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

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Chapter 5

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

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

5.1 Introduction

Biosensors have by means the potential to monitor analytes continuously in vivo. An ideal biosensor can be used in the ambulant patient to monitor metabolism and warn in case of life-threatening emergency. Major problems associated with biofouling such as the rapid loss of biosensor performance, limited the number of biosensors currently used in clinical applications. To circumvent these problems, microdialysis (MD) and – more recently also – ultrafiltration (UF) has been introduced as an interface between the biological matrix and the measuring device. UF and MD use an implanted semi-permeable membrane to filtrate or dialyse analytes from the biological matrix. The filtrate or perfusate containing analytes is being transported by pumping and analysed outside the body. Because the membrane excludes cells and large molecules, MD and UF are able to deliver a clean matrix for measurement with a biosensor. A major drawback of conventional MD is that no quantitative recoveries of the analyte in vivo is obtained and excessive (in vivo) calibration procedures are required for accurate monitoring. Unlike MD, UF-sample concentrations are independent on probe diffusion characteristics and analytes with low molecular weight are quantitatively recovered. Quantitative recoveries are also obtained with ultraslow MD. In
this case dialysate is collected by means of underpressure at extremely low flow rates (less than 300 nl.min⁻¹) instead of the conventional way of pushing the carrier solution through the probe. To achieve this, a disposable pump capable of producing a stable low flow rate (50 – 300 nl.min⁻¹) for several days has been developed in our laboratory. To enable, however, real-time continuous monitoring at these very low flow rates, the construction as well as the performance of the biosensor needs special attention. Critical parameters, such as cell volume (≤ 25 nanoliter), low dead volume connections, biocompatibility of the materials, and low backpressure is of importance. In addition, to allow the reproducible production of these measuring devices, the final construction of the biosensor will more or less depend on today's available manufacturing procedures. Based upon these criteria, a miniaturised flow-through cell with a maximum volume of 20 nanoliter has been developed in our laboratory. Thanks to the construction, this flow-through cell, as schematically demonstrated in figure 1, can be directly coupled to an UF or MD probe.

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

Figure 1:
Schematic demonstration of the flow-through cell in detail; position of the electrodes in (a) in the centre, or (b) partly in the wall of the tygon tubing.
peroxide species is accompanied by large contributions from coexisting oxidizable constituents such as ascorbic acid and uric acid, the specificity of the bio-selector is compromised by the partial selectivity of the electrochemical transducer. To minimise this effect, the enzyme is immobilised on the working electrode preferably by the use of a permselective membrane. An ideal permselective membrane does not only adequately immobilise the enzyme of interest, but also act as a substrate diffusion-limiting barrier and is capable of reducing surface fouling. Via this way the biosensor becomes more membrane diffusion controlled instead of following the Michaelis-Menten kinetics, and as a consequence extends the linear range of the biosensor. To avoid a combination of membranes, which inevitably extend the response time and lower the response, these functions are preferably combined in a single membrane. To achieve high solute fluxes and maintain good chemical selectivity, ultra-thin polymer-films are used for this reason. Much attention has been given to the application of electropolymerised conducting films of polypyrrole, poly(N-methylpyrrole) and polyaniline and recently of the non-conducting polymers, such as polyphenol, poly(2-naphthol) and poly(o-phenylenediamine). Biosensors using these polymers exhibit several advantages, such as they are effective in preventing the biosensor from fouling and eliminating the interference from electroactive species, whereas an enhanced sensitivity and electrocatalytic action for poly(1,3-phenylenediamine) permselective films was observed, which was attributed to the high efficiency of transformation of enzymatically generated \( \text{H}_2\text{O}_2 \) to an electrical signal. Thanks to the self-controlling film thickness of the non-conducting polymer during electropolymerisation, a thin and uniform film can thus reproducibly be obtained. In addition, it has to be emphasised that the introduction of the biorecognition part of the biosensor on submicron levels and/or immobilisation of biomolecules in these closed micro-channels can only be performed via this way. Based on these advantages, the applicability of poly(1,3-phenylenediamine) as a permselective membrane in our miniaturised flow-through biosensors has been investigated. The present paper reports the studies conducted and results obtained with these miniaturised flow-through biosensors. To investigate and improve the performance of the biosensor, the conditions during electropolymerisation have been investigated and optimised. The linearity, precision and sensitivity as well as the selectivity and stability of various biosensors thus produced has been investigated and will be described in detail. Finally, to demonstrate the potential respectively possibilities of this instrumental set-up, results of some ongoing studies will be shown.
5.2 Experimental

