RESEARCH ARTICLE

Circular permutated PQQ-glucose dehydrogenase as an ultrasensitive electrochemical biosensor

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Abstract: Protein biosensors play an increasingly important role as reporters for research and clinical applications. Here we present an approach for the construction of fully integrated but modular electrochemical biosensors based on the principal component of glucose monitors PQQ-glucose dehydrogenase (PQQ-GDH). We designed allosterically regulated circular permutated variants of PQQ-GDH that show large (>10-fold) changes in enzymatic activity following intramolecular scaffolding of the newly generated N- and C termini by ligand binding domain:ligand complexes. The developed biosensors demonstrated sub-nanomolar affinities for small molecules and proteins in colorimetric and electrochemical assays. For instance, the concentration of Cyclosporine A could be measured in 1 μl of undiluted blood with the same accuracy as the leading diagnostic technique that uses 50 times more sample. We further used this biosensor to construct highly porous gold bioelectrodes capable of robustly detecting concentrations of Cyclosporine A as low as 20 pM and retained functionality in samples containing at least 60% human serum. These experiments suggest that the developed biosensor platform is generalizable and may be suitable for Point-of-Care diagnostics.

Introduction

Allosterically regulated proteins have the remarkable ability to specifically recognise analytes ranging from ions to large polymers such as nucleic acids and proteins, and convert these recognition events into a range of biochemical activities.[1] This property has multiple biological utilities and underlies the ability of biological systems to respond in complex and adaptable ways to physiological and environmental changes. The ability to create systems with inputs and outputs of choice holds the promise of transforming the way we design biotechnological processes, engineer cells and diagnose diseases. Regardless of the application, the most common approach for biosensor construction is to render a constitutively active protein conditionally inhibited, and then find a way to relieve this inhibition with an input of choice. Conditional inhibition can be achieved by mutationally separating the reporter protein into inactive fragments, distorting its structure through the introduction of regulatory domains, or by blocking the active sites of the reporter with inhibitory domains.[2][3][4][3] An alternative approach, that is specific to light emitting biosensors, is based on changes in fluorescence resonance energy transfer or bioluminescence resonance energy transfer efficiency as a result of ligand-induced distance change between the light donor and acceptor.[5] Another way of classifying the biosensor architectures is to categorise them as multi-component or fully integrated single component systems.[6] In multicomponent systems the analyte drives an increase in the local concentrations of the components, thereby increasing their encounter frequency and causing formation of the active reporter. The single component biosensors rely on analyte-induced shifts in conformational state distribution preferencing the active conformation of the reporter.[1]-,, The concentration driven two-component biosensors are easier to develop but are inherently influenced by the absolute and relative concentrations of the components.[7] They also often susceptible to a “hook effect” (a decreasing signal at high analyte concentration) when non-cooperative analyte binder pairs are used.[8] The fully integrated biosensors do not suffer from these shortcomings but require extensive optimisation to reconcile relative orientations of structural elements, affinities, and dynamics to achieve the desired sensitivity, kinetics, and the dynamic range.[9]

Although in Point-of-Care diagnostic applications electrochemical measurements absolutely dominate all other readouts, the enzymes used for this purpose have attracted little attention from protein engineers. This is despite the fact that electrochemical readouts allow the use of disposable electrodes that can be manufactured for less than $US 0.02.[10] We recently reported several approaches for converting the principal component of glucose monitoring systems, PQQ-glucosethe the dehydrogenase (PQQ-GDH) from *Acinetobacter calcoaceticus,* into two component biosensors. The sensor architecture was based on either a split enzyme or on calmodulin-GDH chimer activated by a ligand-controlled calmodulin binding peptide.[11][12] Our analysis demonstrated that the developed biosensors could be used to detect clinical biomarkers in biological fluids with the same accuracy as the current clinical methods[12]. To address the inherent complexities and limitations of two component systems we now present an approach for the construction of fully integrated artificial allosteric biosensors based on the circular permutated version of PQQ-GDH. We show that the introduction of two conformation-changing switches (thus forming a biochemical logic AND gate) resulted in biosensors with the

largest dynamic range and lowest detection limits that could be used to construct sensory bioelectrodes.

