3 Microdialysis based glucose sensor

3.1 Introduction
In vivo studies performed by Lönnroth et al. [221] among others [223, 224, 226, 237-239] have demonstrated that changes in the subcutaneous glucose concentration can be monitored with the microdialysis method. A dialysis tube is placed in the periumbilical subcutaneous tissue or in the subcutaneous tissue of the forearm and perfused with isotonic saline. Small molecules, including glucose, diffuse from the surrounding of the probe into the perfusion fluid as a consequence of the concentration gradient existing between the perfusion fluid and the surrounding of the probe [240-242]. In clinical practice this method has two main advantages over the traditional glucose determination in samples of drawn blood, especially when measurements for longer periods are needed. First, the presence of a dialysis tube in the subcutaneous tissue is less dangerous and more comfortable than the presence of an indwelling catheter in a vein. Second, in contrast to glucose measurements in blood no additional operations are needed to purify the sample because the dialysis tube prevents the diffusion of large molecules into the perfusion fluid. As a consequence, the dialysate samples are relatively clean and glucose determination in the dialysate can be performed at once. In these in vivo studies the dialysate was collected in fractions and later analysed with a standard glucose analysis method. An improvement of this discontinuous measurement is the “on-line” detection of glucose in the dialysate by a glucose sensor. Pfeiffer et al. [66, 67, 228, 229, 231, 232, 243] and Shichiri et al. [69, 233] made a combination of their previously designed needle-type glucose sensor and a microdialysis system to monitor glucose in real-time. Laurell developed a glucose measurement system where the per-
fusate passed through an enzyme reactor containing immobilised glucose oxidase [68]. An oxygen electrode was placed in line with the reactor to monitor the enzymatic conversion of glucose. The common denominator of these designs is the use of an open perfusion system. This is necessary because recovered glucose is only partially converted in the enzymatic reaction with the immobilised \textit{god}. Closing the perfusion system would ultimately lead to accumulation of glucose within the system. However, the use of two perfusion fluid reservoirs, one for supply and the other for waste, impede the miniaturisation of these systems. This in contrast to the microdialysis based glucose sensor developed by Schoonen and Schmidt [1]. Their sensor used a closed perfusion system to circulate a \textit{god}/catalase solution. Due to the excess of dissolved \textit{god}, all recovered glucose was converted which enabled the short circuit of the perfusion system provided that oxygen used in the enzymatic conversion of glucose was replenished. Moreover, the stability of the sensor was increased by the excess of \textit{god}; thus made continuous glucose measurements for two weeks possible. Several in vivo studies have been performed successfully with this glucose measurement system [65, 234, 235]. Both, the closed perfusion system and excess of enzyme enabled the development of a miniaturised version of the glucose sensor, a condition for the long-term ambulatory use of such a system. Yet the use of a \textit{god}/catalase solution as perfusion fluid introduces at the same time a potential danger of enzyme leakage through the dialysis tube. Enzyme leakage makes the clinical use of such systems not appropriate. Therefore, we redesigned the perfusion system to minimise the risk of enzyme leakage without losing the benefits of a closed perfusion system. A solution was found in the replacement of the enzyme perfusion fluid by isotonic saline. Several adaptations have been made to the existing perfusion systems to prevent both enzyme leakage from and glucose accumulation within the system.
3.2 Materials and methods

In the following sections, two newly designed perfusion systems are described that have a minimised risk of enzyme leakage. Glucose measurements of both systems were based on the enzymatic conversion of glucose by glucose oxidase (GOD - See reaction 2-2 on page 20). To monitor the reaction, oxygen used in the conversion was measured by an O₂-electrode. In the first section a design is described that made use of a dual circulation system composed of a physiological salt and an enzyme solution. In the subsequent section a single circulation system is described where also a physiological salt solution was used as perfusion fluid, but where unlike the dual circulation system, the enzyme solution was immobilised in a reactor.

![Figure 3-1. Schematic overview of the dual circulation system: one circulation with saline and one with GOD solution. At the mixing point the two solution are mixed. GOD converts glucose and the diminution of dissolved oxygen is measured by the O₂-electrode. The separator is used to separate the two solutions.](image)

### 3.2.1 Dual circulation system

In the dual circulation system the microdialysis probe was perfused by isotonic saline (Figure 3-1). After the probe outlet the saline was mixed with a
solution of glucose oxidase and catalase. Glucose present in the saline is after mixing rapidly converted by \textit{god}. To monitor this reaction a Clark-type oxygen electrode measured the diminution of dissolved oxygen in the mix. A complicating factor is that both the diffusion of oxygen from the perfusion fluid through the Teflon membrane into the electrode and the solubility of oxygen in the perfusion fluid depend on temperature. The overall effect of higher temperatures with this type of oxygen electrode is an increase of 2.1 nA with every degree Celsius [236]. To correct for these temperature variations an electronic thermometer measured the temperature. After the $O_2$-electrode, the mix was separated again into a saline and enzyme solution by ultrafiltration. Two small piston pumps were used to circulate the separate solutions. For reasons of safety a filter containing activated carbon was inserted in the saline circulation to adsorb and block enzyme molecules that leaked through the ultrafiltration membranes used in the separation of the two solutions.

**Microdialysis probe**

A microdialysis probe can be designed in several ways and is typified by the position of the inlet and outlet tubes. In general two types of probes can be distinguished: one type with the in- and outlet tubes positioned in a serial arrangement and the other type of probe, like the one used in this system, where the in- and outlet tubes are placed in a parallel arrangement. Consequently, the dialysis tubes were positioned side by side. The construction of the microdialysis probe, which can be subdivided in three stages, is outlined in Figure 3-2, page 52. Gloves were worn during the probe construction to avoid contamination of the materials.