5.2.1 Materials and solutions

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

5.2.2 The flow-through cell

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

5.2.3 The permselective membrane

The flow-through cell is washed with methanol, 10 v/v% hydrogen peroxide solution in water and 0.1 M phosphate buffer pH 7.0 by means of a 1 ml syringe (Becton Dickinson, Etten-Leur, the Netherlands) equipped with a 0.40 x 12 mm Luer Lock needle (B.Braun,
The syringe is filled with a solution containing 2 mg.ml\(^{-1}\) (approximately 500 units per ml) of enzyme and 10 mg.ml\(^{-1}\) (90 mmol.l\(^{-1}\)) of 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9. The syringe is placed in the model 22 syringe pump (Harvard Apparatus, Kent, UK) and the wires outside the tubing are connected with model DECADE electrochemical detector (Antec Leyden, Leiden, the Netherlands) by means of crocodile clips. Electropolymerisation is performed at +0.8 V vs. Ag/AgCl for one hour at a flow rate of 500 nl.min\(^{-1}\) followed by electropolymerisation for an additional 30 minutes using the monomer solution without the enzyme. Before storage and/or use, the biosensors are rinsed with 0.1 M phosphate buffer pH 6.9 for 30 minutes. In between, the biosensors are stored in the refrigerator at 4-8 °C.

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

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

5.2.5 Measurements in the continuous mode

For measurements in the continuous mode, one side of the biosensor is connected to a CMA 60 MD catheter (Aurora Borealis Control, Assen, the Netherlands) whereas the other side of the biosensor is directly connected to a home-made semi-vacuum syringe...
pump (1.2 ml monovette, Sarstedt, Nömbrecht, Germany) as previously described \(^{24}\). Dialysate is continuously analysed at a flow rate of 300 nl.min\(^{-1}\). Accordingly, measurements were carried out at +0.5 V vs. Ag/AgCl. Data is recorded with a model BD 112 flatbed recorder (Kipp & Zonen, Delft, the Netherlands). Before measurements, the biosensor was allowed to settle a stable current over a period of approximately 30 minutes.

### 5.2.6 Electron microscopy

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

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

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

### 5.3 Results

#### 5.3.1 Construction of the miniaturised flow-through cell

By using tygon tubing with a defined internal diameter, a flow-through cell with a defined volume can be easily constructed. With an internal diameter of 0.005 inch (0.127 mm), the tubing has a specified volume of 127 nl.cm\(^{-1}\). If the electrodes are positioned within 1-2 mm of each other, a cell with a total volume of approximately 10-20 nl is obtained. Low dead volume connections between the probe and the semi-vacuum syringe pump are obtained with 150 µm OD x 50 µm ID fused silica capillary. For each centimetre of fused silica used, an additional dead volume of 20 nl is introduced. In practice the biosensor has been connected with approximately 2 cm of fused silica tubing. An instrumental set-up for monitoring in the continuous mode as thus obtained is demonstrated in figure 1. Although for every device the exact delay is determined during calibration prior to \textit{in vivo} measurements, compared to the relatively high dead volume of the CMA 60 MD probe.
(approximately 3 µl), the dead volume of the measuring device is negligible. Delay times in combination with the CMA 60 MD probes are found to be 2-3 minutes, whereas the response time of the biosensor without the MD probe is only in the order of seconds. These delay times are likely to be acceptable for the purpose of clinical monitoring.

