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A label-free fiber-optic Turbidity Affinity Sensor (TAS) for continuous glucose monitoring

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ABSTRACT

In this paper, we describe the concept of a novel implantable fiber-optic Turbidity Affinity Sensor (TAS) and report on the findings of its in-vitro performance for continuous glucose monitoring. The sensing mechanism of the TAS is based on glucose-specific changes in light scattering (turbidity) of a hydrogel suspension consisting of small particles made of crosslinked dextran (Sephadex G100), and a glucose- and mannose-specific binding protein – Concanavalin A (ConA). The binding of ConA to Sephadex particles results in a significant turbidity increase that is much greater than the turbidity contribution by the individual components. The turbidity of the TAS was measured by determining the intensity of light passing through the suspension enclosed within a small semi-permeable hollow fiber (OD: 220 μm, membrane thickness: 20 μm, molecular weight cut-off: 10 kDa) using fiber optics. The intensity of measured light of the TAS was proportional to the glucose concentration over the concentration range from 50 mg/dL to 400 mg/dL in PBS and whole blood at 37 °C ($R > 0.96$). The response time was approximately 4 min. The stability of the glucose response of the TAS decreased only slightly (by 20%) over an 8-day study period at 37 °C. In conclusion, this study demonstrated proof-of-concept of the TAS for interstitial glucose monitoring. Due to the large signal amplitude of the turbidity change, and the lack of need for wavelength-specific emission and excitation filters, a very small, robust and compact TAS device with an extremely short optical pathlength could be feasibly designed and implemented for in-vivo glucose monitoring in people with diabetes.

1. Introduction

The International Diabetes Federation (IDF) has predicted that the prevalence of diabetes will further increase from 6% (246 million people) in 2007 to 7.3% (380 million) in 2025 worldwide. The fastest increase in diabetes prevalence is expected to occur in developing countries. Type 1 diabetes is expected to rise at an alarming rate of 3% each year (Shaw et al., 2010). External restoration of glycemic levels through diet, lifestyle, and insulin therapy have been shown to dramatically reduce associated comorbidities (The Diabetes Control and Complication Trial Research Group, 1993). Tremendous progress has been made with regard to the development of glucose-monitoring technologies, and control algorithms for insulin infusion ("Artificial Pancreas") (Hovorka, 2006, Place et al., 2013, Russell et al., 2012). The current crop of commercially available Continuous Glucose Monitoring Sensors (CGMS – Seven by Dexcom, Paradigm by Medtronic-Minimed) are based on electro-enzymatic detection technology. They require several recalibrations per day to avoid sensor drift, and can be prone to certain electrode-sensitive compounds (e.g. acetaminophen). In order to overcome these issues, novel glucose-sensing technologies are warranted, which may provide a superior robustness, thus safety, while maintaining high accuracy levels during in-vivo operation. This is especially of utmost importance when integrated within a closed-loop system. Various optical affinity-based glucose sensing technologies have been successfully demonstrated as potential alternatives for glucose-monitoring in people with diabetes (Schultz et al., 1982, Ballerstadt et al., 2012, Colvin and Jiang, 2012, Meadows and Schultz, 1993, Romey et al., 2012, Tolosa et al., 1997, Worsley et al., 2007). The shortcomings of most fluorescence-based sensing technologies are potential signal degradation due to gradual destruction of the fluorescent dyes via photobleaching (Ballerstadt et al., 2004), or chemical attack from Reactive Oxygen Species (ROS) (Colvin and Jiang, 2012). To overcome the weakness of the fluorescence sensor approach, our group has demonstrated proof-of-concept of a label-free optical glucose sensor (Ballerstadt et al., 2007a,b). The mechanism was based on measuring glucose-sensitive changes in light scattering (turbidity) with optical coherence tomography (OCT) (Ballerstadt

