DEVELOPMENT OF THE SENSING SYSTEM FOR AN IMPLANTABLE GLUCOSE SENSOR

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EXECUTIVE SUMMARY

We have successfully completed Phase II of our GlucoChip development program. Phase II was intended to develop the glucose sensing system, which is the mission critical component of the implantable glucose sensor for use by diabetic patients. This proof-of-principle demonstration shows that the closed-cycle continuous glucose-sensing system can produce a consistent, measurable response to physiologically relevant levels of glucose while functioning under biologically relevant conditions. The demonstration sensing system meets our primary stability, sensitivity and specificity criteria. These proof-of-principle results, accompanied by the synthetic materials and relational database, provide a firm foundation upon which to optimize the glucose sensing system and incorporate it into the implantable glucose sensor device.

GOAL

Diabetes is one of the greatest worldwide health challenges of the 21st century.[1] It is one of the leading causes of death and according to the Centers for Disease Control and Prevention (CDC), a major contributor to heart disease and stroke, and the leading cause of kidney failure, non-traumatic lower-limb amputation and new cases of blindness in the United States. Currently, diabetes is not curable and requires proper blood glucose management, which includes accurate monitoring of blood glucose levels in order to improve lifestyle and lifespan. Effective and consistent testing for blood glucose levels is a barrier to proper management due to the invasive and costly nature of the available monitoring devices.

Currently, the self-monitoring blood glucose test is a cornerstone of self-management for patients with diabetes. Unfortunately, this test requires that the patient extract a small drop of blood through a painful finger pricking method three to four times daily for type 1 diabetes, according to the American Diabetes Association. In addition to this motivational barrier, high out-of-pocket expenditures for device test strips are also cited for non-compliant testing.[2] Overtime, suboptimal testing frequency leads to out of range blood glucose levels. As a result, positive societal and economic impact can be achieved with the development of an easy-to-use, implantable glucose monitoring system.

There have been numerous efforts over the past several decades to successfully build an *in vivo* glucose sensor. These efforts have not been successful due to deficiencies in designing one or more of the critical device components, deficiencies that include: external reagent or renewable reagent requirements, device size and limited communication capabilities, and adverse interactions with the biological system. In previous approaches, lack of component integration has been the fundamental flaw in the design approach. Consequently, development of one component has typically led to an *ad hoc* approach to integration of the entire system. Our goal was to overcome these previous failures to build the first successful, *in vivo* chemical sensing system. Our strategy for successful development was (and is) based on an in-depth consideration of all the critical components and how they relate to the system as a whole. The key components of this system are the bioselective interface between the *in vivo* environment and the sensing system, the mission critical closed-cycle sensing system and a mass sensitive glucose-to-signal transduction interface that is coupled to the RFID enabled data communication component.

PLAN

Device design is based on the creation of an integrated, self-contained sensing system that produces an RFID communication read-out that provides mg/dL (milligrams per deciliter) values and whether the glucose level is increasing or decreasing. This combination of information can be used by the diabetic patient to determine if their glucose levels are currently "LOW-SAFE-HIGH".