5.3.2 Production of the miniaturised flow-through cell

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

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

Although in this case m-phenylenediamine is used, even the formation of the frequently used poly(o-phenylenediamine) films has never been fully understood. To our knowledge, a polymerisation mechanism of o-phenylenediamine has only been proposed by Jang et al.\textsuperscript{26}. They suggested that during electropolymerisation, at least two different polymeric
components which are responsible for electron mediation are produced: an active component containing phenazine, formed mainly in the early stage of polymerisation, and an inactive component containing noncyclically coupled species. Chiba et al.\textsuperscript{27} investigated the structure of a 2.5 µm thick electropolymerised poly(o-phenylenediamine) films by means of infrared (IR) spectroscopy. They proposed a 'ladder' polymer with an asymmetrical 'quinoid' structure, which is partially ring-opened and involves moieties of oxidised forms of the quinone-imine type. To investigate the properties of the poly(m-phenylenediamine) films as described here, a previously washed 1 mm thick platinum electrode with a total surface area of 40 mm\textsuperscript{2} was coated with electropolymerised m-phenylenediamine. For this reason, the electrode was dipped in a 0.1 M phosphate buffer pH 6.9 solution containing 10 mg.ml\textsuperscript{-1} 1,3-phenylenediamine, a platinum counter electrode and a Ag/AgCl reference electrode, and electropolymerised at +0.8 V for 60 minutes. A brownish coloured electrode was obtained, which was completely coated with polymer with a thickness of approximately 1 µm, as investigated with electron microscopy. Structural information about the polymer was obtained by means of Total Reflectance Fourier Transform Infrared Spectroscopy. Although limited information can be obtained from the fingerprint area (1200 - 650 cm\textsuperscript{-1}), the stretching vibration of the N-H bonds (3300 – 3500 cm\textsuperscript{-1}), C-H bonds (2930 cm\textsuperscript{-1}) and C-C bonds (1500 – 1600 cm\textsuperscript{-1}) are clearly distinguished, whereas absorption peaks observed at \sim 1650 cm\textsuperscript{-1} and around 1600 cm\textsuperscript{-1} can be ascribed to C=O stretching vibrations of the quinone respectively C=C stretching vibrations (see figure 2). Although no full qualitative analyses of the polymer is possible with these thin polymer films, the spectra thus obtained are comparable with those found by Chiba et al.\textsuperscript{27}. This means that the formation of m-phenylenediamine based polymer films is comparable with o-phenylenediamine based polymer films.

\textbf{Figure 2:} Infrared spectrum of an electropolymerised poly(m-phenylenediamine) film on a platinum disk electrode (film thickness of \sim 1 µm).
5.3.4 Dynamic range of the poly(m-phenylenediamine) based biosensor