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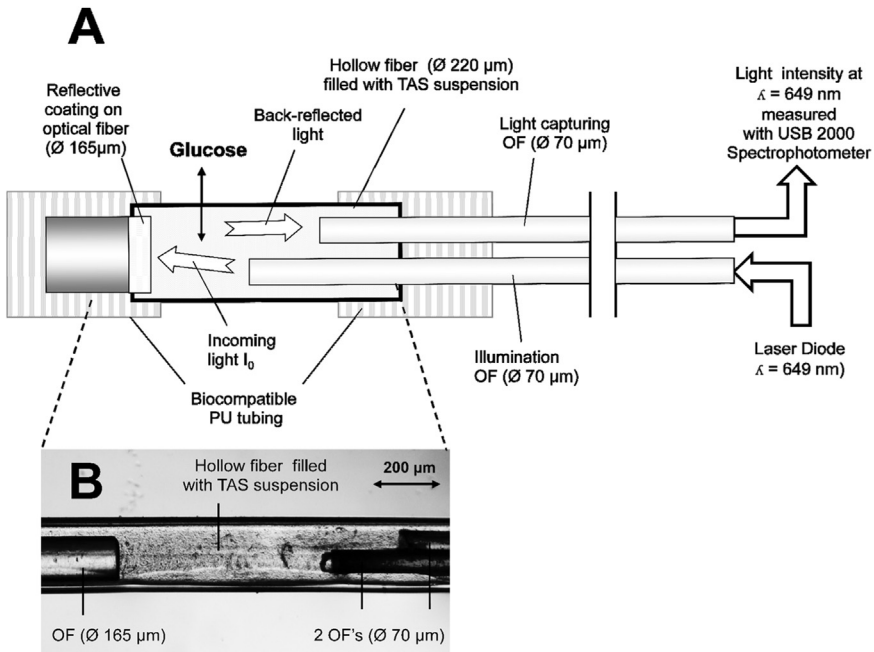

Fig. 1. *Illustration (A) and Photographic Image (B) of the TAS.* See text for further explanation.

et al., 2007a,b). The chemistry was composed of small crosslinked dextran-particles (Sephadex) and the glucose-specific protein Concanavalin A (ConA). The binding of ConA to the hydrogel particles resulted in a light scattering coefficient that was 8 times higher than the sum of the scattering coefficients of the individual components. The addition of glucose led to the reversible decrease in turbidity. Despite the promising data, the complexity of OCT operation to facilitate turbidity measurements through skin can become extremely challenging, because of strong signal attenuation by skin and due to the difficulty to reproducibly implant the turbidity sensor at less than 500–300 µm into subcutaneous tissue. Therfore, a practical solution may still take years. Therefore, in this paper the objective of the study was to simplify the optical interrogation technique by measuring the intensity of light passing through the glucose-sensitive suspension within a small – potentially implantable – semipermeable hollow fiber. At low glucose levels the intensity of light passing through the hydrogel is low due to high light scattering. However, in the presence of high glucose levels, the intensity of light passing through the suspension is high due to low light scattering. The central element of the TAS is a semipermeable hollow fiber containing the ConA/Sephadex hydrogel suspension (see Fig. 1A). The hollow fiber has an outer diameter of 220 µm and a membrane thickness of approximately 20 µm. The molecular weight cutoff is approximately 10 kDa. It allows glucose to diffuse in and out of the lumen of the hollow fiber, but prevents ConA and the hydrogel beads from leaking out. To enable light measurements in a transmission mode, two 70-µm optical-fibers are located inside the hollow fiber – one fiber for coupling incident light into the lumen using a laser diode (λ=649 nm), and the other to couple back the light passing through the TAS suspension when returning from the reflection against the mirror-like surface at the distal end of the hollow fiber. Both optical fibers were positioned apart in order to minimize the background signal by optical "bleeding". A microscopy photographic image of a mock sensor is shown in Fig. 1B. The experiments in this study were designed to establish proof-of-concept data of the TAS by assessing the in-vitro performance of the sensor device under physiological conditions for its potential use as an implantable glucose sensor for people with diabetes.

2. Material and methods

2.1. Chemicals

Concanavalin A (ConA, Type VI, Sigma-Aldrich, St.Louis, MO), Sephadex G100 (fine 20–80 µm, dry), dextrose and phosphate buffered saline (PBS) were purchased from Sigma Aldrich (USA).

