Glucose Response of Near-Infrared Alginate-Based Microsphere Sensors Under Dynamic Reversible Conditions

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Abstract

Background: Minimally invasive optical glucose biosensors with increased functional longevity form one of the most promising techniques for continuous glucose monitoring, because of their long-term stability, reversibility, repeatability, specificity, and high sensitivity. They are based on the principle of competitive binding and fluorescence resonance energy transfer. Moving to the near-infrared region of the spectrum has the potential to improve signal throughput for implanted sensors, but requires a change in dye chemistry that could alter response sensitivity, range, and toxicity profiles.

Methods: The near-infrared dissolved-core alginate microsphere sensors were fabricated by emulsion followed by surface coating by layer-by-layer self-assembly. The particles were characterized for sensor stability, sensor response, and reversibility in deionized water and simulated interstitial fluid. The sensor response to step changes in bulk glucose concentrations was also evaluated under dynamic conditions using a microflow cell unit. Finally, in vitro cytotoxicity assays were performed with L929 mouse fibroblast cell lines to demonstrate preliminary biocompatibility of the sensors.

Results: The glucose sensitivity under controlled and dynamic conditions was observed to be 0.86%/mM glucose with an analytical response range of 0–30 mM glucose, covering both the physiological and pathophysiological range. The sensor demonstrated a repeatable, reversible, and reproducible response, with a maximum response time of 120 s. In vitro cytotoxicity assays revealed nearly 95% viability of cells, thereby suggesting that the alginate microsphere sensor system does not exhibit cytotoxicity.

Conclusions: The incorporation of near-infrared dyes shows promise in improving sensor response because of their high sensitivity and improved tissue penetration of infrared light. The sensitivity for the sensors was approximately 1.5 times greater than that observed for visible dye sensors, and the new dye chemistry did not significantly alter the biocompatibility of the materials. These findings provide additional support for the potential application of alginate microspheres and similar systems such as “smart-tattoo” glucose sensors.

Introduction

THE MOST ADVANCED BIOSENSORS on the market include the Medtronic Minimed CGMS[®] Gold System[™] from Medtronic Diabetes (Northridge, CA), the STS[®] sensor from DexCom[™] (San Diego, CA), and the FreeStyle Navigator[®] Continuous Glucose Monitor by Therasense/Abbott Diabetes Care (Alameda, CA). These commercially available implantable sensors are based on an electroenzymatic sensing platform. They generally exhibit excellent sensor properties but have some drawbacks associated with instability of the enzyme electrochemical system, inaccuracy, and low precision, as well as requirements for extended warm-up time and fre-

quent calibration.^{1–4} Therefore, despite the value of trend tracking offered by continuous monitoring, most diabetes patients still prefer to measure their blood glucose using a handheld glucometer.⁴ This is a painful and annoying procedure that results in patients performing fewer than the recommended daily tests. As a consequence, these patients often exhibit periods of extended hyperglycemia that ultimately result in severe complications such as neuro-, nephro-, and retinopathy.^{2,5}

Thus, less invasive approaches to glucose analysis are still desired. A promising optical, minimally invasive technique under development involves the implantation of “stand alone” glucose-responsive materials in the dermis or

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subcutaneous tissue. Implantable fluorescent microparticle sensors (also known as “smart-tattoos”),^{6–12} one embodiment of this, are intended for injection directly into the skin and are exposed to the interstitial fluid, where they can measure local changes in glucose that are correlated with blood glucose levels.^{13–15} Such implants may be interrogated noninvasively using simple optical instrumentation.^{16–19} Towards this goal, several platform technologies have been established using different microsphere and microcapsule materials as well as different transduction approaches.^{6–12}

Our specific team efforts have focused on use of alginate microparticles with competitive binding sensing chemistry, and we recently reported on the response of fluorescent dissolved-core alginate microsphere glucose sensors using visible dyes.^{20,21} A unique aspect of this platform is the use of 10–20- μm in diameter dissolved-core alginate microspheres, which are easily processed and injectable, and the de-cross-linked interior increases molecular transport rates to effectively speed up response time. In addition, we have developed a strategy for improving in vivo compatibility following implantation using a co-immobilized drug–sensor system.²² Again using alginate microspheres as the encapsulating medium, this work demonstrated a system that can concurrently deliver 100% anti-inflammatory drug encapsulated in alginate microspheres over a period of 3–4 weeks for temporarily improving biocompatibility in vivo.²²