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

Table 1: Effect of polymerisation time on the performance of the biosensor

<table>
<thead>
<tr>
<th>Polymerisation time (min)</th>
<th>Peak height (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0.23</td>
</tr>
<tr>
<td>30</td>
<td>0.48</td>
</tr>
<tr>
<td>60</td>
<td>1.83</td>
</tr>
<tr>
<td>90</td>
<td>0.82</td>
</tr>
<tr>
<td>120</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Several biosensors produced accordingly were tested in vitro in the continuous mode. A typical calibration plot as shown in figure 3 was obtained, and linearity was found up to approximately 5 mM of glucose. However, for continuous monitoring of for instance patients suffering from diabetes mellitus, glucose concentrations of up to 30 mM can be expected, which makes this biosensor less suitable for clinical applications. So, in order to
avoid Michaelis-Menten kinetics, and to improve the substrate diffusion-limiting barrier respectively, several options were investigated. By extending the polymerisation time, a ‘densification’ of the polymer layer in combination with a slight thickness increase of the poly(o-phenylenediamine) film was observed by Jobst. In our set-up this was investigated by dipping 0.1 mm previously washed platinum wires in a 10 mg/ml 1,3-phenylenediamine in 0.1 M phosphate buffer pH 6.9 containing a platinum counter electrode and a Ag/AgCl reference electrode, and electropolymerise at +0.8 V for 60 respectively 90 minutes. Although a ‘densification’ cannot be clearly seen with electron microscopy (see figure 4), a slight increase of 200 up to 240 nm of the thickness of the permselective membrane is observed. These layers are clearly thicker than found by others, who reported a thickness of only 10 – 35 nm. Because polymerisation is performed at comparable pH (pH 6.9 versus pH 5.2 - 7.4), the contradicting thickness of the layer cannot be explained by the
pH of the polymerisation solution. More likely, other parameters such as the polymerisation time seems to be of more importance. For instance our polymerisation time is considerably longer (1.5 hours compared to 10-15 minutes). However due to the diversity of protocols and reported contradicting data, no full explanation can be given for this phenomenon. More interesting is, however, whether or not the characteristics of the diffusion-limiting barrier of the permselective membrane can be affected via this way. For this reason, biosensors were produced by electropolymerisation of an additional 30 minutes with a solution containing the monomer only. After testing the biosensors in vitro by means of analysing standard solutions of glucose in the continuous mode, a typical calibration curve, as shown in figure 3, was obtained. Obviously, the linear range (up to 30 mM glucose) is now satisfactory. Although the sensitivity also decreased as expected, with a calculated limit of detection of 0.05 mM of glucose, the sensitivity was found to be adequate enough for our purpose. Also, no significant increase in the response time (only several seconds) was observed. An additional advantage of producing biosensors this way is that the electropolymerisation time is less critical whereas broad dynamic range biosensors can be obtained without the use of complex and/or a diversity of procedures for the fabrication of (multilayers) of permselective membranes.

5.3.5 Enzyme immobilisation

Approximately 3 – 10 U of GOx per cm² of electrode surface can be incorporated into poly(o-phenylenediamine) films. In our set-up, this means that, with a maximum electrode surface area of 0.0571 mm², 0.14 – 0.50 µU of GOx is available, which theoretically can convert 0.0035 – 0.0120 pMol/s of glucose at pH 7.0 at 25 ºC. Based upon the measurements performed, a maximum conversion of approximately 0.0150 pMol.s⁻¹ of hydrogen peroxide has been calculated according to Faraday’s law. The transformation coefficient in poly(o-aminophenol) films, defined as the efficiency of the transformation of H₂O₂ produced by the enzymatic reaction to an electronic signal, varies between 15 – 77%. Although not completely comparable, a maximum experimental conversion of 0.020 – 0.100 pMol.s⁻¹ of hydrogen peroxide can be estimated. Obviously, the amount of active enzyme entrapped in the polymer film is 1 order of magnitude higher than assumed.

5.3.6 Selectivity of the biosensor

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

5.3.7 The stability of the glucose biosensor

To examine the stability of the biosensor as presented here, several experiments have been conducted. Biosensors with only 'one layer' of poly(m-phenylenediamine) (60 minutes of
electropolymerisation) and stored in the refrigerator at 4 °C. The biosensors were tested after respectively 0, 3, 5 and 7 days of storage by analysing a standard solution containing 5 mM glucose in Dulbecco’s buffer by means of FIA and measuring the mean peak height (nA). In contrast, the biosensor tested at t = 0, was kept at room temperature and retested after respectively 1, 2, 3, 7 and 8 days by means of FIA. The results of these tests are shown in table 2.

Table 2: 
Stability of glucose biosensor under different storage conditions, in use and effect of preservative

<table>
<thead>
<tr>
<th>Age of sensor (days)</th>
<th>Storage</th>
<th>4 °C no preservative</th>
<th>Room temperature no preservative</th>
<th>Room temperature with preservative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>22</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>19</td>
<td>80</td>
<td></td>
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<tr>
<td>3</td>
<td>51</td>
<td>17</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>-</td>
<td>58</td>
<td></td>
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<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>7</td>
<td>16</td>
<td>5</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

