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ORIGINAL ARTICLES

Fiber-Coupled Fluorescence Affinity Sensor for 3-Day *in Vivo* Glucose Sensing

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Abstract

Background:

To evaluate the feasibility of an implantable fiber-coupled fluorescence affinity sensor (FAS) for glucose monitoring in humans, we studied the acute and chronic *in vivo* performance in hairless rats and pigs.

Methods:

The implantable fiber-coupled FAS was constructed by filling a dialysis chamber made of a regenerated cellulose membrane mounted to the distal tip of an optical fiber with fluorescent chemistry based on concanavalin A. Blood sugar changes in animals were induced by injections of insulin and dextrose. Determination of interstitial glucose concentrations in skin tissue was facilitated by measuring the fluorescence response of the FAS.

Results:

The acute *in vivo* response of the fiber-coupled FAS exhibited good correlation coefficients (>0.77) with blood sugar changes and minimal lag times (2–10 min) after 2 hours of sensor implantation. Equilibrium of the sensor signal with interstitial fluid was required less than 60 min after implantation. For both rats and pigs, chronic response of the FAS to blood sugar modulations measured during the third day of implantation successfully demonstrated proof-of-concept for short-term glucose monitoring. A slight decrease in sensitivity after 3 days in the small animal model was assumed to be caused by excessive mechanical forces on the implanted device because of high animal motility.

Conclusions:

Overall, the chronic *in vivo* performance of the FAS in two different animal models over 3 days was clinically acceptable and comparable to other continuous glucose monitoring platforms. The major benefit of the FAS is the absence of "autodestructive" side products and any device-related warm-up time after sensor reconnection.

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Abbreviations: (CGMS) continuous glucose monitoring system, (Con A) concanavalin A, (FRET) fluorescence resonance energy transfer, (FAS) fluorescence affinity sensor, (IP) intraperitoneally, (ISF) interstitial fluid, (POF) polymer optical fiber, (STS) short-term sensor

Keywords: affinity, concanavalin A, dextran, diabetes, fluorescence, glucose monitoring, hollow fiber

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Introduction

Diabetes therapy is currently at a turning point, indicative of recent technical and commercial advances.[1] Despite the fact that finger-stick blood glucose sensing will remain the method of choice for the majority of people with diabetes in the coming years, short-term sensors for interstitial glucose monitoring in type I and II diabetes have been gaining significant momentum and will surely gain wider acceptance levels.[2] However, their success will ultimately depend on various factors related to the functionality and reliability of commercially available glucose monitoring systems. For example, the continuous glucose monitoring system (CGMS) System Gold from MiniMed/Medtronic and the short-term sensor (STS) from Dexcom, along with the FreeStyle Navigator continuous glucose monitor by Therasense/Abbott (Food and Drug Administration approval pending), are the most advanced sensor systems to date.[3–6] All three systems are based on a disposable electroenzymatic sensing platform, relying on the amperometric detection of glucose by glucose oxidase immobilized to an electrode. Both the MiniMed/Medtronic CGMS and the STS Dexcom sensor require several calibrations per day for reliable blood glucose detection. More importantly, both sensors require up to a 2-hour warm-up period immediately after implantation or, in the case of the MiniMed/Medtronic CGMS sensor, when the sensor is reconnected to the readout unit (e.g., after bathing). It remains to be seen how these devices will be accepted if they require extended warm-up periods, frequent "finger stick" calibrations, and/or if their performance is unreliable because of shifts in sensor sensitivity.

Over the last several years, research into fluorescence affinity sensors (FAS) for glucose detection has steadily gained acceptance among scientists and clinicians through the introduction of a number of improved glucose-sensitive assays based on either glucose-specific protein concanavalin A (Con A)[7–22] or borate-based artificial glucose-specific receptors.[23,24] Independent of the type of receptor, there are several intrinsic advantages of a fluorescence affinity sensor over electrode-enzymatic sensors in terms of practicality for *in vivo* sensing. For one, the light-based signal detection system does not suffer from the need for warm-up time when the sensor is disconnected from the readout unit, as the light-based signal is immediately available for glucose determination. Second, the absence of an electrode-based system eliminates potential interferences of electrode-active components that may enter interstitial fluid (ISF), such as acetaminophens. Third, the nature of affine interactions in receptor-based sensors eliminates the occurrence of "autodestructive" side products, e.g., hydrogen peroxide, as produced by electroenzymatic sensors. Last but not least, the binding reaction in affinity-based sensors is equilibrium driven, resulting in a signal sensitivity that is independent on the rate of glucose diffusion into the sensor. This is an advantage when compared to electroenzymatic sensors, which are consumptive and, hence, their signal is rate dependent.

