy volunteers during a 75-g oral glucose tolerance
test and in seven Type-2 diabetic patients. The venous blood glucose concentration and subcutaneous sensor signal were followed for a maximum period of 21 hours [67]. After calibration, glucose levels in the dialysate and subcutaneous glucose sensor signal correlated well although a considerable time delay was seen. In following in vivo studies the measuring time was extended up to 48 hours [66, 231] and a portable glucose measuring system called the “Ulm Zucker Uhr System” was introduced [232]. This portable system comprised a microdialysis probe, a glucose sensor, a sender that transferred the glucose concentrations telemetrically and a receiving indicator named the “Sugar Watch”. This system transferred the glucose concentration to the sugar watch once per minute and alarmed the patient by optical and acoustic means, when the tissue glucose was too high or too low.

Shichiri et al. developed a glucose monitoring system by combining their previous developed needle-type glucose sensor with a microdialysis probe for subcutaneous tissue glucose measurements [69, 233]. Subcutaneous tissue glucose concentrations were monitored continuously in 5 healthy and 8 diabetic volunteers for 7 to 8 days. The subcutaneous glucose concentration could be monitored precisely for 4 days without any in vivo calibrations and for 7 days by introducing in vivo calibrations. They found a good correlation between the subcutaneous tissue glucose concentration and venous blood glucose.

Schoonen and Schmidt constructed a glucose sensor combining the microdialysis method and an oxygen electrode [1]. This glucose measure system was the first that made the combination of the microdialysis technique and continuous glucose measurement (“Process for using a measuring cell assembly for glucose determination”, USA patent nr. 5,174,291, 1987). A syringe pump continuously perfused a glucose-oxidase/catalase solution through a hollow microdialysis fiber construction. Glucose that diffused from the outside of the hollow fiber into the perfusate was enzymatically oxidated by GOD. A miniaturised Clark-type oxygen electrode was used to measure the oxygen concentration in the perfusate. The signal output is the difference between the base oxygen level and the level attained as a result of the oxygen depletion by the enzymatic reaction. During in vivo experiments, the flexible microdialysis probe is implanted in the subcutaneous tissue of the abdomen and integrated in a closed flow system (Figure 2-5).
Today, Roche Diagnostics GmbH is developing a commercial glucose measuring system, which is partially based on this system. Major plus point is that enzymes in the perfusate, which were present in excess, prevent that enzyme degradation has an appreciable effect on the stability of the sensor. Recovered glucose is transformed instantly creating sink-conditions in the microdialysis probe. This system was used in 44 healthy volunteers and 24 diabetic patients to measure glucose. The sensor signal correlated well with the blood glucose concentrations without considerable lag-times between changes in blood glucose concentration and sensor measurements, although in most cases the ratio of measured subcutaneous glucose/blood glucose was much lower than 1 (0.43 ± 0.09) [65]. Complementary filtration and equilibration in vivo studies led to the conclusion that the mean glucose concentration in the extra-cellular space in the subcutaneous fatty tissue of humans is approximately 45% of the blood glucose concentration [234]. The duration of probe implant in general ranged from 1 to 4 days although in one experiment the sensor was successfully used for 9 days [235]. The bio-stability of the sensor during the time of implant was sufficiently high and the mean delay times between changes in blood glucose and sensor measurements were well under 10 minutes. The flow system and electronics described in the thesis of Schmidt were not suitable for ambulatory use because of their fragility and substantial dimensions [236].
A potential danger was the use of a GOD/catalase solution as perfusate. Leakage of the enzyme solution through the dialysis membrane into the tissue may cause immunogenic reactions and tissue damage. A precautionary measure applied in the system was a microdialysis probe with a hollow dialysis membrane within another hollow dialysis membrane ("safety-fiber"). If a fiber was damaged, enzyme leakage would still be prevented by the remaining intact fiber. However, the extra hollow fiber membrane increases the diffusional path of glucose into the perfusate, contributing to longer lag-times of the sensor and although the chance of enzyme leakage was reduced, it was still present and could not be excluded entirely.

2.5 Conclusion
The absence today of a glucose sensor for continuous subcutaneous glucose monitoring demonstrates that, in spite of elaborate research efforts during the last four decades, the development of such a sensor is very difficult. The different designs of implantable glucose sensors have their own specific advantages and disadvantages. No ideal configuration or method of in vivo...
glucose sensing can be pointed out. Best approach is to exploit the advantages of a certain measuring technique/configuration and circumvent the disadvantages. In practice, this means that research groups have looked for ways to optimise their existing glucose-sensor designs.

Glucose sensors based on the enzymatic conversion of glucose are still the main focus of the research interest of most groups due to their high selectivity to glucose. The approach of a small needle-type amperometric sensor is most popular among investigators because of their ease of manufacturing and miniaturisation. Major problem is, however, the poor or variable performance in vivo of all these sensors (including potentiometric and electrocatalytic based systems). Although these devices function well in vitro, upon implantation they show a progressive loss of sensor function. Over the past 20 years different design approaches have been proposed to overcome these problems and although some progress has been made using special membranes or mediator techniques, no breakthrough can be reported. In fact, looking at the number of papers published on this type of sensor during the past five years, one may conclude that interest is fading. Only very few research groups have been working on alternative non-enzymatic methods for glucose sensing. It is uncertain whether other concepts such as affinity glucose sensors or coated wire glucose electrodes will be an improvement over actual needle-type glucose sensor designs. Much more work is necessary before these alternative detection principles are completely understood and suitable for in vivo sensing.

A combination of the needle-type glucose sensors and a microdialysis system appear most advantageous and a promising approach for continuous subcutaneous glucose measurements. Using this method it is possible to obtain dialysates from the subcutaneous tissue, which can be analysed ex-vivo. In this case, biocompatibility problems of the sensor interface to the living tissue are minimised.

The effect of sensor or probe implantation on glucose measurements is still partially unclear. In addition, more insight is needed in the physiological processes at the sensor-tissue interface. With better understanding of the processes involved it is possible to develop strategies to improve in vivo sensor performance.