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

Remarkably, although storage at 4 °C does prolong the lifetime of the biosensor, an unacceptable decrease in the performance after 7 days of storage is observed. After 15 days of storage, the performance had grown worse and no signal for a standard solution 5 mM glucose could be measured (data not shown). Because the biosensors tested after respectively 3, 5 and 7 days of storage at 4 °C were not tested before, leaching out of enzyme is probably not the only explanation for stability problems with these biosensors. Due to the fact that the biosensors were not completely stored dry, dissolution of the film into the solution could be an explanation for this phenomenon. However, positive experience with preservatives regarding the stability of enzymes in instrumental set-ups,
led to the next experiment, in which a biosensor with ‘one layer’ of poly(m-phenylenediamine) (60 minutes of electropolymerisation) was kept at room temperature and retested accordingly after 2, 5, 7 and 8 days by means of FIA. However, in this case, 0.1% of Kathon CG (a preservative) was added to the carrier solution. From the results shown in table 2, a significant increase in the stability can be observed. The first drop from 100% (t = 0) to approximately 60% (t = 5 days) of signal is probably due to leaching out of enzyme and/or partly dissolution of the polymer film. After five days, equilibrium is seen whereas the performance of the biosensor gradually decreased and after approximately 30 days (data not shown) no signal could be detected anymore. Although no clear explanation can be given for these observations, it is questionable whether or not the ingredients within the preservative used may affect in a positive way the stability of the biosensor. In order to be able to answer these questions, more research regarding the stability of biosensors during (different) storage conditions will be necessary. Equally important, however, is the stability of the biosensor in practice. During the measurements, gluconic acid as well as hydrogen peroxide is generated. Especially hydrogen peroxide may affect the stability of the enzyme glucose oxidase. To examine these two biosensors, with ‘one layer’ of poly(m-phenylenediamine) (60 minutes of electropolymerisation) and another with an ‘additional layer’ of poly(m-phenylenediamine) (90 minutes of electropolymerisation), were made and tested accordingly by continuously monitoring of a standard solution of 5 mM glucose. The results are shown in figure 6. Again, for both sensors, an initial decrease in sensitivity from 100% to approximately 70% was seen within one day of practice, and is probably caused by leaching out of not fully immobilised enzyme and/or polymer film.\textsuperscript{13,21} However, as demonstrated in figure 6, the biosensor with the ‘additional layer’ retained its activity whereas the performance of the other biosensor gradually decreased within 70 hours. These results suggest that the stability of the biosensor is merely dependent on the denaturation of the enzyme by hydrogen peroxide.\textsuperscript{28} Others\textsuperscript{30} attributed this decline in sensitivity on electrode fouling by small endogenous proteins. To investigate this in our set-up, a serum sample containing approximately 5 mM of glucose was monitored for several days in the continuous mode. The data obtained, as demonstrated in figure 6, are typical for biosensors thus produced. After an initial decrease in sensitivity, the biosensor was stable for at least three days whereas no significant additional decrease in the performance of the biosensor was observed. Based on these ex-vivo results, it might be concluded that the biosensor can be used for up to three days for continuous in vivo monitoring of glucose. Although more stability studies will be conducted in the near future, these results clearly indicate the performance of this type of biosensor.
Obviously, the initial decrease in sensitivity is determined by leaching out of enzyme and/or dissolution of the polymer film followed by biodegradation during storage and/or in practice and denaturation of the enzyme by hydrogen peroxide in practice.

