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Fluorescence Glucose Detection: Advances Toward the Ideal *In Vivo* Biosensor

Elizabeth A. Moschou,[1] Bethel V. Sharma,[2] Sapna K. Deo,[1] and Sylvia Daunert[1,2,3]

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The importance of glucose monitoring for *in vivo* as well as for *ex vivo* applications has driven a vast number of scientific groups to pursue the development of an advanced glucose sensor. Such a sensor must be robust, versatile, and capable of the long-term, accurate and reproducible detection of glucose levels in various testing media. Among the different configurations and signal transduction mechanisms used, fluorescence-based glucose sensors constitute a growing class of glucose sensors represented by an increasing number of significant contributions to the field over the last few years. This manuscript reviews the progress in the development of fluorescence based glucose sensors resulting from the advances in the design of new receptor systems for glucose recognition and the utilization of new fluorescence transduction schemes.

KEY WORDS: Fluorescence; detection; glucose; *in vivo*; sensor.

INTRODUCTION

Glucose is a key physiological analyte affecting a multitude of aspects related to our everyday life. The effect of glucose spans from its importance in the food industry in quality control of food analysis [1] to the monitoring and managing of diabetes [2,3]. The quest for the "ideal" glucose sensor has been a long-time goal of many investigators, and as a result, numerous glucose sensors have been developed thus far. While many glucose sensing systems have found *ex vivo* applications, the need for a reliable glucose sensor that could be trusted to be implanted long-term for the *in vivo* monitoring of physiological levels of glucose, has not yet been fulfilled [4]. The answer to the question "Why is it taking so long?" may reside in the fact that there are many parameters that need to be met collectively for the development of the optimized glucose sensor [5]. These parameters include commonly addressed sensor characteristics, such as selectivity, linear range and response time, as well as other critical parameters that in some instances receive less attention in spite of their great importance. For example, reproducibility and reversibility of signal, reproducibility of sensor fabrication, biocompatibility, operational lifetimes at body temperature, and storage stability of the sensor are of paramount importance when developing reliable *in vivo* sensors.

A number of approaches toward the development of glucose sensors that employ different transduction mechanisms as well as recognition elements for glucose have been undertaken. The glucose biosensors reported in the literature utilize mostly electrochemical signal transduction [6,7], as well as Near-infrared spectroscopy [8], polarimetry [9], Raman spectroscopy[8], and fluorescence spectroscopy [10]. Among these sensors, only a few have been demonstrated to be suitable for *in vivo* applications, with an even smaller number of *in vivo* glucose sensors being commercially available. One example of *ex vivo* sensing coupled with a sampling device is the Glucowatch, which collects interstitial fluid through reverse iontophoresis and measures glucose levels electrochemically displaying the measurement on the watch. This system provides a quick and convenient method of measuring

[1] Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506.
[2] Department of Pharmaceutical Sciences, University of Kentucky, Lexington, Kentucky 40536.
[3] To whom correspondence should be addressed. E-mail: daunert@uky.edu

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glucose in interstitial fluid, but is associated with skin irritations caused by the application of potential on the patient's skin. Another example is the Medtronic MiniMed needle-shaped electrochemical sensor, which is implanted under the abdomen skin of the patients. This sensor is able to monitor glucose levels for 72 hr, but within this period requires frequent calibration, and after 72 hr of service the sensor needs to be replaced by a new one.

Fluorescence-based sensing is a powerful method suitable for fast, reagentless, and noninvasive detection of neutral analytes. The aim of this review is to present recent advances in the development of fluorescence glucose sensors based on various receptor systems, including those that employ synthetic receptors (i.e., boronic acid derivatives), as well as naturally occurring ones (i.e., the enzyme glucose oxidase, the lectin Concanavalin A, and sugar binding proteins), and those based on the rational design of genetically engineered binding proteins (i.e., GBP).

BORONIC ACID-BASED FLUORESCENCE GLUCOSE SENSING

Synthetic boronic acid derivatives have been used as receptors in the development of glucose sensing systems. They are able to complex glucose rapidly and reversibly through covalent interactions. The covalent character of the bonding permits their use in hydrogen bonding solvents, such as aqueous solutions, as opposed to general synthetic systems based on hydrogen bonding. Boronic acid, when it is in the form of a boronate anion, is able to form two covalent bonds with the hydroxyl groups of glucose, creating a cyclic boronate ester. This mechanism of recognition was used by Yoon and Czarnik [11] for the development of the first fluorescent saccharide sensing system. They used the synthetic receptor 1,2-anthrylboronic acid (Fig. 1), with the boronic acid moiety being responsible for the binding of glucose, and the anthracene moiety for the transduction of the fluorescent signal. This work set the basis for the development of fluorescent glucose sensing systems based on boronic acids, even though it suffered from a series of drawbacks. The system usage was limited to the analysis in basic media, as the pH of the sample needed to be higher than 8.8, the pKa of the 2-anthrylboronic acid. In addition, it was characterized by a small analytical signal (with a maximum signal quenching of 10%), and poor selectivity that followed the order: D-fructose > D-allose ≈ D-galactose > D-glucose (characteristic of the monoboronic acid receptors). In spite of all these disadvantages, which render this system unsuitable for *in vivo* applications, a number of groups continued to work toward improving fluorescence-based glucose sensing systems based on boronic acids.

Toward that end, Shinkai and co-workers employed a series of new strategies based on the use of a boronic acid receptor that can work at physiological pH [12]. These investigators postulated that the limitations of the previously developed glucose sensing system were due to the electron poor boronic acid moiety, which they improved by introducing a tertiary amine in the proximity of the boronic acid moiety as originally shown by Burgemeister *et al*. [13]. The new receptor was characterized by a low pKa value of 4.8, making the resulting system usable for glucose determinations in the physiological pH range. Furthermore, the introduction of the amine played another significant role in the receptor design. It offered the ability to use photoinduced electron transfer (PET) as a tool for the transduction of the fluorescence signal. In the absence of glucose, the fluorescence signal of the fluorophore (anthracene) was quenched by the nitrogen atom via photoinduced electron transfer. Upon binding of glucose to the boronic acid moiety, the Lewis acid-base interaction between the boronic acid and the amine was strengthened. This interaction suppressed the PET from the nitrogen to the fluorophore, resulting in the recovery or "switching on" of the fluorescence signal of anthracene (λ_{ex} = 370 nm; λ_{em} = 423 nm). The linear range for the detection of glucose was 0.03–1 mM in 33% MeOH/H_2O pH 7.7, with a maximum relative fluorescence intensity of 7. Another contribution of this work was that it set-up the basis for tuning the selectivity of the synthetic receptors toward glucose. The authors introduced the use of the diboronic acid receptor, with a designed alignment of the two boronic acid moieties. Upon interaction between the two nitrogen with the two boron atoms, the two boronic acid moieties formed are properly aligned to permit selective interaction with the 1,2- and 4,6-hydroxyls of glucose. The system based on this receptor **2** (9,10-Bis[[*N*-methyl-*N*-(*o*-boronobenzyl)amino]methyl]anthracene, Fig. 1), presented the following selectivity series with the binding constants presented in parenthesis: D-glucose (3981 M^{-1}) > D-allose (630 M^{-1}) > D-fructose (316 M^{-1}) > D-galactose (158 M^{-1}), with glucose being the primary saccharide analyzed. Even though the applicability of this system *in vivo* is restricted due to limited solubility, it presented the basis for the improvement of selectivity of the boronic acid receptors.

