4 In vitro characteristics of the SCGM-system

4.1 Introduction
Various characteristics of a glucose sensor are important when applied in vivo [246, 247]. The main function of an in vivo glucose sensor is to help maintain near normoglycaemia in diabetic patients. Malfunction of the sensor can have far-reaching consequences for the patient if glucose regulation is based on the sensor measurements. Important sensor properties, which can be determined in vitro, are the accuracy of measurement, sensitivity of the sensor, the response time of the sensor and the stability of the sensor. Chapter 3 describes a glucose measurement system (gms), the single circulation system, which is based on the glucose sensor developed by Schoonen and Schmidt. However, substantial changes have been made to the flow system to minimise the leakage of enzyme from the gms. An enzyme solution of god and catalase, used as perfusion fluid in the flow system of Schoonen and Schmidt, is replaced by saline. The enzyme solution in this newly designed flow system, is retained in an enzyme reactor and separated from the perfusion fluid by a semipermeable dialysis membrane. A carbon filter is inserted in the perfusion flow to retain enzyme molecules that leaked from the enzyme reactor and glucose eliminator. Consequently, the in vitro characteristics of this system are different from the system developed by Schoonen and Schmidt. Therefore, this chapter describes experiments designed to assess the in vitro characteristics of this newly designed single circulation glucose measurement system (sc-gms). We determined the accuracy of the sensor measurements, the sensor sensitivity for glucose, the sensor response times, the influence of temperature on the sensor measurements and the overall stability of the gms.
4.2 Materials and Methods

4.2.1 Experimental setup

For the determination of the various in vitro characteristics, the single circulation glucose measurement system was used (n = 12). The separate flow systems were assembled as described in chapter 2 and placed in the flow system boxes. A personal computer (PC) was used to monitor the glucose measurement systems during the different experiments. The flow system boxes were coupled via an electric cable to a junction box (local electronic workshop) which was connected to an a/d-converter (Daqbook). This junction box contained eight connection sockets in combination with eight potentiostats and pump control units. So in theory a maximum of eight systems could be tested simultaneously. In practice, however, maximally two systems were tested at the same time due to the labour-intensive assembly of the flow systems. The a/d-converter was connected to the PC for communication with the sensor systems. The PC was used for storing the oxygen electrode current, for setting the flow rate and the time interval of measurement storage. The a/d-converter was needed to convert the analogous signals from the potentiostats and the pump units to digital signals so data could be exchanged between the flow system boxes and the PC. Figure 4-1 gives a schematic overview of the experimental set-up.

The flow rate was set to 10µL/min. and the sample storage frequency for most experiments to twice per minute. After a running-in period of 2 to 4 hours, during which the systems were tested on proper functioning, experiments started.
4.2.2 Experiments

- **Assessment of the accuracy of sensor measurements**
  The 12 systems were calibrated by placing the microdialysis probe at room temperature (20 - 22 °C) in saline with different glucose concentrations (0, 1.4, 2.8, 4.2, 5.6, 6.9, 8.3, and 11.1 mM). During the day, one calibration serie was made with every single system. In total, four calibration series were made with every single system. The Saline-glucose solutions were gently stirred to minimise the depletion of glucose from the layer of saline around the dialysis tube. The separate sensors were calibrated by placing the probe in alternately ascending or descending series of glucose concentrations while the resulting oxygen electrode current was stored on the PC. The linear response of the systems was determined by applying the ANOVA F-test (see also “Statistical analysis” on page 79). The slope of the regression line in the linear measuring range were calculated from the separate calibration plots of the 12 sensors.

- **Assessment of the sensitivity (upper and lower detection limit)**
  Of 7 sensors the upper and lower detection limit were determined. For the assessment of the lower detection limit, the microdialysis probe was placed in stirred saline-glucose solutions with concentrations of respectively 0.28,
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0.6, 0.8 and 1.1 mM. For the assessment of the upper detection limit saline-glucose solutions of respectively 8.3, 11.1, 13.9, 19.4 and 22.2 mM were used. The highest glucose concentration within the linear measuring range of each system was taken as the upper detection limit of that system.

Assessment of response times
Of 7 sensors the T₉₀%, i.e. the time to reach 90% of the plateau value if a sudden change in glucose concentration occurs, and the lag-time, i.e. the time between the alteration of the glucose concentration and the sensor reaction, was determined. The environment of the microdialysis probe was changed from saline to a saline-glucose solution of 5.6 mM and vice versa. Both T₉₀% and lag-times were calculated from the calibration plots of each 7 sensors.