5.3.8 Performance characteristics of the lactate biosensor

Calibration curves of the lactate biosensor were performed by analysing standard solutions of lactate by means of FIA, and linearity was found up to approximately 5 mM of lactate. Reference values of lactate in blood range from 0.5 till 2.2 mM, which means that the lactate biosensors produced here are sufficiently linear for this purpose. Although similar characteristics were observed regarding the selectivity (see figure 5), response time (in the order of seconds) and reproducibility (4% as determined by FIA and expressed as the relative standard deviation in the peak height after six consecutive injections), the stability of the lactate biosensor was of our most concern. In figure 7, the stability data are demonstrated which were obtained after analysing by means of FIA a standard solution of 2 mM of lactate in Dulbecco’s buffer. From this figure a 60% decrease in the performance of the biosensor can be seen within 5 hours of practice. Experiments with these biosensors
in the continuous mode showed a rapid decrease in signal to almost zero within one hour of practice. Based upon these results it is evident that both the stability towards denaturation as well as immobilisation within a poly(m-phenylenediamine) polymer film is significantly poorer for LOx compared to GOx. From work by others it is known that the potential scan range for polymerisation can affect the film morphology, and thus the performance of the biosensor. Zhang et al\textsuperscript{21} demonstrated that electropolymerisation by cyclic voltammetry generates a more uniform and compact insulating film. For this reason, a lactate biosensor was produced by sequentially potentially cycling from –0.2 to +1.0 V vs. Ag/AgCl at a scan rate of 50 mV.s\textsuperscript{-1} for at least 20 potential sweeps in a 0.1 M phosphate buffer solution pH 7.0, containing 2 mg.ml\textsuperscript{-1} of enzyme and 10 mg.ml\textsuperscript{-1} of 1,3-phenylenediamine. Although, as can be seen from figure 7, the stability of the lactate biosensor produced in this way is significantly increased, during continuous monitoring however, these biosensors still show an unacceptable decline in the performance of the biosensor. For \textit{in vivo} studies by means of continuous monitoring of lactate, more work needs to be done to improve the stability of these biosensors.

5.3.9 Applications

To demonstrate the applicability of the biosensors described in this article, some results of our ongoing \textit{in vivo} studies will be presented here. Only a few studies reported the use of these devices \textit{in vivo} neurochemical studies. Besides the fact that the extracellular fluid (ECF) contains several potential electrocatalysts (e.g. glutathione and ascorbic acid), which might affect the performance of the biosensor; also the restriction of mass-transport to the electrode surface by brain tissue, reflects the difficulties associated with performing direct neurochemical measurements in this complex environment. J.P. Lowry \textit{et al.}\textsuperscript{17-19} and Cass
et al.\textsuperscript{31} demonstrated the use of such a biosensor for the monitoring of brain extracellular glucose in rat striatum in awake freely-moving rats. As the reported ECF glucose contents differed significantly (0.5 – 2 mM), we decided to investigate these ECF glucose concentrations in rat striatum. Small I-shaped home-made MD probes, as described earlier in detail\textsuperscript{32}, were implanted in rat striatum of anaesthetised rats. Immediately after implantation, the biosensor was coupled to the probe and glucose was monitored continuously at a flow rate of 100 nl.min\textsuperscript{-1} in dialysate. One day respectively 3, 5 and 6 days after implantation, glucose was monitored similarly in rat striatum of freely moving rats. Although the study is still ongoing, tentative results (see figure 8) have been obtained which are in accordance with reported preliminary results. In our set-up, directly after implantation of the probe, a concentration of approximately 0.7 mM of glucose is found; this concentration decreases to about 0.2 mM within 3 days followed by an increase to above 1 mM 6 days after implantation. These levels are among the lowest reported levels and illustrate both sensitivity and selectivity of the biosensor.

5.4 Conclusion

A miniaturised device, comprising a sampling unit (UF or MD probe), a biosensor and a semi-vacuum syringe pump, has been developed and tested for the continuous on-line monitoring of glucose or lactate in biological matrices at very low perfusion flow. The permselective membrane of the bio-selector, based on a ‘double layer’ of electropolymerised m-phenylenediamine provided good performance characteristics for \textit{in vivo} studies. The good selectivity, sensitivity and quick response (several seconds) enabled
us to develop a miniaturised flow-through nanoliter biosensor. Although still made by hand in the laboratory, a cheap and reliable biosensor can thus reproducibly be fabricated. The linearity as well as stability was extended by increasing the time of electropolymerisation with m-phenylenediamine. The stability of lactate based biosensors, however, is still cumbersome. Although good performance is demonstrated in the FIA mode, in order to apply these lactate biosensors in the continuous mode more work needs to be done to improve the stability of the lactate biosensor. The results obtained so far with the glucose biosensors from in vivo studies demonstrate the potential of the measuring device presented here.

5.5 References

Chapter 5