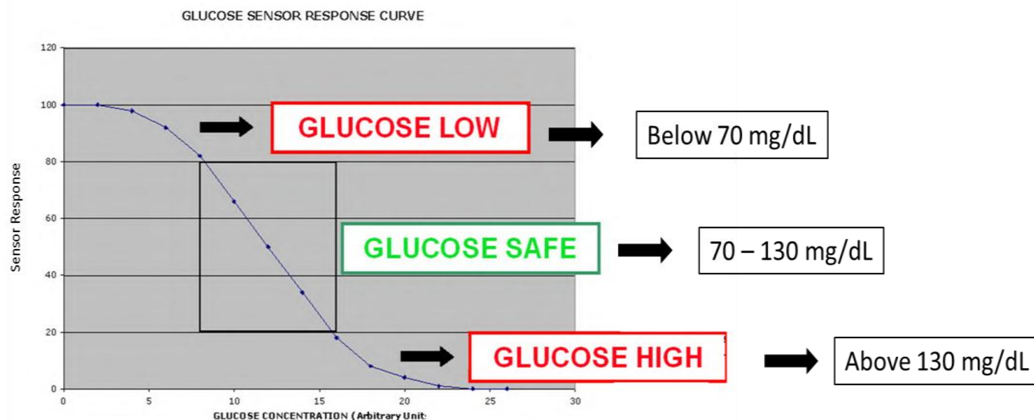

FIGURE 1. **Glucose Chemical Sensing System Design**. Bloodstream glucose levels will produce a response in the glucose sensing system and, correspondingly, in the electronics that are interfaced to the RFID communication read-out. LOW, SAFE, HIGH display readings correspond to adult glucose levels of below 70 mg/dL, between 70 and 130 mg/dL, and above 130 mg/dL, respectively, as they pertain to fasting glucose levels.[3]

Self-Contained, Closed-Cycle Glucose Sensing System

Demonstration of the self-contained chemical sensing system requires the interaction of two components. These components are: 1) the competitive agent/signaling component, which is a dendrimer-boronic acid construct (DBA, Figure 2) and 2) the glucose competitive binding environment, which consists of an immobilized monosaccharide mimic (iDIOL, Figure 2). The interaction of glucose, the signaling component and the competitive binding environment will produce a signal that is proportional to glucose levels (Figure 3). The design of the glucose sensing system is based on our evaluation of the structural compatibility and interaction potential of the components. A combination of RECEPTORS' experience in both analytical systems development and artificial receptor technology has led to the successful development of the glucose measurement system.

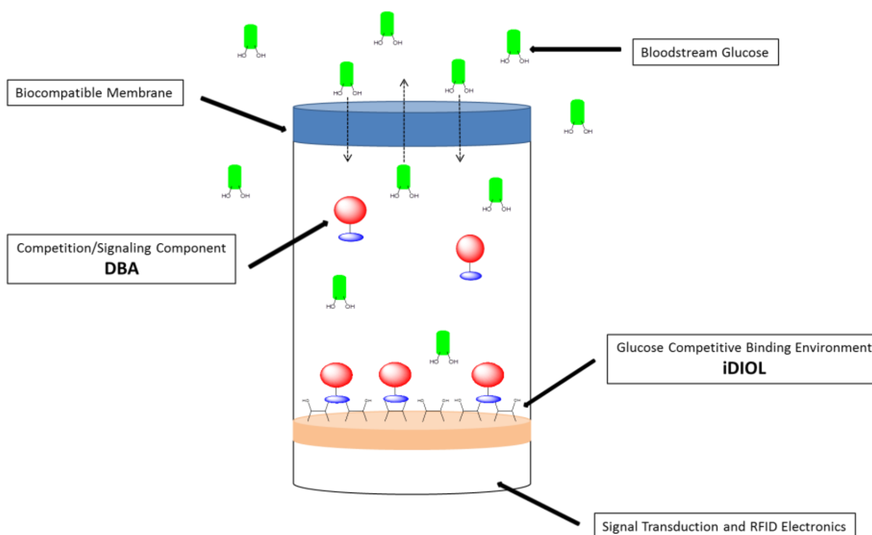

FIGURE 2. **Integrated Sensor Device Design**. The sensor device design includes the bioselective interface, the mission critical closed-cycle glucose sensing system including the glucose competitive binding environment (iDIOL) and the competition/signaling component (DBA), the mass sensitive glucose-to-signal transduction interface and the RFID enabled data communication components; integrated into a millimeter scale, implantable package.