Assessment of sensor stability
Two sensor systems were kept operational for 28 days at room temperatures (20 - 22 °C). During this period base-line values (probe in saline) of both sensors were recorded on the PC (30 measurements stored per hour). The sensitivity of both sensor systems was determined by placing the microdialysis probe regularly in a stirred 5.6 mM saline-glucose solution. The base-line shift and the change in sensitivity for glucose of both systems during this period were calculated using the sensor measurements. To assess the influence of base-line shift of the separate oxygen electrodes on the total base-line shift of the systems, the two electrodes were perfused with saline for 28 days at room temperature while the output current was recorded (30 measurements stored per hour). For this purpose a perfusion system was used that consisted of a micro-pump (Parker) which circulated saline, via the oxygen electrode and oxygen permeable tubes (Teflon, Zeus Industrial Products, i.d. 0.7 mm, o.d. 1.5 mm), at a flow rate of 10µL/min.

The effect of temperature changes on the sensor measurement at 0 mM glucose was determined by placing 6 flow systems in an incubator (Model KBP 6087, Termacks, Kipp & Zonen, programmable temperature range: 0-70 °C). The temperature of the incubator during the temperature experiments was increased from 5 °C to 35 °C in steps of 1.8 °C/hour.
Results

1) Statistical analysis
Data are reported as mean ± SD. The linear association between variables is expressed by the Pearson’s product-moment correlation coefficient. To assess whether the various glucose concentrations (mM) and the accompanying system response (nA) complied with a linear relation ($I_{\text{cur}} = \beta_1 + \beta_2 C_{\text{gluc}}$), the *anova* F-test was performed. Of every batch of measurements, the “lack of fit” F-value ($F_{\text{lof}}$) and the inherent P-values of the separate regression lines were calculated. The P-values found indicate how well the measured currents and presented glucose concentrations comply with a linear relationship. When P-values equally or higher than 0.95 were found, a linear relationship was assumed.

4.3 Results

2) Accuracy of the sensor
The measurement series of four systems complied with a linear relation (Table 4-1, p < 0.95, page 83). The calibration series of the remaining eight systems showed a deviation of the linear model (Table 4-1, p < 0.95). The current output of three systems did not change proportionally at glucose concentrations higher than 8 mM (Table 4-1). The corresponding correlation coefficients of the regression lines show that the output current of the systems is reasonably correlated with the different glucose concentrations. Base-line values of the oxygen electrodes used, i.e. at 0 mM glucose, are different for each system and range from 420 to 185 nA. The slopes of the calibration plots are different for each system. Figure 4-2 shows a typical in vitro calibration plot of two of the systems in the range of 0 to 11.1 mM (page 80). Figure 4-3 shows the corresponding response of one of these systems to glucose concentrations of 4.2 and 8.3 mM (page 80).

3) Sensitivity and response time of the sensor
The lower detection limit of the 7 tested systems was 0.58 ± 0.15 mM. The signal to noise ratio of most systems became too small for reliable measurements when a glucose concentration of 0.28 mM was tested. The upper detection limit of the systems was 14.3 ± 4.9 mM ($n = 7$).
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Figure 4-2. In vitro calibration plot of two glucose measurement systems.

Figure 4-3. Typical in vitro response of one of the glucose measurement systems to glucose solutions of respectively 4.2 mM, 8.3 mM and 0 mM.
The mean $T_{90\%}$-down of the 7 systems was $7.6 \pm 0.9$ min. and the $T_{90\%}$-up $9.6 \pm 1.5$ min. By switching the microdialysis probe of each system from saline to a saline-glucose solution of 5.6 mM and vice versa, lag-times of $1.5 \pm 0.4$ min. and $1.8 \pm 0.4$ min. ($n = 7$) for respectively the descending and ascending response curve, could be calculated. Figure 4-4 shows one of the response curves made to assess the lag-times and $T_{90\%}$'s.
Stability of the sensor

Figure 4-5 shows the measurements of one of the two systems during 28 days of continuous operation. The mean base-line shift of the two glucose measurement systems during the 28 days is -0.98 ± 0.14%/24h. The mean decline in sensitivity for glucose during this period was -0.79 ± 0.11%/24h (n = 2). The mean base-line shift of the two oxygen electrodes during 28 days was -0.87 ± 0.1%/24h (n = 2).

Temperature influence on sensor measurement

The increase in temperature by 1.8 °C/hour of 6 glucose measurement systems resulted in a linear increase in output current (Table 4-2). The mean increase in output current was 4.7 ± 0.9 nA/°C.
## Table 4-1. In vitro calibration values of single-circulation glucose measurement systems.