Results

Our earlier work demonstrated that PQQ-GDH from *Acinetobacter calcoaceticus* is amenable to significant structural modifications including enzyme splitting and domain insertions. We have identified one split and three domain insertion sites in this protein that do not destroy the enzyme's catalytic activity (Fig. S1).[11–13] We conjectured that one could exploit the malleability of this enzyme to construct a fully integrated single-component biosensor where a reversible OFF state is generated through entropically driven local structural disorder. Such disorder is then reversed by ligand-mediated intramolecular scaffolding, resulting in restoration of the ON state. We decided to test if we can introduce activity modulating structural disorder by adding long unstructured linkers into the sites previously identified as suitable for splitting or domain insertion. The success of this approach is expected to depend on the ability of the secondary structure elements to maintain at least a partially folded state when separated from the rest of the molecule. -the was One can view such a system as a protein with intrinsically disordered elements sharing similarity with a rapidly growing class of natural allosterically regulated systems.[14][15] We, therefore, circularly permutated GDH by fusing its native N- and C–terminus with a flexible linker and created new termini at the loop connecting β-strands A and B (D154 in the nomenclature of the wild type enzyme PDB:1c9u) that was previously used as a split site (Fig. 1 and Table S2).[11] The resulting open reading frame was recombinantly produced in *E. coli* and showed activity similar to the wild type, confirming that circular permutation did not significantly interfere with protein integrity.

Next, we wanted to test if this circular permutated protein could be made switchable by introducing flexible linker into position N404 that was previously used for domain insertion.[12] We expected that increasing the linker length would lead to progressively larger entropically-driven disordering of connected GDH fragments, creating an OFF state (Fig. 1C,D,G). Therefore, we designed constructs with serine and glycine linkers containing 10, 20, 30 and 50 amino acids. Then, the sequences coding for rapamycin-binding domains FRB and calcineurin-A fusion with calcineurin-B were added to the C- and N-terminus of the open reading frames respectively (Table S2). The recombinant products of these constructs were tested for GDH activity in the presence and absence of the macrocyclic compound tacrolimus (FK-506) that induces complex formation between FK506-binding protein (FKBP) and calcineurin-A/calcineurin-B fusion (Fig S3). [16] Analysis of the data revealed that tacrolimus dose-dependently increased the activity of all four constructs, and the dynamic range increased with the length of the linker. The fit of the data showed that the K_d values of the interactions were in the low nanomolar range (Fig. 2B,D). However, the observed maximal catalytic activity of the biosensors decreased with the increase in the length of the linker (Fig. 2E and S4). We interpreted this observation as an indication of a decrease in the efficiency of enzyme reconstitution with the increase of the sphere that could be sampled by the fragments when connected with a flexible tether. We speculated that introducing a constraint in the linker that decreases this radius might improve the efficiency of reconstitution. Previous work by our and other groups have demonstrated that the calmodulin:calmodulin binding peptide pair can be used as a conditional conformation switch regulating protein activity.[12][17]Therefore, we decided to replace the unstructured linker in our GDH chimers with the sequence encoding calmodulin, thus allowing us to test two different conformations of the same construct by just adding the ligand peptide (Fig. 3A). The resulting protein termed circular permutated GDH-CaM (cpGDH-CaM tacrolimus) could be recombinantly produced at a good yield and displayed low background GDH activity. Addition of calmodulin binding peptide (CaM-BP) increased the background activity only slightly (Fig. 3B and S4D). However, addition of tacrolimus in the presence of calmodulin binding peptide resulted in dose dependent increase of the biosensor activity (Fig. 3B, S8). When compared with the variants bearing unstructured linker, this variant demonstrated significantly higher dynamic range (>10-fold), and also much higher maximal catalytic activity (Fig. 2E and S4A). As in the earlier reported calmodulin-operated GDH biosensors, presence of Ca^{2+} ions was required for efficient association of CaM-BP with the biosensor and its activation (Fig. S4D). The fit of tacrolimus titration data led to a K_d value of 1 nM, representing over 10-fold affinity improvement compared to the two-component version (Fig. 3D).[12]

