A percutaneous device as model to study the *in vivo* performance of implantable amperometric glucose sensors

M. GERRITSEN[1], A. KROS[2], J. A. LUTTERMAN[3], R. J. M. NOLTE[2], J. A. JANSEN[1]
[1]*Department of Biomaterials, University of Nijmegen, Nijmegen, The Netherlands*
E-mail: J.Jansen@dent.kun.nl
[2]*Department of Organic Chemistry, NSR Center, University of Nijmegen, Nijmegen, The Netherlands*
[3]*Department of Internal Medicine, Academic Hospital St. Radboud, Nijmegen, The Netherlands*

Glucose kinetics were investigated in subcutaneous tissue of rabbits, in which a percutaneous device was implanted. The device was used for collection of tissue fluid and as carrier of an amperometric glucose sensor. Changes in glycaemia were reflected in subcutaneous tissue fluid. However, a limited number of responses of the implanted sensors were observed. Histologic evaluation showed thin fibrous capsules surrounding the implants. Accumulations of inflammatory cells were observed inside the subcutaneous chamber. The experiments again showed that changes in blood glucose concentration can be measured in subcutaneous tissue fluid collected with a percutaneous device. Nevertheless, implanted glucose sensors could not reliably monitor these changes. Supported by our histological observations and sufficient *in vitro* performance, we suppose that the cellular reaction to the sensor plays an important role in this poor *in vivo* performance. In combination with adsorption of tissue fluid proteins, this results in a reversible deactivation of implanted sensors. The exact mechanisms involved in this process are currently unknown and need further investigation.

1. Introduction

It is generally accepted that implantable glucose sensors can be of great importance for the clinical management of diabetes mellitus. Subcutaneously implanted sensors, however, showed a significant drift in sensor signal over the implantation period. Until now, this poor *in vivo* stability limited their effectiveness after implantation and, to date, it is still not clear whether their response can be used for long-term continuous glucose monitoring [1–4]. Deactivation mechanisms have been related to sensor properties, biofouling, actions of inflammatory cells, and the tissue response to the implanted sensor [2, 5–7]. The exact reason for the long-term failure of subcutaneously implanted glucose sensors, however, has not yet been elucidated.

To investigate the mechanisms involved in the deactivation of implanted sensors, a model is needed in which the sensor can easily be changed. Ideally, this should occur without influencing the environment surrounding the sensor and without inducing a trauma upon re-insertion. A percutaneous device (PD), which provides a permanent connection between the exterior and interior of the body, can perhaps solve the above mentioned problem [8]. As carrier of an implantable sensor, such a device could provide an excellent means of examining a functioning sensor *in vivo*. It exposes the sensor to tissue fluid and inflammatory cells, but protects the implant from direct contact with healing subcutaneous tissue. In addition, this system also allows the periodical withdrawl of tissue fluid for glucose measurements.

In a previous experiment we already investigated glucose kinetics in subcutaneous tissue fluid collected with a PD containing a subcutaneous tissue chamber. We found that the use of porous membranes to separate the interior of the chamber from the subcutaneous tissue should be avoided. Obstruction of these membranes resulted in an additional barrier to the diffusion of glucose and prevented rapid adjustment of subcutaneous glucose concentration to changes in glycaemia. In uncovered devices, on the other hand, changes in glycaemia were reflected in subcutaneous tissue fluid.

The objective of this study is to investigate whether the previously developed animal model can be used for *in vivo* measurements with subcutaneously implantable glucose sensors. A percutaneous device containing a subcutaneous tissue chamber was implanted in rabbits. Glucose kinetics in subcutaneous tissue fluid were

*Author to whom correspondence should be sent.

determined after aspiration of tissue fluid from the chamber and with implantable amperometric glucose sensors.