Tony James and co-workers continued the studies on the design of a glucose selective fluorescent sensor, by examining the distance between the two boronic acids and the proper selection of the fluorescent moiety. They observed that the use of hexamethylene as a linker between the two boronic acid moieties enhanced the glucose

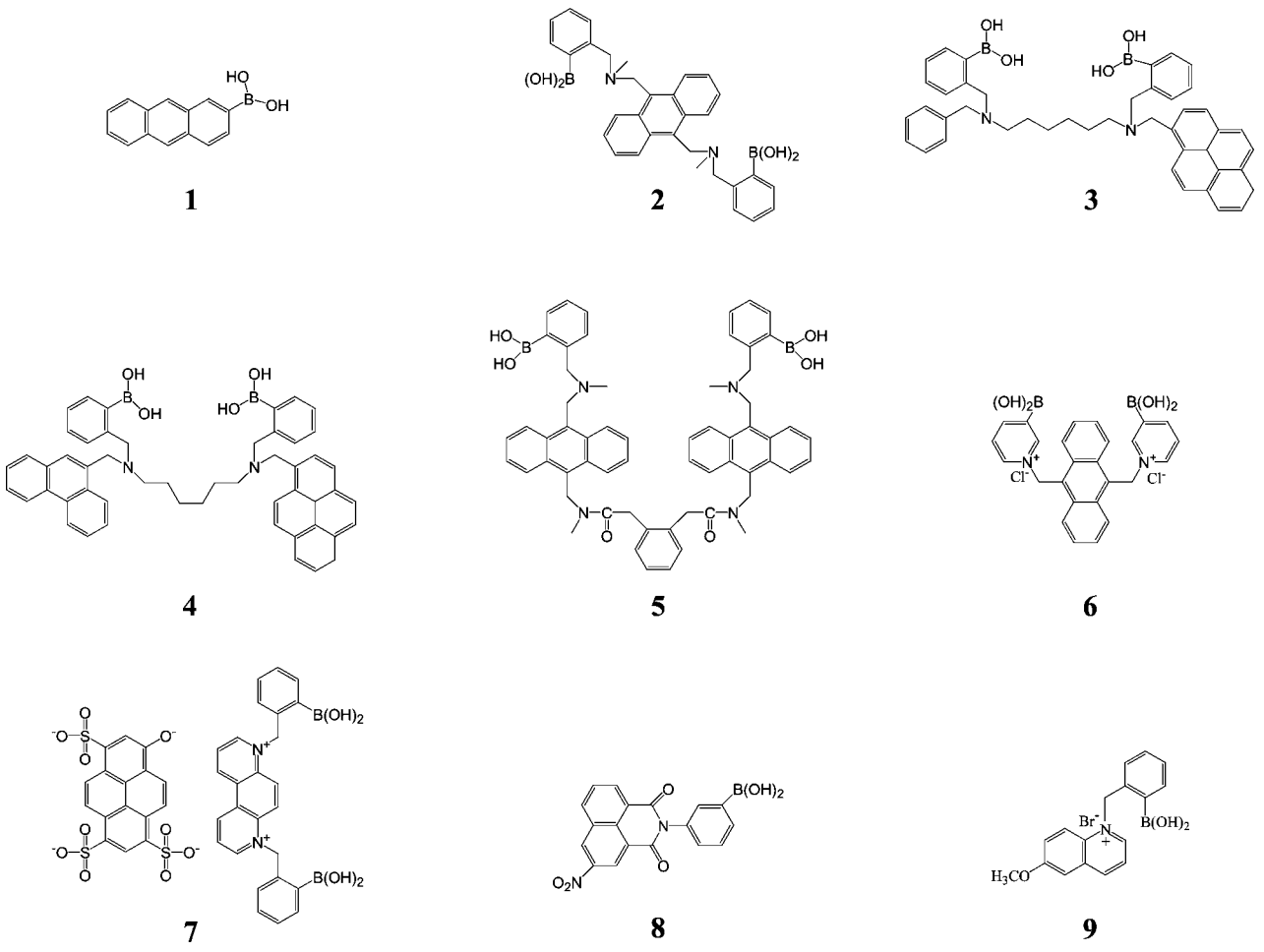

Fig. 1. Structures of fluorescence glucose receptors based on various boronic acid derivatives.

sensitivity of the receptor [14]. In addition, they showed that the selectivity of the saccharide sensor was influenced by the specific fluorophore used [15]. The pyrene moiety, with the largest size of π-surface and the smallest steric crowding, provided better glucose selectivity than the naphthalene, phenanthrene, and anthracene fluorophores examined. The resulting PET glucose sensor based on receptor **3** (Fig. 1) showed a linear range of response 0.1–5 mM glucose in 52% methanol, and the following series of selectivity with the binding constants presented in parentheses: D-glucose ($962 \pm 70\ M^{-1}$) > D-fructose ($784 \pm 44\ M^{-1}$) > D-galactose ($657 \pm 39\ M^{-1}$) > D-mannose ($74 \pm 3\ M^{-1}$). The same group also tested the use of the diboronic acid receptor comprising two different fluorophores, one phenanthrene and one pyrene fluorophore per receptor molecule [16]. In the absence of glucose, the excited phenanthrene fluorophore ($\lambda_{ex} = 299$ nm) emitted light at 369 nm, which excited the pyrene fluorophore ($\lambda_{ex} = 342$ nm) and resulted in the emission of light from the excited pyrene at 417 nm. Upon interaction with glucose, receptor **4** (Fig. 1), underwent a conformational change that resulted in the spatial separation of the two fluorophores, which eliminated the intramolecular energy transfer. This system was selective to glucose (binding constant K: $142 \pm 12\ M^{-1}$) over galactose (K: $74 \pm 7\ M^{-1}$) and fructose (K: $76 \pm 10\ M^{-1}$), with a fluorescence enhancement of 3.9 after excitation at 299 nm. Although the use of these systems *in vivo* is restricted due to limited solubility, this work demonstrated the capability of tuning the characteristics of boronic acid based systems.