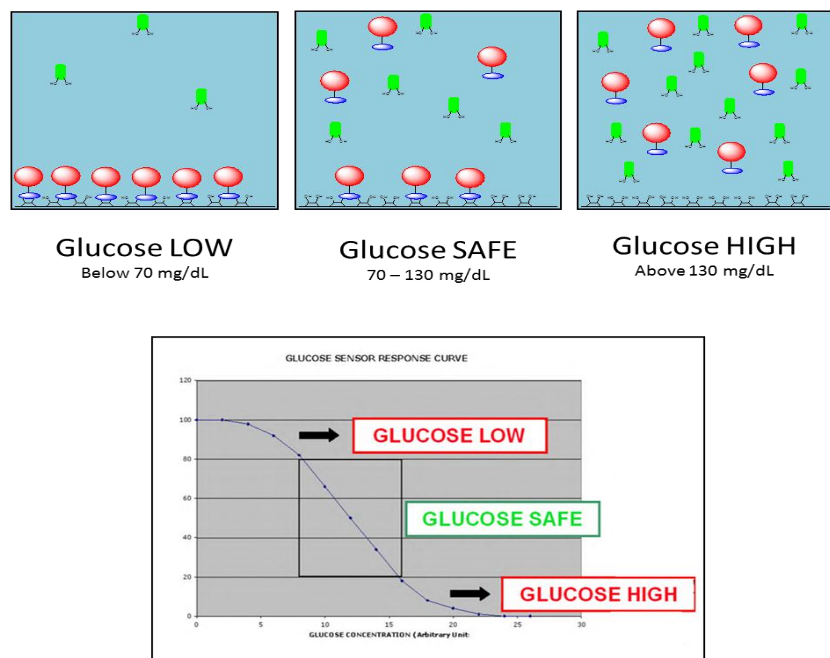

FIGURE 3. **The Sensing System's Competitive Interaction with Glucose**. The competitive interaction of glucose with the DBA and the iDIOL produces an inverse, proportional signal that is responsive to the fluctuating levels of glucose in the *in vivo* environment. Due to the higher optimized affinity of the DBA competitor for glucose over that of the immobilized iDIOL, glucose will preferentially displace the DBA from the iDIOL resulting in a mass change on the surface and signal.

RESULTS

The critical steps required for the demonstration of the self-contained glucose sensing system were the construction of the competitive agent/signaling component (DBA), the selection and immobilization of the glucose competitive binding environment (iDIOL), and the coordinated identification of DBA:iDIOL leads that demonstrate the proof-of-prototype for the glucose sensing system. The key steps in this process include:

1) Synthesis of the DBA Competitive Agent/Signaling Component. Selection and preparation of DBA candidates were based on a literature and in-house developed critical parameter (e.g. pK) database. These data were combined with an initial screening process to define the chemical and structural capability of both the selected boronic acids and the constructed DBAs to competitively interact with glucose.
2) Selection of the iDIOL Glucose Competitive Binding Environment Component. Selection of iDIOL candidates was based on a combination of molecular modeling and an initial screening process to define the interaction between the candidate DBAs and iDIOLs.

3) Coordinated Identification of Lead DBA:iDIOL Pairs. Combination of the interaction database and selective screening to identify specific DBA:iDIOL combinations that interact with glucose to meet the required sensitivity and selectivity criteria.

The design and development plan approach used for the proof-of-prototype allowed us to successfully overcome specific technical challenges that had to be met for development of a functional glucose sensing system. The steps involved in this process were:

1) Synthesis of the Competitive Agent/Signaling Component

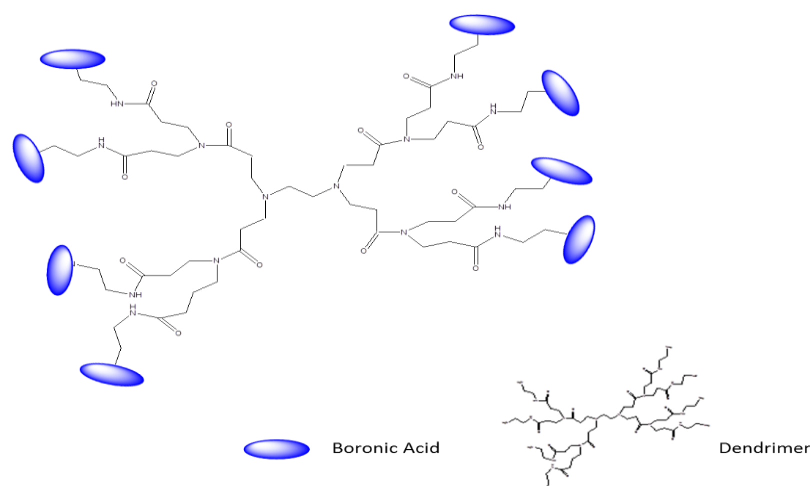

FIGURE 4. **Dendrimer-Boronic Acid (DBA)**. The competitor agent was prepared using a dendrimer as a core carrier or scaffold with boronic acid analogue moieties covalently bound to the appendages of the dendrimer.