Since 2002 our group has made fast and steady progress toward improvements and optimization of a Con A-based fluorescence affinity sensor for *in vivo* glucose monitoring.[25,26] To briefly review our progress, which we have reported previously,[25] we demonstrated significant improvements in the chemical stability of the FAS over earlier Con A-based sensors by immobilizing Con A to a macroporous hydrogel, such as Sepharose, which eliminates precipitation of Con A and increased *in vitro* functionality of the FAS over a time period from 3 to 6 months at 37°C. The thermostability of bare Con A Sepharose at 37°C was maintained over 450 days with only 20% loss of activity. In addition to our research on studying a transdermal FAS for long-term glucose monitoring in type I and II diabetes,[26] we have been concentrating our interest on developing a fiber-coupled FAS for short-term (3–5 days) interstitial glucose monitoring. The basic design of this prototype sensor is facilitated by interrogating a hollow dialysis fiber containing the fluorescent Con A-based assay with an optical fiber (see **Figure 1**). This concept was first described by Schultz and co-workers,[7] who demonstrated measurement of blood sugar in the jugular vein of a dog. However, their sensor chemistry was ill-suited for longer *in vivo* interrogation as a consequence of strong photobleaching of fluorescent dyes (fluorescein-dextran) and elaborate and inferior assay chemistry. In contrast, we have employed much brighter and more photostable dyes (Alexa 647 and Alexa 750), enabling us to detect fluorescence with off-the-shelf photodetectors instead of cumbersome and power-consuming photomultipliers. **Figure 2** illustrates the mechanism of fluorescence signal generation in the sensor. The absorption spectrum of the acceptor chromophore overlaps with the fluorescence emission spectrum of the donor. When the donor dye bound to the glucose analogue (dextran) is in proximity to the acceptor dye attached to immobilized Con A, the fluorescence decreases because of fluorescence resonance energy transfer (FRET) (see **Figures 2A** and **2B**.) Upon diffusion of fluorescent dextran from the Con A after

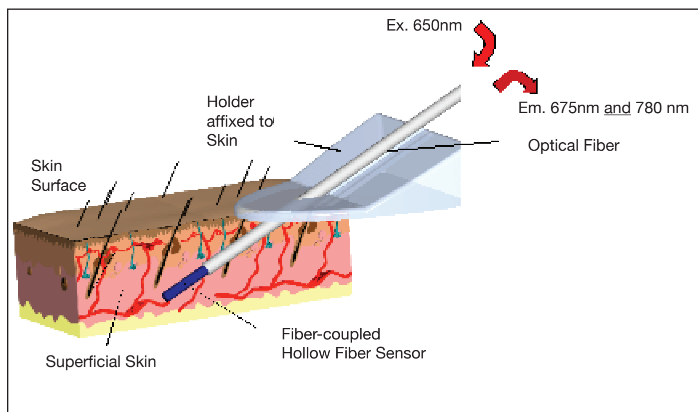

Figure 1. Conceptual illustration of a fiber-coupled fluorescence affinity sensor (FAS). The actual sensor device, which is attached to the distal tip of an optical fiber, resides in the subdermal tissue that is filled with interstitial fluid and is well vascularized. A hollow fiber made of a cellulose acetate membrane provides a housing that allows glucose to diffuse through but prevents Con A, dextran, or beads from leaking out of the sensor. The presence of glucose leads to an increase in sensor fluorescence, which is measured by guiding laser light through the optical fiber into the sensor and measuring the fluorescence returning back through the same fiber with a linear charge-coupled device array spectrometer.

competitive displacement by glucose, an increase in fluorescence is observed (see **Figures 2C** and **2D**). The signal ratio of FRET-based glucose-sensitive fluorescence to the reference dye provides a light-intensity independent sensor output.

This article assesses the feasibility of using the fiber-coupled FAS for 3-day glucose monitoring in *in vivo* experiments in small and large animal models.

Materials and Methods

Preparation of FAS Assay Suspension

The procedure was described in another paper.[25] Briefly, Alexa 750 succinimidyl ester (Invitrogen, Carlsbad, CA) was conjugated to Con A Sepharose (GE Bioscience). Alexa 647 succinimidyl ester (Invitrogen) was conjugated to 70,000 Da amino-dextran (Invitrogen). Conjugation reactions were performed according to the supplier's protocol. The conjugation buffer was 12 m*M* phosphate-

Figure 2. Mechanism of fluorescence signal generation in the FAS. The absorption spectrum of the acceptor chromophore (Alexa 750) overlaps with the fluorescence emission spectrum of the donor. When the donor dye bound to the glucose analogue (dextran) is in proximity to the acceptor dye attached to immobilized Con A, the fluorescence decreases because of FRET (see **A** and **B**). Upon diffusion of fluorescent dextran from the Con A after competitive displacement by glucose, an increase in fluorescence is observed (see **C** and **D**). The signal ratio of the FRET-based, glucose-sensitive fluorescence to the reference dye provides a light-intensity independent sensor output.

buffered saline solution (pH 7.1, 0.9% NaCl, 1 m*M* $CaCl_2$) without NaN_3. To make a sensor suspension, Alexa 647 dextran was then mixed with Alexa 750 Con A Sepharose at a final concentration of 1 to 2 mg/ml and stored in the dark at 4°C.