2. Materials and methods

2.1. The percutaneous device

Implantable percutaneous devices as shown in Fig. 1 were used. The devices consisted of a KEL-F body (poly-chloride-trifluoro-ethylene) in which a subcutaneous chamber was formed with a volume of 500 µl connected with a percutaneous part that penetrated the skin. The access to the subcutaneous chamber was closed with a stainless steel screw. A sintered titanium fiber mesh sheet (volumetric porosity 80%, fiber mesh weight 600 g/m^2, fiber diameter 50 µm, Bekaert Fiber Technologies, Belgium) was used for subcutaneous anchorage of the device. In previous experiments, this material showed good biocompatibility and anchorage in soft tissue [9, 10]. The bottom of the device was left uncovered.

2.2. Animals and surgical procedure

The percutaneous devices were inserted in the dorsum of female New Zealand white rabbits ($n = 15$), age three months, weight approximately 2.5–3 kg. One percutaneous device was implanted in each rabbit. Before insertion, the implants were sterilized in an autoclave. Surgery was performed under general anaesthesia by intravenous injection of Hypnorm® (0.5 ml/kg) and atropine (0.5 mg).

The backs of the rabbits were shaved, depilated, washed and disinfected with iodine on both sides of the spinal column. The percutaneous devices were placed using a one-stage implantation procedure, i.e. placement of the subcutaneous part and creation of the percutaneous exit site in the same session. Therefore, paravertebrally a longitudinal incision was made through the full thickness of the skin. Subsequently, lateral to the incision, a subcutaneous pocket was created by blunt dissection with scissors. This was followed by the creation of an exit site lateral of the first longitudinal incision. After placement of the implant, the wound was carefully closed with sutures. To reduce the perioperative infection risk, an antibiotic (Terramicine®) was administered postoperatively by a subcutaneous injection. The animals were separately housed in cages. After surgery, the animals were left for three weeks to allow for wound healing. Guidelines for the care and use of laboratory animals were observed.

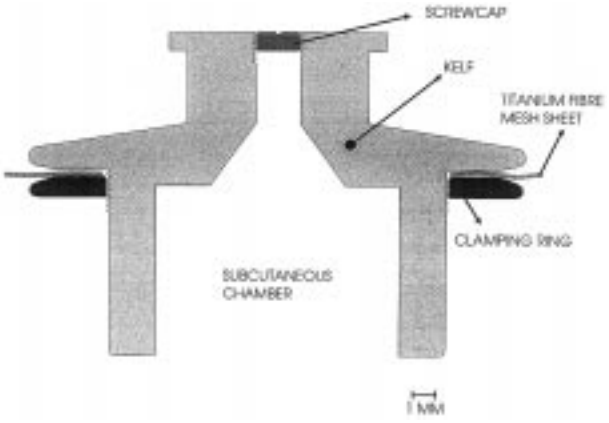

Figure 1 Graphical cross-section of the percutaneous device used in the experiment.

2.3. Preparation and characteristics of the glucose sensor

A platinum wire (diameter 1 mm) and a silver wire (diameter 0.5 mm) were placed in a stainless steel tube (outside diameter 3.175 mm) and sealed with an epoxy resin (Araldite). The probe was polished with lapping film (3 M) to 0.3 µm and sonicated in water for 1 min. After rinsing with aceton/ethanol, silver/silverchloride paint (Acheson Colloiden B.V. Scheemda, The Netherlands) was applied to the silver wire and dried at 333 K for at least 1 h. The probe was poised at 1.1 V vs Ag/AgCl for 15 min in 0.1 M HCl and subsequently placed in 0.1 M phosphate buffered solution (PBS, pH 7.4, room temperature), containing 15 MM 1,3–diamino benzene, 15 MM resorcinol [11], and 4 mg/ml Glucose oxidase ((E.C. 1.1.3.4) type II (265.8 IU/mg) from Aspergillus niger, Genzyme) for 15 min. Ten scans from 0 to 0.8 V vs Ag/AgCl at a rate of 2 mV/sec were applied to immobilize the enzyme using an autolab PGSTAT–10 (Eco Chemie, The Netherlands). For obtaining a coated sensor, the probe was dipcoated at 278 K in a 2.5 wt % cellulose acetate solution in aceton/Nafion (1 : 1) and stored for 10 min. After dipping in 5% Nafion and drying for 10 min the probe was stored in PBS at 278 K when not in use. The probe was placed in 0.1 M PBS (pH 7.4) and a potential of 700 mV vs Ag/AgCl was applied using an Antec EC Controller or a home-built potentiostat. After the background current diminished, glucose was added. The response times of both uncoated and coated sensors, determined as rise to 95% response for a concentration step from 0 to 5 MM glucose, were < 1 min. The sensors exposed a linear response range from 0 to 15 MM glucose. When not used, the sensor was stored at 278 K.