Boronic Acid Selection

Selection of boronic acid analogues for incorporation onto the dendrimer core as the recognition component of the competitor agent was based on our extensive evaluation of the interaction between boronic acids and saccharide compounds including glucose and other diol containing species. It is known that boronic acids rapidly and reversibly bind with diols through boronate ester formation in aqueous environments.[4,5] This favorable interaction occurs with such high affinity that boronic acids are used as recognition moieties in the construction of sensors for saccharides.[6-15] The manner in which dendrimers and boronic acids are used as recognition moieties in our design of a sensing system is unlike any detection system currently known. Our focus on developing a glucose specific sensor that utilizes boronic acid based dendrimers as a competitive component prompted us to select a variety of chemically and structurally diverse boronic acid ligands as receptor recognition moieties for the design of DBAs. A library of readily available boronic acid candidates that possess different functional moieties were

strategically selected as candidates because they contained diverse and unique chemical and structural characteristics that in turn provided a range of desired saccharide binding characteristics.

Dendrimer Selection

Owing to their physical and chemical properties, dendrimers are advantageous for the construction of synthetic receptor materials and stable sensing system applications. Readily available in a number of different generations, these macromolecules have specific size and mass characteristics. Size and mass are critical in the selection of a competitor agent for this system. The components must be large enough in mass to create a differential with glucose in order to generate a detectable signal. Uniquely shaped, dendrimers also have a 3-D architecture that gives them desirable polyvalency characteristics. The highly functionalized terminal surfaces allow for control over which surface recognition elements are being displayed as well as how they are pre-organized around the surface of the scaffold.

DBA Construction

Alizarin Red S. (ARS) competitive assays were performed in a physiological environment at neutral pH to confirm that the boronic acid ligand could adequately bind glucose with the required sensitivity and selectivity. Following the initial competition screening, the candidates were subsequently conjugated to a dendrimer scaffold using a robust and reproducible DBA synthetic technique.[16] A library of DBA competitor agents, each different in physical and chemical properties and linked to our properties database, were available as part of a toolbox that enabled us to "pick-and-choose" the optimal signaling component for the desired sensing system environment.

2) Selection of Glucose Competitive Binding Environment

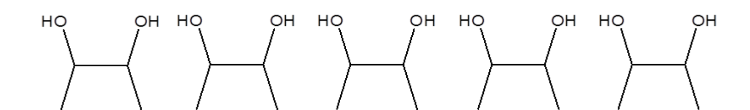

FIGURE 5. **Immobilized Sugar Surface Sensing Mimics (iDIOLs)**. The glucose competitive binding environment is prepared by covalently immobilizing a diol-containing monosaccharide mimic to the platform surface.

Selection of sugar surface mimics for the glucose competitive binding environment was based on our database that quantified the reversible interactions between our kit of boronic acids and diols. The hydroxyl groups on saccharides, preferably 1,2-diols or 1,3-diols, are known to covalently bind with boronic acids to form five- or six- membered ring structures.[17] The conformational change that is associated with this binding event alters the charge of the boronic acid moiety allowing it to function as a competitive chelator for saccharides in water.[18] Our focus on developing a glucose specific sensor that utilizes a diol functional group as a

competitive component prompted us to select iDIOLs containing 1,2- and 1,3-diols with various molecular structures that are capable of selectively and reversibly binding with the boronic acid functional groups of DBAs. Subsequently, a library of iDIOLs were selected as candidate glucose competitive binding environments because they possessed unique chemical and structural characteristics that, in turn, provided a desired range of DBA binding capabilities.