Manufacture of Fiber-Coupled FAS

One end of a 175-μm-diameter multimode polymer optical fiber (POF) was mechanically spliced to two 105-μm-diameter silica optical fibers. The proximal ends of the two smaller fibers were terminated with SMA-905 connectors. One of the fibers was attached to a collimated laser diode at 650 nm (Thorlabs, Newton, NJ) and the other was attached to a miniature spectrometer (USB-2000, Ocean Optics, Dunedin, FL). An individual hollow dialysis fiber (diameter 210 μm, length 5 mm) was carefully pushed onto the end of the 175-μm POF. The hollow fiber was then filled with sensing suspension by aspiration and sealed with cyanoacrylate (Loctite) at both ends. An additional bonding sleeve made of thin-walled polyimide tubing was then attached over the junction between the optical fiber and the sensor fiber. A microphotograph of the FAS tip is shown in **Figure 3**. The entire fiber sensor assembly fits inside a 20-gauge hypodermic needle for insertion into skin tissue.

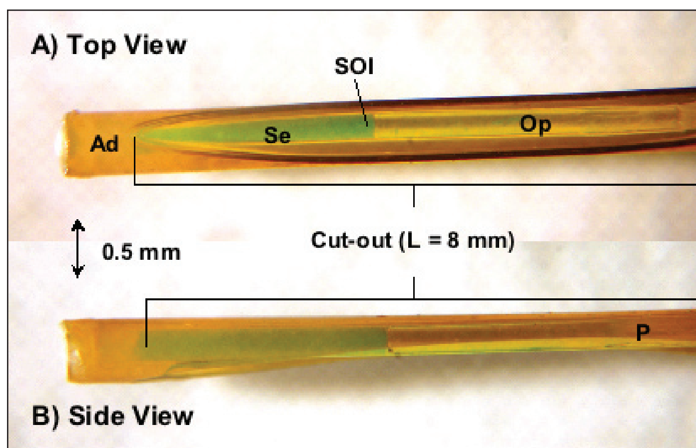

Figure 3. Photomicrography of the distal end of the prototype FAS. The sensor (Se) is mounted on the distal end of an optical fiber (Op) and sealed on its distal and proximal ends with adhesive (Ad). To enhance the physical integrity of sensor, the FAS tip is protected with polyimide tubing having a cut-out length of approximately 8 mm. SOI, sensor/optical fiber interface.

FAS Implantation and Testing in Hairless Rats

Nondiabetic male hairless rats of approximately 300 g were anesthetized and maintained with isoflurane by inhalation. Before implantation the sensors were bathed for 10 min in sterile saline. A 20-gauge hypodermic needle containing the fiber-coupled FAS was inserted intradermally at a shallow angle on the dorsum of the animal 1 to 4 cm from the midline. After the sensor and needle were pushed approximately 2 cm into the skin, the hypodermic needle was entirely withdrawn, leaving the sensor exposed to skin tissue. A MiniMed/Medtronic CGMS sensor (Northridge, CA) was coimplanted in each rat for comparison purposes. After one hour of baseline acquisition by the implanted FAS, "regular" fast-acting insulin (0.5 unit/kg) was administered subcutaneously. After another hour, a bolus glucose injection (50% dextrose, 3 ml/kg) was given intraperitoneally (IP). Serial blood samples from the tail vein were taken approximately every 5 to 10 min over the 2- to 3-h period and measured using a FreeStyle glucometer (Abbott, IL). At the end of the experiment, blood glucose was normalized by an IP bolus injection of 50% dextrose and the animal was returned to its cage. When a 3-day experiment was performed, the sensor sites were protected with bandages and masking tape to prevent sensor rupture or removal by the animal. On day 3, an identical modified glucose tolerance test was performed. At the end of the experiment, the FAS was carefully removed from the site and the animal was returned to its cage.

FAS Implantation and Glucose Testing in Pigs

Juvenile nondiabetic farm pigs (25–30 kg) were preanesthetized with ketamine (1 ml/50 kg), scrubbed thoroughly with povidone and warm water, and then maintained with 1–4% isoflurane by mask. After a saline rinse for 10 min, up to three fiber-coupled FAS were inserted in the upper back using a hypodermic needle as described previously. We implanted four sensors for redundancy and to maximize our chances that sensors would remain in the animals. Sensors were secured with tape and covered with sterile Tegaderm bandages and masking tape when not in use. A MiniMed/Medtronic sensor was coimplanted in each pig for comparison purposes. To modulate blood glucose, 25 ml of 50% dextrose was infused intravenously through an ear vein. Blood samples were collected from a vein in the contralateral ear. On day 3, all sensors remained implanted and one was chosen for monitoring. After the experiments on day 3, sensors were carefully removed and the pig was returned to its cage.

All animal studies were carried out at an Association for Assessment and Accreditations of Laboratory Animal Care-accredited facility and in accordance with an Institutional Animal Care and Use Committee-approved protocol.

