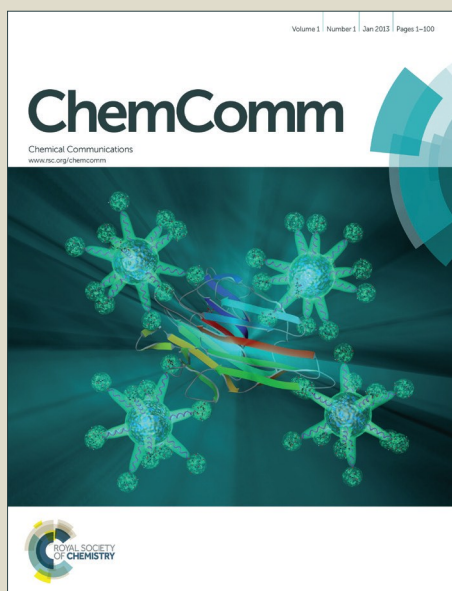


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Engineering PQQ-glucose dehydrogenase into an allosteric electrochemical Ca^{2+} sensor

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Electrochemical biosensors convert biological events to an electrical current. To date most electrochemical biosensors exploit activities of naturally occurring enzymes. Here we demonstrated that insertion of a calmodulin domain into the redox enzyme PQQ-glucose dehydrogenase resulted in a selective Ca^{2+} biosensor that could be used to rapidly measure Ca^{2+} concentrations in human biological fluids. We were able to convert a point-of-care glucometer into Ca^{2+} monitor by refurbishing it with the developed biosensor. We propose that similar engineering strategies may be used to create highly specific electrochemical biosensors to other analytes. Compatibility with cheap and ubiquitous amperometric detectors is expected to accelerate progression of these biosensors into clinical applications.

Over the past three decades biosensors have become a practical alternative to the complex and expensive analytical instruments used in healthcare and biotechnology¹. Among several currently used detection technologies such as optical, acoustic and piezoelectric sensors, electrochemical sensors feature prominently due to their simplicity, specificity and high performance². Electrochemical blood glucose sensors are the most commercially

successful biosensors accounting for nearly 90% of the biosensor market³. The success of these sensors is due to their high selectivity and sensitivity, combined with simplicity of design and ease of manufacturing. These sensors are based on the amperometric monitoring of glucose oxidation by either recombinant glucose oxidase or glucose dehydrogenases⁴. Here, a dry analysis chamber in the disposable sensor is filled with the sample setting off an enzymatic reaction with high current density. Unlike original sensor designs based on glucose oxidase (GOx), modern glucose sensors that utilize pyrroloquinoline quinone glucose dehydrogenase (PQQ-GDH) are also independent of oxygen and electron transfer mediators making the system less drift-prone^{5,6}. The simplicity and robustness of the design enables manufacturing of disposable glucose sensing electrodes for less than US\$0.1⁷. Yet, the technological and commercial success of glucose sensors has not been paralleled by other electrochemical biosensors despite both the clinical demand and the commercial potential of point-of-care diagnostics. This can at least in parts be explained by the unique features of glucose sensing where the analyte is present at high concentrations (4–20 mM) and also provides the source of energy for a selective, physically stable electrochemical receptor with high turnover rate. In one recent example, highly specific DNA switches were developed that triggered the release of invertase to catalyze the conversion of sucrose to glucose that could in turn be read-out by standard glucose monitors⁸. However, these biosensors are not integrated with the electrode and require external sample processing. In an alternative approach several groups exploited the fact that PQQ-GDH is stable albeit inactive in its apo form. This was used to devise ways to couple the diagnostically relevant molecular interactions to enzyme reconstitution and activation^{9,10}.

We conjectured that instead of exploiting glucose sensors in an indirect manner through coupled biosensing reactions, it could be possible to reengineer glucose sensing enzymes such that they could directly detect analytes that are not structurally related to glucose. This approach would allow direct utilization of the existing amperometric detector architectures with minimal adjustment. As the starting point for biosensor design, we chose PQQ-GDH of *Acinetobacter calcoaceticus* which has become an enzyme of choice in recent years^{11,12}. However, rather than seeking to modify the substrate specificity of the enzyme, we chose to endow the enzyme with an allosteric receptor domain that controls its catalytic activity in a ligand dependent fashion. To achieve this, we analyzed the high resolution structure of *A. calcoaceticus* PQQ-GDH (PDB: 1CQ1) for possible sites in the vicinity of the active center that would be close enough to transmit the conformational changes into the active center, and at the same time far enough to tolerate insertion of a receptor domain. Our choice fell on the loop connecting strands A and B of the β -sheet 3 (Fig. 1A and B). The beginning of strand A harbors His144 which acts as the general base abstracting a proton