3) Coordinated Identification of DBA:iDIOL Lead Pairs

Keq Determination and Application

Although it is ultimately necessary to actually screen candidate DBA:iDIOL pairs to determine their response to glucose, even a limited set of boronic acids (e.g. n = 50) incorporated into dendrimer DBA constructs (e.g. n = 5) and evaluated relative to iDIOL candidates (e.g. n= 50) for glucose competition gives a formidable number (e.g. n= 50 x 5 x 50 = 12,500) of possible combinations. We solved this screening problem by building a binding affinity model and database based off of a three component (DBA:iDIOL:glucose) interaction mathematical model. This approach proved to be critical to furthering our efforts in designing and developing a system that utilizes a DBA and an iDIOL in coordination to produce lead glucose sensing systems. Applying a method for measuring the binding equilibrium constants (Keq) of DBA:Glucose and DBA:iDIOL complexes was critical to understanding how each component interacts in relation to the system as a whole. Not only did this method significantly limit the number of DBA:iDIOL candidate combinations that needed to be screened, it quantified and directly compared the mechanism and the process of complexation between each DBA with Glucose relative to the same DBA with other iDIOLs.

Binding constants (Keq) of DBA:iDIOL combinations were calculated based on a literature procedure describing a three component competitive assay.[19] Using Alizarin Red S (ARS) as a fluorescent reporter, the equilibrium or binding strength between each respective DBA:Glucose and/or DBA:iDIOL pair could be determined. This method took advantage of the fluorescence spectral changes that the reporter exhibits upon binding to diols. Within this system there are two competing equilibria that are measured. The first equilibrium measured (Keq1) is between the DBA and the ARS compound. The second equilibrium (Keq2) is between the DBA and the iDIOL. Fluorescence intensity changes as they relate to the formation and perturbation of each equilibrium were used to calculate the binding strength of glucose and the iDIOL relative to the DBA. These data were, in turn, used to estimate the relative glucose competition for each DBA:iDIOL pair as a tool for the selection of which DBA:iDIOL pairs to move forward into the actual screening process.[20]

These relative binding affinities of each DBA:iDIOL combination were subsequently used to generate a scatter plot of the interaction data (Figure 6). Based on the location of a representative data point on the interaction graph, the response of glucose and/or each iDIOL for each DBA could be evaluated and the particular DBA:iDIOL pair scored for inclusion into the screening process. For example, if a data interaction point is located along the 1:1 line in Figure 6, this indicates that the relative binding strength of a DBA for Glucose is similar to the binding strength of that same DBA for the iDIOL of the DBA:iDIOL pair. If a data interaction point is located along the 2:1 line, the binding strength of a DBA for the iDIOL is approximately twice the binding strength of that same DBA for Glucose. If a data interaction point is located along the 1:2 line, the strength of binding of a DBA for Glucose is approximately twice the

strength of binding of the same DBA for the iDIOL. Thus, these data and the relative strength of the DBA:iDIOL:glucose interactions can be used to select candidate DBA:iDIOL pairs for evaluation in immobilized iDIOL glucose competition systems.

The coordinated identification of a DBA:iDIOL pair that meets sensitivity and specificity requirements necessary to initiate a competitive interaction with glucose is fundamental for system design. In general, *in vivo* detection of normal levels of glucose requires sensitivity and selectivity. The affinity of the competitor agent for glucose must be sensitive enough to give a usable change in signal while at the same time be specific enough to allow for selective competitive binding to occur between glucose and the iDIOL. The construction of the Keq interaction graph assisted our efforts in identifying the optimal pair by enabling us to "read" which candidate matches met the desired sensitivity and selectivity criteria to enable successful competition with glucose.