2.2. Shredding of Sephadex hydrogel particles

We determined in previous studies (Ballerstadt et al., 2007a,b) that wet hydrogel particles with bead sizes ranging from 1 to 30 µm were optimal for measuring high glucose-dependent light scattering changes of the suspension. To achieve this, Sehadex G100 (dry diameter 20–80 µm) was swollen in distilled water for 2 h. Then 600 µl of the swollen suspension was transferred to 1.5-ml Eppendorf tubes, and 0.19 mg of ZrO*x* beads with an average size 0.15 mm and 0.19 mg with a size of 2 mm were added to the suspension. The tubes were then placed in a bullet blender (Next Advance Inc., Averill Park, NY, USA), and shredded at 4 °C for a total of 20 min. After completion, the shredded Sephadex G100 hydrogel particle were separated from the ZrO*x* beads by gravity and removed from the tube. Shredded hydrogel material was then stored at 4 °C until use.

2.3. Preparation of sensor components

A solution containing ConA was slowly added to a suspension of shredded G100 hydrogel beads to a final ConA-concentration of 20 mg ml^{-1}. To fill short cellulose-based hollow fiber segments (Membrane GmbH, Wuppertal, Germany) at a length of approximately 5 cm with this suspension, dextrose at a concentration of approximately 500 mg/dL was added to the suspension to lower its viscosity for easier filling. The suspension was aspirated with an automatic pipette (tip volume 1–200 µl), and tightly pressed into a 1–10 µl tip which had a short hollow fiber segment adhered by glued to its distal end. By slowly turning the plunger screw on the pipette the hollow fiber was filled with the TAS suspension by visual observation. Then sections filled with the suspension that were free of air-bubbles were cut and sealed with loctite 4013 glue

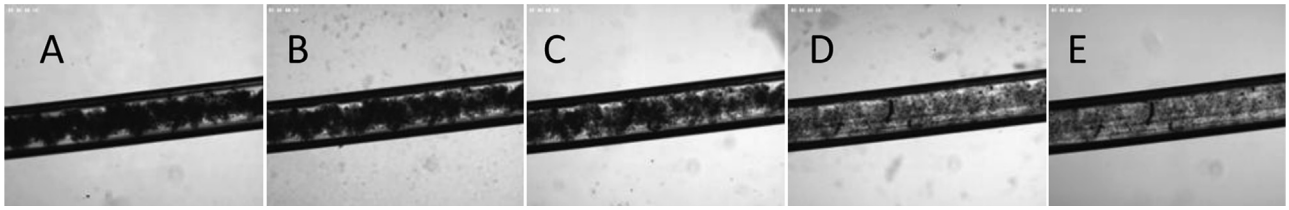

Fig. 2. Microscopy images of the turbidity change of the TAS at gradually increasing glucose concentrations. The turbidity decreases as the results of dissociation of ConA from Sephadex by glucose. The TAS chemistry was enclosed in a 200-μm OD hollow fiber made of cellulose. Glucose concentrations: A – 0 mg/dL, B – 50 mg/dL, C – 100 mg/dL, D – 200 mg/dL, E – 400 mg/dL. Magnification: 10 × . *T*: 25 °C.

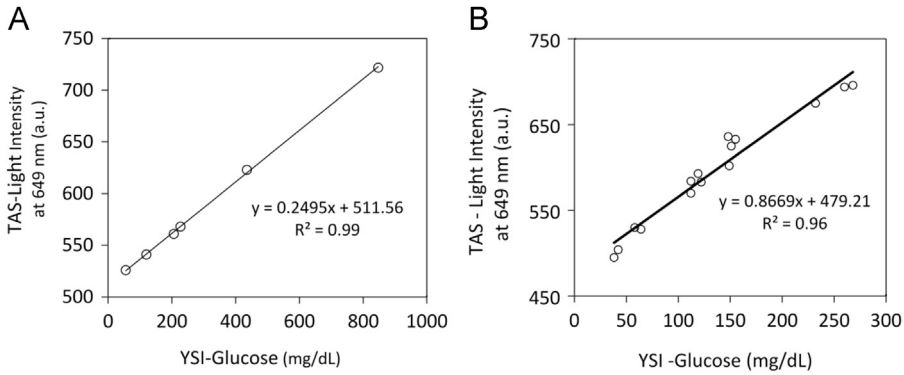

Fig. 3. *Glucose Calibration Curves of TAS.* Sensor I response was measured from 50 to 800 mg/dL glucose (A). Sensor II was measured from 50 to 250 mg/dL (B). *T*=37 °C.

on both ends. The prepared hollow fibers were then incubated for 60 min in excipient solution, and submersed in liquid nitrogen, followed by freeze-drying over 15–20 h. The freeze-dried TAS segments were stored in pouches with silica-bags under vacuum until use.