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from the glucose O1 atom¹³. As His144 is critical for catalysis, we conjectured that its dislocation introduced by separation of strands A and B could lead to a change in GDH catalytic activity. As the loop connecting strands 3A and 3B faces away from the rest of the structure and the second subunit in the homodimer, its modifications are unlikely to introduce steric clashes. We decided to test this idea by inserting a protein domain known to undergo large conformational changes upon ligand binding into the chosen site of GDH (Fig. 1B).

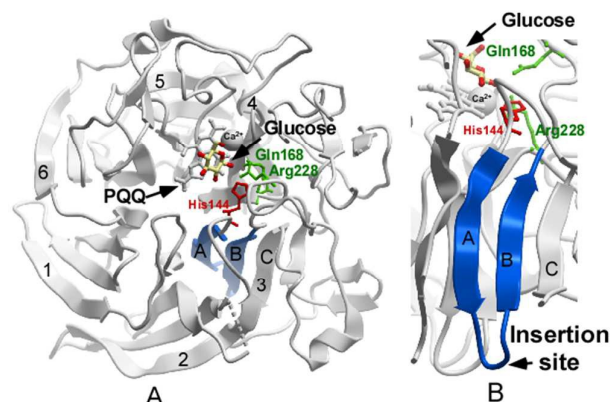


Figure 1. Structure of *A. calcoaceticus* PQQ-GDH and identification of calmodulin insertion site. (A) Ribbon representation of the enzyme in complex with PQQ and glucose. The PQQ cofactor is displayed in ball and stick representation while glucose is colored in atomic colors. The bound Ca^{2+} is displayed as space filling object. The β -sheets are marked with respective numbers and the β -strands of sheet 3 are marked by letters. Strands 3A and 3B are colored in blue and the active site residues involved in coordinating glucose are displayed in ball and stick. The catalytic His144 is colored in red. (B) Side view of GDH displaying the loop connecting strands A and B. The structure is displayed and colored as in A. Structure analysis and graphics preparation was performed using ICM Browser Pro 3.8.

Specifically, we chose calmodulin (CaM), a 17 kDa protein which plays a key role in the transmission of calcium signals to target proteins in eukaryotes¹⁴, as an insertion domain. The binding of Ca^{2+} to four EF hands of CaM results in large conformational changes that open up a peptide binding pocket between the two lobes of CaM. This feature of CaM has been repeatedly exploited for constructing genetically encoded Ca^{2+} sensors based on either spectral changes or FRET intensity of fluorescent proteins or activity of β -lactamase^{15,16}. Based on the available structural information, we designed a chimeric protein where residues of mouse CaM 2-148 were inserted between residues 153 and 155 of PQQ-GDH¹⁴. In order to reduce structural tension and clashes, we also introduced several glycine residues at the N-terminus of CaM and between the C-terminus of CaM and the junction site. The resulting protein was recombinantly produced in the periplasm of *E. coli* and purified to homogeneity by Ni-NTA affinity chromatography. We then used an established colorimetric assay to analyse the activity of the GDH-CaM chimeric protein¹⁷. As can be seen in figure 2, in the absence of Ca^{2+} ions, the GDH-CaM

displayed virtually no enzymatic activity. Addition of CaCl_2 resulted in dose-dependent activation of the chimeric protein while having negligible effect on the wild-type enzyme. The activation was reversible as addition of the Ca^{2+} chelator EDTA returned the enzyme to the inactive state (Fig. 2A). Analysis of Ca^{2+} titration data revealed a non-linear response to Ca^{2+} concentrations which has been described for CaM previously and is reflective of cooperative Ca^{2+} binding (Fig. 2B)¹⁸.

The data demonstrated that the developed sensor displays the largest signal change between 0 and 100 μM of Ca^{2+} (Fig. 2B). The comparison of Ca^{2+} sensitivity of the obtained biosensor and the wild type enzyme reveals the key difference in their response to this ion. While the activity of the wt enzyme is only partially enhanced by the Ca^{2+} ion though polarization of the PQQ C5–O5 bond, the activity of the developed biosensor was strictly dependent on the presence of the ion (Fig. S1A)^{13,19}. Furthermore, the wt GDH displayed much steeper response to the Ca^{2+} ion than the chimeric enzyme (Fig. S1B,C). These results strongly suggest that the rate limiting step regulated by Ca^{2+} differs between wild type and the chimeric GDH.

