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Generalizable protein biosensors based on synthetic switch modules

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ABSTRACT: Allosteric protein switches are key controllers of information and energy processing in living organisms and are desirable engineered control tools in synthetic systems. Here we present a generally applicable strategy for construction of allosteric signaling systems with input and output of choice. We demonstrate conversion constitutively active enzymes of choice into peptide-operated synthetic allosteric ON switches by insertion of a calmodulin domain into rationally selected sites. Switches based on EGFP, glucose dehydrogenase, NanoLuciferase, and dehydrofolate reductase required minimal optimization and dynamic response ranging from 2-fold in the former case to nearly 200-fold in the latter case. The peptidic nature of the calmodulin ligand enables incorporation of such synthetic switch modules into higher order sensory architectures. Here, a ligand-mediated increase in proximity of the allosteric switch and the engineered ligand peptide modulates biosensor's activity. Such biosensors were used to measure concentrations of clinically relevant drugs and biomarkers in plasma, saliva and urine, performing with the accuracy comparable to that of the currently used clinical assays. The approach presented is generalizable as it allows rapid construction of efficient protein switches that convert binding of potentially any ligand into a biochemical activity of choice enabling construction of artificial signaling and metabolic circuits of, potentially, unlimited complexity.

INTRODUCTION: Allosteric regulation of proteins has been referred to as the 'second secret of life' as it controls every aspect of information and energy in biology¹. Allosterically regulated proteins use often subtle structural rearrangements to convert binding events or chemical modifications into changes in their biochemical activity. This endows the protein-based signaling and metabolic networks with multiple layers of regulation that allows them to respond to environmental changes in

complex but robust and rapid ways. In the recent years our understanding of how the energetic coupling between sites of the allosterically regulated proteins has significantly advanced due to a large number of computational and experimental studies (²and references therein). Not surprisingly, given the importance of allosteric regulation proteins in biology, construction of artificial allosteric proteins converting one type of biochemical signal into another became a central goal of synthetic biology and protein engineering. Furthermore, sensing systems based on artificial allosteric proteins, often referred to as biosensors, hold a promise of transformative changes in biotechnology, healthcare, food industry and agriculture³.

Despite significant efforts and considerable academic and commercial interest, our ability to construct allosteric protein switches remains underdeveloped. While there are several impressive cases of *de novo* design of allosteric switches through computational design or high throughput screening they remain to be isolated examples (⁴and references therein). Presently, construction of chimeric receptor-reporter proteins by domain insertion is the most successful approach for the development of artificial allosteric modules⁴. The main challenge of this approach is in finding analyte binding domains that undergo conformational changes sufficiently large that they can be transferred onto the actuator domain. This largely disqualifies most commonly used binding modules such as antibodies or their synthetic analogues as they often display only minor structural changes upon ligand binding. The second challenge is in finding an appropriate insertion site in the reporter domain that can both tolerate the domain insertion and at the same time operably connect it to the reporter's activity. Often both random and structure-guided domain insertion yield switches with small dynamic ranges that have to be further optimized by mutagenesis⁵.

Compared to colorimetric, luminescent and fluorescent biosensors, electrochemical biosensors feature prominently due to their simplicity, low cost and connectivity with portable electronic devices⁶. Electrochemical blood glucose sensors are the most commercially successful biosensors, accounting for nearly 60% of the US\$15 billion global biosensor market. The sensors are based on amperometric monitoring of glucose oxidation by recombinant glucose oxidase or glucose dehydrogenase (GDH)⁷. Remarkably, while a number of attempts have been made to build additional bio-sensing application on top of the glucometer technology⁸, only very recently GDH itself was successfully engineered to detect analytes other than glucose⁹.

Here we use a combination of structure-based protein engineering to convert pyrroloquinoline-quinone (PQQ)-GDH into an allosteric peptide sensor. This basic switch module was then used to construct a two-component biosensor systems that can be targeted to practically any analyte. We demonstrate that this approach can be applied to other reporter domains and discuss the path toward broad application of this new class of molecular switches.