Wang's group also followed the strategy of designing an appropriate linker for the proper positioning and orientation of the two boronic acid moieties in order to achieve selective bidentate binding of glucose. They found that employing a two acetamide ortho-substituted phenyl ring as a spacer, provided the right spatial separation and orientation to the two boronic acid moieties, thus allowing them

to interact with the two diol in the glucose molecule [17]. The system based on receptor **5** (Fig. 1) showed a 43- and 49-fold selectivity for glucose over fructose and galactose, respectively and an enhanced 3.5-fold fluorescent intensity for 1 mM glucose in 50% methanol solutions.

One of the main factors restricting the application of the PET glucose sensing systems discussed so far is their limited solubility in pure aqueous solutions. Norrild and co-workers worked towards the development of water-soluble boronic acid receptors, introducing the ionic receptor **6** (Fig. 1) [18]. This receptor comprised of two zwitterionic pyridinium hydroxyboronates, which provided the boronic acid moieties for glucose recognition, and an anthracene moiety for transduction of the fluorescent signal. The ionic character and the low pKa of 4.0 of the receptor increased its solubility in aqueous solutions of neutral pH. This system showed a linear range of 0.1–1 mM glucose, but its sensitivity to glucose was significantly deteriorated in the presence of 0.1 mM of either fructose or galactose, limiting its use for *in vivo* glucose measurements.

Singaram's group introduced another aqueous soluble system (**7,** Fig. 1), comprised of two distinct parts, a pyranine playing the role of the fluorescent transducer, and a discrete diboronic acid receptor [19]. The boronic acid molecule was linked to a zwitterionic phenanthrolinium viologen. In the absence of glucose, the cationic viologen interacted with the anionic pyranine through ionic association, suppressing the fluorescence signal of the later. When glucose was added in the solution, the viologen receptor bound to glucose to form the negatively charged boronate ester. The electrostatic attraction between the dye and the quencher was lost, resulting in the increase of the pyranine fluorescence. Both fluorophore and receptor molecules employed were zwitterionic, thus enhancing the solubility of this system in aqueous solutions. This system showed a 45% increase in fluorescence signal in the presence of 3 mM glucose at neutral pH, relative to the fluorescence recovery of 32 and 25% generated by the presence of 3 mM fructose and 3 mM galactose, respectively.

Heagy and coworkers developed a ratiometric sensing system for the fluorescence sensing of glucose. They designed a dual-fluorescence assembly that promoted charge-transfer excited states. The authors used receptor **8** (Fig. 1) that contained a monoboronic acid moiety linked to a 3-nitronaphthalic anhydride, which displayed two emission bands (at 430 and 550 nm) after excitation at 370 nm [20]. This system showed a decrease in the ratio of the fluorescence intensities at 430/550 nm when increasing the glucose levels in aqueous solutions of neutral pH. Although this system showed limited sensitivity at physiological glucose levels, it utilized the advantages of a ratiometric system, overcoming problems from excitation source fluctuations and background fluorescence of interferents.

Lakowicz's group presented another approach for the fluorimetric determination of glucose, namely lifetime-based sensing of glucose employing phase-modulation methods [21]. Upon interaction of the boronic acid receptor **2** with glucose, there is a shift toward low-modulation frequencies indicating longer mean lifetimes. Furthermore, the apparent glucose binding constants were found to be 5- to 10-fold smaller with phase-modulation or mean lifetime than that with the measurement of intensities, shifting the glucose detection range in the low millimolar range, within the physiological concentrations of glucose. Even though this specific system utilizes receptor **2** with limited water solubility, it makes use of the fluorescence intensity decay times, which are independent of the intensity of the signal and the concentration of the probe.

Lakowicz and co-workers are working toward the development of glucose sensing contact lenses for *ex vivo* monitoring of glucose in tears. They have designed receptor **9** (Fig. 1), based on a quaternized form of the quinolinium nucleus, which was found to respond to physiological tear glucose within disposable contact lenses [22]. Even though this receptor shows comparable selectivity for both glucose and fructose, the authors state that the physiological concentration of fructose is 10 fold lower than glucose minimizing the fructose interference for such an application.

GLUCOSE OXIDASE-BASED FLUORESCENCE GLUCOSE SENSING

The inherent ability of proteins to interact selectively with specific molecules has been long utilized in the design of selective chemical sensors. Enzymes are the most commonly used proteins in the development of biosensors. In that respect, the enzyme glucose oxidase (GOx) has been widely employed in glucose sensing. GOx catalyzes the conversion of D-glucose and oxygen to D-glucono-1,5 lactone and hydrogen peroxide. The detection of oxygen consumption, hydrogen peroxide production, or local pH change has been widely utilized in the development of GOx-based glucose sensors as they correlate with the levels of glucose present in a given sample. GOx sensors utilizing electrochemical signal transduction form a large and important category of glucose sensors that demonstrate good analytical performance [6,7]. The widespread use of GOx as a model enzyme in the development of biosensors is mainly due to its high glucose selectivity over other sugars, *ex vivo* enhanced enzyme stability, its well-established mechanism of activity, commercial availability, and low cost.

The simplest strategy employed for the development of a fluorescent glucose sensing system based on GOx took advantage of the intrinsic fluorescence of the biomolecule. GOx exhibits an intense fluorescence signal with excitation at 224 and 278 nm, and emission at 334 nm, mainly due to the tyrosine and tryptophan residue in the molecule. Another fluorescence band arises at 520 nm due to the fluorescence of the cofactor of GOx, FAD (flavin adenine dinucleotide). The visible fluorescence of FAD is quenched by adjacent amino acids, but the intrinsic fluorescence of GOx at 334 nm can be used for the signal transduction in glucose determination. During the enzymatic reaction, the change in the fluorescence of the enzyme over time is correlated to the glucose levels in the sample [23]. The signal is therefore transduced without the need of any other steps or the utilization of other reagents. The disadvantage of using the intrinsic fluorescence of the enzyme is the short excitation and emission wavelengths used, where a large number of compounds fluoresce as well.