<table>
<thead>
<tr>
<th>System</th>
<th>( C_{\text{max}} ) (mM)</th>
<th>Baseline (nA)</th>
<th>Slope (nA/mM)</th>
<th>( r ) (-)</th>
<th>( F_{\text{lof}} ) (% of ( C_{\text{max}} ))</th>
<th>( P_{\text{cmax}} ) (%)</th>
<th>( F_{\text{lof all conc.}} ) (%)</th>
<th>( P_{\text{all conc.}} ) (%)</th>
<th>( \text{var}<em>{C</em>{\text{max}}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.3</td>
<td>303</td>
<td>-21.1</td>
<td>-0.996</td>
<td>0.29</td>
<td>0.95</td>
<td>2.13</td>
<td>0.08</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>11.1</td>
<td>256</td>
<td>-12.0</td>
<td>-0.996</td>
<td>0.94</td>
<td>0.50</td>
<td>–</td>
<td>–</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>11.1</td>
<td>420</td>
<td>-16.5</td>
<td>-0.999</td>
<td>1.37</td>
<td>0.27</td>
<td>–</td>
<td>–</td>
<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>8.3</td>
<td>253</td>
<td>-22.8</td>
<td>-0.996</td>
<td>0.10</td>
<td>0.91</td>
<td>10.3</td>
<td>0.01</td>
<td>4.6</td>
</tr>
<tr>
<td>5</td>
<td>11.1</td>
<td>185</td>
<td>-10.9</td>
<td>-0.993</td>
<td>1.00</td>
<td>0.45</td>
<td>–</td>
<td>–</td>
<td>4.1</td>
</tr>
<tr>
<td>6</td>
<td>11.1</td>
<td>199</td>
<td>-12.6</td>
<td>-0.998</td>
<td>0.84</td>
<td>0.56</td>
<td>–</td>
<td>–</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>11.1</td>
<td>342</td>
<td>-25.2</td>
<td>-0.998</td>
<td>0.76</td>
<td>0.62</td>
<td>–</td>
<td>–</td>
<td>3.5</td>
</tr>
<tr>
<td>8</td>
<td>8.3</td>
<td>335</td>
<td>-30.0</td>
<td>-0.999</td>
<td>0.24</td>
<td>0.95</td>
<td>2.58</td>
<td>0.04</td>
<td>2.4</td>
</tr>
<tr>
<td>9</td>
<td>11.1</td>
<td>301</td>
<td>-12.7</td>
<td>-0.984</td>
<td>0.82</td>
<td>0.58</td>
<td>–</td>
<td>–</td>
<td>4.6</td>
</tr>
<tr>
<td>10</td>
<td>11.1</td>
<td>418</td>
<td>-31.9</td>
<td>-0.997</td>
<td>1.22</td>
<td>0.33</td>
<td>–</td>
<td>–</td>
<td>4.5</td>
</tr>
<tr>
<td>11</td>
<td>11.1</td>
<td>209</td>
<td>-9.7</td>
<td>-0.984</td>
<td>0.39</td>
<td>0.99</td>
<td>–</td>
<td>–</td>
<td>4.4</td>
</tr>
<tr>
<td>12</td>
<td>11.1</td>
<td>285</td>
<td>-16.1</td>
<td>-0.993</td>
<td>1.26</td>
<td>0.31</td>
<td>–</td>
<td>–</td>
<td>3.8</td>
</tr>
</tbody>
</table>

## Table 4-2. Influence of temperature increase on single-circulation glucose measurement system.

<table>
<thead>
<tr>
<th>System</th>
<th>Current 5° C</th>
<th>Current 35° C</th>
<th>Slope (nA/° C)</th>
<th>( r ) (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>115</td>
<td>268</td>
<td>5.1</td>
<td>0.999</td>
</tr>
<tr>
<td>B</td>
<td>130</td>
<td>277</td>
<td>4.9</td>
<td>0.998</td>
</tr>
<tr>
<td>C</td>
<td>187</td>
<td>295</td>
<td>3.6</td>
<td>0.999</td>
</tr>
<tr>
<td>D</td>
<td>243</td>
<td>429</td>
<td>6.2</td>
<td>0.998</td>
</tr>
<tr>
<td>E</td>
<td>194</td>
<td>329</td>
<td>4.5</td>
<td>0.999</td>
</tr>
<tr>
<td>F</td>
<td>250</td>
<td>330</td>
<td>4.0</td>
<td>0.999</td>
</tr>
</tbody>
</table>
4.4 Discussion