Analysis of in Vivo FAS Response

Both the glucose-sensitive emission at 675 nm and the reference dye emission at 780 nm were recorded

simultaneously with a rate of 10–15 measurements h^{-1} and stored on a notebook computer. After each experiment, the 675- and 780-nm signals were analyzed for drift due to photobleaching and normalized accordingly. Normalization was performed by determining the slope of the change in emission at 675 and 780 nm, respectively, before and after the experiment (usually 3–4 hours) at the same glucose concentration. Then the corrected ratio signal was calculated. Glucose concentrations measured by FAS were determined retrospectively. The initial baseline period during which no changes in blood glucose were measured (usually 50 to 60 min) was used for one-point blood glucose calibration. If the one-point glucose calibration reading was not in agreement with the *in vitro* calibration curve for FAS obtained 24 hours before the *in vivo* experiment, the calibration curve was shifted along the *y* axis accordingly with the slope remaining constant. The correlation coefficient *R*, describing the linear relationship between venous blood glucose and FAS signal, was calculated by minimizing the lag time between venous blood glucose and FAS output. MiniMed/Medtronic CGMS data were extracted from MiniMed Solutions CGMS software (MMT-7310; version 3.0B) into Windows XP Excel by a Matlab application (version 4.2c.1, The MathWorks, Inc., Natick, MA).

Results

Operational in Vitro Stability of Fiber-Coupled FAS

In order to validate stable performance of the prototype fiber-coupled FAS over several days, we measured the fluorescence response during random glucose concentrations cycling at 37°C over a period of 3 days. **Figure 4A** shows the ratio response measured at the same glucose concentration over 3 days. The corresponding calibration curve shown in **Figure 4B** has a linear slope ranging from 0 to 540 mg/dl glucose and a correlation coefficient of 0.99. The sensor showed excellent signal stability during the test period. The day-to-day variation of the ratio signal did not exceed 3%.

Acute and Chronic in Vivo Response of Fiber-Coupled FAS in Small Animal Model

Results of four independent animal experiments in anesthetized hairless rats are shown in **Figure 5**. **Figures 5A** and **5B** show the performance of two fiber-coupled FAS in two different rats, while **Figures 5C** and **5D** depict the results of two sensors implanted in the same rat. A functional delay associated with a one-hour warm-up period of the MiniMed/Medtronics CGMS is evident in **Figures 5A** and **5B**. However, in **Figures 5C** and **5D**, implantation of the fiber-coupled sensor overlapped with the end of the CGMS warm-up phase. As can be seen in **Figures 5A** and **5B**, the fiber-coupled sensor required less than 60 min of equilibration time after implantation to reach operational functionality. Overall, the ratio response of our prototype FAS correlated very well with changes in blood glucose levels. The correlation coefficients ranged from 0.88 to 0.94 with minimal lag times of less than 5 min.

For an animal carrying the FAS for 3 days, the response is shown in **Figure 6**. The correlation coefficients of 0.94 and 0.81 on days 1 and 3, respectively, indicate good stability of the FAS over the test period. The slightly lower correlation coefficient on day 3 was probably due to

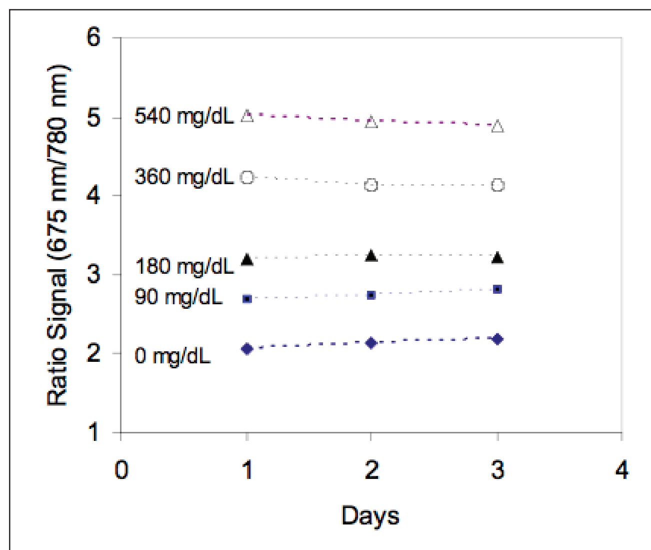

Figure 4A

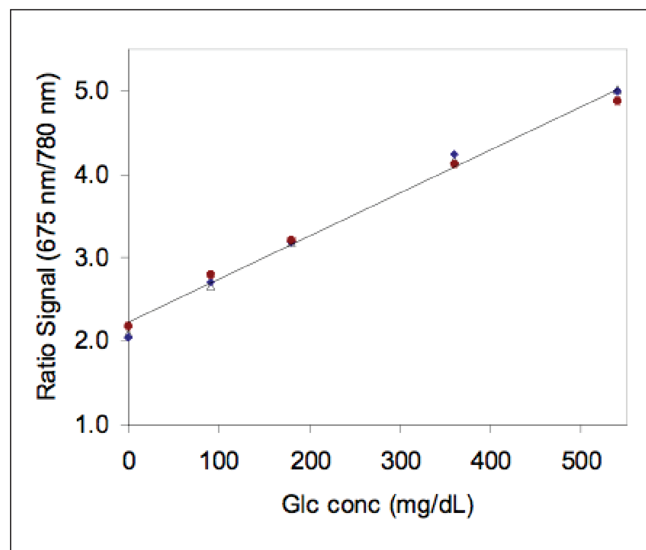

Figure 4B

Figure 4. *In vitro* response of fiber-coupled FAS to physiological glucose concentrations over 3 days (**A**) and corresponding calibration curve (**B**). The line represents linear regression of individual FAS ratio signal data. T = 37 ° C.