2.4. *In vitro* sensor evaluation

The *in vitro* sensitivity to glucose of both uncoated and coated sensors was determined at 700 mV vs Ag/AgCl in PBS (0.1 M, pH 7.4, room temperature) at 5 MM glucose before implantation and immediately after use in the percutaneous device. Sensitivity to glucose was also determined in fetal bovine serum (Gibco) and subcutaneous tissue fluid collected from the percutaneous device. The influence of oxygen tension on the performance of the cellulose acetate/Nafion coated sensor was evaluated *in vitro* with a Clark-type oxygen sensor [12]. The sensor was placed in an air-saturated PBS with a glucose concentration of 5 mmol/1. After application of 700 mV vs Ag/AgCl, sensor current was determined. Argon was bubbled through the solution until a zero oxygen concentration had been reached. Subsequently, air was bubbled through the solution until saturation of the oxygen concentration. In both situa-

tions, simultaneous recording of the sensor current and the oxygen tension was performed.

2.5. Direct collection of tissue fluid

Glucose kinetics in collected subcutaneous interstitial fluid were investigated. Experiments were performed in duplicate. At least 24 h before each experiment, the devices were filled with sterile 0.9% NaCl to facilitate glucose diffusion into the subcutaneous chamber. The animals were sedated by intramuscular injection of 0.8 ml Hypnorm®. After removal of the stainless steel screw, the chamber was accessed through the percutaneous part of the device. Samples of approximately 20 µl were collected using sterile capillary tubes (Ciba Corning Diagnostics Limited, UK). The sampled volume was not replaced with saline. Only clear samples, free of haemoglobin, were used for analysis. Upon microbial examination, the tissue fluid was shown to be sterile. Blood glucose levels were elevated by subcutaneous injection of 6 µg octreotide and intramuscular injection of 0.15 mg glucagon. At regular intervals up to 3 h after injection, subcutaneous glucose concentrations were measured and related to glycaemia. Therefore, blood samples were obtained from a cutaneous aural vein. Glucose analysis in tissue fluid was performed by an APEC glucose analyzer (Stam Instruments B.V., The Netherlands). Blood glucose measurements were performed using a Gluco-Touch portable blood glucose meter (Lifescan, Belgium).

2.6. *In vivo* sensor evaluation

The *in vivo* sensitivities of both uncoated and coated probes were determined in duplicate in each animal. The measurements with uncoated and coated probes were performed one to three months and four to five months after implantation, respectively. Before implantation, both uncoated and cellulose acetate/Nafion coated probes were sterilized in 70% ethanol for 45 min before usage *in vivo*. During the experiments the animals were sedated by intramuscular injection of 0.8 ml Hypnorm®. After removal of the stainless steel screw, subcutaneous tissue fluid was sampled for glucose measurements. Basal blood glucose concentrations were also determined. Subsequently, sensors were placed in the subcutaneous chamber at the diffusional interface of subcutaneous tissue and tissue fluid. To achieve this, the sensors were carefully lowered into the chamber until resistance was felt. Because of a precise fit in the percutaneous access, fixation of the sensor during the measurements was warranted. After a stabilization period of 20 min, blood glucose levels were elevated by subcutaneous injection of 6 µg octreotide and intramuscular injection of 0.15 mg glucagon. Sensor currents were monitored up to 90 min after injection. At regular intervals, blood samples were drawn to determine blood glucose concentrations. The *in vivo* lag-time corresponds to the time after injection of glucagon and octreotide that an increase in sensor current was observed. After removal of the probes, subcutaneous tissue fluid was sampled for glucose measurements. Results are given as mean and standard deviation. Sensor characteristics, blood and tissue glucose values were compared by paired t-test.