**Assessment of the accuracy of sensor measurements**

The in vitro experiments done with the newly developed glucose measurement system show that the system is suitable to measure glucose within a defined glucose concentration range. The calibration data of four systems illustrates that it is possible to have a good correlation between the output current and the glucose concentration, although the mutual characteristics, including the linear measuring range, differ for each system. The calibration data of the remaining eight systems, however, show that the linear relationship between the offered glucose concentrations and the output current is not significant (Table 4-1, p < 0.95). This can be explained by the influence of additional parameters on the measurements. Because the calibration plots were made on separate days, a combination of changes in air pressure, perfusion flow and electrode basal values may have contributed to deviations in the separate calibration series during these days.

Nine of the tested systems gave a near linear response up to 11 mM glucose, whereas of three systems the output current reached a plateau value at a glucose concentration lower than 8 mM. This linear response of each system is valid until the glucose concentration recovered in the microdialysis process exceeds the actual concentration of dissolved oxygen present in the perfusion fluid. Also the detection range of the oxygen electrode influences the linear measuring range.

At similar conditions, the basal current of the electrodes used in this study varies considerably for each electrode. A higher basal current extends the detection range of the electrode. These mutual differences are most likely a result of the manual manufacturing process. For example, a larger platinum cathode will increase the basal current relatively. The nominal disadvantage of a higher basal electrode current is the accelerated consumption of electrolyte, which shortens the total lifetime of the glucose sensor.

To expand the linear measuring range, the relative recovery has to be reduced. This can be achieved by increasing the flow rate and/or by reducing the surface area of the dialysis tube. This is illustrated in equation 3.1
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(page 70) which describes the recovery by a microdialysis probe. Inherent to a wider dynamic range is the loss in resolution because the detection range of the oxygen electrode does not change. For in vivo studies in non-diabetic human subjects, a linear measuring range of 0 to 11.1 mM glucose is sufficient. In literature there has been some debate about the required detection range in diabetic patients [73, 75]. The blood glucose concentration in “treated” diabetics can range from 1 up to 30 mM. Velho et al. [73] proposed that a linearity of response in the range of 1 to 15 mM would be required for a glucose sensor. They argued that the upper limit of 15 mM was sufficient high to be useful in the management of blood glucose levels. Coming from normal levels, there would be enough time to avert too high blood glucose levels. Kraeger and Chisholm [75] have suggested that a sensor need only respond linearly up to 8 mM in “non-meal” periods. They stated that the patient should inject insulin before meals and use the sensor as an alarming device for too high blood glucose levels. However the general opinion in the field is that a sensor should respond over the entire concentration of 1 to 30 mM commonly observed in diabetics [74]. This means that the range of our glucose measurement system has to be enlarged when used in diabetic patients. One way to achieve this is by decreasing the size of the dialysis membrane in the probe. At the same flow rate, the recovery of glucose will be decreased [248] and thus result in a wider detection range. The implied disadvantage will be the decrease in sensor sensitivity.

Sensitivity of the sensor

The mean resolution of the tested systems is 0.58 mM. With smaller glucose concentration differences it is not clear whether the response of the systems is a result of the recovered glucose or due to the inherent noise of the systems. A longer dialysis tube and/or a lower perfusion rate will increase the relative recovery and as a consequence, the resolution. However, it will also decrease the linear measuring range and result in longer lag-times and $T_{90\%}$’s if a lower perfusion rate is used. Depending on the intended in vivo application, the relative recovery should be adjusted by changing the length of the dialysis tube rather than increasing the perfusion flow. Halving the length of the dialysis tube will result in twofold increase of the linear measuring range (equation 3.1, page 70). An increase in perfusion flow, higher
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than the 10 µL/min. used in the present experiments, may result in ultrafiltration of perfusion fluid through the dialysis tube, affecting the recovery of glucose.