Thus, previous work provides solid hope that such implantable sensor materials can be useful as long-term continuous monitoring devices. In our quest to further improve these systems for in vivo application, we have modified the current competitive-binding scheme to incorporate near-infrared (NIR) dyes. Fluorescent dyes that emit in the NIR region, which covers the range of 600 to 1,300 nm, are considered superior to visible dyes for optical diagnostic applications when operating in biological medium. Emission in the NIR is relatively immune to tissue scattering because of the “optical window” of tissue¹ that results in 90–95% of NIR light passing through the stratum corneum and epidermis into the subcutaneous space—independent of skin pigmentation. Therefore, NIR dyes are generally independent from biological interferences such as tissue autofluorescence and scattering. In addition, they typically have higher detectability because of high absorbance ($>10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at long wavelength peak) and higher quantum yield. It has been observed that the use of short wavelength excitation (488 nm) inhibits signal acquisition and signal correlation to blood glucose.¹ Instead, the NIR light, which optically penetrates several millimeters of tissue, was hypothesized to increase the signal-to-noise ratio and improve fluorescent peak detection.^{1,23}

The Alexa Fluor® (Molecular Probes, Eugene, OR, a division of Invitrogen) (AF) dyes used in this sensor design are reported to possess better signal-to-noise ratio under varying skin depths, with photon counts of the longer wavelength dyes being over four times that of similar lower-wavelength dyes.²⁴ The quencher dye used in this work offers the advantage of eliminating the potential problem of background fluorescence resulting from direct acceptor excitation (non-sensitized). However, ratiometric sensor systems are preferred because they are less sensitive to noise and fluctuations in measurement apparatus properties. Therefore, in this scheme, the sensors are rendered ratiometric by inclusion of a

complementary reference fluorophore via polyelectrolyte–dye conjugates, as has recently been demonstrated in a similar hybrid microcapsule system.¹²

In summary, this work tries to overcome the shortcomings of the previous approaches in attempting to build “smart-tattoo” glucose sensors using fluorescence resonance energy transfer and a competitive binding transduction mechanism by extending the operating wavelength while retaining the sensitivity and nontoxic nature of the materials.

Sensor Design and Description

The sensing scheme used in this study is based on the principle of competitive binding and fluorescence resonance energy transfer,^{25–27} illustrated in Figure 1. When AF-647-dextran-amino is bound to QSY-21-apo-glucose oxidase (GOx), there is energy transfer from AF-647 to QSY-21, whereby fluorescence of AF-647 is quenched. When glucose is introduced into the system, it displaces the AF-647-tagged dextran amino from QSY-21 dye-conjugated apo-GOx, resulting in an increase in the AF-647 fluorescence. The AF-647 fluorescence is normalized with respect to the fluorescence of a reference dye (AF-750) incorporated in the polyelectrolyte coatings. The relative increase in the AF-647 fluorescence with respect to the reference dye fluorescence is then quantitatively related to the glucose concentration.

Experimental Procedures

Materials

Alginate (low viscosity, 2%), GOx (160 kDa, type VII from *Aspergillus niger*), β -D-glucose (mol wt 180 Da), sodium poly(styrene sulfonate) (PSS) (70 kDa), poly(allylamine hydrochloride) (PAH) (70 kDa), phosphate-buffered saline tablets, dimethyl sulfoxide (formula weight 78.13), dimethylformamide (molar mass 73.09 g/mol), SPAN 85 (sorbitane trioleate), Tween 85 (polyoxyethylene sorbitan trioleate), and 2,2,4-trimethylpentane (iso-octane) were purchased from Sigma-Aldrich (St. Louis, MO). AF-647, AF-750, dextran amino (500 kDa), and QSY-21 were purchased from Molecular Probes (Invitrogen). NAP5 columns were purchased from GE Healthcare (Little Chalfont, UK). Bovine albumin fraction V powder was purchased from Sisco Research Laboratories (Mumbai, India). Calcium chloride, ammonium sulfate, and sodium hydroxide were purchased from Merck (Mumbai). Sodium acetate was purchased from Ranbaxy Fine Chemicals Ltd. (Mumbai). Sodium bicarbonate, sodium chloride, potassium bicarbonate, magnesium sulfate, and dipotassium hydrogen orthophosphate were purchased from SD Fine Chemicals Ltd. (Mumbai). Calcium gluconate was purchased from Loba Chemie (Mumbai). All chemicals were reagent grade and used as received.