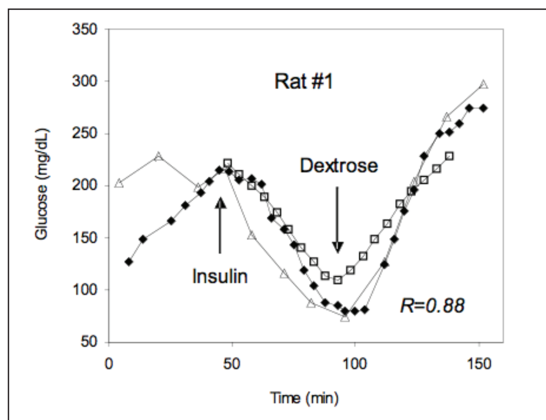

Figure 5A

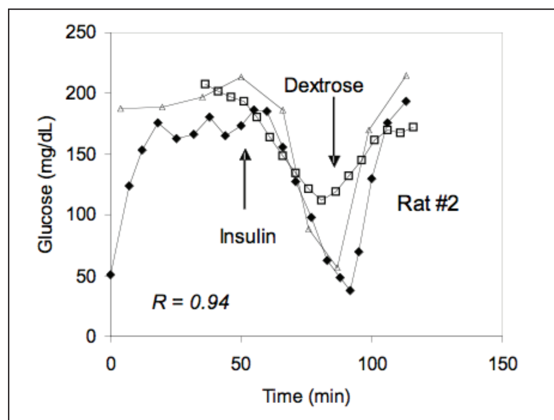

Figure 5B

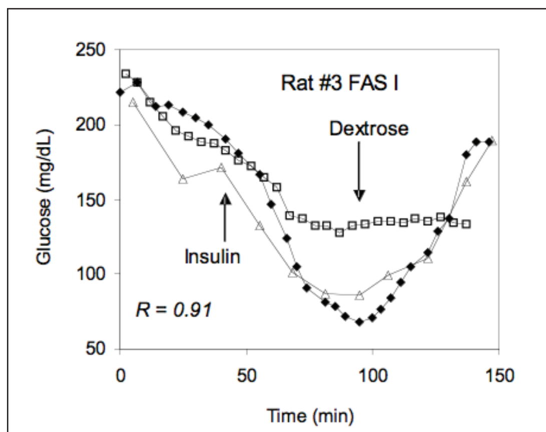

Figure 5C

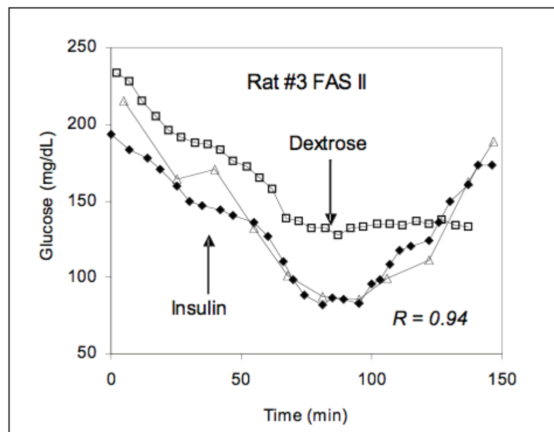

Figure 5D

Figure 5. Acute *in vivo* performance of four different fiber-coupled sensors in hairless rats. ♦, FAS; □, MiniMed/Metronics CGMS sensor; Δ, blood glucose. The time of 0 min on the *x* axis was time of implantation of the fiber-coupled sensor. Arrows indicate introduction of respective blood glucose level modulator (dextrose or insulin). *R* denotes correlation coefficient between blood glucose values and FAS signal. *R* values of MiniMed CGMS are 0.8 (**A**), 0.81 (**B**), and 0.7 (**C** and **D**).