Assessment of response times
The system T_{90\%}-up/-down are higher than the T_{90\%} values Hashiguchi et al. [69] and Laurell [68] found with their microdialysis based glucose measurement systems (respectively 5.6/7.4 min. at flow rate 2 µL/min. and 2.0/2.0 min. at flow rate 25 µL/min.). Found T_{90\%}-up/-down can, however, be compared with the values reported by Keck et al. -T_{90\%}-up: 6.7 min. at flow rate 10 µL/min. [228, 229].
The mean T_{90\%}-down of the tested systems is 2.4 times larger than the T_{90\%} Schoonen & Schmidt found with their glucose measurement system -7.6 min. versus 3.1 min. [1]. Although the mean lag-time of both type of systems differ to some extent (1.5 min. versus 1 min.), the main cause for the smaller T_{90\%} is that recovered glucose in the system of Schoonen and Schmidt is converted immediately by \textit{god}, which is dissolved in the perfusion fluid. In the newly developed system, however, recovered glucose has to diffuse from the perfusion fluid into the enzyme reactor, before conversion takes place. Consequently, at equal perfusion rates, higher values of T_{90\%} will be found. The higher T_{90\%}-up of the tested systems can be explained by the fact that after switching the dialysis probe from a glucose concentration to saline, first all glucose present in the perfusion fluid has to be converted and oxygen used in this conversion, has to be replenished.

The T_{90\%}'s and the lag-times can be reduced by increasing the flow rate and reducing the total volume of the tubing, including the enzyme reactor, between the dialysis tube and oxygen electrode. However, higher flow rates and smaller tubing create a build-up of fluid pressure within the flow system and can result in fluid “sweating” through the dialysis tube due to ultrafiltration. This disturbs the environment near the dialysis tube and results in misinterpretation of measured glucose concentrations. Also the total length of the tubing between the microdialysis tube and the oxygen electrode can be shortened to improve the lag-time and T_{90\%} of the systems. Yet, for practical reasons, the length of the tubing can not be made too short without giving in ease of use by the patient. This because the oxygen electrode is
positioned in the flow system box and there must be some flexibility between the microdialysis probe, inserted in the tissue, and the flow system box.

When a microdialysis system is designed, compromises have to be made. On the one hand there is the wish of small delay times and on the other hand there is the question of what is technically possible without undermining the reliability of the sensor measurements. So, an optimum has to be found within the sensor design between these parameters.

Most important criterion is of course, how the $T_{90\%}$’s found relate to the time parameters of physiological blood glucose concentration changes in diabetic patients. Especially, a rapid fall in the blood glucose concentration causes an acute danger. If the concentration falls below 2.6 mM it can result in a hypoglycaemic coma of the patient. It is therefore essential that the sensor is producing a distress signal (“hypo-alarm”) well in time. In this situation the relationship between decline rate in glucose concentration and the sensor lag-time is a very important factor.

The rate of decline in blood glucose concentration will differ between individuals. Alders et al. [235] observed in a study in type-1 diabetics, a mean shift of 13 minutes between the venous blood glucose concentration levels and the subcutaneous sensor levels when a rapid fall in the blood glucose concentration was induced by the intravenous administration of insulin. Hashiguchi et al. found in a comparable in vivo study, a mean delay time of $8.8 \pm 1.6$ minute [69]. It is therefore important that a distress signal is already given at a glucose concentration higher than the critical glucose concentration in vivo. This, to compensate for the total lag-time between glucose change and corresponding sensor signal.

Stability of the sensor and temperature influences
The overall in vitro stability of the systems mainly depends on the basal drift of the oxygen electrodes as can be concluded when the mean basal drift of the oxygen electrodes is compared to the mean basal drift of the complete glucose measurement systems. Factors that also may influence the system stability when glucose is measured are changes in the perfusion flow of the microdialysis probe and the dialysis membrane permeability. The overall
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Effect in time is a decrease in sensor sensitivity for glucose. The mean calibration factor during this period changed by \(-0.95%/24\text{h}\).

For reliable sensor functioning, measurements in time should be compensated for the decrease in sensor sensitivity. The sensor measurement should also be compensated for temperature changes. Increasing temperatures will not only induce a diminution of oxygen solubility in the perfusion fluid but also in higher diffusion rates across the Teflon membranes used in the electrodes. The overall effect of temperature rise is an increase in output current of \(4.7 \text{nA}/\text{°C}\).

In summary, the in vitro characteristics of the \(\text{sc-gms}\) presented here show that it is possible to measure glucose reliably with the new designed flow system. Although properties like dynamic range and resolution are system dependent, it is very well possible to apply these systems in vivo after separate calibration. Because it is the intention to use the system in diabetic patients, widening of the linear measuring range to at least 30 mM is necessary. The most suitable solution to expand the linear measuring range would be by reducing the length of the dialysis tube in the probe. The in vitro drift of the total system is acceptable to measure during a period of at least 2 weeks. However, sensor measurements should be compensated for temperature changes and the overall sensor drift in order to be reliable.