To overcome this drawback, a fluorophore that emits in the visible region was coupled to the enzyme GOx. Galban's group labeled GOx with a fluorescein derivative (GOx-FC), which has an excitation wavelength at 489 nm and an emission wavelength at 520 nm [24]. The mechanism of signal transduction was based on the inner filter effect between FAD and GOx-FC. The molecular adsorption of FAD overlapped with the molecular adsorption of GOx-FC (creating the inner filter effect), but not with that of $FADH_2$. In the presence of glucose, the inner filter effect was cancelled due to the conversion of FAD to $FADH_2$, increasing the fluorescence signal of GOx-FC. The sensor was constructed by the immobilization of GOx-FC in a polyacrylamide polymer, and was incorporated in a flow injection analysis system. This system was able to analyze 30 samples of 300 μL volume per hour, with a linear range of detection 2.2–11 mM glucose, reproducibility of 3% RSD, and a sensor lifetime of more than 3 months. The applicability of this system was evaluated by the real sample analysis of glucose in drinks. The increase of the range of detection to that of physiological glucose levels is desired for further use of this system in blood glucose determinations.

An innovative approach for the development of a reversible GOx fluorescent sensor was presented by D'Auria *et al*. [25]. This approach used apo-glucose oxidase (apo-GOx), resulting from the removal of FAD, which could still bind glucose with comparable affinity to that of the holoenzyme. The apo-GOx interacted non-covalently with the fluorophore ANS (8-anilino-1-napthalene sulfonic acid), with excitation wavelength at 325 nm and an emission wavelength at 480 nm. The fluorescence intensity and mean lifetime of ANS were found to decrease with the increase in glucose concentration in the sample. The advantage of this system is reversibility without the consumption of glucose, but for most practical applications, as the determination of glucose *in vivo*, the dynamic range for glucose detection needs to be improved to lower than the reported 5 mM glucose.

McCurley proposed a new alternative for the development of a glucose fluorimetric sensor. GOx was physically entrapped in a pH sensitive polyacrylamide polymer gel that was chemically functionalized with the fluorescent tetramethylrodamine cadaverine (TMRC) [26]. The hydrogel, which was applied to the tip of a fiber optic probe, swelled in the presence of glucose, due to the pH decrease accompanying the enzymatic reaction (Fig. 2A,B). This swelling resulted in the increase of the fluorescence signal measured from the hydrogel, and was correlated to the amount of glucose present in the sample. This sensor presented a dynamic response within physiological glucose levels by utilizing longer wavelengths of excitation ($\lambda_{ex} = 540$ nm) and emission ($\lambda_{em} = 580$ nm). However, improvements towards the linearity of the response and the decrease in the analysis time to less than 10 min are needed for the practical application of the sensor *in vivo*.

The group of Otto Wolfbeis utilized a different transduction scheme for their planar thin-film sol-gel based glucose sensor. This sensor was based on the entrapment of GOx in the sol-gel doped with the oxygen-sensitive fluorophore ruthenium(II)(4,7-diphenyl-1,10-phenanthroline)$_3$-(dodecylsulfate)$_2$ (Ru(dpp)) [27]. The quantity of the oxygen consumed by the enzymatic reaction was monitored by the fluorescence of the oxygen-sensitive fluorophore, at high excitation wavelength ($\lambda_{ex} = 465$ nm) and emission wavelength ($\lambda_{em} = 610$ nm). The sensor presented a linear range of detection from 0.1 to 4 mM glucose, with a response time of 4 min, with return of the signal to baseline within 6 min, good signal reproducibility (2% RSD), and storage stability higher than 4 months. In practical applications, this sensor should be accompanied by an oxygen sensor, to compensate the partial oxygen pressure variations from sample to sample, as well as during the continuous monitoring of glucose.

An innovative technique for the development of polyacrylamide-based ratiometric fluorescent glucose nanosensors was presented by Kopelman and coworkers [28]. The PEBBLEs (probes encapsulated by biologically localized embedding) with an average diameter of 45 nm, incorporated GOx and an oxygen sensitive fluorescent dye, $Ru[dpp(SO_3Na)_2]_3)Cl_2$ (4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt), (Fig. 2C). The dye was used for the detection of the local oxygen depletion that results from the enzymatic reaction. In addition, the nanosensors also contained an oxygen insensitive fluorescent dye, used as a reference for the ratiometric measurements. The nanosensors presented a linear

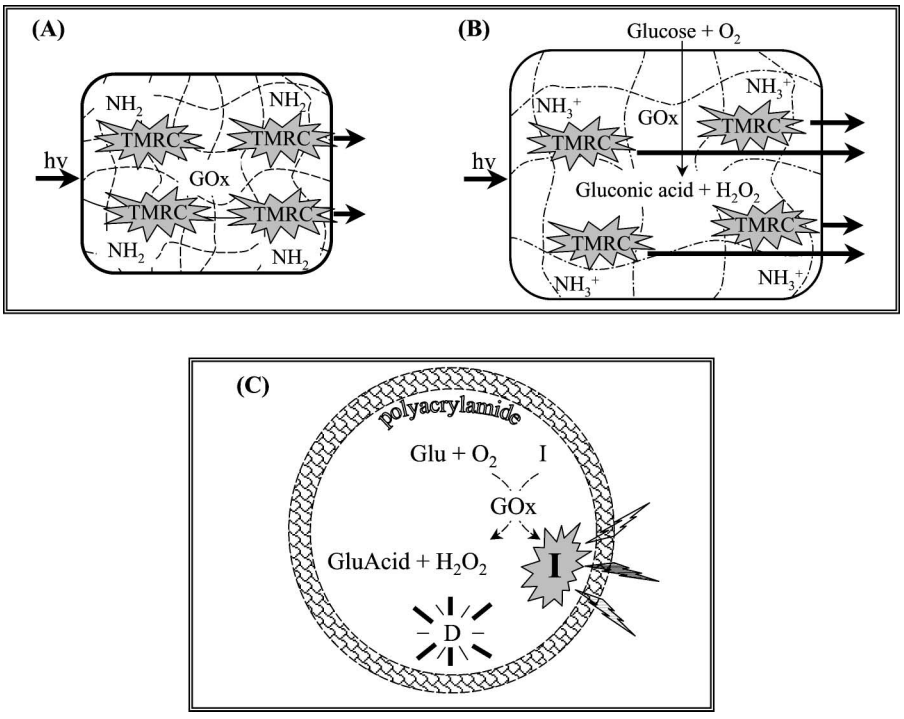

Fig. 2. Response mechanism of polyacrylamide hydrogel doped with GOx and TMRC in the absence (A) and in the presence (B) of glucose [26]. The decrease in the pH accompanying the enzymatic reaction results in the swelling of the hydrogel and the increase of the fluorescence signal measured. (C) Response mechanism of PEEBLEs, incorporating GOx, the oxygen sensitive fluorophore $Ru[dpp(SO_3Na)_2]_3)Cl_2$ (symbolized as I), and an oxygen insensitive dye (D) [28]. Glu and gluacid stand for glucose and gluconic acid, respectively. I monitors the local oxygen depletion accompanying the enzymatic reaction within the polyacrylamide nanosensors.

