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Glucose Sensors Based on Microcapsules Containing an Orange/Red Competitive Binding Resonance Energy Transfer Assay

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Abstract

Fluorescent sensing systems offer the potential for noninvasive monitoring with implantable devices, but they require carrier technologies that provide suitable immobilization, accessibility, and biocompatibility while maintaining adequate response characteristics. A recent development towards this goal is a highly specific and sensitive competitive binding assay for glucose using apo-glucose oxidase (apo-GOx) as the recognition element and dextran as the competing ligand; this has been demonstrated as a glucose sensor system by encapsulating the competitive binding assay in semipermeable microcapsule carriers. This paper describes the extension of this sensor design to longer wavelengths in an attempt to increase the applicability to in vivo monitoring. The glucose sensitivity of the tetramethylrhodamine isothiocyanate-dextran (TD) and cyanine Cy5-apo-GOx (CAG) complexes showed five to 10 times greater specificity for β -D-glucose over other sugars. Microcapsules loaded with TD/CAG complexes exhibited a linear, totally reversible response in the range of 0–720 mg/dL, with a sensitivity (percent change in intensity ratio) of 0.06%/(mg/dL). The decrease in sensitivity observed with the use of longer-wavelength dyes is most likely to be compensated with the deeper penetration of light and reduced tissue scattering. These findings imply that the encapsulation of sensing assay elements in microcapsules is a simple and translatable method for the fabrication of stable biosensors, and optimization of resonance energy transfer pairs and assay component preparation will further improve the response to approach clinically relevant performance.

INTRODUCTION

It is well known that frequent monitoring of glucose concentrations and appropriate countermeasures can help in achieving euglycemia and minimizing secondary complications.^{1,2} Optical sensors enabling glucose testing without any need for blood extraction are an attractive alternative to the current standard of painful finger-stick testing procedures.^{3,4} In particular, implantable fluorescent sensors, which can be remotely monitored using light, would be an ideal solution^{4–9}; such sensing mechanisms have been

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intensely pursued in recent years using fluorescent spectroscopic techniques, because of the high sensitivity that can be obtained.^{6–12}

One of the approaches that has been deeply explored for glucose monitoring using fluorescence techniques is based on the changes in resonance energy transfer (RET) that occur due to the competitive binding between a fluorescent ligand and an analyte to occupy binding sites on a fluorescent receptor. Schultz was the pioneer in this area, and has reported various sensors based on this concept, i.e., glucose competes with fluorescein isothiocyanate (FITC)-dextran (FD) to occupy binding sites on tetramethylrhodamine isothiocyanate (TRITC)-Concanavalin A (Con A) because of higher binding affinity.^{9,13} Thus, glucose concentration has been estimated by measuring the changes in distance between FD and TRITC-Con A in terms of RET efficiency. Recently, this work has been extended into the near-infrared (NIR) by labeling the dextran and Con A with an NIR donor–acceptor pair.^{14,15} Because there are concerns over Con A toxicity and nonspecific binding, alternative RET-based glucose assays have also been developed using other glucose-binding proteins and genetically engineered proteins.^{16–18} However, the sensitivity of these assays is too high for monitoring glucose levels typically found in the blood and interstitium. Therefore, we developed a novel RET assay that employs an inactive form of the enzyme glucose oxidase (apo-GOx) as the glucose-binding protein,¹¹ which is highly specific to β -D-glucose.¹⁹ Unlike the low dissociation constant (K_D) values (~ 0.1 – $1 \mu M$) observed for glucose-binding proteins,^{16–18} the sensitivity for apo-Gox was observed to be in the millimolar range, making it more suitable for monitoring physiological glucose levels.

To make such assays amenable to in vivo clinical use, the fluorescent chemistry must be placed within a carrier system that provides accessibility to the analyte while preventing movement of the molecules outside of the region of intended use. Towards this goal, a novel “smart tattoo” system was proposed based on the entrapment of glucose-sensitive Con A/dextran assay in poly(ethylene glycol) microspheres, which could be implanted in the skin and interrogated remotely using harmless visible or NIR excitation light.^{6,20–22} This general concept of entrapping glucose sensing chemistry in biocompatible microspheres is an attractive approach to exploiting the sensitivity of fluorescence spectroscopy and the specificity of molecular recognition agents such as proteins for in vivo use. There are several other investigations of smart tattoo devices, with variation in the sensing mechanism, e.g., optical glucose sensor based on the encapsulation of the GOx and oxygen-quenched fluorophore in polymer-coated alginate microspheres^{12,23}; however, the competitive binding approach carries a unique requirement that at least one of the assay molecules (receptor or ligand) must be free to diffuse away from the other upon glucose binding to the receptor, while the free molecules then also must be restricted to a local environment and cannot diffuse away so far that no further interaction with the receptor is possible.