RESULTS: Rational design of a peptide-responsive PQQ-glucose dehydrogenase. Allosteric protein switches rely on a protein's ability to reversibly adopt alternate conformations that display different levels of activity. Binding of a ligand leads to structural rearrangements, shifting the equilibrium from one state to another. In most cases such conformational states are mediated by complex interaction networks between the allosteric and the active site¹⁰. Replacement of the ligand binding part of the switch typically destroys the ability of the catalytic site to adopt active and inactive conformations in a ligand dependent fashion⁵. This creates a major barrier to predictable construction of efficient protein switches. We speculated that

this problem could be addressed by first creating a generic autonomous, peptide-controlled allosteric ON switch with the desired reporter activity. Such a switch could be then integrated into higher order protein signaling systems where ligands control local concentrations of the activator peptide in the vicinity of the allosteric reporter. The main challenge of this approach is in finding a peptide-controlled regulatory protein domain that in apo- form could render the host domain inactive, while the conformational transition upon ligand binding should restore its activity. One way of achieving this would be using a domain with a large peptide-induced conformational transition that bring the termini of the domain closer together, reducing the distortion of the host protein. We and others have previously exploited the large conformational change of calmodulin in response to calcium to construct Ca^{2+} biosensors^{9,11,12}. Yet peptide-ligand mediated structural transition of calmodulin has received much less attention in biosensor research despite the demonstration that it can modulate the activity of a chimeric protein¹³. Importantly, in response to the peptide ligand, calmodulin transits between two well-ordered states, which is consistent with goal of creating two distinct structural states of the chimeric protein.

To test the general applicability of this concept we chose PQQ-GDH from *Acinetobacter calcoaceticus*, which is ubiquitously used in electrochemical point-of-care glucose monitoring systems¹⁴. We inspected the structure of the GDH molecule for a potential insertion site from which conformational changes could be relayed to the GDH active site (Fig. 1B). This identified a loop connecting β -sheets 4 and 5 as a suitable insertion site as it harbors Trp346 and Thr348 that form a part of glucose binding site and Thr348 that is involved in coordination of the PQQ cofactor¹⁵ (Fig 1A). We expected that displacement of these residues from their native position would reduce activity of PQQ-GDH.