Intrigued by this finding, we wanted to test if the developed biosensor architecture was generalizable. To this end, we fused cyclophilin and a fusion of calcineurin A with calcineurin B to the N- and C-termini of cpGDH-CaM chimera and produced the resulting polypeptide recombinantly (Table S2, Fig. S2, S5). These domains form a ternary complex with Cyclosporine A and have been previously used to construct Cyclosporine A biosensors.[18];[11] As can be seen in Figure 3E and F, the biosensor demonstrated excellent sensitivity with a K_d value of 0.28 nM and a limit of detection of (at least) 50 pM. The observed affinity is nearly 20-fold higher than in the case of a two component biosensor based on the same Cyclosporine A binding domains.[12] The fully activated biosensor displayed approximately 25% of the wild type GDH activity and required a 30-minute exposure to the ligand to reach full activity that is in the range observed for other GDH-based biosensors (Fig. S9). [7] As the binding of a ligand is expected to occur on a much faster time scale (milliseconds to seconds) this delay is likely to reflect local structural rearrangements of the GDH molecule.

To assess the extent of structural changes during biosensor activation, we performed analytical gel filtration in the presence and absence of Cyclosporine A (Fig. S10). We observed no change in retention times, indicating that activation led to no changes to the oligomerisation state of the biosensor. We further recorded Circular Dichroism spectra of cpGDH-CaM Cyclosporine A biosensor immediately after the Cyclosporine A and CaM-BP addition, and after 45 minutes. As can be seen in Figure S11 there were only minor changes in the spectrum suggesting that the biosensor does not undergo global unfolding which is in accord with the results of the gel filtration experiments (Fig. S10). The caveat of these experiments is in the large contribution of binding domains that are expected to remain structurally autonomous, and thus may mask subtle but

functionally significant local structural rearrangements in the GDH domain. Given that the binding domains of tacrolimus and cyclosporine A differ significantly, it appeared that the developed system could be activated by complexes of diverse sizes and topologies.[18];[16] To determine whether the observed general activation was not a result of similarities of analytes, we decided to construct a biosensor of a protein biomarker. To this end, we fused two VHH domains recognising non-overlapping epitopes of α-amylase[11] to the N- and C-terminus of cpGDH-CaM assembly. As shown in Figure 3F, the solution of the resulting recombinant protein displayed a dose-dependent increase in GDH activity leading to an apparent K_d value of 5.6 nM and the limit of detection of 500 pM.

We next assessed the ability of the developed biosensors to detect their cognate analytes in biological fluids. We first tested the ability of the developed α-amylase biosensor to quantitatively detect its target in human saliva, where it represents a well-established marker of psychological stress.[19] Figure 4 shows that the obtained quantitation matched closely the data obtained using conventional activity-based α-amylase assay. The accuracy of the data has improved compared to the earlier reported results obtained using the two-component α-amylase biosensor (compare Figure 4A and Figure 3A in [12]).

Encouraged by these results, we decided to test the performance of the developed biosensor in a more challenging application. Cyclosporine A is an immunosuppressant with the main application in organ transplant management. Due to a narrow therapeutic window, the concentration of the drug in patient blood needs to be monitored either immunochemically or, more recently, mass spectrometrically.[20] Due to the hydrophobic nature of the compound, more than half of the drug is sequestered in red blood cells where it is presumed to be protein bound. In all currently used diagnostic methods Cyclosporine A is extracted using organic solvents prior to the analysis. Given the high affinity of the developed Cyclosporine A biosensor for the drug, we decided to test whether the biosensor is able to efficiently compete with cellular proteins for Cyclosporine A binding. To determine this, we collected the blood of kidney transplant patients undergoing Cyclosporine A treatment and measured the activity of cpGDH-Cyclosporine A biosensor in the presence of 1 μl of EDTA-stabilised blood sample. The resulting data were plotted against the concentrations of Cyclosporine A in the same samples quantified using mass spectrometric analysis (performed as described under the Experimental procedures). As can be seen in Figure 4B, there was an excellent correlation between both assays. To further corroborate this notion, we compared the performance of the developed biosensor with the previously described two component GDH-based Cyclosporine A biosensor.[12] We repeated assays using this biosensor on the same blood samples and obtained the apparent concentrations of the drug using a calibration assay. The accuracy of the assay was significantly lower and gave an R^2 value of only 0.52 (Figure 4C). It is noteworthy that to obtain a sufficient signal, the assay required the addition of 50 μl of blood. While it is reassuring that the assay still could be performed under such conditions, it is not surprising that 50-fold increase in blood protein and lipids adversely influenced the performance of the assay. These experiments suggest that the presence of binding domains in the same biosensor molecule leads to avidity effects that enable the biosensor effectively to withdraw the drug from cellular and plasma binding proteins.