**Stage A:**

Two dialysis tubes with the length of 65 mm were cut out of a large bundle of dialysis tubes (Cellulose, Spectra/Por RC, Spectrum Medical Ind., LA, USA, i.d. 150 µm, o.d. 180 µm, 18,000 molecular weight cut-off). Of both dialysis tubes, one end of 5 mm was fixed with cyanoacrylate glue ($\text{C}$â 1500, Ruplo lijmtechniek, The Netherlands) into the in- and outlet tubing (Pol-
yethylene, Rubber BV, The Netherlands, i.d. 0.40 mm, o.d. 0.80 mm) of the probe (Figure 3-2A).

Stage B:
After drying, both parts were positioned parallel and fixed together by means of a polyethylene tube (Rubber BV, The Netherlands, i.d. 1.40 mm, o.d. 2.00 mm, length 10 mm) which was placed over the interface of both the in- and outlet tubing and the dialysis tubes. To provide the microdialysis probe with sufficient firmness and flexibility a tungsten wire (T 5-3, Clark Electromedical Instruments, UK, Ø 0.12 mm) with a length of 55 mm was placed between the dialysis tubes with one end in the 10 mm polyethylene tube. This tube was subsequently filled with cyanoacrylate glue (C â 1500). A butterfly, cut from a dwelling catheter (Venofix, Braun, Germany, 25G), was fixed on the outside of the tube (Figure 3-2B).

Stage C:
After hardening of the glue, a 30 mm tube (Teflon, Zeus, USA, i.d. 0.50 mm, o.d. 0.57 mm) was pushed over the tungsten wire and dialysis tubes until it touched the 10 mm polyethylene tube and was fixed with cyanoacrylate glue (C â 1500, Ruplo lijmtechniek, The Netherlands). At the tip of the probe a turning point was placed. This turning point consisted of a 5 mm long tube (Teflon, Zeus, USA, i.d. 0.50 mm, o.d. 0.57 mm) which had one end previously closed with glue (C â 1500). The turning point was pushed over the tip of both the tungsten wire and dialysis tubes and fixed with cyanoacrylate glue (C â 1500). Special attention was paid when the turning point was positioned on the probe to minimise dead volume. After fixation of the turning point, a total of 30 mm of dialysis tubing was left uncovered (Figure 3-2C).

Usually the microdialysis probes were made in batches of five to ten. When all glue was hardened, the probes were tested for congestion or leakage, first by flushing with air and subsequently with water. Probes that functioned properly were kept in a flask filled with distilled water to prevent dehydration of the dialysis tube and stored at 5º C to inhibit bacterial growth. Upon use, the microdialysis probe was rinsed successively with an ethanol solution (70%) and sterile water.
O2-electrode
For the on-line measurement of dissolved oxygen in the enzyme/saline mix, a Clark type oxygen electrode was used (Figure 3-3). The electrode was made in the local workshop of the university. It was constructed of a silver case (i.d. 4.2 mm, o.d. 5.4 mm) with a centrally placed glass isolated platinum wire where the tip of the wire was left free (Pt wire Ø 1.4 mm, Pt wire & glass isolation Ø 2.5 mm). The platinum wire was fixed in the silver case by filling the case with two-component epoxy resin (Pattex® super-mix, Henkel) leaving at one end an electrolyte cavity of about 5·10⁻² ml resin free. The silver case and platinum wire were subsequently connected to electric wires and mounted in a housing of pvc. For the measurement of dissolved oxygen a flow chamber was used consisting of a pvc top that could be screwed on the electrode housing. In this top two stainless-steel in- and outlet tubes (i.d. 0.3 mm, o.d. 0.5 mm) were connected to the tubing.
of the perfusion system. Prior to use the electrode cavity was filled with a 0.5 M KCl/K$_2$HPO$_4$ electrolyte solution and covered with a Teflon membrane (High sense, Yellow springs Inc., Ohio, USA). For the specific detection of oxygen, the platinum wire was polarised to a fixed negative potential of -600 mV/SCe using a potentiostat (CTI, the Netherlands). Lower oxygen concentrations resulted in a decrease in currents crossing the electrode cell. Currents could be read from a liquid display, recorded on a flatbed chart recorder (Kipp & Zonen, The Netherlands) or stored for future data operation in a microchip (H8, Hitachi, Japan).

![Figure 3-3. Clark type oxygen electrode.](image)

**Pumps**

Two micro-pumps (Parker Micro-pump Ambulatory Medication Infuser, Parker Hannifin Corporation, Irvine, CA, USA, 51 x 76 x 18 mm, weight 70 gr.) were used for the circulation of enzyme solution and the perfusion of the microdialysis probe with saline (Figure 3-4, page 54). The working of this pump is based on magnetically actuated pulse infusion. A piston is pulled by an electromagnetic pulse and pushed backward by two return
springs (one pump cycle). Two valves positioned on either side of the pumping chamber force the fluid in one direction. In one pump cycle 5.0 µL of fluid is moved. The flow range (5.0-30.0 µL/min.) of the pump could be adjusted by changing the frequency of the electromagnet activation. If not mentioned otherwise, a flow rate of 10 µL/min. was used. A fluid reservoir of 2 ml was attached to the pump containing a wicking filter to prevent air entering the pumping chamber. As far as energy consumption was concerned, the pump could operate, together with accompanying electronics, at a perfusion rate of 10 µL/min. for at least a month on an ordinary 9-Volt battery. Before use, the pumps were thoroughly rinsed with an ethanol solution (70%) and sterilised water.