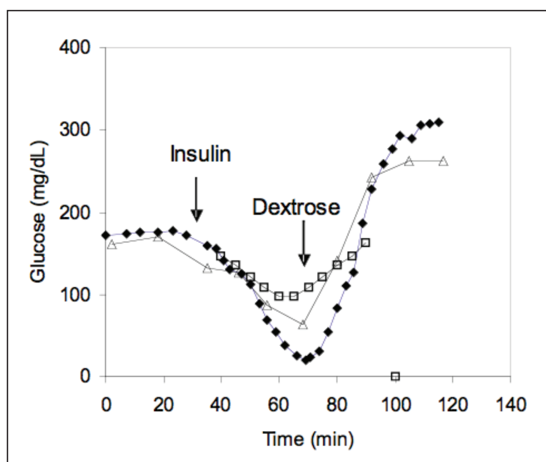

Figure 6A

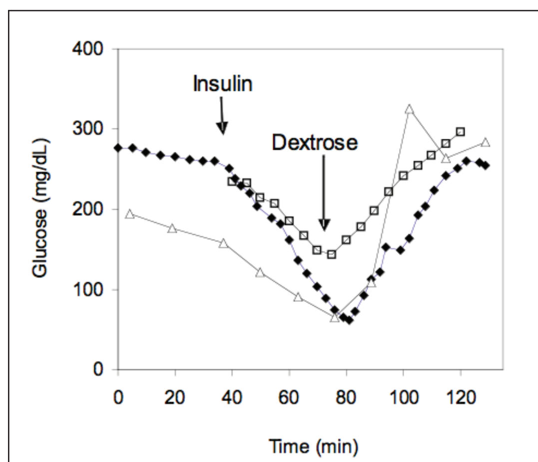

Figure 6B

Figure 6. Stability of FAS response over a 3-day period in a hairless rat. The sensor was implanted on day 1 in an anesthetized rat, and its acute response to variation in blood glucose due to injection of insulin and dextrose was measured (**A**). The animal was allowed to move around freely on day 2. On day 3, the sensor response was tested again (**B**). ♦, FAS; □, MiniMed/Metronics CGMS sensor; Δ, blood glucose. *R* denotes correlation coefficient between blood glucose values and FAS signal. *R* values of MiniMed CGMS are 0.56 (**A**) and 0.75 (**B**).

excessive movement of the implanted sensor by the highly agile animals, which might have led to bending of the flexible FAS sensor chamber relative to the field of view of the optical fiber or perhaps even causing microruptures at the membrane/sealant interface, which may allow material to leak out of the sensing chamber. This, in conjunction with an increase in trauma, might have possibly resulted in attenuation in sensitivity of the sensor.

Acute and Chronic in Vivo Performance of FAS in Large Animal Model

Results of acute implantation and blood glucose tolerance tests of two sensors implanted in two different pigs are shown in **Figure 7**. Approximately one hour after implantation, a bolus injection of dextrose was given via the ear vein, resulting in the large spike in measured blood sugar. In both animals the FAS responded after a short delay (less than 15 min) with an increase in the ratio signal. In the second animal (**Figure 7B**), a second bolus injection of dextrose was given to the animal at 100 min, upon which the FAS responded almost immediately with a delay of 5 min. Because glucose was administered intravenously rather than intraperitoneally in pig experiments, it is not surprising that the interstitially implanted FAS and CGMS sensors showed a dampened response, as the rate of clearance of glucose from blood by the liver may be higher than the rate of glucose diffusion from blood into ISF. However, the experiments showed acceptable correlation of the FAS response with blood glucose changes during the initial period after implantation.

Figure 8 shows results of one sensor during a 3-day implantation experiment. The correlation coefficients of 0.89 and 0.88 for days 1 and 3, respectively, demonstrate good stability of the sensor response over the target period of 3 days. Sensors were generally well tolerated by the animals. Visual assessment of one implantation site after explantation on day 3 showed only slight redness at the insertion site (see **Figure 9**), which subsided after a few days, possibly the result of minor bleeding after FAS insertion on day 1. No evidence of inflammation, irritation, or infection was observed at any of the implantation sites.

Discussion

The objective of this study was to demonstrate feasibility of a fiber-coupled FAS for glucose monitoring over several days in small and large animal models. In general, the stability of the FAS response obtained *in vitro* over 3 days in prior studies was confirmed in *in vivo* studies in rats and pigs. Implanted FAS exhibited good overall correlation with blood sugar manipulations. It is relevant to mention that the successful operation of FAS in pigs is significant, as the pig model is similar to humans in terms of skin anatomy and general physiology. The shift from small animal model to large animal model also improved the rate of success of *in vivo* experiments, as the sensor was much less susceptible to destruction by the animal when implanted in a pig (back). This will enable us to perform ambulatory experiments to assess the accuracy of our device during longer periods of continuous glucose monitoring.