2.7. Histologic procedure and evaluation

Six months after the start of the experiment the animals were sacrificed using an overdose of Nembutal®. The implants with their surrounding tissues were excized immediately and fixated in 4% buffered formalin. After dehydration, excess tissue was removed and the samples were embedded in methyl methacrylate (MMA). After polymerization, histologic sections of approximately 10 µm thickness were prepared using a sawing microtome [13, 14], stained with methylene blue and basic fuchsin and investigated with light microscopy. The tissue reaction to the percutaneous device was evaluated histologically by examining the epithelial attachment and the quality of the tissue inside the titanium fiber mesh. The quality of the fibrous capsule covering the access to the subcutaneous chamber was also examined.

3. Results

3.1. Macroscopic clinical findings

Four rabbits had to be sacrificed during the experiment because of the appearance of a pasteurella abcess underneath the implant. The other animals appeared in good health during the six-month implantation period ($n = 11$). All implants showed good healing with the surrounding soft-tissue with various degrees of limited epithelial migration. No clinical signs of inflammation were observed.

3.2. Direct collection of tissue fluid

The glucose concentration in the subcutaneous chamber was monitored at regular intervals up to 3 h after injection of octreotide and glucagon. In one animal the experiment could not be performed because of a persistent infection inside the subcutaneous chamber. In the remaining 10 animals duplicate measurements were performed. In these 20 experiments, in all cases a clear and prolonged increase in glycaemia was observed, which was followed by an increase in the tissue fluid glucose concentration. The subsequent decrease of the blood glucose concentration was also reflected in the tissue fluid. An example is shown in Fig. 2.

3.3. *In vitro* sensor evaluation

Table I shows the *in vitro* sensitivities of both uncoated and coated sensors in PBS, fetal bovine serum, and sampled subcutaneous tissue fluid. Sensitivity of uncoated probes to glucose before and directly after implantation was $29.8 \pm 13.8\,\mathrm{nA/mM}$ and $10.8 \pm 6.3\,\mathrm{nA/mM}$, respectively ($p < 0.01$) in PBS. The sensitivity of all explanted probes gradually returned to pre-implantation values within 24 h after explantation. The sensitivity of coated sensors was lower, 8.7 ± 8.2 before vs $3.1 \pm 2.5\,\mathrm{nA/mM}$ directly after explantation ($p < 0.01$). Both uncoated and cellulose acetate/Nafion coated sensors produced stable currents in fetal bovine serum and in sampled subcutaneous tissue fluid. The

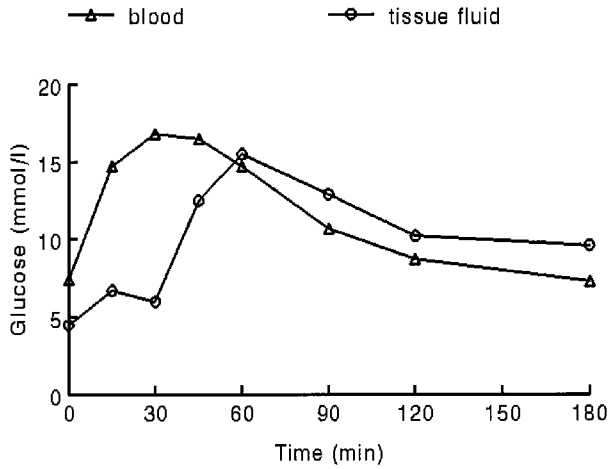

Figure 2 Blood glucose and subcutaneous tissue fluid glucose concentration after injection of octreotide and glucagon. Example of tissue chamber in which the increase as well as the decrease in glycaemia was reflected in the subcutaneous tissue fluid.