One important feature of the developed biosensors is the ability to generate an electric current which can be measured using cheap disposable screen-printed electrodes interfaced to portable amperometers. This approach has been widely adopted for blood glucose and cholesterol monitoring, and expansion of this technology to other biomarkers could lead to transformative diagnostic applications. To this end, we tested the suitability of amperometric measurements for cpGDH-CaM Cyclosporine A biosensor in the presence of excess glucose and CaM-BP, and in the presence and absence of Cyclosporine A. As shown in Figure 4D there was an approximately 10-fold signal difference in electric current in the absence and presence of the analyte, indicating that the activity of the developed GDH-based biosensors could be monitored by conventional Point-of-Care type electrochemical testing. Limitations of this approach include comparatively low sensitivity resulting from an inefficient election transfer in solution, as well as difficulties associated with the construction of multiplexed electrodes. We previously demonstrated that PQQ-GDH-based switch modules can display direct electron transfer when immobilised on the gold or carbon nanotube electrodes.[21][22] We, therefore, tested whether immobilisation of the cpGDH-CaM-Cyclosporine A biosensor on a highly porous gold electrode would enable sensitive and specific electrochemical detection of its ligand (Fig.S12-17). As seen in Figure 4E, cyclic voltammetry of the developed bio-electrode revealed a saturable increase in current following the electrode's exposure to increasing concentrations of Cyclosporine A. A plot of the measured currents as a function of the ligand concentration is shown in Figure 4F and its fit leads to an apparent K_d value of 0.6 nM, which is close to that measured using a solution colorimetric assay (Fig. 3F). It is also worth noting that the functionalised electrode allowed recording much higher currents than in the case of solution-based experiments (>10 μA as opposed to the <1 μA respectively) (Fig.4 D-G). The electrode could reliably detect 20 pM concentrations of Cyclosporine A, and we were able to carry out experiments at concentrations of human serum up to 60% (Fig.4G, S17). While higher concentrations of serum should also be accessible to this method, the viscosity of more concentrated serum presented a technical challenge in the employed experimental setup.

Discussion

The results presented here demonstrate the construction of synthetic allosteric biosensors based on an electrochemically active reporter enzyme PQQ-GDH. This biosensor architecture is based on the idea that a conditional OFF state of the GDH could be created by introducing entropically driven local structural disorder. The return to the active state is achieved by constraining the molecule through intramolecular interactions between two ligand binding domains. One can view this process as non-covalent intramolecular cyclisation that favours intramolecular interactions of biosensor structural elements over their entropically driven dissociation (Fig. S18). It is intuitively obvious that if the linker is unstructured, its length will determine the volume of the sphere that the fragments can occupy thereby

directly determining the local concentrations of the components and their propensity to spontaneously associate. From this perspective it appears logically that an increase in linker length is accompanied by a corresponding decrease in the background activity of the biosensor as fragments occupy a larger volume. The dynamic range also increases with the length but, peaks at 4.5-fold with a 30 amino acid linker. However, the linker increase is also accompanied by an unwanted progressive decrease in the maximal activity of the biosensors. This can be interpreted that longer linkers do not provide sufficient constraint, and despite the presence of the ligand that promotes association of the binding domains, the system remains flexible and adopts catalysis-competent conformations with decreasing frequency. This helps to rationalise the observation that chimeras contining calmodulin domain as a linker displaye the largest dynamic ranges. In the absence of the calmodulin binding peptide the system remained inactive regardless of the presence of the analyte, which is not surprising as the extended conformation of calmodulin would function as a wedge keeping the fragments apart. In the presence of calmodulin binding peptide, the system is constrained on one side but apparently not sufficiently to result in a catalytically active conformation of GDH. Hence, the biosensor functions like a logic AND gate where two biochemical signals are required to activate the system, which may contribute to its large dynamic range. Remarkably, the ligand-mediated association of the ligand binding domains drives the most efficient reconstitution of the GDH activity relative to any other variant. This could be potentially explained by the relatively rigid structure of calmodulin:CaM-BP complex that provides sufficient constraint, thus enabling GDH to adopt a near native structure.