**Figure 3-4. Micro piston pump (Parker Hannifin Corp.).**

- **Separator**

  The separation of the enzyme/saline mix into separate solutions was done by ultra-filtration (Figure 3-5). For this purpose 8 hollow ultra-filtration membranes made of acrylonitril-natrium methallylsulfonate (Filtral 12, Hospal, Bologna, Italy, i.d. 0.22 mm, o.d. 0.31 mm) were cut in lengths of 6 cm. The membranes were subsequently positioned parallel and glued (CA 1500) together with a tube (Polyethylene, Rubber BV, The Netherlands, i.d. 0.4 mm, o.d. 0.8 mm) for drainage of filtered fluid, into a polyethylene tube (Rubber BV, i.d. 2.8 mm, o.d. 4.0 mm, length 75 mm) leaving at both
sides 7.5 mm free for fixation of connective tubing. After drying, the connective tubing (Polyethylene, Rubber BV, i.d. 0.4 mm, o.d. 0.8 mm) was fixed with cyanoacrylate glue (CA 1500).

**Activated carbon filter**
For the adsorption of enzyme molecules leaking through the ultra-filtration membranes of the separator, a filter was positioned in the saline circulation containing activated carbon (Figure 3-6, page 56). To prevent that carbon particles being washed away, six hollow ultra-filtration membranes (AN 69HF, Hospal) each with a length of 120 mm, were glued (CA 1500) in a tube (Polyethylene, Rubber BV, i.d. 3.2 mm, o.d. 3.9 mm) with the in- and outlet positioned in the same direction forming consequently a loop. After drying, the fiber bundle was glued (CA 1500) in a polyethylene tube (Rubber BV, i.d. 2.8 mm, o.d. 4.0 mm, length 80 mm) which was subsequently filled up with activated carbon (Norit, Norit Farma, The Netherlands) leaving 10 mm free for connective tubing. After drying, the connective tubing (Polyethylene, Rubber BV, i.d. 0.4 mm, o.d. 0.8 mm) was secured (CA 1500) on both sides of the carbon filter. The carbon filter was positioned in the saline circulation in such manner that the saline first had to flow through a part of the carbon bed and subsequently was pressed through the ultra-filtration membranes.

![Figure 3-5. Separator; see text for description.](image-url)
Enzyme solution
During the enzymatic conversion of glucose by \textit{god}, oxygen is used and hydrogen peroxide produced. It is well known that hydrogen peroxide has a negative influence on the \textit{god} activity. A second enzyme (catalase) was used to convert the produced hydrogen peroxide. The enzyme solution was prepared dissolving 10,000 unit’s glucose oxidase (Grade II, Boehringer Mannheim, Mannheim, Germany) and 90 mg NaCl in 8 ml H\textsubscript{2}O under gently stirring. The solution was subsequently filled up to 10 ml with a filtered catalase solution (45 µm filter, Inacom Instruments, The Netherlands; Catalase from bovine liver dissolved in an ethanol solution, Boehringer Mannheim, Mannheim, Germany). The solution obtained was filtered (0.2 µm filter, Inacom Instruments) to make the solution free from bacteria and stored at 5 °C for a maximum of three months.

Ultra-filtration membranes

Carbon particles

\textbf{Figure 3-6.} Active carbon filter.

Connective tubing
The separate parts of the perfusion system were connected with polyethylene tubing (Polyethylene, Rubber BV, i.d. 0.4 mm, o.d. 0.8 mm). The Y-joint in the systems was made of two inlet tubes and one outlet tube (Polyethylene, Rubber BV, i.d. 1.4 mm, o.d. 2.0 mm) fixed with cyanoacrylate glue (CA 1500) in a 10 mm polyethylene tube (Rubber BV, i.d. 2.8 mm, o.d. 4.0 mm). Because \textit{god} uses oxygen during the enzymatic conversion
of glucose, precautionary measures had to be taken to prevent oxygen deficiency within the perfusion system. For this purpose an oxygen permeable tube (Teflon, Zeus Industrial Products, NJ, USA, i.d. 0.7 mm, o.d. 1.5 mm) connected the enzyme pump with the Y-joint.

Operational glucose measurement system

To make the system operational, the microdialysis probe, O₂-electrode, the two pumps, the separator, and carbon filter were connected with tubing in accordance with the outline of figure 3-1 (see page 49). Both saline and enzyme pumps, together with accompanying tubing, were filled with respectively a sterile physiological salt solution and an enzyme solution. Since the perfusion system contained teflon tubing and an enzyme solution it was not possible to autoclave the system or use gamma radiation for sterilisation. Therefore, the only way to disinfect the interior of the perfusion system was by flushing with an ethanol solution (96%). Perfusion systems used for in vivo studies were put together in a laminar air flow cabin to prevent bacterial contamination. When all parts were connected, the perfusion system was ready for placement in a box containing the electromagnets for pump operation and electrode connection.