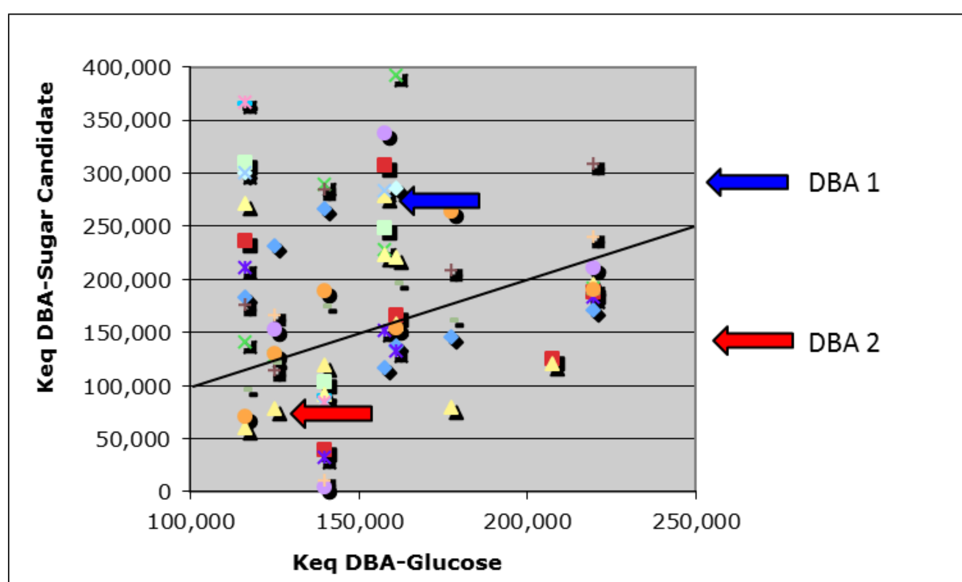

FIGURE 6. **Keq Interaction Graph**. Comparison of DBA-to-Glucose binding affinity (X-axis) to DBA-to-Saccharide Mimic binding affinity (iDIOL; Y-axis). Keq interaction data showing the location of the Keq data point for DBA 1 (blue arrow) and the Keq data point for DBA 2 (red arrow) with respect to the 1:1 binding affinity interaction line. The respective glucose competition curves are shown in Figure 7.

Glucose Competition Binding Assays

Competitive binding assays are established analytical detection techniques that are currently gaining notoriety in medicinal and clinical chemistry.[21] A typical competitive binding assay constitutes a receptor and a signaling unit that also serves as a surrogate substrate. The signaling unit possesses an observable and quantifiable property, which is regulated in response to competitive binding with an analyte.[22]

The assembly of the screening assay was done in such a way as to accurately represent the dynamics of an actual chemical sensing system. Specifically, previously selected iDIOLs that exhibited appropriate binding affinity (Keq) values with select DBAs were covalently immobilized onto a microarray surface. A series of mixtures that contain a fixed concentration of the fluorescently labeled DBA with varying amounts of glucose present, including the concentration range encompassing physiologically relevant glucose levels (40-400 mg/dL), were incubated on the iDIOL functionalized microarray surface. Loss of the fluorescent signal from the iDIOL environment following exposure to glucose confirmed successful competition. A plot of the increasing loss of fluorescent signal in response to increasing concentrations of glucose produced a response curve that indicated the sensitivity of the DBA for glucose versus the DBA:iDIOL interaction.

Response curves for multiple DBA conjugates with a reference iDIOL competitive binding environment were also generated in a physiological matrix consisting of physiological saline buffer and fractionated plasma, both within a biologically relevant range. Reproducible competition curves for the library of DBAs were successfully generated utilizing multiple DBA species with inherently different binding affinities for glucose with respect to a reference iDIOL. These curves show diversity in response with respect to sensitivity for glucose versus the reference iDIOL. In a more specific example, the ability of DBA 1 and DBA 2 to compete with glucose versus a specific iDIOL were compared (Figure 7). Both DBAs were able to successfully compete with glucose within a biologically relevant range with different sensitivities in physiological relevant matrices. From visual inspection of the slope of each curve, it is apparent that DBA 2 exhibits much greater glucose sensitivity relative to DBA 1.