As an example of a realistic possibility for in vivo sensing that meets these needs, the apoenzyme competitive binding RET assay described previously [FD/TRITC-apo-GOx (TAG) complexes]¹¹ has been encapsulated in polyelectrolyte microcapsules.⁸ In this system, the use of microcapsules enables the free movement of the ligand, receptor, and analyte during the process of competitive displacement, while the semipermeable capsule walls entrap the larger sensing assay elements in the capsule interior and yet allow free

glucose transport through capsule walls. The main disadvantage of the prototype microcapsule-based glucose sensors previously reported is the use of FITC and TRITC as the energy transfer pair; while more photostable analogs of these dyes are available, these visible excitation/emission systems operate at short wavelengths where tissue is highly scattering. Even if fluorescence lifetime measurements are used for in vivo interrogation, it is desirable to replace the visible-range fluorescent dyes with NIR fluorophores to increase penetration depth and decrease scattering losses. Therefore, this paper describes the effect of using a longer-wavelength energy transfer pair on the construction and response of microcapsule-based apo-enzyme fluorescent glucose sensors.

SENSOR DESIGN

A schematic of the transduction mechanism used in this sensor is illustrated in Figure 1. The basic procedure for apo-GOx preparation was followed as previously described.¹⁹ The apo-GOx prepared by this method was observed to maintain its reduced catalytic activity and preserve its binding capacity for at least 4 weeks.¹¹ The close proximity of Cy5-apo-GOx (CAG) and TRITC-dextran (TD) in the absence of glucose results in the strong fluorescence peaks due to energy transfer from TRITC to Cy5, as indicated in Figure 1, step 3. As apo-GOx has higher affinity for glucose over dextran, the addition of glucose results in the displacement of dextran from apo-GOx, accompanied by a decrease in the energy transfer efficiency (Fig. 1, step 4) as indicated by a stronger TRITC peak relative to Cy5. It is noteworthy that this apo-enzyme sensing approach retains the advantages of competitive binding assays (no analyte consumption) and RET-based sensors (ratiometric, high sensitivity) while adding the high specificity of enzymatic sensors and reduced scattering due to the employment of longer-wavelength fluorophores.

This approach employs the elegant layer-by-layer self-assembly²⁴ approach for the fabrication of microcapsules comprising photocross-linkable materials [diazoresin (diazotized-10,4-diazodiphenylamine/formaldehyde condensate hydrogen sulfate-zinc chloride salt) (DAR) and sodium poly(styrene sulfonate) (PSS)] in the shell structure, which are used for stable entrapment of RET assay elements. In this study, TD/CAG complexes were trapped in microcapsules using the photosensitive properties of the materials coated in the capsule walls. A detailed description of fabrication of microcapsules and encapsulation of sensing elements was reported elsewhere.⁸ The resulting loaded microcapsule comprises an optical glucose sensor, with sensing materials in the hollow capsule interior. This report describes the investigation of glucose sensitivity of TD/CAG complexes encapsulated in polyelectrolyte microcapsules using fluorescence spectroscopic and imaging techniques, and compares their performance characteristics with those obtained for the FITC/TRITC energy transfer pair⁸ to determine the sensor response characteristics after replacing the green/orange dyes with the orange/red fluorescence RET pair.

EXPERIMENTAL METHODS

Materials

Amino-dextran (molecular size 500 kDa), GOx (G-2133), PSS (molecular size ~1 MDa), poly(allylamine hydrochloride) (PAH) (molecular size 70 kDa), β -D-glucose, mannose, α -D-

glucose, sucrose, sodium bicarbonate, dimethyl formamide, ammonium sulfate, and sodium acetate buffer were obtained from Sigma (St. Louis, MO). TRITC and cyanine bis-(*N*-hydroxysuccinimide) ester (Cy5) were obtained from Molecular Probes (Eugene, OR) and conjugated to amino-dextran and apo-GOx, respectively, using standard amine labeling procedures.²⁵ DAR was purchased from PC Associates (Livingston, NJ). All reagents were used as received. MnCO_3 (5- μm -diameter) particles were prepared as previously described as templates for the polymer microcapsules.²⁶