Figure 3-7. Schematic overview of the singular circulation system.
3.2.2 Single circulation system

In this section a description is given of the single circulation system. This system was developed after the previously described dual circulation system. The main difference of the single circulation system when compared to the dual circulation system was the absence of an enzyme circulation. This was done to further decrease the risk of enzyme leakage. In the single circulation system (Figure 3-7, page 57) the glucose oxidase/catalase solution was retained in an enzyme reactor. Isotonic saline was used to perfuse the microdialysis probe. A glucose eliminator was developed to prevent the accumulation of glucose in the perfusion system. An $O_2$-electrode was positioned in line with the enzyme reactor to monitor the oxygen concentration in the perfusion fluid. Before re-entering the microdialysis probe the saline was filtered by carbon filter to adsorb enzyme molecules that leaked out of the enzyme reactor. Placing a fluid equaliser after the pump reduced fluid pulsations caused by the piston pump. The pump, $O_2$-electrode, carbon filter and connective tubing used in this system and disinfection procedures were the same as described in the section of the dual circulation system.

Microdialysis probe

The microdialysis probe used in this system was an enhanced version of the microdialysis probe described previously in the dual-circulation system section. The construction of the probe can also be subdivided in three stages and is outlined in figure 3-8, page 60.

Stage A:

Two dialysis tubes with a length of 20 mm were cut out a large bundle of dialysis tubes (Cellulose, Spectra/Por RC, i.d. 150 µm, o.d. 180 µm, 18,000 molecular weight cut-off) and glued (CA 1500) on respectively a 40 mm and 300 mm long silica tube (SGE Scientific Pty. Ltd., Sydney, Australia, i.d. 0.11 mm, o.d. 0.18 mm). Instead of the separate in- and outlet tubing used in the dual circulation probe, the in- and outlet of this probe were combined in one triple lumen tube (Polyethylene, Dural Plastics & Engineering,
Auburn, Australia, i.d. 0.35mm, o.d. 1.0 mm, length 250 mm). The silica tube (300 mm), used as probe outlet, was threaded through one of the triple lumen (Figure 3-8A.1) leaving 30 mm of silica tubing free. Next the 40 mm silica tube, used as probe inlet, was positioned in one of the remaining two lumen (Figure 3-8A.2), leaving also 30 mm of silica tubing free. At both ends of silica the two dialysis tubes were glued (CA 1500). In the third lumen a tungsten wire (TW5-3, Clark Electromedical Instruments, UK, Ø 0.12 mm) was positioned to give the probe the required firmness as well as flexibility (Figure 3-8A.3). The two silica tubes and tungsten wire were subsequently fixed to the triple lumen tube with cyanoacrylate glue (CA 1500).

Stage B:

Once the glue had dried, a tube (Teflon, Zeus Industrial Products, i.d. 0.50 mm, o.d. 0.57 mm, length 30 mm) was slipped over both the dialysis tubes and the tungsten wire until it touched the triple lumen tube. Subsequently it was fixed with glue (CA 500). A second tube (Polyethylene, Rubber BV, i.d. 1.40 mm, o.d. 2.00 mm) was positioned over the interface of the triple lumen tube (Figure 3-8B.1) and the microdialysis probe and fixed with glue (CA 1500). At the tip of the microdialysis probe a turning point was placed (Figure 3-8B.2) as described previously in the dual-circulation system section, leaving in total 30 mm of dialysis tubing uncovered.

Stage C:

The final stage involved the fixation of a butterfly and tubing for the probe in- and outlet. For future fixation of the probe on the skin, a butterfly cut from a dwelling catheter (Venofix) was glued on the interface of the triple lumen tube and the microdialysis probe (Figure 3-8C.1). At the other end of the triple lumen tube connective tubing was fixed, serving as in- and outlet tubes. In one of the lumen, 20 mm of silica tubing was left uncovered and was used for the connection with the enzyme reactor (Figure 3-8C.2). In the other lumen, used for the inlet of the microdialysis probe, a silica tube of 20 mm was fixed (CA 1500) and connected to a polyethylene tube (Rubber BV, i.d. 0.4 mm, o.d. 0.8 mm).
CHAPTER 3  
Single circulation system

The microdialysis probes were usually made in batches of five to ten and tested and stored as described previously.

![Figure 3-8](image)

**Figure 3-8.** Construction of the enhanced version of the microdialysis probe (stages A, B and C). For explanation of parts, see “Microdialysis probe” on page 58.

- **Enzyme reactor**

An enzyme reactor was used for the enzymatic conversion of glucose recovered in the perfusion fluid (Figure 3-9A, page 62). For the exterior of the reactor, a 50 mm long stainless-steel tube (i.d. 0.5 mm, o.d. 0.70 mm) was used. Two dialysis tubes (Cellulose, Spectra/Por RC, i.d. 150 µm, o.d. 180 µm, 18,000 MW CO) with a length of 60 mm were threaded through this metal tube leaving 5 mm of dialysis tube free on both sides. A turning point, made of a 5 mm long tube (Polyethylene, Rubber BV, i.d. 0.28 mm, o.d. 0.61 mm) with one end previously sealed (CA1500), was fixed on one side of the two dialysis tubes with glue (CA 1500) leaving a minimum of death volume. Two tubes (Silica, SGE Scientific Pty. Ltd., i.d. 0.11 mm, o.d. 0.18 mm, length 20 mm) necessary for filling of the reactor with enzyme solu-
tion, were pushed in for 10 mm on both sides of the metal tube. Next the dialysis- and silica tubes were carefully glued to the metal tube which had both ends made rough for better glue fixation. Both open ends of the dialysis tubes were glued to connecting tubing (Polyethylene, Rubber BV, i.d. 0.28 mm, o.d. 0.61 mm) for placement in line with the microdialysis probe and the oxygen electrode. After drying of the glue the reactor was tested by flushing with air and water. Before filling with enzyme solution, the reactor was rinsed with ethanol (70%) and sterile water.