range of 0.3 to 5 mM glucose with a response time of 150 s, reversibility, and good signal reproducibility (2% RSD). As with all sensors detecting oxygen depletion, this system also needs to be accompanied by another oxygen sensor serving as a reference, for the compensation of oxygen pressure variations in the samples and limited oxygen diffusion at the site of the sensor. These polyacrylamide nanosensors offer an attractive approach for glucose determination *in vivo*, exploiting the benefits of the ratiometric technique. In addition, PEEBLEs also offer the advantages of both the matrix protection of GOx and dyes from protein interferences in the cells, as well as minimization of the dye toxicity to the cells.

LECTIN-BASED FLUORESCENCE GLUCOSE SENSING

A number of binding proteins exist in nature that possess highly specific interactions with their ligands. These interactions between the binding protein and its corresponding ligand have been utilized in a number of biomedical applications, such as in drug design and the development of biosensors. One characteristic example is the plant sugar-binding protein Concanavalin A (Con A) isolated from the Jack bean. Con A is one of the best characterized lectins, and it is mannose and glucose-specific [29]. Due to its four-site, reversible binding of sugars, this particular plant lectin has been exploited for use in different types of glucose sensing systems. The most recent methods of glucose sensing utilizing Con A are based on fluorescence resonance energy transfer (FRET) and fluorescence intensity measurements.

The glucose-specific FRET assay that Ballerstadt and Schultz engineered, made use of two sets of dextran polymers, one labeled with rhodamine, and the other labeled with fluorescein. In the absence of glucose, a multivalent lectin interacted with the dextrans of both types of polymer beads bringing them close to each other. The close proximity of the two fluorescent labels caused FRET to occur when the fluorescein donor was excited, thus decreasing the measurable signal emitted by fluorescein.

This "fluorescence affinity sensor," was based upon fluorescence quenching of the donor emission by FRET [30]. When a high enough concentration of glucose was added to the system, the lectin would become bound to the glucose instead of the dextran beads and the FRET signal decreased, thus giving an increase in donor fluorescence emission. The excitation and emission wavelengths used were 495 and 520 nm, respectively. Both a cuvette assay and a hollow fiber optic sensor were described. The initial response time was around 60 s, but the minimum hollow fiber response time, corresponding to changes in the external concentration of glucose, was 4 min. The dynamic signal range was from 0 to 30 mM. Greater than 60% of the fluorescent signal could be measured in response to 20 mM glucose for a period of 80 days. With the incorporation of fluorophores in the near-infrared range this sensor could be amenable to measurement through skin tissue.

Tolosa *et al*. illustrated how the attachment of ruthenium to Con A to form a metal-ligand complex (RuCon A) can permit the selective detection of glucose [31]. Their competitive assay is a FRET based system using RuCon A as the donor and a maltose-insulin-malachite green (MIMG) complex as the acceptor (Fig. 3). The maltose component was included to offer affinity of the acceptor for Con A. The method was designed to utilize the long-lifetime of a ruthenium probe, so that changes in the decay time of the complex due to the presence of glucose could be measured. The emission spectra of the RuCon A overlapped with the absorbance of the MIMG. The RuCon A showed a large Stokes shift, which is ideal in order to distinguish the emission from the excitation signal. Frequency domain intensity decays showed that the presence of the MIMG decreased the average lifetime of the RuCon A emission, which was attributed to energy transfer. The steady-state fluorescence measured for 2 μM RuCon A and 4 μM MIMG demonstrated a consistent increase in the RuCon A emission signal as the concentration of glucose increased up to 150 mM. In another study, binding of Cy5 labeled Con A was combined with malachite green labeled insulin and maltose for the purpose of transdermal detection applications [32]. The donor/acceptor pair employed in this FRET assay was Cy5-Con A/malachite green-insulin-maltose. The Cy5 can be excited by long-wavelength red laser diodes and has a considerably higher quantum yield than RuCon A, thus it is potentially suitable for tissue glucose sensing. However, Cy5 has a small Stokes shift and a considerably shorter lifetime than the previously described RuCon A system. Competitive displacement of the insulin complex from the labeled Con A resulted in less intensity and smaller decay times of the Cy5 label. This sensor was determined to be completely reversible, but the excitation wavelength used would not be ideal for transdermal measurements.

A UV-polymerized hydrogel containing fluorescein isothiocyanate (FITC)-labeled dextran that was conjugated to a labeled Con A was described by Russell *et al*. [33]. The principle of this FRET-based system is the

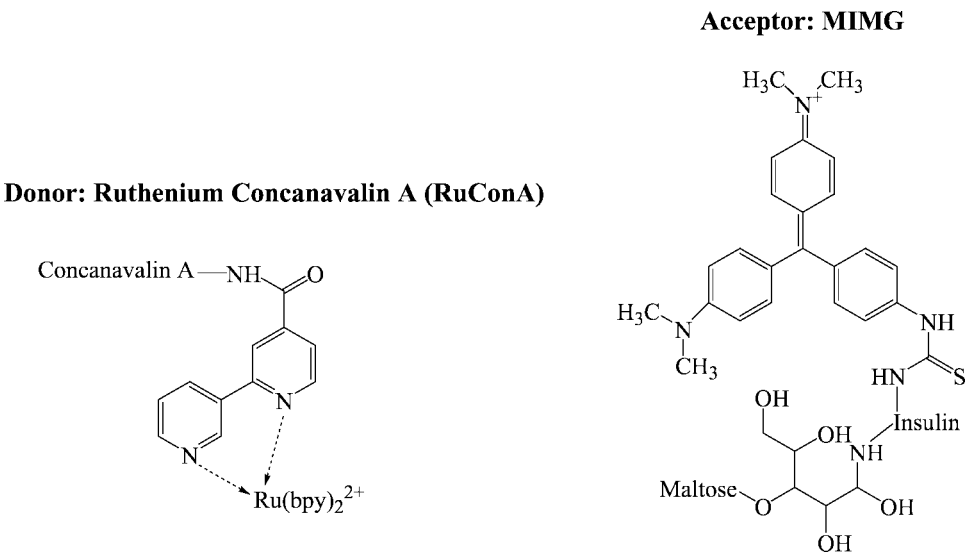

Fig. 3. Glucose sensor based on FRET between Con A derivatized with a Ru-metal complex, serving as the recognition element and the donor of the fluorescence energy transfer, and MIMG (maltose-insulin-malachite green), which plays the role of the energy transfer acceptor [31].