Instrumentation

A Nikon (Tokyo, Japan) YS 100 microscope and a Zeiss (Oberkochen, Germany) optical microscope with a digital camera were used for microscopic studies. A Hitachi (Tokyo) U-2900E UV-Vis spectrophotometer (double beam) was used to collect absorbance spectra. A fluorescence spectrophotometer (model F-2500, Hitachi) was used for collection of emission scans. A fluorescence microscope (model TE2000U, Nikon) was used for imaging alginate microspheres loaded

FIG. 1. Principle of glucose sensing. AF, Alexa Fluor; GOx, glucose oxidase.

with the sensing assay. A Hitachi quartz LC microflow cell unit (90 μ L) for fluorescence spectrophotometer (model 650-0163) was used for dynamic testing. A syringe pump (model NE-1000Multi-PhaserTM, New Era Pump Systems, Wantagh, NY) was used to pump the fluid into and out of the flow cell.

Preparation of apoenzyme

Apo-GOx is an inactive form of the GOx enzyme. It does not catalyze glucose oxidation, but retains its glucose binding affinity.²⁸ Apo-GOx is used as the glucose binding protein, so as to avoid analyte consumption and prevent by-product formation. Apo-GOx was prepared by removal of FAD from glucose oxidase, using the Swoboda²⁹ protocol as reported previously.^{20,21} The Hitachi UV-Vis spectrophotometer was used to confirm the removal of FAD and to calculate to concentration of the apoenzyme.

Preparation of NIR dye-conjugated sensing reagents

Dextran amino is a glucose analog that has affinity for the glucose binding element, apo-GOx. For the NIR sensor system, dextran amino was labeled with AF-647 donor dye, whereas the prepared apo-GOx was labeled with QSY-21 quencher acceptor dye, and PAH polyelectrolyte was labeled with AF-750 reference dye using the standard amine labeling procedure.³⁰ The relative molar concentration of dye to protein was 30:1. The dye solution was prepared in dimethylformamide and then slowly added to the protein solution with constant stirring. The reaction mixture was then

incubated in the dark at room temperature for 1 h with continuous stirring. Finally, the mixture was eluted through NAP5 columns to separate out the labeled sample from the unconjugated dye. The concentration of the labeled apoenzyme was calculated to be 1.3 μ M with a degree of labeling of 2, whereas the concentration of labeled dextran amino was calculated to be 2 μ M with a degree of labeling of 2.

Preparation of NIR dye-loaded alginate microspheres

Both AF-647-dextran amino (2 μ M) and QSY-21-apo-GOx (1.3 μ M) were mixed together in a 4:1 volume ratio under stirring for 30 min in the dark. The sensing reagents were then mixed in 2% (wt/vol) sodium alginate solution in a ratio of 1:10 by volume. The solution was gently agitated for about 30 min in the dark to prevent photobleaching of the dye while allowing complete mixing in the precursor solution.

Calcium alginate microspheres were then prepared using the emulsification technique reported earlier.³¹ Furthermore, the layer-by-layer self-assembly technique was used for deposition of multilayered nanofilm coatings over the alginate microspheres to prevent leaching of the sensing assay. Subsequently, the core of alginate microspheres was partially dissolved using citrate treatment (0.1 M sodium citrate in Tris HCl) to provide free space inside the alginate microspheres required for competitive binding. The characterization of the dissolution process has already been reported in our previous work.²¹ The polyelectrolyte coatings do not dissolve and thus stabilize the de-cross-linked alginate microspheres while

simultaneously preventing the leakage of the sensing chemistry.

Stability of encapsulation

For potential use as glucose biosensors, it is important for the sensing chemistry to be stably retained in the partially dissolved alginate microspheres over time. In this regard, the Hitachi UV-Vis spectrophotometer was used to measure absorbance of leached products from the polyelectrolyte-coated alginate microspheres, and the data were used to estimate the loss of encapsulated material from these coated microspheres. All samples were covered and stored under dark conditions at room temperature.

Sensor response

The glucose response of alginate microspheres was tested in deionized (DI) water and simulated interstitial fluid (SIF), after storage of the microspheres in SIF for 2 days. The SIF had almost equal ion concentrations as those of the human interstitial fluid³² and was buffered to pH 7.4. The percentage change in fluorescence intensity ratio (relative to the initial value without glucose) after titration of β -D-glucose in increasing concentrations of 3 to 60 mM was calculated from each spectrum and plotted with respect to glucose concentrations.