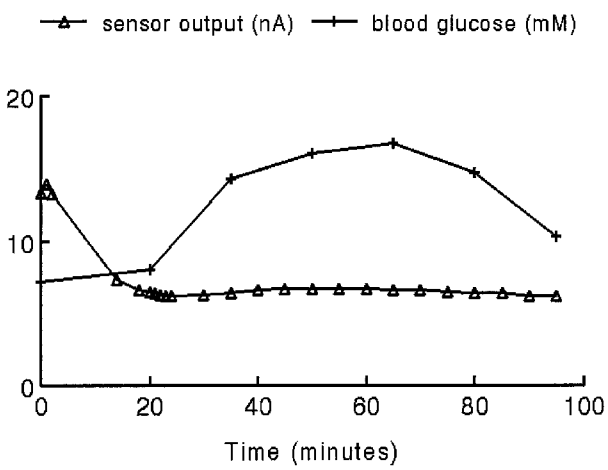

Figure 3 Blood glucose concentration and output of an uncoated sensor after injection of octreotide and glucagon. A decrease in sensor current to background levels was observed after implantation in the percutaneous device, despite an increase in tissue fluid glucose.

sensitivity of uncoated sensors was 14.7 ± 5.6 nA/MM in serum and 4.7 ± 1.7 nA/MM in subcutaneous tissue fluid. Coated sensors showed lower sensitivities in these media, 1.02 ± 0.67 nA/MM and 0.50 ± 0.49 nA/MM, respectively. Measurements of the oxygen dependence of cellulose acetate/Nafion coated glucose sensors revealed that sensor currents were not influenced by oxygen tensions above 25 mm Hg.

3.4. *In vivo* sensor evaluation

The response of both uncoated and cellulose acetate/Nafion coated probes was evaluated *in vivo*. In one animal the sensor measurements could not be performed because of the earlier mentioned persistent infection inside the subcutaneous chamber. In the remaining 10 animals, duplicate measurements were performed. A significant increase in blood glucose concentration was obtained by injection of glucagon and octreotide, 5.8 ± 0.7 mmol/l at implantation vs 14.6 ± 3.1 mmol/l at 45 min after injection ($p < 0.01$). This increase was followed by an increase in sampled tissue fluid glucose from 3.6 ± 3.0 mmol/l before implantation to 7.2 ± 4.2 mmol/l immediately after explantation for the uncoated sensors ($p < 0.05$). In only two experiments was a response of the implanted sensor observed with lag-times of 5 and 8 min and *in vivo* sensitivities of 0.4 and 0.25 nA/MM. In the other cases, sensor output gradually decreased to background currents. Fig. 3 shows an example of a non-responding sensor. For the coated sensors, the increase in glycaemia resulted in an increase of tissue fluid glucose from 0.9 ± 0.5 mmol/l before implantation to 3.8 ± 2.5 mmol/l after implantation ($p < 0.05$). This time, five responses of implanted sensors were observed with lag-times ranging from 5 to 50 min. *In vivo* sensitivities ranged from 0.1 to 9 nA/MM. Fig. 4 shows the response curve of one of the coated sensors. In the other cases, sensor current stabilized at values well above basal currents.

3.5. Histological evaluation

In one animal with a persistent bacterial infection inside the subcutaneous chamber, the tissue reaction was not evaluated. Light microscopic evaluation of the tissue surrounding the other 10 percutaneous devices revealed that the tissue reaction was relatively uniform. Only limited epithelial downgrowth was observed. The titanium fiber mesh sheets were almost all filled with immature connective tissue, free of inflammation. In one specimen, an inflammatory reaction was seen inside the titanium fiber mesh. This reaction was characterized by the presence of large numbers of inflammatory cells, but was not related to the presence of infection in the subcutaneous chamber. Further, all the implants were surrounded by a relatively thin fibrous capsule with a thickness of approximately 15 to 20 layers of fibroblasts, containing small blood vessels and few inflammatory cells (Fig. 5). Accumulations of inflammatory cells were observed inside the subcutaneous chamber in every specimen (Fig. 6).