As a redox enzyme, the most efficient integration of GDH-CaM into a POC device is via an electrochemical interface. The performance of the enzyme in this setting is demonstrated in chronoamperometric experiments (Fig. 2C) that similar to the spectroscopic data confirmed that engineered GDH-CaM sensor was activated by Ca^{2+} in dose dependent fashion.

While the observed dynamic range reflected affinity of CaM for ionized Ca^{2+} in the intracellular environment, the extracellular concentration of this ion is much higher and the bodily fluids such as blood, urine and saliva feature Ca^{2+} concentrations between 1 and 2 mM²⁰. The ability to rapidly assess these parameters is important in clinical practice since deviations from this concentration range is often reflective of pathological states such as endocrine disorders, osteoporosis, cancer, sepsis and acute renal failure²⁰. Therefore an inexpensive, rapid and accurate test for ionized calcium would be of significant value for point of care (POC) diagnostics. While we could demonstrate that this could be achieved by diluting the biological sample with the buffer until Ca^{2+} concentration falls into the response range of the sensor, this protocol inevitably introduces pipetting errors and requires complex engineering solutions to become suitable for POC applications (Fig. S2 A,B). Instead, we tested whether we could buffer Ca^{2+} to bring its free concentration into the response range of the sensor. To this end, we repeated our experiments in the presence of the well-characterized and specific Ca^{2+} chelator 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA)²¹. Addition of 1.1 mM BAPTA to the sample allowed us to shift the optimum of the sensor's response into the range of the physiological Ca^{2+} concentration in biological fluids (Fig. 2C). We tested, whether the presence of the closely related ion Mg^{2+} would affect the release of Ca^{2+} from BAPTA and demonstrated that its presence had no impact on the apparent K_d (Fig. 2C). These results support the notion that the developed GDH-CaM chimeric protein is suitable for construction of the POC biosensors.

One of the key features that enabled the development of inexpensive glucometer strips was the ability of GDH to retain its

activity following de- and rehydration. We subjected our chimeric protein to drying and re-hydration and confirmed that, similar to wild type GDH,

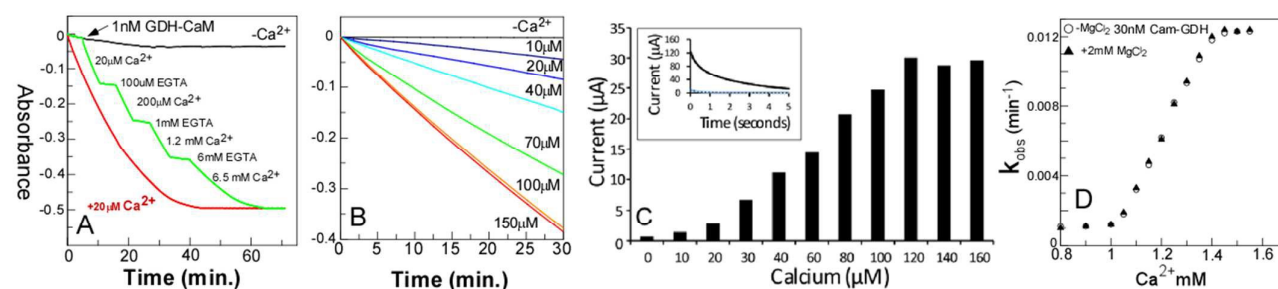


Figure 2. Spectrometric analysis of PQQ-GDH-CaM activity at different concentrations of Ca^{2+} . (A) Time resolved changes in absorption of 60 μM electron accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulphate were measured at 600 nm in the presence of 20 mM of glucose and 1 nM GDH-CaM. (B) As in A, but using 3 nM GDH-CaM exposed to the increasing concentrations of CaCl_2 . (C) Performance of GDH-CaM chimer as a sensor in electrochemical systems. Main plot; response of GDH-CaM chronoamperometric electrode to increasing Ca^{2+} concentrations. Current measured after 5 s at +0.4V versus imbedded Ag reference strip, GDH-CaM present at 300 nM, PMS mediator at 3 mM, glucose at 50 mM. Inset plot; current versus time after polarization at +0.4V versus imbedded Ag reference strip at two representative calcium concentrations (0 and 100 μM). (D) A plot of the observed initial reaction rates of 3 nM GDH-CaM in the presence of 20 mM glucose and 1.1 mM of the specific Ca^{2+} chelator BAPTA. The experiments were performed as in (B). Filled triangles represent experiments in which titration was performed in the presence of 2 mM MgCl_2 .