Reversible sensor response under dynamic conditions

The reversible sensor response to step changes in the bulk glucose concentrations was tested in both DI water and SIF. A Hitachi quartz LC microflow cell unit equipped with an inlet and an outlet that allows continuous flow of the sensor suspension within the chamber was used for the test. A syringe pump equipped with a 10-mL syringe attached to silicone tubing was used to pump the sensor suspension into and out of the flow cell chamber. The experimental setup is illustrated in Figure 2. The flow rate of the pump was maintained at 4 mL/min. Glucose was added into the microsphere suspension to a desired concentration, and the equilibrated suspension was then passed through the flow cell chamber after

2 min of reaction. Fluorescence spectra were continuously recorded for 5 min during flow. The concentration of glucose was then returned to 0 mM by continuously flushing DI water or SIF, and fluorescence emission spectra were again recorded for 5 min. Measurements were performed on the same batch of microspheres so as to demonstrate the reversible nature of the response. The response data were collected for the same set of sensors that were exposed to random bulk glucose levels ranging from 0 to 30 mM glucose.

Sensor stability

The repeatability of the alginate microsphere glucose biosensors was obtained by testing the response of the sensors to the same glucose concentration for five successive readings under dynamic conditions. Similarly, to evaluate reproducibility, three different sets of the biosensors were independently prepared and tested using the same conditions. Additionally, the 1-month stability of the biosensors was examined by intermittently measuring the dynamic reversible response to successive glucose concentrations at every 7th day for over a 30-day period. The biosensors were stored in SIF at room temperature in a dark place when not in use.

In vitro cytotoxicity studies

In vitro cell culture tests are considered as one of the most fundamental tests for biocompatibility and are used to screen the biocompatibility of implantable devices. The preliminary cytotoxicity of the uncoated alginate microspheres and [PAH/PSS]₂-coated alginate microspheres loaded with the fluorescent glucose sensing assay was evaluated by using the sulforhodamine-B semiautomated assay. L929 (mouse fibroblasts) cell lines obtained from the National Centre for Cell Science, Pune, India, were used for the assay. The cells were grown in modified Dulbecco's modified essential medium (Sigma, St. Louis) supplemented with 10% fetal bovine serum (Sigma) and 1% antibiotic/antimycotic solutions (Himedia, Mumbai) and incubated at a temperature of 37°C under a saturated humidity atmosphere of 5% CO₂. Nearly confluent cells in 25-cm² tissue culture flasks were trypsinized by trypsin-EDTA solution and centrifuged at 1,000 g for 10 min. The cell pellet was then resuspended in fresh medium. Cells were counted, and the cell count was adjusted accordingly to the titration readings so as to give an optical density in the linear range (from 0.5 to 1.8). Samples were tested in 96-well plates in three triplicates, with each well receiving 90 μ L of cell suspension with a concentration of 1×10^4 cells per well. The plate was then incubated at 37°C in a CO₂ incubator for 24 h. Afterward, 10 μ L of diluted (10^5 microspheres/100 μ L) plain, polyelectrolyte-coated, and alginate microsphere glucose biosensors were added to the 96-well plate and further incubated for 48 h. Finally, the experiment was terminated by gently layering the cells in the wells with 50 μ L of chilled 50% trichloroacetic acid for cell fixation. Plates were kept in a refrigerator (4°C) for 1 h. The plates were washed thoroughly with tap water at least five times and air-dried. For the assay, plates were stained with 50 μ L of 0.4% sulforhodamine-B for 20 min, then washed with 1% acetic acid at least five times, and air-dried. Finally, the bound sulforhodamine-B was eluted with 100 μ L of Tris (10 mM, pH 10.5) for 10 min. Thereafter, the plates were shaken for 1 min using an automated shaker, and the absorbance (optical density) of each

FIG. 2. Experimental setup for glucose sensing under dynamic reversible conditions.

FIG. 3. Fluorescence microscopy image of alginate microspheres containing near-infrared dye sensing assay after citrate treatment.

well was read in a microplate reader (Thermo Electron Corp., Waltham, MA) at 540 nm with reference to 690 nm against blank (culture medium without any cells). Plain uncoated microspheres were used as control in the study.