TABLE I *In vitro* characteristics of uncoated and cellulose/acetate coated first-generation sensors in PBS: pre-and post-implantation, bovine calf serum, and subcutaneous tissue fluid. Sensitivity to glucose in nA/mM

Probe	PBS: pre-implantation	PBS: post-implantation	Bovine calf serum	Subcutaneous tissue fluid
Uncoated	29.8 ± 13.8 (n = 21)	10.8 ± 6.3 (n = 13)	14.7 ± 5.6 (n = 4)	4.7 ± 1.7 (n = 3)
Coated	8.7 ± 8.2 (n = 19)	3.1 ± 2.5 (n = 20)	1.0 ± 0.67 (n = 8)	0.50 ± 0.49 (n = 3)

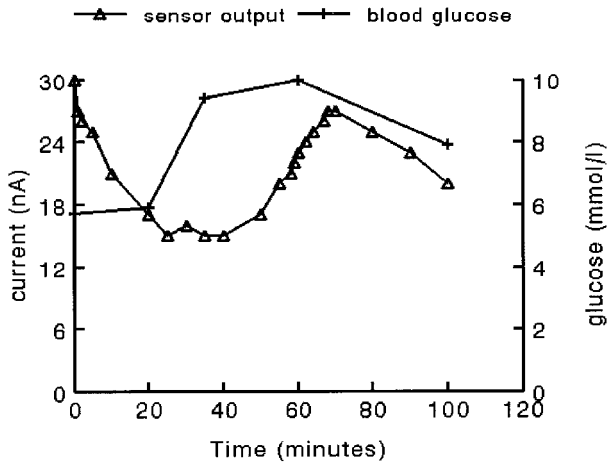

Figure 4 Blood glucose concentration and output of a coated sensor after injection of octreotide and glucagon. A response of the implanted sensor was observed with a lag-time of approximately 20 min and an *in vivo* sensitivity to glucose of 2.8 nA/mM.

Figure 5 Histological cross-section showing the formation of a thin fibrous capsule containing small blood vessels and few inflammatory cells around a percutaneous device. Accumulations of inflammatory cells (arrow) are observed inside the subcutaneous chamber (A). Original magnification × 267, bar 37 μm.

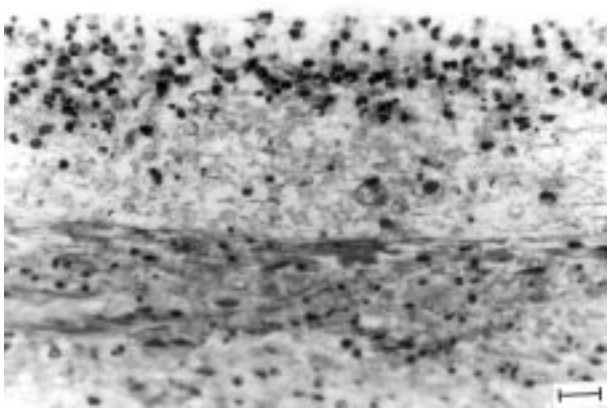

Figure 6 Histological cross-section showing in detail the accumulation of inflammatory cells inside the subcutaneous chamber. Original magnification × 600, bar 17 μm.

4. Discussion and conclusions

The results of this study again confirmed that changes in blood glucose concentration can be measured in subcutaneous tissue fluid collected with a percutaneous device. On the other hand, our implanted glucose sensors could not reliably monitor these changes. With both uncoated and coated first generation glucose sensors only a limited number of responses were observed during the *in vivo* measurements. Still, there was a difference. In contrast to uncoated sensors, the output of coated ones did not decrease to background currents upon implantation. This difference in *in vivo* behavior of uncoated and coated sensors can be attributed to the use of the cellulose acetate/Nafion membranes.