**Glucose eliminator**

Not all the recovered glucose is converted in the enzyme reactor. Therefore a glucose eliminator was developed to prevent accumulation of glucose within in the perfusion system. Elimination of glucose from the perfusion fluid by the eliminator is based on the enzymatic conversion of glucose by \textit{god}. Both its functioning and design are based on the glucose reactor. The eliminator was made of two dialysis tubes (Cellulose, Spectra/Por RC, i.d. 150 µm, o.d. 180 µm, 18,000 \textit{MW CO}) with a length of 160 mm lying side by side in a Teflon tube (Zeus, i.d. 0.70 mm, o.d. 1.50 mm, length 150 mm) with one end of the dialysis tube fixed in a turning point (Polyethylene, Rubber BV, i.d. 0.28 mm, o.d. 0.61 mm, length 5 mm, one side sealed). Two tubes (Polyethylene, Rubber BV, i.d. 0.28 mm, o.d. 0.61 mm, length 30 mm) necessary for filling the eliminator with enzyme solution, where each positioned on both sides of the Teflon tube. The dialysis tubes and enzyme filling tubes were fixed in the Teflon tube with glue (CA 1500). After drying each of the dialysis tubes open ends were glued (CA 1500) to connective tubing (Polyethylene, Rubber BV, i.d. 0.40 mm, o.d. 0.80 mm). Upon use, the glucose eliminator was rinsed with an ethanol solution (70%) and sterile water after which the eliminator was filled with enzyme solution.

**Equaliser**

The dialysis flow should be regular because it is an important parameter in the recovery of substances. Changing flows will result in variable recoveries. Protracted fluid pulsation may deform the ultra-filtration membranes used
in the carbon filter, resulting in an increase in flow and thus in a change in recovery. To reduce the effect of fluid pulsation an equaliser was constructed and inserted in the perfusion system after the piston pump (Figure 3-9B). It was made of a glass tube (i.d. 3.5 mm, o.d. 4.0 mm, length 75.0 mm) where one end was sealed. Two connective tubes (Polyethylene, Rubber BV, i.d. 0.40 mm, o.d. 0.80 mm) were glued (CA 1500) into the glass tube opening. When the equaliser was inserted in the perfusion system an air bubble was trapped in the dead end of the glass tube and consequently absorbed the fluid pulse caused by the piston pump. Before use the equaliser was rinsed with an ethanol solution (70%) and sterile water.

![Figure 3-9. Construction of the enzyme reactor (A) and equaliser (B).](image)

- **Enzyme solution**

  The preparation of the enzyme solution used in this system was the same as described in the dual-circulation system section except for the amount of glucose oxidase used. Instead of the 1,000 units \( \text{god} / \text{ml} \) present in the enzyme solution of the dual circulation system, 10,000 units \( \text{god} / \text{ml} \) were used to fill the enzyme reactor and glucose eliminator.
3.2.3 Electronics and software

**Electronics**

A condition for ambulatory in vivo studies is that the accompanying electronics for pump control and data storage are manageable. The electronic equipment (CTI, Groningen, The Netherlands) developed for the dual circulation system was incorporated in two separate boxes made of PVC (Figure 3-10). One box (162 x 84 x 21 mm) contained the potentiostat, the microprocessor (H8, Hitachi, Japan) and electronics used for pump control and data storage as well as a liquid display for direct data read out and a standard 9 Volt battery for power supply. To prevent data loss when the main power supply failed, a small silver oxide battery was integrated in the electronics and used as back-up power supply for the microprocessor. For data interchange, the computer box could be coupled to a conventional PC by a flat-cable. The second box (103 x 62 x 21 mm), the flow system box, contained the perfusion system as well as the two electromagnets needed for pump operation, the electrode connector and an electronic thermometer for correcting temperature influences on the working of the O₂-electrode. Both boxes were coupled with an electric cable of 50 cm long.

![Figure 3-10. Schematic overview of the dual circulation system with accompanying electronics as used in the experiments.](image-url)
CHAPTER 3  
Electronics and software

A) box with potentiostat, power supply and microprocessor for data storage, B) flow system box, C) microdialysis probe.

Although the electronics developed for the dual circulation system could also be used for the single circulation system, we re-designed the existing electronics because the two separate boxes and especially the electronic cable between them was a source of errors when used in vivo. In order to improve the robustness of the system, the existing electronics were re-designed (cti, Groningen, The Netherlands) to fit together with the single perfusion system in one box (pvc, University workshop, 125 x 80 x 20 mm). The liquid display was omitted from the electronics for further miniaturisation of the glucose measurement system (Figure 3-11).

![Figure 3-11. Single flow system and electronics in one box (A), microdialysis probe (B) and battery (C).](image)

- Software
  A pc-software program (cti, Groningen, The Netherlands) was used to adjust the frequency of pumping and data storage, change the time of the built-in clock or to retrieve stored data. To do this the computer box had to be coupled with a flat-cable to the pc and upload or download respectively the adjusted parameters or the measurements to or from the microprocessor in the computer box. The software program allowed the user to set the
pumping frequency and change accordingly the flow rate of the perfusion fluid (5 to 30 µL/min.). Also the time between the pump pulse and O₂-measurement storage could be adjusted to minimise the effect of the perfusion pulse on the O₂-measurement. In practice, the oxygen measurement was stored 5 seconds after every pump pulse. The microprocessor could store a total of 16,000 measurements in its memory. One measurement contained the values of the O₂-electrode current (nA), the temperature (°C) and the time (hh:mm). Down loaded measurements were saved on the PC as ASCII-files and could be imported into a spreadsheet program (MS Excel for Windows, version 4.0 to 8.0) for further data processing.