Instrumentation

An ultraviolet-visible spectrometer (Lambda 45, Perkin Elmer, Boston, MA) was used to collect absorbance spectra. The slit size (4 nm) and scanning speed (480 nm/min) were held constant throughout all the experiments. A scanning fluorescence spectrometer (QM1, Photon Technology International, Birmingham, NJ) was used to collect fluorescence emission spectra by exciting the sample at 543 nm. A 100-W long-wave ultraviolet lamp (Blak-ray® Model B 100AP, Entela, Grand Rapids, MI) was used to irradiate the microcapsules for photocross-linking PSS and DAR layers in the capsule walls. Confocal images were taken with a Leica (Bannockburn, IL) TCS SP2 microscope equipped with a 63 $\times$ oil immersion objective and green and red He-Ne excitation lasers. Counts and sizes of microcapsules were obtained with a Beckman (Palo Alto, CA) Coulter Counter (Z2) using a 100- μm aperture. A YSI (Yellow Springs, OH) biochemical analyzer was used to measure the glucose concentrations.

Procedures

Solution-phase experiments—All glucose sensitivity experiments were performed in phosphate-buffered saline (PBS) (0.01 *M* phosphate buffer, 0.0027 *M* potassium chloride, and 0.137 *M* sodium chloride) solution maintained at pH 7.4. The apo-GOx and amino-dextran used in all experiments were conjugated to Cy5 and TRITC, with a labeling ratio of 1.14 and 1.12, respectively. In all experiments, the changes in energy transfer from TD to CAG were monitored at every step using a fluorescence spectrometer to collect emission across the range of 560–725 nm. Initially, 17 pmol of TD (donor) was added to 0.4 mL of PBS, which was followed by the stepwise addition of 80 pmol of total CAG into the sample solution. To observe the dissociation of dextran and apo-GOx complexes with the addition of glucose, RET changes were observed with the titration of 100 mg/mL β -D-glucose solution aliquots into the sample containing TD/CAG complexes.

It is known that GOx exhibits at least 20 times higher specificity for β -D-glucose over other sugars (e.g., α -D-glucose, D-mannose, D-fructose).²⁷ In our previous report,⁸ the specificity of the glucose sensor based on FD/TAG was found to be five to 10 times more specific for β -D-glucose compared with other sugars. The specificity of the new sensor was investigated in this report, because the amine conjugation efficiency of Cy5 is much greater than TRITC, which could result in a different degree of labeling near glucose binding sites, and this could ultimately change the binding properties. The specific nature of the sensor for glucose was demonstrated by performing similar RET measurements with the titration of β -D-glucose, α -D-glucose, and mannose into the solution of TD/CAG complexes, maintaining all other parameters constant. Sensitivity curves were obtained by plotting the percent change in

TRITC/Cy5 peak ratio versus analyte concentration, and the slope of the linear region was calculated as a measure of response sensitivity.

Formation of hollow microcapsules—Solutions of PSS (anionic), PAH (cationic), and DAR (cationic) used for assembling {PSS/PAH} and {PSS/DAR} multilayers were prepared in deionized water at 2 mg/mL. As described in detail elsewhere,⁸ the positively charged manganese carbonate (MnCO_3) particles were coated with the ionic polymers to obtain a final architecture of {(PSS/PAH)(PSS/DAR)}₃ (PSS/PAH/PSS). Hollow microcapsules were then obtained by dissolving the MnCO_3 cores using 0.1 M HCl solution.

Encapsulation of sensing elements—The microcapsules prepared as described above contain porous walls, which are permeable to sensing elements (TD and CAG) prior to ultraviolet irradiation, but become impermeable after photocross-linking.⁸ The microcapsule suspension was incubated in the mixture of 1 μM TD and 6 μM CAG for 60 min, which allowed sufficient time for diffusion of loading molecules into the capsule interior. In order to encapsulate the TD/CAG molecules in microcapsules, DAR and PSS layers in the capsule walls were cross-linked by exposing the microcapsules in loading solution to ultraviolet light. As the final step, the capsules were rinsed in deionized water to remove excess TD/CAG complexes, yielding microcapsules loaded with the sensing assay elements, with a final form as illustrated in Figure 2.

Sensor response to random glucose concentrations in microcapsules—

Glucose sensitivity experiments were performed on the microcapsules loaded with TD/CAG complexes to demonstrate a reversible sensor response. The sensing assay-loaded microcapsules were exposed to random glucose concentrations, after an initial fluorescence spectrum was collected with TD/CAG-loaded microcapsules in PBS buffer. The random glucose concentrations were achieved by repeating two steps several times: (1) increase the glucose concentration with the addition of stock solution to microcapsules in buffer and then collect the fluorescence spectrum; and (2) reduce the glucose levels in the suspension by centrifuging and removing the supernatant (glucose) solution, measuring the actual glucose concentration of this solution with the biochemical analyzer, and then adding an equal volume of fresh PBS buffer without glucose. Fluorescence spectra were collected after each addition and removal of glucose solution from the microcapsules, and the corresponding changes in TRITC/Cy5 peak ratios were calculated for changing glucose concentration. This procedure was repeated to cover the glucose concentration in the range of 0–2,800 mg/dL, in the order of 0, 164, 56, 108, 632, 433, 813, 1,980, and 2,800 mg/dL.