The developed biosensor architecture appears generally applicable as we were able to construct biosensors to both small molecules and proteins. This indirectly supports the idea of the intramolecular non-covalent cyclisation leading to a decrease in system's entropy and a shift to the more structured and active conformation. Here the exact size and structure of binding domains is expected to play a smaller role, potentially explaining system's tolerance to the type of the analyte:binding domain combination. An additional benefit of this architecture is in its very high affinity for the cognate analytes that is, presumably, a result of cooperative interactions of the binding domains. For instance, Cyclosporine A and Tacrolimus biosensors displayed affinities more than an order of magnitude higher than their two component versions utilising the same ligand binding domains[12]. One can expect that, similar to other fully integrated biosensors, linker optimisation may be particularly beneficial for optimising performance of cpGDH biosensors[23];[24].

We tested the developed α-amylase and Cyclosporine A biosensors on biological and clinical samples and demonstrated that they perform as accurately as the currently used clinical diagnostic methods. To our knowledge, detection of Cyclosporine A in whole blood without extraction has not been achieved to date. Therefore, the developed biosensor-based assays can be used to measure this and potentially many other analytes on plate readers or open clinical chemistry analysers as opposed to dedicated immunochemistry stations and mass spectrometers. This would allow these tests to be performed in regional and makeshift laboratories that do not have access to high-end equipment. This is further supported by the fact that the developed assay Cyclosporine A required as little as 1 μl of blood while a typical blood draw for this analysis is 2 ml. This potentially allows the assay to be performed on samples collected through a finger prick rather than via presently used venal puncture.

The demonstrated ability of the developed Cyclosporine A biosensor to function in solution electrochemical assays, and to maintain its functionality when immobilised on gold electrodes, points to a range of a potentially exciting analytic and diagnostic applications. The ability of the developed biosensor to reliably detect concentrations of the ligand as low as 20 pM and perform in the presence of high concentrations of serum is particular important. This holds promise for the development of highly selective and sensitive bioelectrodes capable of detecting a broad range of small molecules and proteins in biological fluids. Given that blood, lymphatic and interstitial fluids contain millimolar concentrations of glucose, such biosensors could be suitable for continuous monitoring of biological markers and xenobiotics that has so far been demonstrated in a very limited number of cases. This undoubtedly will require further elucidation of the mechanisms underlying the functioning of these biosensors, as well as further protein engineering to optimise their response rates and remove their reliance on the calmodulin binding peptide. Clearly, the potential benefits of a generalizable electrochemical detection platform make such efforts worthwhile.