3.2.4 Validation of carbon filter functioning

To validate the functioning of a carbon filter and to determine if the filter was capably to block enzyme molecules present in the perfusion fluid, three parameters were examined. First, the extent of god leakage through the dialysis tube of the enzyme reactor was determined. Second, the amount of god absorbed per milligram carbon was determined at a flow rate of 10 µL/min. Finally, complete carbon filters were tested on their capability to block the total amount of god used in the enzyme reactor.

To determine the number of god units that leaked through the dialysis tubes per unit time, enzyme reactors containing dialysis tubes (Cellulose, Spectra/Por, i.d. 150 µm, o.d. 180 µm) with different lengths were examined. The dialysis tubes had lengths of successively 2.5 cm, 5.0 cm, 7.5 cm and 10.0 cm and were perfused with water at a flow rate of 10 µL/min. using a syringe pump (Braun perfusor IV, Germany). All enzyme reactors were filled with a god solution of 10,000 units/ml. During 24h, one-hour samples were collected of the out-going perfusion fluid and examined on god using the UV detection method as described by Foulds et al. [244]. god concentrations in the samples were calculated using a calibration curve of know god concentrations determined with the above mentioned UV god-detection method. The minimal god concentration that could be detected with this method was 0.01 units god/ml. From the measured god concentration, the absolute amount of god units leaking through the separate dialysis tubes per hour could be calculated. Knowing the absolute amount of god units leaking through the dialysis tubes and the total
CHAPTER 3
Validation of carbon filter functioning

amount of \textit{god} units present in the separate enzyme reactors, the percentage of leaked units \textit{god} per hour could be calculated.

The number of \textit{god} units adsorbed per milligram activated carbon was determined by perfusing columns of activated carbon at a flow rate of 10 µL/min. with a \textit{god} solution of 12 units \textit{god} /ml using a syringe pump (Braun Perfusor IV). The columns were made as follows; in one end of a polyethylene tube (Rubber BV, i.d. 3.2 mm, o.d. 3.9 mm) a cotton wool ball was placed and the tube was filled with a known amount of activated carbon. Subsequently, a second cotton wool ball was placed in the other end of the tube. No external pressure was applied for the packing of the carbon particles. The cotton wool balls prevented that carbon particles were washed away during the perfusion of the column. Connective tubing (polyethylene, Rubber BV, i.d. 0.4 mm, o.d. 0.8 mm) was glued (CA 1500) at both ends of the tube. After drying, the separate columns were coupled to the syringe pumps. The experiments started when the first fluid left the outlet of the column \((t = 0)\). Samples at different time intervals of the out-going perfusion fluid were collected and the remaining \textit{god} concentrations in these samples were measured using the above-mentioned \textit{uv-god} detection method. The amount of \textit{god} adsorbed per milligram carbon could be calculated from the difference between the absolute number of the in- and out-going \textit{god} units and the amount of carbon used in the columns.

For the assessment of the carbon filter functioning, 6 carbon filters as described in section “dual circulation system”, were perfused during sixteen hours with water at a flow rate of 10 µL/min. After \(t_{30}\) min. \(4.4\times10^{-3}\) ml of a \textit{god} solution (10,000 units/ml) was introduced into the perfusion fluid. This amount is corresponding with the amount of \textit{god} units present in the enzyme reactors. For sixteen hours, one-hour samples were collected and tested on the presence of \textit{god} using the \textit{uv-god} detection method.

\section*{Statistical Analysis}
Experimental data are reported as mean ± SD.
### Table 3-1. Characteristics of GOD leakage from enzyme reactors of different length.

<table>
<thead>
<tr>
<th>Dialysis tube length (cm)</th>
<th>Conc. GOD in perfusion fluid Units/L (mean ± SD, n=24)</th>
<th>Abs. number units GOD in enzyme reactor</th>
<th>Abs. number units GOD in perfusion fluid</th>
<th>% units GOD leaked from enzyme reactor per hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>6.4 ± 2.1</td>
<td>42.7</td>
<td>3.8·10^{-3}</td>
<td>9.0·10^{-3}</td>
</tr>
<tr>
<td>5.0</td>
<td>14.1 ± 2.2</td>
<td>85.5</td>
<td>8.5·10^{-3}</td>
<td>9.9·10^{-3}</td>
</tr>
<tr>
<td>7.5</td>
<td>21.2 ± 3.5</td>
<td>128.2</td>
<td>12.7·10^{-3}</td>
<td>9.9·10^{-3}</td>
</tr>
<tr>
<td>10.0</td>
<td>26.1 ± 3.9</td>
<td>170.9</td>
<td>15.7·10^{-3}</td>
<td>9.2·10^{-3}</td>
</tr>
</tbody>
</table>