2.4. Sensor manufacture

The design of the TAS used in this study is shown in Fig. 1A. Freeze-dried hollow fiber segments filled with the TAS suspension were cut to a length of 6–7 mm, and pushed over a short piece of de-cladded optical fiber (length 3–4 mm, OD after de-cladding=165 μm), and sealed with UV-sensitive epoxy-based adhesive (Dymax Corporation, Torrington, CT, USA). 2–3 mm of this optical fiber was then cut off. Two 70-μm optical fibers (Molex Fiber Optics, Downers Grove, IL, USA) were carefully inserted through the other end of the hollow fiber in such a way that the distal end of the one 70-μm fiber was slightly set apart from the distal end of the other fiber by approx. 1–2 mm (see Fig. 1A and B). This is to minimize the effect of light "bleed-over" from the illumination optical fiber to the optical fiber that couples the light back to the light detector. The end of the hollow fiber was then sealed with UV-sensitive adhesive. The end of the one 70-μm optical fiber was linked via SMA-connector to the light source (Laser diode, λ=649 nm), and the other to the light detector (USB2000 spectrophotometer, OceanOptics, Inc., xDunedin, Fl, USA). After sensor assembly was completed, the sensor was placed in degassed phosphate-buffered solution (PBS) containing 400 mg/dL glucose for rehydration. Then the sensor was placed in a flow-through chamber that was connected to a peristaltic water pump and a temperature-controlled heating system. This allowed flushing glucose-containing media at physiological temperatures (37 °C) through the flow cell.

2.5. Glucose response studies

To measure the response of the TAS to glucose, PBS and whole blood were spiked with glucose at various levels and calibrated with the YSI-glucose analyzer (YSI, Inc., Yellow Springs, OH, USA). Sensors were retrospectively calibrated using linear regression analysis. In blood, non-linear polynomial regression analysis was performed. Accuracy was analyzed by calculating mean absolute relative error (MARE).

3. Results

3.1. Glucose response, detection range, and kinetics

In preliminary experiments, the TAS design and chemistry composition was optimized to enable fast sensor kinetics and a large dynamic range. Fig. 2 shows a sequence of photographic images of the turbidity change of the TAS ConA/Sephadex suspension inside a hollow fiber.

The images clearly illustrate the large bulk change in turbidity of the ConA/hydrogel suspension within the physiological range (50–400 mg/dL) of glucose. With the increase in glucose concentration the suspension turns from very opaque to modestly transparent. Also, a slight decrease in packing density with increasing glucose level can be observed (compare Fig. 2A with Fig. 2E). These observations are evidence for the reversible association/dissociation reaction of ConA with terminal glucose residues of shredded G100 particles. In the absence of glucose, ConA forms tight inter- and intra-particle bonds, resulting in a dense ConA/hydrogel network that is also highly viscous. With gradual addition of glucose, those ConA/hydrogel complexes appear to break apart, causing the suspension to take up a larger volume within the lumen of the fiber, and rendering it optically more transparent. Also, the viscosity at higher glucose levels decreased significantly.

To quantify the change in light intensity of the TAS and assess the detection range, we performed glucose response experiments with the sensor shown in Fig. 3A and B over a wide glucose concentration range. Fig. 3A shows the turbidity change measured in PBS from 50 to 800 mg/dL, and Fig. 3B the change from 50 to 250 mg/dL in a different sensor.

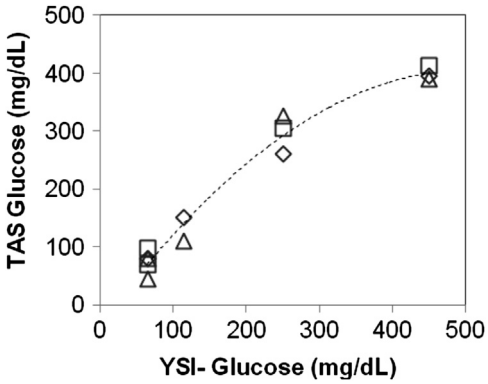

Fig. 4. Glucose calibration curve of TAS in porcine blood. Blood was spiked with glucose. Symbols represent different sensors ($n=3$). Sensors were retrospectively calibrated. Broken line – polynomial regression. Correlation coefficient (R): 0.99. MARE (> 75 mg/dL): 17.3%. MARD (< 75 mg/dL): 9.2 mg/dL. T: 37 °C.