Results and Discussion

Alginate microsphere biosensor fabrication

The alginate microspheres produced via emulsion were in the size range of 10–20 μm . A representative fluorescence microscopy image of the alginate microspheres loaded with

NIR sensing assay after citrate treatment is shown in Figure 3. The uneven distribution of fluorescence is indicative of a change in the structure and fluorescence sensing assay distribution, confirming the dissolution of the alginate microsphere core.²¹

Stability of encapsulation

Loss of AF-647-dextran amino and QSY-21-apo-GOx molecules from the layer-by-layer coated microspheres was quantified using absorbance studies. The increase in the absorbance of the supernatant was used to quantify the release of the co-encapsulated molecules, as depicted in Figure 4. The percentage leached indicates the percentage of initially encapsulated sensing assay molecules lost from the microspheres to the supernatant solution. There was only a small amount ($\sim 4\%$) leaching observed during the first 15 h for the encapsulated assay, after which point no additional loss was observed.

Sensor response

For the glucose sensitivity experiments, the AF-647 fluorescence was normalized with respect to the AF-750 peak fluorescence. It was observed that with the addition of glucose, there is an increase in the AF-647 fluorescence, attributed to the increase in distance between AF-647 and QSY-21 such that quenching of AF-647 is decreased. It can be observed from Figure 5 that the glucose sensitivity of the NIR sensor system suspended in DI water was estimated to be 0.73%/mM glucose with an analytical response range of 0–50 mM glucose, whereas that in SIF was observed to be 0.8%/mM glucose with an analytical response range of 0–50 mM glucose. In both cases, the response is observed to be linear up to 12 mM glucose concentration (as illustrated in the inset of the sensitivity graphs), which covers the range required to accurately predict glucose concentrations in hypo- and normoglycemia. In addition, the sensitivity of the assay using NIR dye sensors is approximately 1.5 times the sensitivity that was observed for similar sensors using visible dyes, wherein the

FIG. 4. Leaching curve for [poly(allylamine hydrochloride)/sodium poly(styrene sulfonate)]₂-coated near-infrared dye-loaded alginate microspheres. Data are mean $\pm$ SD values ($n = 5$). AF, Alexa Fluor; GOx, glucose oxidase.

FIG. 5. Glucose sensitivity curve for near-infrared dye sensors in (a) deionized water and (b) simulated interstitial fluid. Data are mean $\pm$ SD values ($n = 5$).

glucose sensitivity in DI water was estimated to be 0.33%/mM glucose, whereas that in SIF was observed to be 0.5%/mM glucose.²¹ This improved sensitivity is attributed to the increased Förster radius of the NIR fluorescence resonance energy transfer, which results in greater quenching when dextran is in the bound state.

Reversible sensor response under dynamic conditions

The dissolved-core alginate microspheres were designed to increase the sensitivity and time response of the sensor system. As seen in Figure 6, the sensor response was observed to reach steady state in approximately 2 min (worst-case response time), which matches observations for visible dye systems in our previous work.^{21,33} In our previous work with visible dye sensors, we had illustrated that when glucose was added to the microsphere suspension (not exposed to glucose previously), the response time was slightly faster compared with during subsequent additions of glucose concentrations. The increase in response time in subsequent steps is due to the basal level of glucose now already present in the microsphere suspension. Similarly, when a 3 mM glucose concentration was added to the microsphere suspension, the response time observed was less compared with subsequent steps of glucose addition. In subsequent glucose additions steps (6 mM glu-

cose to 50 mM glucose), the response time was, however, observed to be approximately 2 min. Therefore, the time response for the subsequent steps was observed to be almost identical, and it was concluded that the sensor response was observed to reach steady state in approximately 2 min (maximum response time) for the visible dye sensors.^{21,33}

Figure 7 demonstrates real-time continuous acquisition of emission spectra with constant irradiation under dynamic glucose concentration changes in DI water and SIF (Fig. 7a and c, respectively). The percentage change in peak ratio, calculated relative to the baseline (no glucose) fluorescence ratio, is plotted as a function of time at different incremental glucose concentrations from 0 to 30 mM glucose. It can be clearly observed from the graph that the step changes in glucose concentration elicit a significant increase in fluorescence intensity ratio. Furthermore, exposing the sensors to glucose-free buffer caused the peak ratio to return to the baseline value, demonstrating reversible sensor response.