### 3.3 Results
The dialysis tubes used in the enzyme reactor leak GOD molecules. The percentage of GOD units present in the enzyme reactors that leaked through the dialysis tubes of different length is $9.5\times10^{-3} \pm 4.7\times10^{-4}$ % (mean ± sd) per hour. The absolute number of units GOD leaked through per dialysis tube length per hour is shown in table 3-1. The amount of units GOD leaked through the dialysis tubes is positively correlated with the tube length ($r = 0.9953$). The amount of GOD that is adsorbed per milligram activated carbon per hour at a flow rate of 10 µL/min. is $1.54 \pm 0.20$ units GOD/mg per hour ($n = 20$). We could not find any units of GOD in the perfusion fluid leaving the intact carbon filters during 16h when the filter was perfused with a solution of 12 units GOD per litre.
3.4 Discussion
This chapter describes two glucose sensors that are based on the glucose measurement system developed by Schoonen and Schmidt. However, unlike the glucose measurement system of Schoonen and Schmidt, the risk of enzyme leakage from the perfusion system of these sensors into the body is minimised. We could accomplish this by replacing the enzyme solution that perfused the microdialysis probe with isotonic saline. In addition, we inserted a carbon filter in the perfusion system to filter the saline from enzyme molecules present. Our second condition was to maintain a closed perfusion system to make future miniaturisation of the glucose sensor possible. Therefore, the existing perfusion system of Schoonen and Schmidt was re-designed. Two different approaches were chosen to use the enzyme solution for both the measurement of glucose and for the prevention of glucose accumulation within the perfusion system. In the dual circulation system the enzyme solution is part of the flow system in the sense that the solution is circulated by a pump and is mixed with saline containing recovered glucose. The enzyme solution used in the single circulation system on the other hand is immobilised in respectively the enzyme reactor and a glucose eliminator, which are perfused with saline containing recovered glucose.

The use of saline to perfusate the microdialysis probe has made a safe in vivo application of the dual and single circulation system possible. An accidentally disrupted dialysis tube during in vivo use will now result in the leakage of the harmless saline into the body instead of the GOD/catalase solution used in the system described by Schoonen and Schmidt [1, 65]. Although there was no immediate danger of leakage of a highly concentrated enzyme solution into the body, enzyme solutions were still present in both systems. Since in the dual circulation system the mix of enzyme- and salt solution was separated by ultra-filtration, some enzyme molecules could leak through membrane “pin-holes” into the saline as a result of the imposed pressure. Pinholes are membrane pores far larger than the mean membrane pores and are caused by irregularities in the production process of these membranes. Leakage of enzyme into the saline circulation also occurred in the single circulation system. Although, the pressure of the perfusion fluid is directed inward in both the enzyme reactor and the glu-
cose eliminator, diffusion of some enzyme molecules through the membrane pinholes into the saline could not be prevented. Experiments showed that per hour 9.5x10^{-3}\% of the \textit{god} present in the enzyme reactor is leaking away. This means that after 14 days of continuous working, about 3.2\% of the initial \textit{god} concentration have leaked from the enzyme reactor into the perfusion fluid. Therefore, as an extra safety measure we inserted a carbon filter in the saline circulation of both systems. Before the saline enters the microdialysis probe it has to pass the carbon filter. Enzyme molecules present will be adsorbed by the carbon particles or be blocked by the ultrafiltration membranes. Results from the experiments of \textit{god} absorption to carbon shows that at a flow rate of 10 \mu L/min., ± 1.5 units of \textit{god} is absorbed by 1 mg of carbon. When an enzyme reactor is used as described in the single-flow system section, ± 1.4 units has leaked into the perfusion fluid during 14 days of functioning. In theory 1 mg of activated carbon should be sufficient to absorb all leaked \textit{god}. In practice, however, not all fluid containing \textit{god} molecules can be forced to come in contact with 1 mg of carbon. Therefore in practice at least 40 mg of carbon is used to form a bed of carbon around six ultra-filtration membranes which form an extra barrier for enzyme molecules. The leakage of catalase, which is also used in the enzyme solution, was not examined. However, the size of catalase is about 1.5 times greater than that of \textit{god} [93]. We assumed that the extent of catalase leakage is at most equal to, but probably lesser than the leakage of \textit{god} from the enzyme reactor. The surplus amount of activated carbon in the filter as well as the presence of the ultra-filtration membranes was sufficient to block all \textit{god} present in the enzyme reactor. No \textit{god} could be demonstrated in the out-flow fluid of the carbon filter when an enzyme solution, with an equal number of \textit{god} units as in the enzyme reactor, was introduced to the in-flow of the carbon filter. It is therefore not likely that leaked \textit{god} or catalase molecules from the enzyme reactor can enter the body when used in vivo. Yet, the advantage of an enzyme solution as perfusion fluid is that recovered glucose is converted immediately and completely [1, 236]. The perfusion fluid can be circulated without danger of glucose accumulation so miniaturisation of the perfusion system is possible. In the dual circulation system saline was mixed with the enzyme solution. Between the mixing point and separator \textit{god} instantly converted
glucose, present in the saline. Since, \( \text{god} \) in the enzyme circulation was present in excess and the solutions were considered well mixed, all glucose recovered by the dialysis probe is converted before the separation in the two solutions. Therefore, it is not likely that glucose will accumulate in this system.

This in contrast to the single circulation system where without the necessary precautionary measures glucose accumulation would occur. The enzyme solution in this system is immobilised in an enzyme reactor that is placed in-line with the microdialysis probe. Saline containing recovered glucose flows through the dialysis tube positioned inside the enzyme reactor (Figure 3-9, page 62). The dialysis tube was situated in an enzyme solution with an excess of \( \text{god} \). Glucose diffused into the enzyme solution as a result of the concentration difference between the perfusion fluid and saline (Fick’s law). There exist a dynamic balance between the glucose concentration in the perfusion fluid and the enzyme compartment of the reactor. If we consider the flow in the dialysis tube to be laminar (diameter tube = 0.15 mm) and ignore diffusion in the direction of the flow, the following equation gives a simple diffusion model for microdialysis [245]:

\[
\left( \frac{C_t - C_e}{C_i - C_e} \right) = \exp \left( -\frac{P \cdot S}{F} \right)
\]

**Equation 3-1.** Simple model of microdialysis; \( C_t \) denotes the concentration of glucose in the tube, \( C_o \) the uniform concentration of glucose in the enzyme solution, \( C_i \) the inlet concentration, \( P \) the permeability coefficient of the dialysis tube, \( S \) the surface area of the dialysis tube and \( F \) the flow rate.