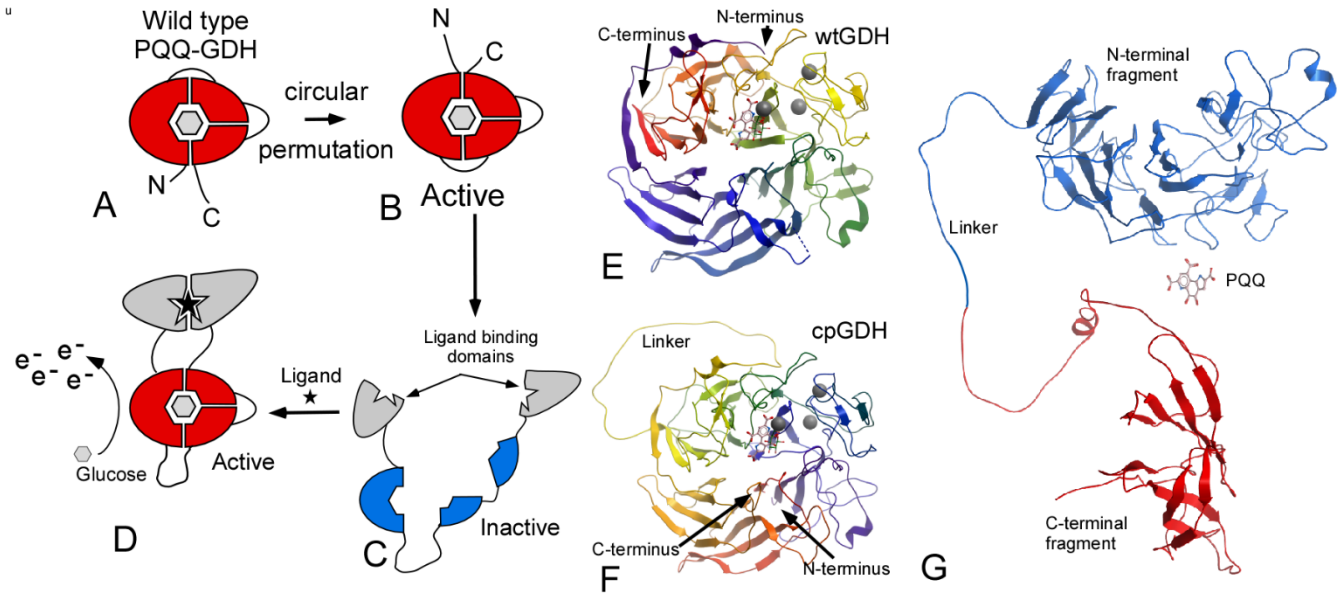

Figure 1. Design of circular permutated PQQ-GDH. (A) Graphic representation of a wild type PQQ-GDH and its circular permutated variant (B) that is rendered inactive by insertion of a linker and attachment of ligand binding domains at N- and C- termini (C).(D) Addition of ligand leads to intramolecular rearrangement and reconstitution of the enzyme activity. (E) Ribbon representation of PQQ-GDH structure (PDB: 1cq1). The molecule is shown blue to red from N- to C-terminus. The PQQ and glucose in the active site are shown in a ball and stick representation with bound Ca^{2+} as gray spheres. (F) A model of circularly permutated PQQ-GDH. The molecule is displayed and coloured N-C terminus as in D and the termini are indicated. (G) A model of cpGDH with dissociated N (blue) and C (red) termini linked via the unstructured linker. The position of the active site is marked by PQQ displayed in ball and stick representation.

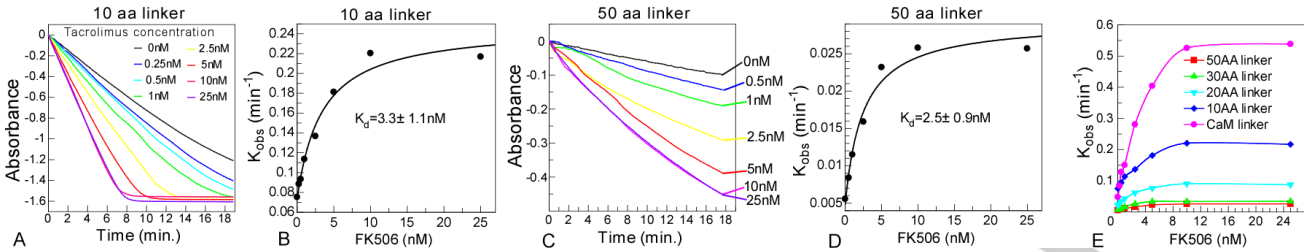

Figure 2. Response of circular permutated (cp)GDH-tacrolimus biosensors with different linker lengths, plotted against increasing concentration of the drug. (A) GDH activity of 10 nM solution of calcineurin A/B-cpGDH-FKBP fusion carrying 5 GS repeats between S403 and N405 in the presence of increasing concentrations of tacrolimus. The activity of the enzyme was monitored by changes in absorption of 60 µM electron accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulphate, 20 mM of glucose, 1 µM PQQ and 1 mM $CaCl_2$. (B) The fit of the data shown in A leading to a K_d value of 3.3 nM. (C) GDH activity of 10 nM solution of CaA/CalB-cpGDH-FKBP fusion carrying 50 amino acid linker. (D) The fit of the data shown in C leading to a K_d value of 2.5 nM. (E) Comparison of dynamic ranges of (cp)GDH-tacrolimus biosensors developed in this study.