The reversibility of the sensors was assessed by calculating the baseline change before and after exposure to glucose. The average baseline shift was determined (for five steps) to be 0.85% with an SD of 0.5 in DI water and 1% with an SD of 0.56 in SIF, thus demonstrating a highly reversible sensor response. The small initial drift observed may be attributed to a small amount of leaching of the free dye or the dye-

FIG. 6. Time-response curve. Data are mean $\pm$ SD values ($n = 5$).

macromolecule conjugate from the microspheres, due to some irreversible binding. Any instrumental variations in the form of source fluctuations and minor changes in sensor concentration were accounted by the fluorescent ratiometric analysis. As seen in the repeated measurements, the response appears very stable. The response time of a glucose sensor is critical as sensors must respond within the physiological time scale of glucose fluctuations to ensure accuracy. The sensor response time under dynamic reversible conditions was observed to be 2 min (maximum response time), which is identical to that observed in steady state, making these sensors adequate to monitor fluctuations in blood glucose, which usually occur over a period of 30 min time.³⁴

As illustrated in Figure 7b and d, the glucose sensitivity under dynamic conditions was estimated to be 0.79%/mM

glucose in DI water and 0.86%/mM glucose in SIF, with an analytical response range of 0–30 mM glucose in both cases. The NIR dye sensor response is approximately 1.5 times that of the visible dye sensor response, wherein the glucose sensitivity under dynamic conditions was estimated to be 0.52%/mM glucose in DI water and 0.6%/mM glucose in SIF.³³ This range of response covers the complete expected physiological range (normoglycemia) and pathophysiological range (hyperglycemia and hypoglycemia). The response is observed to be linear up to 6 mM glucose concentration, which is the range required to accurately predict glucose concentrations in hypoglycemia.

The glucose response sensitivity under dynamic conditions was found to be statistically equivalent to the steady-state glucose response (Student's paired t test, $\alpha = 0.05$). These findings suggest that these dissolved-core alginate microsphere glucose sensors are stable under both static and dynamic flow conditions, further supporting their potential for monitoring glucose under physiological conditions.

Sensor stability

The repeatability of the alginate microsphere glucose biosensors was obtained by testing the response of the sensors to same glucose concentration for five successive readings under dynamic conditions. The SD was calculated to be 1.5%. Additionally, to evaluate reproducibility, three different sets of the biosensors were independently prepared and tested using the same conditions; these experiments yielded an SD value of 1.3%, proving that the fabrication process and outcomes in terms of glucose measurements are highly consistent.

FIG. 7. Continuous sensing profile and sensitivity curves of near-infrared dye-loaded alginate microsphere glucose sensors under dynamic conditions in (a and b) deionized water and (c and d) simulated interstitial fluid.

TABLE 1. SENSOR CHARACTERISTICS FOR APO-GLUCOSE OXIDASE-BASED NEAR-INFRARED SENSORS

	Glucose sensitivity (% change/mM glucose)
Successive repeatability measurements	
1	0.838
2	0.849
3	0.855
4	0.845
5	0.872
Average	0.8518
Relative SD	1.5%
Reproducibility measurements	
Sensor batch 1	0.871
Sensor batch 2	0.848
Sensor batch 3	0.858
Average	0.859
Relative SD	1.3%
Days	
0	0.868
7	0.851
14	0.872
21	0.848
28	0.875
Average	0.8628
Relative SD	1.4%

The long-term stability of the biosensors was further examined by intermittently measuring the dynamic response to successive glucose concentrations at every 7th day for over a 30-day period. The biosensors were stored in SIF at room temperature in a dark place when not in use. The average glucose sensitivity was observed to remain constant (0.8%/mM) over this entire time, with an SD of 1.4%. Therefore, it can be concluded that the alginate microspheres were stable in the SIF when tested over a period of 1 month, confirming that the sensing assay was stabilized in a favorable microenvironment. It is also noteworthy to state that the data were not corrected for photobleaching, indicating the system exhibits a high degree of photostability. These sensor characteristics are summarized in Table 1.

FIG. 8. Cytotoxicity results of plain, polyelectrolyte-coated, and near-infrared (NIR) dye-loaded alginate microspheres (MS). Data are mean $\pm$ SD values ($n = 5$). PAH/PSS, poly(allylamine hydrochloride)/sodium poly(styrene sulfonate).

In vitro cytotoxicity studies

Preliminary cytotoxicity studies were performed using the L929 mouse fibroblast cell lines. The results, depicted in Figure 8, indicate that the viability of cells was approximately $96 \pm 1.3\%$ with plain alginate microspheres compared with the control (cells without microspheres sample), indicating that there was no significant cytotoxicity to cells. In the case of [PAH/PSS]₂-coated alginate microspheres, the viability was again observed to be around $94 \pm 2.5\%$. Finally, the dissolved-core alginate-templated microspheres loaded with the fluorescent NIR dye sensing assay also demonstrated $95 \pm 1.7\%$ viability of cells. No significant differences were observed among the three situations. Therefore, it is concluded that the microsphere glucose biosensors exhibit no significant cytotoxicity, to allow their further in vivo evaluation.