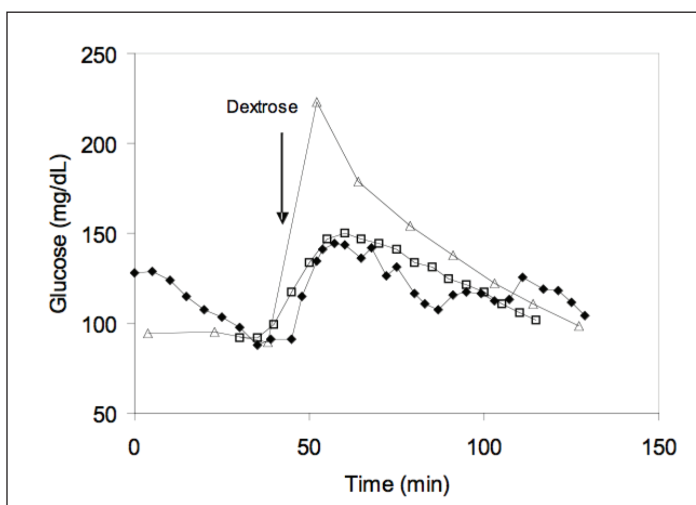

Figure 7A

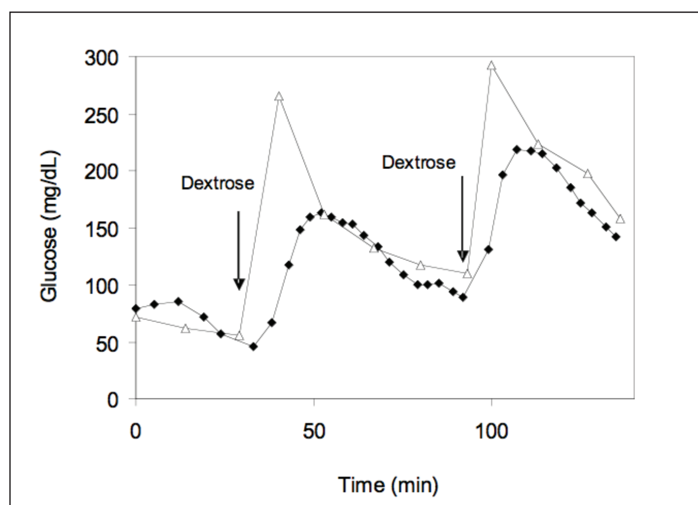

Figure 7B

Figure 7. Acute sensor response of FAS in two different pigs. ♦, FAS; □, MiniMed/Metronics CGMS sensor; Δ, blood glucose. The time of 0 min on the *x* axis was time of implantation of the fiber-coupled sensor. Arrows indicate introduction of respective blood glucose level modulator (dextrose). *R* denotes correlation coefficient between FAS signal and blood glucose. The R value of MiniMed CGMS is 0.68 (**A**).

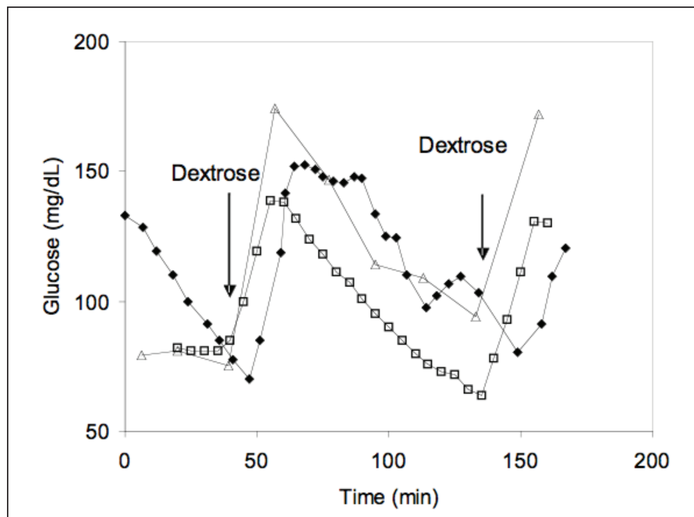

Figure 8A

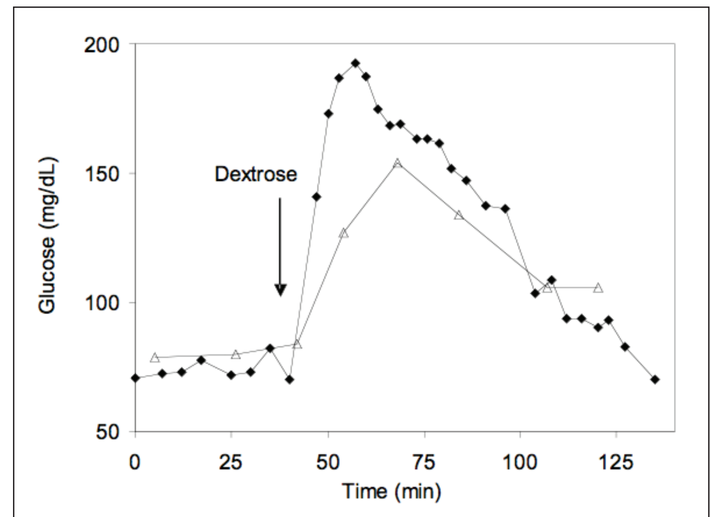

Figure 8B

Figure 8. Stability of FAS response over a 3-day period in a pig. The sensor was implanted on day 1 in an anesthetized pig, and its acute response to variation in blood glucose due to injection of dextrose was measured (**A**). The animal was allowed to move around freely on day 2. On day 3, the sensor response was tested again (**B**). ♦, FAS; Δ, blood glucose. *R* denotes correlation coefficient between blood glucose and FAS signal. The *R* value of MiniMed CGMS is 0.83 (**A**).