the chimeric protein retained its activity (Fig. S2C). To further demonstrate the compatibility of the developed biosensor with the established glucose monitor platform, we dismantled the commercially available glucose monitoring Accu-Chek electrodes and replaced the wild type GDH with the GDH-CaM sensor. We tested the ability of the refurbished electrode to be activated by Ca^{2+} present in human saliva (Movie S1). Exposure to undiluted human saliva resulted in generating an electric current that could be detected using the standard Accu-Chek detector. Although the internal calibration and numerous built-in safety features of the device prevented us from accurately determining Ca^{2+} concentrations in the sample, the result demonstrates that it can communicate with the standard potentiostat-based POC device.

In summary, we present here the first example of successful structure-guided engineering of a Ca^{2+} -gated electrochemical sensor. We identified an insertion site in the vicinity of the catalytic center that tolerates the insertion of large autonomously folding protein domains. The domain insertion strategy has been previously employed on several occasions to create artificial allosterically regulated enzymes^{22,23,24}, but has never been applied to electrochemically active enzymes. The described approach changes the operational mode of the GDH biosensor where its electron output is no longer limited by the glucose concentration, but is gated by the chosen analyte. The high turnover rate of GDH makes it a very attractive electrochemical actuator for engineering further biosensors, and the identified insertion site in GDH can now be exploited with alternative sensory domains. While this process is largely empirical and involves a significant amount of optimization, the available data suggests that once insertion sites in a protein have been identified, they can be readily exploited to insert

alternative domains¹⁶. This, at least in principle allows construction of the autoinhibited version of GDH which can then serve a starting point for engineering of a broad range of sensory systems²⁴. Given the ubiquitous use of GDH-based glucose sensors and availability of end point and continuous measurement devices, we anticipate the rapid translation of such biosensors into clinical practice.

Material and methods

Chimeric gene construction and protein expression and purification

The chimeric GDH-CaM gene was generated by Gibson Assembly method according to the manufacturer's instruction (New England Biolabs) and cloned into pET28a vector. The full sequence is available in Supplementary information section. The gene fragments for this assembly were either generated by PCR or ordered as synthetic gBlock gene from IDT (Integrated DNA Technologies). The protein expression and purification were described²⁵. After Ni-NTA purification of both GDH wt and GDH-CaM the pooled enzyme-containing fractions were supplemented with EDTA to the final concentration 5 mM and dialyzed against buffer containing 20 mM KH_2PO_4 pH 7.0 and 5 mM EDTA for 10 hours. Subsequently EDTA was removed by dialyzing the sample against the buffer containing 20 mM KH_2PO_4 pH 7.0 only. Purified GDH was reconstituted with its co-factor by adding PQQ in a 1:1.5 molar ratio.

Spectrophotometric analysis of GDH enzymatic activity

The GDH enzyme assay was performed as described²⁶. Briefly, the 1.5 mL reaction volume comprised 20 mM glucose, 0.6 mM phenazine methosulfate (PMS), 0.06 mM 2,6-dichlorophenylindophenol (DCPIP), 10 mM MOPS (pH 7.0), and chosen concentrations of MgCl_2 and enzyme. The enzymatic assay

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was performed at 25 °C by monitoring the decrease in absorbance of DCPIP at 600 nm.

Electrochemical analysis of GDH-CaM activity

Chonoamperometric measurements were carried out using a Digilyvy DY2116B 3-electrode mini-potentiostat interfaced to DropSens disposable screen printed gold electrodes (Cat#DRP-C220BT). Electrodes were washed with 98°C milliQ water between scans to ensure no bound active enzyme was present. Reactions contained 2 mM PMS mediator, 1.1 mM calcium chelator BAPTA, 100 nM GDH-CaM and variable calcium concentration in 50 µl total volume at pH 7.6 PBS. Reactions were started with the addition of 40 mM glucose and incubated at room temperature for 1 minute before being pipetted onto the electrode surface. Chonoamperometry was carried out for 5s at +0.4 V versus the imbedded silver strip on the screen printed electrode, with data generally reported as current at the 5 s time point versus calcium concentration.

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