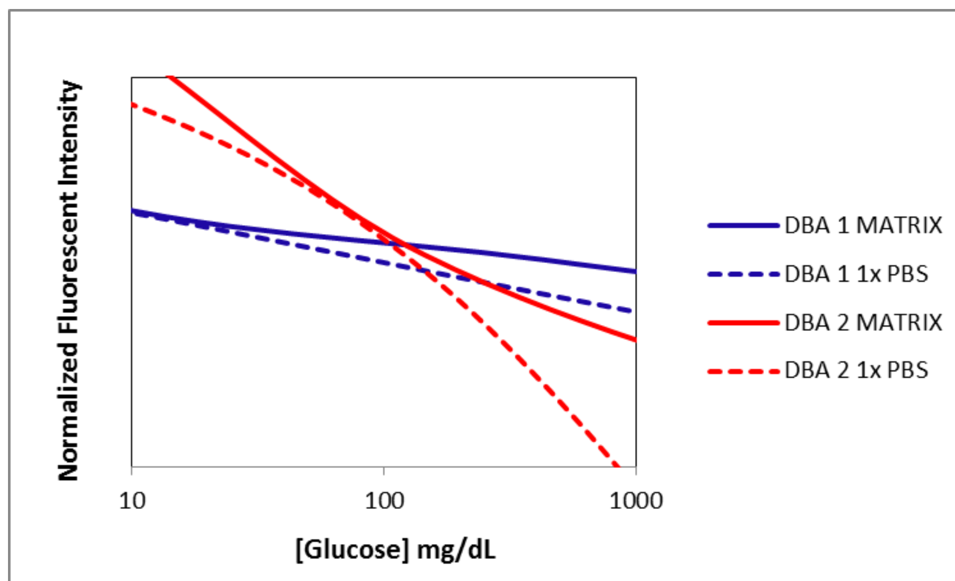

FIGURE 7. Glucose competition curves showing the normalized fluorescence intensity vs. glucose concentration profile of DBA 1 and DBA 2 in fractionated plasma (matrix) and 1x phosphate buffered saline on an iDIOL modified microarray surface.

Significantly, the diversity in response can also be correlated to the location of the respective Keq data points on the interaction graph. Figure 6 shows where the Keq values for DBA 1 and DBA 2 are located with respect to one another. DBA 1, which lies within proximity of the 2:1 line, exhibits a greater binding strength for the immobilized iDIOL than for glucose. As predicted from our database and mathematical model, DBA 1 is less sensitive to the interaction of glucose and is not efficiently competed from the immobilized iDIOL. A response curve with a smaller slope, showing less signal change as glucose concentration changes, was predicted and is observed. On the other hand, DBA 2, which lies within proximity of the 1:2 line, exhibits a greater binding strength for glucose than for the immobilized iDIOL. As predicted, DBA 2 is more sensitive to the interaction of glucose and is more efficiently competed from the iDIOL. A response curve with a greater slope, showing more signal change as glucose concentration changes, was expected and is observed.

We have successfully demonstrated competitive binding assays for glucose based on fluorescently labeled DBA conjugates that show competitive binding between the DBA, iDIOL and glucose (Figure 7). Critically, these assay demonstration results establish that the binding database (Figure 6) is an effective guide to defining DBA:iDIOL pairs for screening. Significantly, these results demonstrate the competitive response between glucose and the DBA:iDIOL components that were specifically designed for the development of the glucose sensing system. <u>The proof-of-prototype demonstration assays show that the DBA:iDIOL system can produce a measurable response to changing levels of glucose within a physiologically relevant range while functioning under biologically relevant conditions.</u>

CONCLUSION

A chemical sensing system that can be integrated into an implantable glucose device has been successfully developed. Each component of the system was constructed of materials that competitively interacted with glucose to produce a proportionate response to physiologically relevant glucose levels in biologically relevant matrices. Not only are these components capable of generating reproducible glucose response curves, they also provide a library of structurally and chemically diverse materials that can be used to optimize the unique set of sensitivities and selectivities required by the dynamic nature of a physiological system. We have demonstrated that Keq constants are capable of predicting what type of competitive response will occur with glucose upon selection of a particular DBA:iDIOL pair. <u>These proof-of-prototype results, accompanied by the synthetic materials and relational database, provide a firm foundation upon which to optimize the glucose sensing system and incorporate it into the implantable glucose sensor device.</u>

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