The glucose concentration in the perfusion fluid will decrease when the perfusion fluid flows through the enzyme reactor. The extent of concentration decrease can be manipulated by adjusting the dialysis tube surface area and flow rate of the perfusion fluid. In theory, given a certain inlet concentration, the glucose concentration in the perfusion fluid can approach zero when there is the right relationship between the dialysis tube surface area and the perfusion flow for that certain inlet concentration. In practice, given
the highest recovered glucose concentrations seen under physiological conditions (up to 10 mM with $F = 10 \mu$L/min. and $S_{dialysis} = 30$ mm), the area of the tube has to be large enough and the flow rate of the perfusion fluid low enough to achieve near zero glucose concentrations. However, this means a prolonging of lag-times, as the distance between the microdialysis probe and oxygen electrode is increased and the flow rate is lowered. An enzyme reactor placed in the single circulation system that is long enough to remove most of the glucose from the perfusate at the flow rate of 10 µL/min., leads to an unacceptable increase in lag-times and $T_{90}$’s. A second condition regarding the dimensions of the enzyme reactor is that the layer of enzyme solution around the dialysis tube should not be too wide. If the volume of enzyme solution around the dialysis fiber is enlarged, diffusion of oxygen within the enzyme layer would lead to tailing of the oxygen electrode signal. Therefore, an acceptable length and diameter for the enzyme reactor was sought to remove a major part of recovered glucose but at the same time produce a perfusion system that would still have lag-times below 10 minutes (see also chapter 4). A glucose eliminator, which is in fact an enlarged version of the enzyme reactor, was inserted in the perfusion system in line with the oxygen electrode. The eliminator removed a substantial part of the remaining glucose from the perfusion fluid. The residual glucose concentration in the perfusion fluid after it passed the glucose eliminator is low (< 3% of recovered glucose). This made the closing of the perfusion system possible and thereby supplied the basis for the miniaturisation of the system.

Preferably the perfusion flow ($F$ in equation 3.1) of a microdialysis probe should be regular to prevent fluctuations in glucose recovery. A strict requirement which, for the moment, only can be met by a high precision syringe pump. Unfortunately, a syringe pump is not suitable for closed perfusion systems as described in this chapter. Both flow systems use a piston pump to circulate the perfusion fluid. The flow produced by the piston pump pulsates with each pump cycle. Fortunately, the resulting flow had a constant oscillation due to the precisely constructed pump-chamber and the accurate functioning of the pump. In addition, both the carbon filter and the equaliser are dampening the pulse. The size of the pump and its ability...
CHAPTER 3
Discussion

to create enough pressure to perfuse the carbon filter makes this pump suitable for use in both these ambulatory perfusion systems.

The microdialysis probes used in these systems have the dialysis tubes positioned “side by side”. For glucose measurement in the subcutaneous tissue this type of probe is most suitable. Both probe in- and outlet tubes were positioned in the same direction so insertion of the probe needed only one point of entrance. Implantation of the probe is rather straightforward and could be done with a slightly modified catheter (20 Gauge, Intraflon 2, Vygon, France). After implantation the probe can be fixed on the skin using the butterfly and adhesive tape. Important for user comfort during ambulatory experiments is the flexibility of the probe. Rigid probes may cause irritation when the subject is moving, so the probes used in these perfusion systems were made of flexible and inert materials. The length of the dialysis tubes can be varied depending upon the desired recovery. Longer dialysis tubes will lead to higher recoveries at the same flow rate.

The choice for application during in vivo studies and further development of one of the discussed perfusion systems depends on the advantages and disadvantages of each system. The dual circulation system has the advantage over the single circulation system that all recovered glucose is converted and no extra measures are needed to prevent glucose accumulation. This, in contrast to the single circulation system which needs a glucose eliminator to prevent glucose accumulation. However, the other features of the single circulation system makes this system the first in choice for ambulatory in vivo studies. First, the enzyme solution is immobilised in a reactor instead of being a part of the actual perfusion fluid seen with the dual circulation system. The pressure on the ultra filtration membranes used in the dual circulation system may promote leakage of enzyme molecules through the membrane pinholes. Second, in the single circulation system only one pump is used to maintain the fluid circulation, which is an advantage for the miniaturisation of the system. Third, an additional advantage of the use of a single pump is that the energy consumption of the glucose measurement system is reduced.
In summary, both the dual circulation system and the single circulation system described in this chapter are designed to minimise the risk of enzyme leakage compared to the perfusion system used by Schoonen and Schmidt. A glucose measurement system based on this perfusion system is safe to use in vivo because the enzyme solution used as perfusion fluid is exchanged for saline. Additional filtering of the saline by a carbon filter contributes to an enzyme free perfusion fluid. For both in vivo studies and further development, the single circulation system is most suitably. This, because of both described perfusion systems, the single circulation system has the lowest risk of enzyme leakage, lowest energy consumption and most promising possibilities for further miniaturisation.