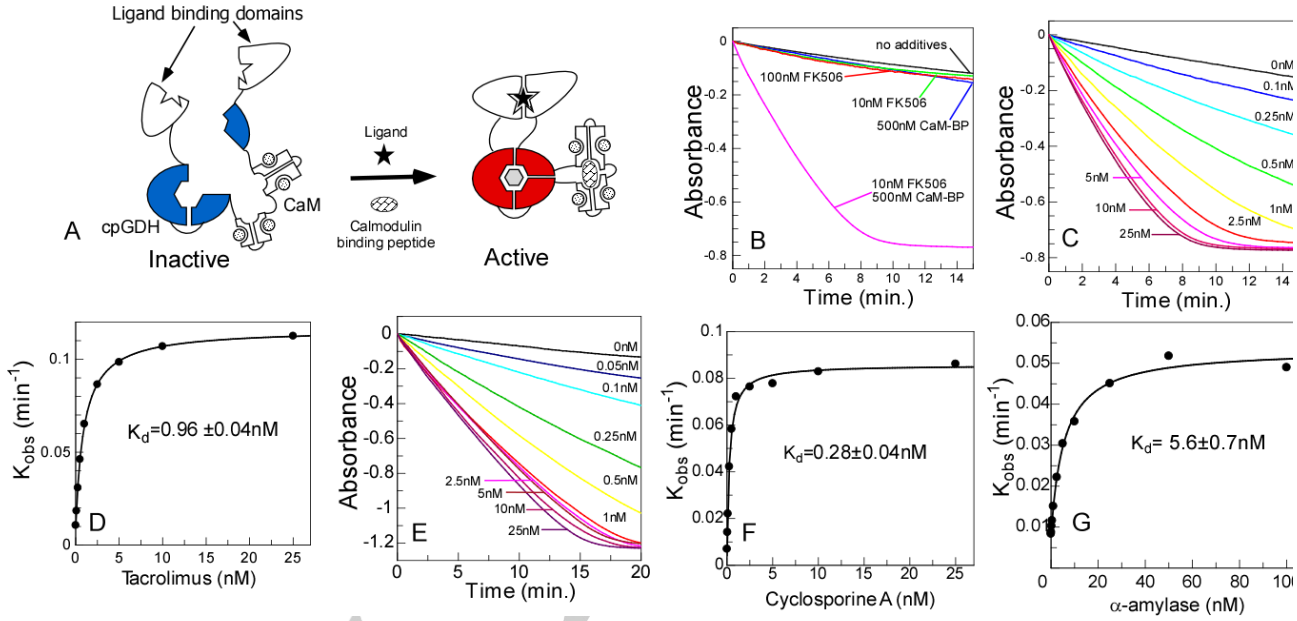

Figure 3. Structure and activity analysis of cpGDH biosensor with calmodulin domain linker. (A) Schematic representation of biosensor architecture and proposed mode of activation. (B) Activity of 2.5 nM solution of cpGDH-CaM tacrolimus biosensor in the presence or absence of indicated concentrations of calmodulin-binding peptide and (or) tacrolimus. The activity of the enzyme was monitored by changes in absorption of 60µM electron accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulphate, 20 mM of glucose, 1 µM PQQ, 1 mM $CaCl_2$ and 500 nM M13 calmodulin binding peptide. (C) Activity of 2.5 nM solution of cpGDH-CaM tacrolimus biosensor in the presence of 0.5 µM calmodulin-binding peptide , 1µM PQQ, 1mM $CaCl_2$ and increasing concentrations of tacrolimus. (D) Fit of the data shown in C to a quadratic equation. (E) Activity of 2.5 nM solution of cpGDH-CaM-Cyclosporine A biosensor in the presence of the increasing concentrations of Cyclosporine A. (F) A fit of the data shown in leading to a K_d value of 0.28 nM. (G) A fit of the K_{obs} values obtained from titration of 5 nM solution of cpGDH-CaM-α-amylase biosensor with increasing concentrations of α-amylase.