Conclusions

The longer-wavelength glucose sensors described herein exhibited improved response sensitivity to add to the advantage of superior tissue penetration, while maintaining an analytical response range of 0–30 mM glucose that covers both the physiological (normoglycemia) and pathophysiological (hyperglycemia and hypoglycemia) range. The response was found to be comparable under static and dynamic conditions, with sensitivity of 0.79%/mM glucose in DI water and 0.86%/mM glucose in SIF, values approximately 1.5 times greater than what was observed for similar sensors using visible dyes. The sensors demonstrated a reversible, repeatable, and reproducible response when evaluated under dynamic flow conditions and were found to be non-cytotoxic in preliminary in vitro culture studies. Therefore, these findings continue to support the potential of such sensors for application as “smart-tattoo” glucose sensors to monitor glucose under physiological conditions. In vivo biocompatibility and glucose sensing studies are currently underway to assess the practical efficacy of this promising technology.

Despite several commercially available continuous glucose monitoring systems, glucometers still remain the current state of art for self-monitoring of blood glucose, which intermittently assess glucose and only provide a snapshot of the glucose concentration. Therefore, there is still an unmet clinical need to continuously monitor glucose levels inside the body in a way that is less invasive and more accurate than the currently existing techniques. Such continuous glucose sensors can be combined with an insulin delivery system to implement the closed-loop control, which is considered to be the “Holy Grail” of diabetes management. Therefore, continuous glucose sensing represents the next step in the evolution of self-monitoring of glucose.

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Author Disclosure Statement

No competing financial interests exist.