quenching of fluorescence emission of FITC when the dextran interacts with the Con A in the absence of glucose. When glucose is added to the system, the fluorescence of FITC increases in a manner proportional to the amount of glucose present in the sample. This system is intended for subcutaneous glucose monitoring. The optimal concentration chosen for the lectin:sugar mass ratio was 100:1 of Con A:FITC-dextran. The results of this system were similar to those performed in aqueous solution measurements. The response time for a change in glucose concentration from 0–11 mM was at least 10 min. The authors attributed this slow response time to the size of the spheres and proposed that a reduction in their size should result in a decrease of the time necessary to see a change in the fluorescence signal. Another system designed for transdermal sensing was presented by Rolinski *et al*. [34]. This system was based on near-infrared (NIR) FRET using the fluorescent protein allophycocyanin (APC) (quantum yield ~0.8, pH independent from 5 to 9) to label Con A. Malachite green (MG)-labeled dextran served as the FRET acceptor. The kinetics of the FRET interaction were well characterized. A drawback of the system was that 50 hr were needed for complexation of the APC-Con A/Dextran-MG. Frequency domain and time domain measurements were used to generate calibration curves for glucose in the millimolar range, reflective of physiological glucose levels.

Another study by Rolinski *et al*. addressed the donor/acceptor distribution in FRET assays [35]. The authors used the same affinity sensor that was previously described [34] as an example to compare with computational results of their model. They approached the problem assuming that the donor-acceptor distribution function remains unchanged throughout a monitored FRET process, in this case glucose sensing. The authors propose that by measuring the fluorescence decay with nanosecond resolution, structural information can be obtained. They refer to this method as "distribution sensing." The changes in the distribution function for the APC/MG, donor/acceptor pair, were calculated at a single Förster radius for glucose concentrations ranging from 0–30 mM. The theoretical results were in reasonable agreement with the experimental values.

Hamachi *et al*. have designed a novel fluorescent saccharide biosensor based upon a natural saccharide binding protein [36]. A fluorescent Con A was created by first designing a photoaffinity labeling reagent, which consisted of a mannoside group and a phenyldiazyrine group. The Con A was then labeled when the mannoside group became bound to the binding pocket of Con A. The labeling occurred in the presence of UV irradiation, and the resulting labeled Con A was subsequently purified by affinity chromatography. The authors noted that the presence of different saccharides could be observed by a shift in the wavelength of the fluorescence intensity. Concentrations of the same saccharide could be monitored by a change in intensity of the fluorescence at the wavelength of interest. The sugar selectivity of the fluorescent labeled Con A was determined to be nearly identical to that of native Con A. As expected, mannose derivatives had a slightly higher affinity than glucose derivatives based upon results of fluorescence titration experiments.

Ballerstadt and Schultz developed a glucose sensor employing a fluorescence affinity hollow fiber that was intended for transdermal applications. This approach uses Alexa 488-labeled Con A that is sequestered inside dyed porous beads, contained within a hollow dialysis membrane with a molecular weight cut off of 10 kDa [37]. The beads were selected to allow the movement of up to 100 kDa species into the pores and were colored to prevent light from entering their interior. In the presence of glucose, the labeled Con A would diffuse out of the beads and into the hollow tube, at which point the excitation light could reach the labeled Con A and an increase in the fluorescence signal could be observed. This system showed a response time of about 4 min and a glucose response range from 0.15 to 100 mM (within the physiogical range) during *in vitro* experiments. A set-up to evaluate the performance of the sensor over time was constructed by connecting tubes to each end of the hollow fiber sensor. One tube led to a 20 mM glucose solution and the gravity-generated flow continued through to the other tube, which led to a waste container. The fluorescence change of the sensor in response to 0 and 20 mM glucose solutions at ambient temperature was tested. Unfortunately, the stability of this sensor decreased within the second month of testing.

GLUCOSE SENSING BASED ON CONFORMATIONAL CHANGES OF GBP

Protein-based biomolecular recognition events are typically accompanied by a protein conformational change upon binding to the corresponding target ligand. To use these proteins as biorecognition elements in biosensor development, the signal transduction function is directly incorporated into the protein in order to simplify the detection scheme. This is achieved by incorporating reporter groups into the protein structure, which respond to the binding of the ligand. An environment sensitive fluorophore can be positioned strategically on the protein to respond to the conformational changes accompanying the binding events. Another approach includes the arrangement of a pair of fluorophores on selected sites of the

protein to allow for detection of the binding of the ligand by energy transfer measurements.

Our group was one of the pioneers in the rational design of molecular biosensors by genetically manipulating proteins that undergo conformational changes upon binding to a target ligand [38,39]. The strategy involved attachment of an environment-sensitive fluorophore at a unique site on the protein. This site is selected by examining the X-ray crystal structure of the protein such that the binding of the ligand is allosterically linked to this site, causing a change in the fluorescence intensity of the fluorophore when there is a change in conformation of the protein. The successful application of this strategy was first demonstrated by Daunert *et al*. for the detection of calcium using the calcium-binding protein calmodulin [38]. The advantages of this rational design approach include the high reproducibility in the production of biosensing reagents since, they are prepared from a single gene followed by site-specific modification, the inherited selectivity provided by these naturally occurring systems, the capability of working under physiological conditions since the proteins are stable at 37°C and at pH 7.4, and no need for addition of substrates to trigger signal production.

The glucose/galactose-binding protein (GBP) is found in the periplasm of *E. coli* and has a binding affinity for glucose in the micromolar range. The periplasmic space of *E. coli* contains several binding proteins whose mission is the transport of nutrients from the outside of the cell to the cytoplasm. These periplasmic binding proteins share a similar overall structure consisting of a single polypeptide chain folded into two domains connected by flexible hinge region of typically 12–18 amino acids in length. The ligand-binding site is located between the two domains. The proteins adopt two conformations, an "open" conformation in which the domains are apart and a "closed" conformation in which the domains come together due to ligand binding. The conformational change that occurs upon binding of the protein to the ligand forms the basis for the development of the biosensor.