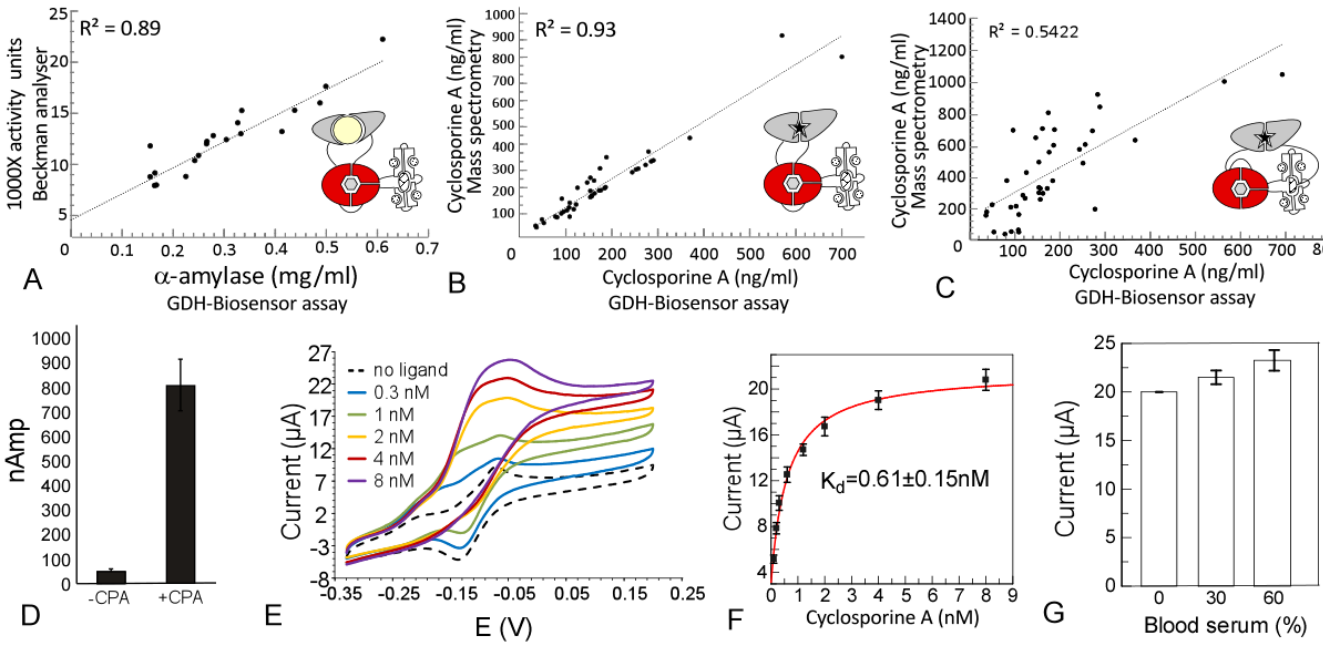

Figure 4. Quantification of biomarkers in human fluid samples using assays based on cpGDH-CaM biosensors. (A) Detection of α-amylase in human saliva α-amylase biosensor (X-axis) and its comparison with the clinical α-amylase activity assay (Y-axis). (B) Quantification of Cyclosporine A in blood of kidney transplant patients using cpGDH-CaM biosensor-based assay (X-axis) and its comparison with the clinical Cyclosporine A LC-MS assay (Y-axis). (C) The blood samples from B were retested using the assay based on a two component GDH biosensors of Cyclosporine A[12]. (D) Electrochemical response of 200 nM single component Cyclosporine A (CPA) sensor to absence and presence of 250 nM Cyclosporine a (average of n=3 plotted, error bars are SD). Sensor enzymatic activity is reported as maximum nA increase per minute using disposable DropSense gold electrode polarized at +0.1 V *vs* Ag reference strip and 1-methoxy-5-methyl phenazinium methylsulfate as an electron mediator. (E) Typical voltammograms of GDH-CaM- Cyclosporine A functionalized gold electrode upon exposure to different concentrations of Cyclosporine A. The scan rate was 2 mV·s^{-1}; the potentials were measured *vs*. Ag/AgCl/3MKCl reference electrode. (F) A plot of values obtained in three independent replicates of the experiment shown in E. The data was fit to a quadratic equation leading to a K_d value of 0.6 nM. (G) Analysis of the impact of the serum addition to the sample on the performance of GDH-CaM-Cyclosporine A biosensor functionalized bioelectrode. In the experiment, the electrode was activated by addition of 10 nM of Cyclosporine A to a sample containing indicated concentrations of human serum.

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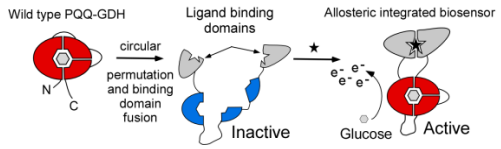

Circular permutation of PQQ-glucose dehydrogenase creates an artificial allosteric enzyme controlled by ligand binding to N- and C-terminal domains.

Key Words:

Protein circular permutation, protein biosensors, electrochemical biosensors, cooperative binding, Cyclosporine A, point of care diagnostics.