References

- Pickup J, McCartney L, Rolinski O, Birch D: In vivo glucose sensing for diabetes management: progress towards non-invasive monitoring. *BMJ* 1999;319:1289.
- Pickup JC, Hussain F, Evans ND, Sachedina N: In vivo glucose monitoring: the clinical reality and the promise. *Biosens Bioelectron* 2005;20:1897–1902.
- Wickramasinghe Y, Yang Y, Spencer SA: Current problems and potential techniques in in vivo glucose monitoring. *J Fluoresc* 2004;14:513–520.
- Newman JD, Turner AP: Home blood glucose biosensors: a commercial perspective. *Biosens Bioelectron* 2005;20:2435–2453.
- Nathan DM: Long term complications of diabetes mellitus. *N Engl J Med* 1993;328:1676–1685.
- McShane MJ: Potential for glucose monitoring with nanoengineered fluorescent biosensors. *Diabetes Technol Ther* 2002;4:533–538.
- Brown JQ, Srivastava R, McShane MJ: Encapsulation of glucose oxidase and an oxygen-quenched fluorophore in polyelectrolyte-coated calcium alginate microspheres as optical glucose sensor systems. *Biosens Bioelectron* 2005;21:212–216.
- Chinnayelka S, McShane MJ: Microcapsule biosensors using competitive binding resonance energy transfer assays based on apoenzymes. *Anal Chem* 2005;77:5501–5511.
- Brown JQ, McShane MJ: Modeling of spherical fluorescent glucose microsensor systems: design of enzymatic smart tattoos. *Biosens Bioelectron* 2006;21:1760–1769.
- Chinnayelka S, McShane MJ: Glucose sensors based on microcapsules containing an orange/red competitive binding resonance energy transfer assay. *Diabetes Technol Ther* 2006;8:269–278.
- Stein EW, Grant PS, Zhu H, McShane MJ: Microscale enzymatic optical biosensors using mass transport limiting nanofilms. 1. Fabrication and characterization using glucose as a model analyte. *Anal Chem* 2007;79:1339–1348.
- Chinnayelka S, Zhu H, McShane MJ: Near-infrared resonance energy transfer glucose biosensors in hybrid microcapsule carriers. *J Sens* 2008;2008:1–10.
- Freeland AC, Bonnez RT: Inference of blood glucose concentrations from subcutaneous glucose concentrations: applications to glucose biosensors. *Ann Biomed Eng* 1999;27:525–537.
- Thennadil SN, Rennert JL, Wenzel BJ, Hazen KH, Ruchti TL, Block MB: Comparison of glucose concentration in interstitial fluid, and capillary and venous blood during rapid changes in blood glucose levels. *Diabetes Technol Ther* 2001;3:357–365.
- Caplin NJ, O'Leary P, Bulsara M, Davis EA, Jones TW: Subcutaneous glucose sensor values closely parallel blood glucose during insulin-induced hypoglycaemia. *Diabet Med* 2003;20:238–241.
- McShane MJ, Russell RJ, Pishko MV, Coté GL: Optical system for implantable analyte sensors. *Conf Proc IEEE Eng Med Biol Soc* 1999;2:804.
- McShane MJ, Rastegar S, Pishko M, Coté GL: Monte Carlo modeling for implantable fluorescent analyte sensors. *IEEE Trans Biomed Eng* 2000;47:624–632.
- Long R, McShane MJ: Modeling of selective photon capture for collection of fluorescence emitted from dermally-implanted microparticle sensors. *Conf Proc IEEE Eng Med Biol Soc* 2007;2007:2972–2975.
- Long R, McShane M: Optical instrument design for interrogation of dermally-implanted luminescent microparticle sensors. *Conf Proc IEEE Eng Med Biol Soc* 2008;2008:5656–5359.
- Chaudhary A, Srivastava R: Glucose sensing using competitive binding assay co-encapsulated in uniform sized alginate microspheres. *Sens Lett* 2008;6:253–260.
- Chaudhary A, Raina M, Harma H, Hanninen P, McShane MJ, Srivastava R: Evaluation of glucose sensitive affinity binding assay entrapped in fluorescent dissolved-core alginate microspheres. *Biotechnol Bioeng* 2009;104:1075–1085.
- Srivastava R, Jayant RD, Chaudhary A, McShane MJ: "Smart tattoo" glucose biosensors and effect of coencapsulated anti-inflammatory agents. *J Diabetes Sci Technol* 2011;5:1658–1667.
- McCartney LJ, Pickup JC, Rolinski OJ, Birch DJ: Near-infrared fluorescence lifetime assay for serum glucose based on allophycocyanin-labeled concanavalin A. *Anal Biochem* 2001;292:216–221.
- Ibey BL, Cote GL, Yadavalli V, Gant VA, Newmyer K, Pishko MV: Analysis of longer wavelength AlexaFluor dyes for use in a minimally invasive glucose sensor. *Conf Proc IEEE Eng Med Biol Soc* 2003;2003(4):3446–3449.
- Schultz JS, Mansouri S, Goldstein IJ: Affinity sensor: a new technique for developing implantable sensors for glucose and other metabolites. *Diabetes Care* 1982;5:245–253.
- Ballerstadt R, Schultz JS: Competitive-binding assay method based on fluorescence quenching of ligands held in close proximity by a multivalent receptor. *Anal Chim Acta* 1997;345:203–212.
- Ballerstadt R, Gowda A, McNichols R: Fluorescence resonance energy transfer-based near-infrared fluorescence sensor for glucose monitoring. *Diabetes Technol Ther* 2004;6:191–200.
- D'Auria S, Herman P, Rossi M, Lakowicz JR: The fluorescence emission of the apo-glucose oxidase from *Aspergillus niger* as probe to estimate glucose concentrations. *Biochem Biophys Res Commun* 1999;263:550–553.
- Swoboda BE: The relationship between molecular conformation and the binding of flavin-adenine dinucleotide in glucose oxidase. *Biochim Biophys Acta* 1969;175:365–379.
- Svensson HP, Kadow JF, Virudhula VM, Wallace PM, Senter PD: Monoclonal antibody- β -lactamase conjugates for the activation of a cephalosporin mustard prodrug. *Bioconjug Chem* 1992;3:176–181.
- Srivastava R, Brown JQ, Zhu H, McShane MJ: Stable encapsulation of active enzyme by application of multilayer nanofilm coatings to alginate microspheres. *Macromol Biosci* 2005;5:717–727.
- Doherty GM, Way LW: Fluid and electrolyte management. In: *Current Surgical Diagnosis and Treatment*, 12th ed. San Francisco: McGraw-Hill Medical, 2005:95–106.
- Chaudhary A, McShane MJ, Srivastava R: Glucose response of dissolved-core alginate microspheres: towards a continuous glucose biosensor. *Analyst* 2010;135:2620–2628.
- Kulcu E, Tamada JA, Reach G, Potts RO, Lesho MJ: Physiological differences between interstitial glucose and blood glucose measured in human subjects. *Diabetes Care* 2003;26:2405–2409.

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