RESULTS AND DISCUSSION

Solution-phase glucose response tests

Titration of TRITC-dextran up to 43 nM into 200 nM CAG in PBS solution led to strong fluorescence emission peaks corresponding to TRITC and Cy5 under 543 nm excitation, implying significant energy transfer from TRITC to Cy5. To observe the displacement of dextran from apo-GOx with the addition of glucose, spectra were measured after the titration of 100 mg/mL β -D-glucose solution aliquots into the sample containing TD/CAG

complexes. As hypothesized, the glucose in the solution displaced dextran from apo-GOx, resulting in decreased energy transfer from TRITC to Cy5, as indicated by the increase in TRITC peak relative to Cy5 (Fig. 3a). The percent change in TRITC/Cy5 peak ratio (relative to the baseline ratio, at zero glucose) as a function of glucose concentration is shown in Figure 3b, from which it can be observed that there is a total change of 15% in energy transfer due to 360 mg/dL glucose. The data clearly show that the response is linear in the range of 0–540 mg/dL, with a sensitivity of 0.06%/(mg/dL). These measurements confirm that apo-GOx and dextran molecules were dissociated in the presence of glucose, though the overall change in signal is only about one-fourth of that observed when FITC and TRITC were used as the donor and acceptor, respectively.

Sensor specificity

To demonstrate the specificity of the TD/CAG-based glucose sensor, the RET measurements were performed with the titration of β -D-glucose, mannose, and α -D-glucose into solutions of 50 nM:200 nM TD:CAG complexes. The corresponding percent change in TRITC/Cy5 peak intensity ratio versus analyte concentration is plotted in Figure 3b, from which it can be observed that the maximum sensitivity was achieved with β -D-glucose, and there was about five to 10 times lower sensitivity for mannose and α -D-glucose. This agrees with the previous results,⁸ and proves that the apo-GOx retains its binding specificity for β -D-glucose, in spite of exposing the sensing elements to several chemical processes, such as protein precipitation, conjugation, etc.

Encapsulation of sensing chemistry in microcapsules

Microcapsules with {(PSS/PAH)(PSS/DAR)₃ (PSS/PAH/PSS)} shell architecture were prepared as described and used for encapsulating TD/CAG complexes. Confocal images of polyelectrolyte microcapsules loaded with the glucose assay are shown in Figure 4, from which it can be observed that the TRITC and Cy5 fluorescence is concentrated in the capsule interior and walls, indicating the immobilization of TD/CAG complexes inside and on the capsule walls. High encapsulation in the walls could be due to a combination of electrostatics (residual charges of polyelectrolytes), van der Waals forces, different solubility/partitioning, and physical entrapment of the sensing elements (upon cross-linking). It is noteworthy that association with capsule walls occurs prior to wall cross-linking, so the contribution of the latter effect is expected to be minimal. A detailed discussion of the stability of the encapsulated materials appears elsewhere.⁸

Demonstration of reversible sensor response

To test the reversibility of these sensors, loaded microcapsules were exposed to random glucose concentrations. The fluorescence spectra were collected after each addition and removal of glucose solution from the microcapsules, and the corresponding changes in TRITC/Cy5 peak ratios are plotted in Figure 5. It is clear that there is a linear response in the 0–720 mg/dL range, with a sensitivity of 0.05%/(mg/dL), which is comparable to the sensitivity obtained in solution-phase experiments. The dissociation constant in the case of TD/CAG was observed to be 202 mg/dL, which is comparable to that obtained with FD/TAG complexes. These results and our previous results based on FD/TAG complexes⁸

show that the microcapsule-based sensors are completely reversible, without any considerable loss in sensitivity compared with the solution phase.