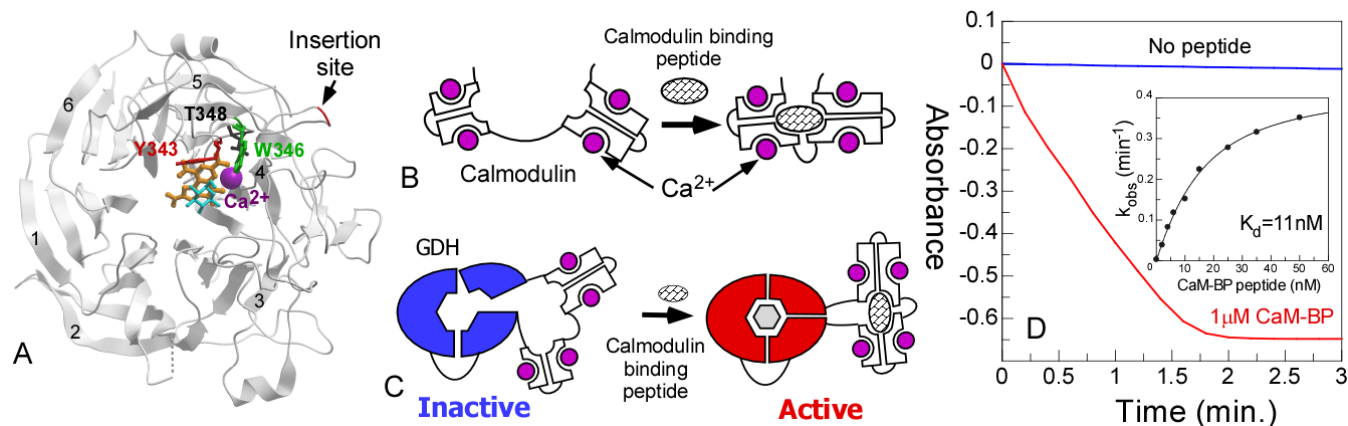


Figure 1. Allosteric peptide biosensors based on PQQ-GDH. (A) Ribbon representation of the PQQ-GDH structure. The arrow indicates the position of the insertion into the loop connecting β -sheets 4 and 5. The residues involved in binding of glucose (blue) are colored in green and red and the catalytic Thr 348 is shown in black. The cofactor PQQ is displayed in gold. (B) Schematic representation of calmodulin and its conformational transitions induced by the calmodulin binding peptide (CaM-BP) (C) Schematic representation of a GDH-CaM chimera and its interaction with CaM-BP where the hexagon represents a glucose molecule. (D) GDH activity of 10nM solution of GDH-CaM chimera in response to CaM-BP. The activity of the enzyme was monitored by changes in absorption of electron accepting dye dichlorophenolindophenol in the presence of 0.6 mM electron mediator phenazine methosulphate, 20mM of glucose and 50 μM CaCl_2 . The inset shows the titration data with the solid line being a fit to a quadratic equation describing the binding curve resulting in a K_d value of 11 nM.

Therefore, we inserted calmodulin into GDH at the position 330 (Fig. 1A, C) and produced a CaM-GDH chimeric protein in recombinant form (Fig.S1). The resulting protein displayed low GDH activity in the absence of the peptide, however, the addition of the M13 calmodulin binding peptide (CaM-BP) increased the CaM-GDH chimer activity in a dose dependent fashion (Fig.1D). The activity of the fully activated chimeric protein was 50% of the activity of wild-type GDH. Fitting of the observed reaction rates showed that the peptide bound to the enzyme with an affinity of 11nM, indicating that the binding affinity of the chimeric protein was reduced by over an order of magnitude. This is not surprising in the light of the bi-stable switch model of allosteric regulation where the allosteric sites are energetically coupled¹⁶.

Two-component biosensors based on the peptide-activated GDH chimera. The fact that both the allosterically regulated reporter switch and its ligand are polypeptides creates an opportunity for their assembly into higher order signaling systems using protein engineering. In such designs, dimerization of ligand binding domains would increase the concentration of the fused CaM-GDH reporter and CaM-BP leading to the activation of the former. Yet, we had to assume that the high affinity of CaM-BP for CaM-GDH would lead to background activation of the reporter at concentrations approaching the K_d of the CaM-GDH:CaM-BP complex. In order to circumvent this problem,

we analyzed the structure of CaM:CaM-BP complex (PDB:2BBM) to design a variant of CaM-BP that would display reduced affinity to CaM but at the same time could induce its structural rearrangement at high concentrations. We speculated that truncating the ligand peptide by 16 amino acids and replacing the last 5 amino acids with an ASASA sequence would on one hand reduce the affinity of the peptide for the CaM but, on the other hand, preserve enough essential sidechains that a CaM:CaM-BP complex with the near-native structure could form. To test the functionality of such architecture, we fused CaM-GDH N-terminally to FK506 binding protein (FKBP) domain and produced the protein in recombinant form. As expected, the protein displayed minimal GDH activity in the absence of the CaM-BP. We then proceeded to construct a fusion between FKBP binding partner FKBP-rapamycin binding domain (FRB) and the low affinity version of CaM-BP, expecting that in the presence of rapamycin the complex formation between FRB and FKBP would increase the concentration of CaM-BP in the vicinity of the CaM-GDH reporter domain leading to its activation (Fig. 2A). Indeed, mixing the solutions of recombinant CaM-GDH- FKBP and FRB fused to CaM-BP mutant induced only a low level of GDH activity (Fig.2B). However, addition of rapamycin dose-dependently induced GDH activity, allowing determination of the concentration and K_d of the compound for the biosensor complex.