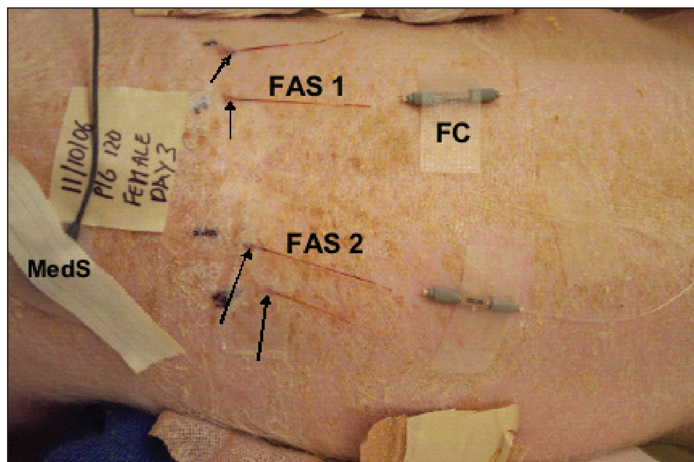

Figure 9. FAS implantation sites in a pig on day 3. A total of four implanted fluorescence affinity sensors (arrows) with two active sensors (FAS 1 and FAS 2) were tested. Insertion sites can be identified by a slight reddish spot (diameter 3 mm), probably the result of slight bleeding after FAS insertion on day 1. The proximal ends of the sensors were connected to the distal ends of bifurcated fibers (Bif) by mechanical fiber splice couplers (FC). MedS, MiniMed/Medtronic sensor.

Our *in vivo* studies demonstrated fast equilibration of the FAS response after implantation. We found that it took on average not more than 60 min and, in some cases, less than 30 min before the sensor signal adjusted to the baseline blood glucose level. These results can be corroborated with results from a study performed with an implanted viscometric affinity sensor with a similar short equilibration time after implantation in humans.[28] This is in noticeable difference to reports of electroenzymatic sensors, which require 1–3 hours of warm-up time or even more due to the need of the enzyme electrode to electrically equilibrate with the ISF. The absence of a warm-up time of the FAS was obvious on day 3 after reconnecting the sensor to the light source and readout device in the large animal experiment (see **Figure 8B**) when the FAS response to glucose was in close approximation to the venous blood glucose levels. Overall, these results strengthen our hypothesis that the intrinsic nature of fluorescence affinity sensors, as explained earlier, appears to be advantageous for *in vivo* glucose monitoring.

The time delay of the FAS in response to blood glucose modulators was modest and did not exceed more than 10 min, even in pigs. We saw very short time lags in rats. In pigs we noticed quite often larger lag times immediately after implantation, which might be due to subacute insertion trauma or other reasons still under investigation. Surely different degrees of vascularization and relative fat content at the implantation site in the pig might have had an impact on the time response of FAS. However, lag times after 100 min of FAS implantation appeared to become progressively shorter (compare **Figures 7B** and **8B**), although more research is needed to confirm this finding further. These preliminary results confirm that initial trauma due to FAS implantation was minimal and that the short-term biocompatibility of the materials of the sensors was more than adequate. In two earlier publications, we also addressed safety concerns over the use of Con A. We demonstrated supportive

experimental and empirical evidence for the absence of systemic toxicity at low Con A doses injected through the subcutaneous route.[29]

This study performed retrospective analysis of the sensor signal obtained *in vivo*. The reason for it was a drift of the sensor ratio signal as a consequence of changes in the fluorescence signal (photobleaching) caused by an excessive illumination regimen (on the average of 10 s per measurement) because of manual data acquisition with our prototype setup. Despite the good correlation of the FAS response to blood glucose, we concede that the one-point calibration model might not have been ideal, considering, for example, the discrepancy of glucose determined by FAS versus venal blood glucose (see **Figure 8B**). Refining the calibration model is under current investigation. However, we believe that real-time glucose monitoring with minimal signal drift is possible by reducing laser power or shortening exposure time to less than one-tenth of a second by implementing automated signal acquisition. By implementing these options, fluorescence-based drift will be minimal during the course of 3 to 5 days, which will enable the FAS to predict blood glucose level changes in real time.

Conclusion

We evaluated the feasibility of a prototype-implantable, fiber-coupled fluorescence affinity sensor for continuous glucose monitoring by studying its acute and chronic *in vivo* performance in hairless rats and pigs. The time lag of the FAS measurements did not exceed 10 min even on day 3 in both animal models. Correlation coefficients of the FAS with blood glucose changes induced by blood sugar manipulations in acute and chronic studies were in the acceptable clinical range. In order to minimize the effect on signal drift of the real-time glucose monitoring FAS, we anticipate reducing light input levels per measurement. Overall, the fiber-coupled FAS performance over a 3-day period compares favorably to other continuous glucose monitoring platforms and indicates its potential value for diabetes therapy.

Acknowledgments:

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