After the comparison of the results obtained with the current sensor design (TD/CAG) and previously reported FD/TAG-based sensor, it was observed that there is a drop in sensitivity by a factor of 3–4 due to the replacement of FITC/TRITC dyes with TRITC/Cy5. This decrease in sensitivity may be attributed to several factors. First, the labeling ratio for dextran varied between the commercially available FD (24.5) and custom-labeled TD (1.12). Considering equal molar concentrations of FD/TAG and TD/CAG complexes, this variation in dextran labeling ratio could logically result in a lower average RET efficiency for bound dextran in the latter case; consequently, with addition of glucose, the total percent change in energy transfer would reduce, translating into lower sensitivity. Second, the TD used in this study was prepared using amino-dextran (cationic), whereas the FD (from Sigma) was anionic. Because apo-GOx is anionic at pH 7.4, a fraction of the cationic dextran used in this study could complex with apo-GOx on the basis of electrostatic forces, in addition to affinity forces; if the ionic complexes do not dissociate with the addition of glucose, this will contribute a strong static baseline signal to for all of the measurements, resulting in decreased sensitivity. Finally, there is a significant variation in the conjugation reaction of apo-GOx with Cy5 compared with TRITC. For this study, the Cy5 bis-(*N*-hydroxysuccinimide) ester can simultaneously bind to two amine groups on apo-GOx, whereas the isothiocyanate group of TRITC can only react with a single amine. Binding multiple amine groups on the protein could partially disrupt the conformation of the apo-GOx conformation and alter binding properties, resulting in a decreased glucose affinity. However, given the agreement between the dissociation constants obtained from the two studies, this last possibility is less likely to be a significant factor. Determination of the major contributing factors and sensitivity optimization will be part of future work.

The system demonstrated here carries the advantages of using (a) prefabricated microcapsules for carrying out the sensing assay, because it allows for the free movement of sensing elements during the competitive binding process, and in maintaining the constant concentrations of the ligand and receptor with continuously varying analyte concentration; and (b) orange/red dyes, which will enable the use of longer excitation wavelengths where tissue scattering is reduced, making the sensor more appropriate for in vivo applications. It is noteworthy that even though there is a threefold drop in sensitivity with use of longer wavelengths, this is not a large concern when comparing the expected improvement in signal intensities measured through the skin. It is also appropriate to note that these sensors could be interrogated using fluorescence lifetime measurements,^{16,28} which will be better suited to in vivo monitoring because they are less affected by the fluorophore concentration and optical properties of the medium. Ongoing studies include complete sensor response characterization, in particular long-term stability analysis, and careful investigation of the reasons for sensitivity loss when longer-wavelength dyes are employed.

CONCLUSIONS

A novel glucose sensing system based on competitive binding, RET, and polymer microcapsule technology operating in the near-infrared region has been demonstrated. This

glucose assay, comprising apo-GOx/dextran complexes, showed about five to 10 times more sensitivity to β -D-glucose compared with other sugars. Microcapsules containing TD/CAG showed a decrease in RET due to the addition of glucose, with a sensitivity of 0.06%/(mg/dL) over the range of 0–720 mg/dL. This sensing system can be used for glucose monitoring in patients with diabetes, based on the sensitivity, specificity, and reversible response in the region of interest (0–360 mg/dL). It is supposed that the threefold loss of sensitivity with the use of longer-wavelength dyes will be compensated for with the increase in the intensity levels and the decrease in the signal-to-noise ratio. Therefore future studies will aim at finding the reasons for sensitivity loss with longer-wavelength dyes and focus more on the improvement of sensor response characteristics. These glucose-responsive systems can also potentially be used in future for “smart” drug delivery studies, e.g., insulin may be released in response to apo-GOx/dextran dissociation. In vitro and in vivo studies to test the response time, stability biocompatibility, and cytotoxicity of these sensors are in progress.

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FIG. 1. Schematic of the RET glucose assay based on the competitive binding between TD and glucose for binding sites on Cy5-apo-GOx: (step 1) holoenzyme; (step 2) apo-enzyme conjugated to Cy5; (step 3) TD/CAGO (**inset** shows significant energy transfer); (step 4) formation of CAG/glucose complexes and free TD molecules with the addition of glucose (**inset** shows a decrease in energy transfer).

FIG. 2.
Schematic of the microcapsules loaded with dextran/apo-GOx complexes.

FIG. 3.
 (a) Normalized fluorescence spectra with the addition of β -D-glucose to TD/CAG complexes in PBS buffer. a.u., arbitrary units. (b) Percent change in TRITC/Cy5 peak ratio with the addition of β -D-glucose, α -D-glucose, and mannose solutions. Error bars show 1 SD of three replicate measurements.

FIG. 4. Confocal fluorescence images of typical microcapsules loaded with TD and CAG: (a) TRITC and (b) Cy5 fluorescence. (c) Overlay of the TRITC and Cy5 fluorescence.

FIG. 5. Percentage in TRITC/Cy5 peak ratio with the addition of random β -D-glucose concentrations to the TD/CAG-loaded microcapsules. Error bars show 1 SD of three replicate measurements.