The Daunert group has utilized GBP in the development of a rationally designed sensing system for glucose by taking advantage of the conformational change occurring in GBP upon binding glucose [40]. In order to incorporate a fluorescent reporter group whose behavior can be correlated to the ligand binding, these authors employed a structure-based rational design approach (Fig. 4A). Since the X-ray crystal structure of GBP in an open form was unavailable, a closely related maltose-binding protein (MBP) and phosphate-binding protein (PBP) were used as models to decide which sites can be manipulated to incorporate the fluorescent reporter on GBP. It was observed with MBP and PBP that some residues in the hinge region are allosterically linked to the ligan D-binding site. Therefore, it was expected that the attachment of a reporter to this site would allow monitoring of the ligand binding. Three different sites were evaluated for the attachment of the fluorophore, namely, amino acid residue 148, 152, and 182 were mutated to a unique cysteine residue. Different environment-sensitive fluorophores were attached to the protein through the

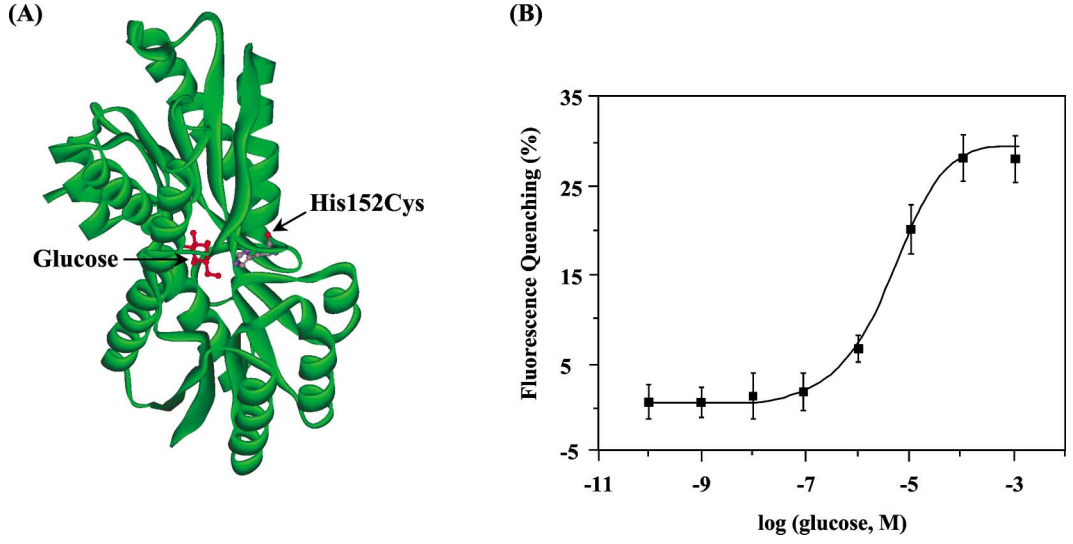

Fig. 4. (A) X-ray crystal structure of Glucose Binding Protein (GBP) showing the mutation site His152Cys and the mutant interaction with glucose. (B) Calibration curve of the fluorescence sensing system based on GBP152 for glucose [40].

unique cysteines. A 30% change in fluorescence was observed using the mutant GBP152 labeled with MDCC (*N*-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide) with a detection limit of 1×10^{-6} M (Fig. 4B). The mutant GBP148-MDCC yielded a detection limit of 5×10^{-8} M glucose, which is the lowest detection limit reported to date for the measurement of glucose. Selectivity studies performed showed that the interference from structurally related sugars is insignificant. Galactose can also bind to GBP, but the affinity of the protein for glucose is higher than that of galactose. Daunert *et al*. have performed extensive studies to optimize the use of this sensing system for glucose in the development of an *in vivo* implantable sensor [40]. In that regard, preliminary studies using a fiber optic probe with incorporated GBP-fluorophore conjugate showed that glucose measurement can be performed in a dynamic environment, which mimics the situation in the human body more closely. Moreover, the labeled GBP mutant was found to be stable at 37°C, which corresponds to body temperature, for over 3 months (unpublished data). In addition, a study performed by Lakowicz's group showed that the secondary structure of the protein is stable up to 64°C [41]. Therefore, the sensing system developed using this protein is ideally suited for developing *in vivo* long-term implantable sensors for glucose.

Hellinga and co-workers also developed a sensing system for glucose using GBP. In this study, amino acid residues 152 and 255 located in the hinge region of GBP were mutated to introduce a unique cysteine in the protein [42]. An environment sensitive fluorophore, IANBD (*N*-(2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole), was conjugated through the sulfhydryl group of the unique cysteine. Upon addition of 10 mM glucose to GBP 152 labeled with IANBD, a four-fold enhancement in the fluorescence signal of the fluorophore was achieved. However, this mutation caused a decrease in the affinity between GBP and glucose. This was attributed to the loss of a hydrogen bond between the sugar and histidine 152 in the wild type protein when the histidine at position 152 was mutated to cysteine. Nevertheless, it was demonstrated that conformational changes in proteins could be exploited to design an integrated sensing system that can allosterically couple ligand binding with a change in fluorescence. The system itself was not optimized for applications in real life glucose sensing.

Lakowicz's group developed a glucose sensor using a rational design approach, in which the fluorophore, 2-(4-iodoacetamido-anilino)naphatalene-6-sulfonic acid (I-ANNS) was conjugated to the position 26 in GBP [43]. A 2-fold decrease in the fluorescence signal was observed upon binding to glucose, with a binding constant of 1 μM. Furthermore, Lakowicz and coworkers have developed a modulation sensor by combining the IANNS-GBP with a long-lifetime ruthenium metal-ligand complex on the surface of a cuvette. Using this system, the binding of glucose resulted in a dramatic change in the modulation at a low frequency of 2.1 MHz. The methods developed using synthetic fluorophores are highly sensitive, but may suffer from the drawback of decreasing the solubility of the protein, and more importantly, of a difficulty in adapting the system to *in vivo* glucose measurement.

In another study, Ye *et al*. used GBP as the recognition element for the development of an energy transfer-based sensor for glucose for *in vivo* measurements [44]. In this work, the green fluorescent protein (GFP) and its mutant yellow fluorescent protein (YFP) were fused to the two termini of GBP. The spatial separation between the two GFPs changed upon binding to glucose, leading to a decrease in the energy transfer from GFP to YFP, which was correlated to the amount of glucose present in the sample. An important consideration while developing an efficient energy transfer system is that the distance between the donor and the acceptor should be within 5–50 Å. The diameter of GBP in a closed form is 50 Å, thus providing appropriate separation between the two reporters for an efficient energy transfer. A linker was engineered between GBP and each of the reporters to allow proper folding of the proteins. By studying the X-ray crystal structure of the closed form of GBP, and by comparing it with the structure of MBP, it was speculated that the two termini of the protein are at the proximal end in the unbound form, and that the hinge motion upon binding to glucose takes them to distal ends. Therefore, in the absence of glucose, the two reporters are close to each other allowing energy transfer to occur, but upon binding to glucose the reporters are pulled apart leading to about 20% decrease in the signal. Using this system, concentrations as low as 10 μM glucose could be accurately determined. Furthermore, a glucose microsensor was developed using a dialysis hollow fiber. For that, this GBP conjugated to GFP and YFP was incorporated into the fiber. The sensor was employed for the continuous measurement of glucose, and showed reversible detection of glucose with a response time of 100 s.