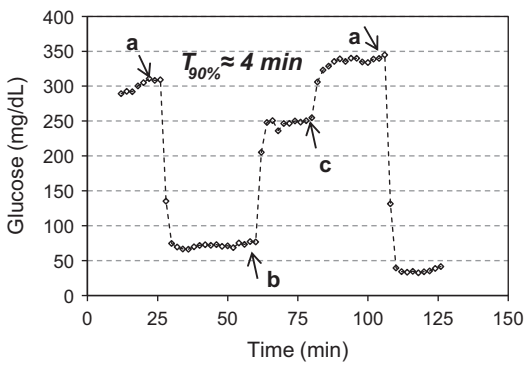

Fig. 5. Dynamics of TAS response to glucose changes. Arrows indicate time at which a new glucose challenge was started. A – 50 mg/dL. B – 250 mg/dL. C – 350 mg/dL. Data acquisition rate: 0.5 min^{-1}. $T_{90\%}$: approximately 4 min. T: 37 °C.

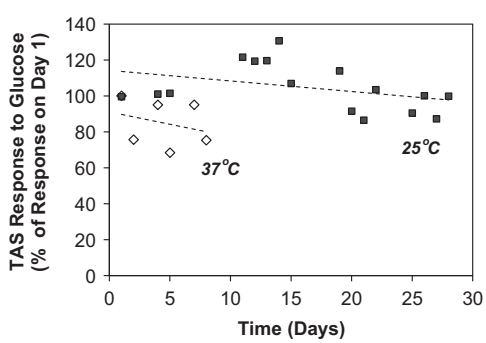

Fig. 6. Stability of TAS response to glucose over time. TAS response to glucose is defined as relative signal change (Δ) between 50 mg/dL and 250 mg/dL. Open diamond: average of sensors ($n=4$, SD = ± 20.8) at 37 °C. Solid square: average of sensors ($n=2$) at 25 °C

The response of the TAS was proportional to glucose levels from 50 to 800 mg/dl ($R=0.99$). We also tested the detection range of the TAS sensor in whole blood (from porcine) that was spiked with different glucose concentrations. Fig. 4 shows the calibration curve. Best fit of the data were achieved with polynomial regression ($R=0.99$). Mean absolute relative difference (MARD) at glucose levels larger than 75 mg/dL in blood was 17.3 ± 10.6% and 14.0 ± 19.4% for glucose levels less than 75 mg/dL. For studying the response time of the TAS device, the kinetics of the optical signal change in response to a glucose challenge was monitored over time. Fig. 5 shows that the TAS device exhibited a response time ($T_{90\%}$) of approximately 4 min at 37 °C.

3.2. TAS response over time

To assess the stability of the TAS response to glucose over time, different sensors were challenged with two different glucose levels (50 and 250 mg/dL) over several days and weeks at different temperatures. The results are shown in Fig. 6. At 25 °C the TAS maintained its operational functionality over approximately one month with only a slight drop in its response to glucose. In contrast, sensors ($n=4$) tested at 37 °C exhibited a small loss in glucose response by approximately 20% over the 8-day study period. Day-to-day variations of sensor readings were relatively large in this experiment for possible reasons that will be discussed in the next section.

4. Discussion

The most significant feature of the TAS is its simplicity due to the lack of the need for wavelength-specific hardware which would significantly minimize the complexity for optical sensing, especially when compared to fluorescence detection (e.g. see Colvin and Jiang, 2012) or fluorescence-life time-based detection principles. Also, there is no conjugation reaction necessary that might deteriorate the bioactivity of the binding ligand. The generation of the turbidity signal of the TAS is based on the affinity interaction between ConA and the glucose-residues of crosslinked dextran hydrogel particles (Sephadex). The large bulk change in turbidity that causes the suspension going from a visibly opaque state to a modestly transparent (see Fig. 2) is in line with the microscopic observation that showed fluorescently labeled ConA being located inside Sephadex beads in the absence of glucose and its appearance in the surrounding of the beads in the presence of saturating levels of glucose (Ballerstadt 2007a). That glucose is able to competitively displace ConA from the pendant glucose-binding sites of dextran hydrogel demonstrates the reversible nature of the TAS mechanism.