Post-implantation *in vitro* behavior of the sensor can often reveal the source of bioinstability [2]. For both uncoated as well as coated sensors, we found that the *in vitro* sensitivity of explanted probes was significantly lower than before implantation. However, sensitivity returned to pre-implantation values in buffered glucose solutions. This phenomenon is also reported by others [15–21]. As confirmed by Fraser [2], it indicates a reversible deactivation of the sensor by protein and cell adsorption, inhibition of peroxide oxidation at the electrode, or oxygen limitation. Oxygen limitation of the enzyme reaction is not very likely in our study. *In vitro* experiments proved that the sensor output was only affected by very low oxygen tensions. In addition, the percutaneous access allows diffusion of adequate quantities of oxygen into the subcutaneous chamber. Apparently, adsorption of leukocytes and proteins blocked the outer membrane in a reversible way thus preventing glucose from entering the sensor. Activation of the leukocytes by the presence of a foreign body in the chamber further increases adherence, resulting in secretion of reactive oxygen species and proteolytic enzymes [22]. Damage induced by these highly reactive oxygen radicals can be excluded in this particular experiment, because it would have resulted in an irreversible deactivation of the sensor. The question remains then to what extent protein adsorption influenced sensor output in our experiment. Currently, we are unable to reveal the exact nature of this process. For example, lower but still sufficient sensitivity in fetal bovine serum and sampled tissue fluid indicates that protein deposition is not the only deactivating mechanism involved. Despite their poor *in vivo* behavior, we have to emphasize that the sensors used were able to measure changes in glucose concentration in tissue fluid collected from the subcutaneous chamber as shown in the *in vitro* part of our experiments. We know from other studies [23–26] that biomaterials placed in so-called tissue cages evoke an inflammatory cellular reaction. The percutaneous device used can also be regarded as a tissue cage. Consequently, we assume that the implantation of a glucose sensor in the subcutaneous chamber triggers a cellular reaction that negatively influences the sensors *in vivo* performance [22, 27, 28]. Our histological results confirm this theory, since we observed leukocytes in the subcutaneous chamber. Besides, preliminary data from *in vitro* experiments to test the possible influence of leukocytes on sensor behavior do also support this hypothesis. Nevertheless, more research has to be done to elucidate the exact mechanism of sensor deactivation by leukocytes.

Another remarkable finding was that the tissue fluid glucose concentration was significantly lower during

measurements with the coated sensors than with the uncoated ones. Rebrin *et al*. [29] suggested that an inflammatory tissue reaction at the measuring compartment alters the effective glucose concentration. Consequently, the presence of inflammatory cells as observed in the subcutaneous chamber could have resulted in these lower tissue fluid glucose concentrations. It is known from other studies that hollow implants promote the influx and persistence of leukocytes in the interior of the implant [27, 28]. In addition, since the measurements were performed in a consecutive way, it can be expected that the repetitve insertion of our sensors, first uncoated sensors followed by coated ones, contributed to the increase of inflammatory cells in the subcutaneous chamber. Further, the diffusional properties of the fibrous capsule surrounding the subcutaneous chamber could have changed over the implantation period. Alterations in density and vascularity during maturation of the capsule are likely to have resulted in slower diffusion rates, and thus in lower glucose concentrations, during the measurements with the coated probes [30].

In conclusion, our results again show that changes in blood glucose concentration can be measured in subcutaneous tissue fluid collected with a percutaneous device. Unfortunately, our amperometric glucose sensors placed in the percutaneous device could not reliably monitor these changes. We hypothesize that a cellular reaction to the sensor plays an important role in the poor *in vivo* sensor performance. In combination with adsorption of tissue fluid proteins, this results in a reversible deactivation of implanted glucose sensors. The exact mechanisms involved in this process are currently unknown and need further investigation.

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