Glucose homeostasis is an important biological function that depends on supply, transport and metabolism of glucose. To monitor perturbations in glucose homoeostasis it would be useful to have a method that would allow glucose measurement in living cells with temporal and cellular resolution. To achieve this, Formmer and coworkers have developed a glucose sensing system, utilizing GBP flanked with the energy transfer pair of yellow fluorescent

protein (YFP)-cyan fluorescent protein (CFP) [45]. Upon binding glucose, a decrease in energy transfer was observed, with a binding affinity for glucose of 0.17 μM. To monitor physiological concentrations of glucose, a mutant of GBP (Phe16Ala) was generated that yielded a glucose binding constant of 0.59 mM when combined with the FRET pair. This mutant allowed sensitive detection of glucose in the range of 0.07–5.3 mM. Free cytosolic glucose concentration in COS-7 cells was monitored employing this system using an external glucose concentration of 0.5 and 10 mM corresponding to typical blood glucose levels. Cytosolic glucose levels dropped upon removal of external glucose and in the presence of a transport inhibitor, cytochalsin B. This demonstrates that the sensor can be used in the real time monitoring of glucose uptake, transport, and metabolism and it works well at physiological levels of glucose.

OTHER PROTEIN-BASED FLUORESCENCE SENSING SYSTEMS FOR GLUCOSE

Proteins isolated from thermostable bacteria that are active at room temperature are an excellent alternative to the proteins that loose activity at shelf temperature. Lakowicz and coworkers have isolated a thermostable glucokinase (BSGK) from the thermophillic microorganism *Bacillus stearothermophilus* [46]. This protein belongs to the family of hexokinases that catalyzes the reaction where hexose is converted to hexose-6-phosphate utilizing ATP. In the absence of ATP, the protein does not consume the substrate and can be used as a binding protein to develop a sensor for the substrate. The X-ray crystal structure of the hexokinase A shows that it consists of two domains, the N-terminal domain and the C-terminal domain. In the absence of ligand, the two domains are apart from each other. Upon binding of the ligand, the N-terminal domain rotates by 12° relative to the C-terminal domain, closing the opening of the cleft formed between the two domains. BSGK has a single cysteine residue near the active site, which was labeled with the fluorophore IAANS (iodoacetamide derivative of anilinonaphthalenesulfonate). The fluorescence intensity of the labeled protein decreased upon addition of glucose, as the fluorophore IAANS that is sensitive to a hydrophilic environment was displaced from the interior of the protein. The change in fluorescence intensity occurred at a glucose concentration of 3 mM, which is within the blood glucose levels.

In order to improve the sensitivity of the system, a resonance energy transfer (RET) study was performed. In this case, intrinsic tryptophan residues were used as the donor and glucose labeled with nitrophenyl group (ONGP) was used as the acceptor. Addition of ONGP quenched about 80% of the fluorescence of tryptophan, whereas addition of free glucose competitively replaced ONPG to recover the fluorescence. At 6 mM glucose concentration, the fluorescence intensity returned back to its initial value, and further addition of glucose did not cause any additional change in the signal. This study demonstrated that the change in fluorescence intensity is only due to glucose concentration and not to non-specific effects caused by ONPG. To overcome the drawbacks of fluorescence intensity measurements, such as the effect of instability of the source and sample perturbation, a polarization-based sensor was developed using thermostable glucokinase. Polarization measurements are self-normalized and allow ratiometric measurements. In this study, the intensity corresponding to the reference containing BSGK was observed through one polarizer, and the sample containing BSGK, ONPG, and various concentrations of glucose was observed through a second orthogonal polarizer. To obtain polarization of the system, the emission from both sides of the sensor was then measured through a vertically and horizontally oriented polarizer. The advantage of using a RET system is that it does not require any conformational change to occur but is rather an effect of spatial interaction. Therefore, any donor-acceptor pair with longer wavelength excitation/emission characteristics can be used to develop sensors for *in vivo* applications. Nevertheless, the system needs the addition of extra reagents, which can be an added complication in the use of this system for *in vivo* applications. The system has not been optimized for miniaturization and use in implantable sensor yet.

CONCLUSIONS

Fluorescence-based sensing is a powerful technique offering fast, noninvasive, and in some cases, reagentless and nonconsumable sensing. Such advantages have prompt the development of a number of fluorescence sensing systems for glucose that can be employed in applications ranging from the food industry to the field of medical diagnostics. There are still a number of limitations that need to be overcome for the development of an ideal fluorescence glucose sensor. Such a sensor should be able to monitor a wide range of glucose levels quickly and accurately, with a high signal response that is reproducible and reversible, its fabrication should be reproducible and it should have extended lifetimes. In addition, the ideal sensor should also be biocompatible, utilizing nontoxic reagents that will not leach out of the system, and be able to function at body temperature. To that respect, the key parameter for the development of the ideal glucose sensor is the utilization of an advanced glucose recognition element, which would combine high selectivity with high

operational and storage stability, for the development of such robust systems with extended lifetimes. Another parameter related to signal transduction is the increase of the detection wavelengths above the "optical window" of the skin, to overcome the interference problems and to take advantage of the most attractive application of fluorimetric glucose determination in the body, transdermal glucose monitoring. In addition, other parameters that need to be taken into consideration for *in vivo* monitoring of glucose are the issues of correlation of glucose levels in blood and interstitial fluid, sensor stability, selectivity, calibration, lifetime, and biocompatibility [5,6]. The progress in synthetic chemistry and molecular biology should allow the design of improved fluorescent probes by the development of selective synthetic receptors and by genetically engineered proteins with exceptional stability and selectivity. The great advances in optical technology in the production of fine fibers will promote the miniaturization of glucose fluorimetric sensors. In addition, the synergistic advancement of other areas of research, such as that of microfabrication, nanotechnology and various engineering disciplines, will provide the means of constructing implantable responsive drug delivery systems that integrate a glucose sensor with a drug delivery system [47,48]. This application will be the ultimate use of technology for the improvement of the quality of life of millions of people suffering from diabetes.

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