To demonstrate proof-of-concept of the TAS, we chose to use a semipermeable cellulose-based hollow fiber for two reasons. First, the hollow fiber is a convenient vehicle to study the dynamics of glucose response in-vitro, and, second, our group has successfully shown proof-of-concept of using hollow fibers for glucose monitoring in large animal models and humans (see Ballerstadt et al., 2012 and Dutt-Ballerstadt et al., 2013). Hollow fibers have a thin membrane which allows for fast glucose kinetics, and the lumen is large enough (OD 220 μm) to fit two 70-μm diameter optical fibers side-by-side (see Fig. 1). During our studies we have recognized that this design was prone to optical signal variations as the result of motion effects – e.g. when fluid was flushed through during glucose challenge experiments in the flow cell. This may have caused minute misalignment of the two optical fibers in relation to each other, and could be a reasonable explanation for slight sample-to-sample and day-to-day variations of sensor readings. However, despite this shortcoming, the study results demonstrate plenty of evidence that the sensor chemistry would be eligible for in-vivo glucose monitoring. The measurable detection range of TAS was within the physiological concentration range of approximately 50–400 mg/dL glucose when studied in buffer and in whole blood. The accuracy (MARD) of TAS in blood at glucose levels larger than 75 mg/dL in blood was 17.3 ± 10.6%, and 14.0 ± 19.4% for glucose levels less than 75 mg/dL. In comparison, MARDs for three commercially available CGMS devices in humans were 11.8 ± 3.8% for Navigator (Abbott Diabetes Care), 16.5 ± 17.8% for SevenPlus (Dexcom), and 20.2 ± 6.8% for Guardian (Medtronic) (Damiano et al., 2013). Further, sensor kinetics would be sufficiently fast (approximately 4 min) to dynamically measure glucose levels in the interstitial tissue, and, the stability of the TAS response for glucose at 37 °C would meet the requirement to monitor glucose levels over 5–7 days. To compensate for the small

loss in sensor response over time, recalibration during in-vivo operation may be necessary.

There are concerns about the biosafety of ConA due to its mitogenicity at high concentrations. To address concerns, we conducted a systemic toxicity study in mice (by Apptec, St.Paul, MN). Concanavalin A as an individual component and in the presence of all sensor components dosed at 5 times the effective sensor concentration in rats, did not show any gross toxic effects in skin, liver, or other organs. The amount of ConA in a miniaturized sensor contained in a semipermeable housing will be eventually even smaller (0.01 mg). This is two orders of magnitude lower than the dose tested in rats. These data support our conclusions about the minimal health risk of ConA at this concentration made from an extensive literature review of in vitro and in vivo ConA toxicity (including the effects of mitogenicity) (Ballerstadt et al., 2006). It is interesting to note that the LD50 reported for glucose oxidase (a widely used protein in glucose sensor implants under research development and commercial in vivo applications) is 3 mg kg^{-1} intraperitoneally (i.p.). The corresponding published LD50 data for ConA is 50 mg kg^{-1} (i.p.) (MSDS, 2014), which is approx 13 times higher than those for glucose oxidase (MSDS, 2013, Sigma Aldrich). This direct comparison with glucose oxidase is not intended to lessen the biotoxic properties of ConA at larger doses, though it indicates that safety concerns must also be expressed when using glucose oxidase in commercial sensor implants.

To conclude, the results of this in-vitro study demonstrate proof-of-concept of the TAS for in-vivo glucose monitoring. To successfully facilitate in-vivo studies a more robust optical TAS design would be required. Due to the large signal amplitude of the turbidity change and the lack of the need for wavelength-specific filters, a very small and compact sensor with an extremely short optical pathlength in the submicron range might be possible to be designed and implemented. Recent advances in the area of optoelectronics, especially in the monolithic integration of optical and electronic components onto silicon-based chips, may allow the implementation of an entirely novel miniature optoelectronic glucose-monitoring chip. Such a miniature device could be feasibly used as a fully-implantable sensor or as a minimally invasive, disposable sensor.

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