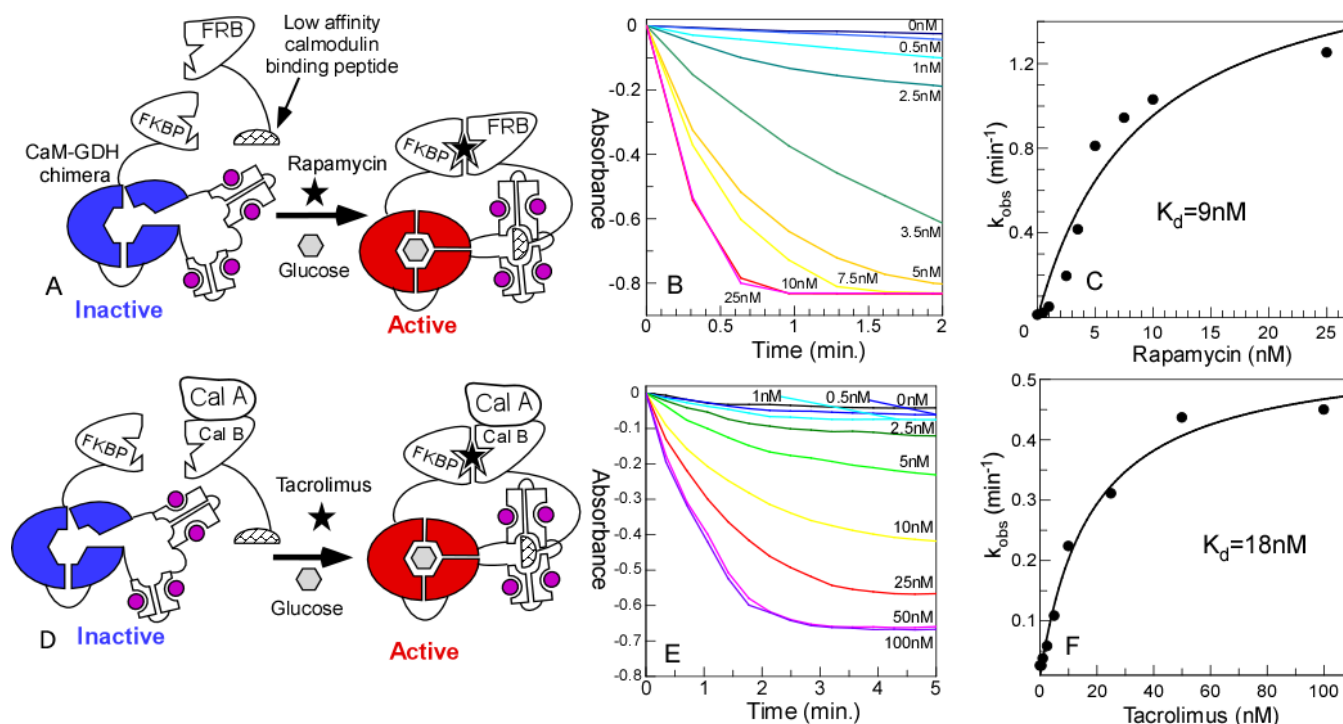


Figure 2. Construction of two component biosensors based of CaM-GDH allosteric switch. (A) Schematic representation of GDH-CaM chimera-based two component biosensor. (B) Titration of a solution of 10nM of GDH-CaM-FKBP and 30nM FRB-CaM-BP with increasing concentrations of rapamycin. The GDH assay was performed as in 1D. (C) Fit of the data shown in B to a quadratic equation as described in 1D. (D) Schematic representation of two component biosensor for tacrolimus. (E) Titration of 10nM of GDH-CaM-FKBP and 30nM of CalA/CalB-CaM-BP with increasing concentrations of tacrolimus. The assay was performed as in B. (F) The fit of the data shown in E leading to a dissociated constant of 18nM.

As analyte-mediated dimerization is the driving force in biosensor activation, we expected the developed architecture to be generally applicable. To test that we set out to construct a bio-

sensor for another immunosuppressant drug, FK506 (tacrolimus), which forms a quaternary complex with FKBP and calcineurin A/B¹⁷. We produced a complex of calcineurin A with

a fusion of calcineurin B and the mutant CaM-BP by co-expression (Fig.2D). When a solution of this complex was mixed with CaM-GDH-FKBP and titrated with tacrolimus, we detected a dose-dependent increase in GDH activity, demonstrating that biosensors of small molecules other than rapamycin could be constructed using the developed biosensor architecture (Fig.2E,F).

We next tested if the approach could be applied to detection of proteins rather than small molecules. To this end we produced fusions of CaM-GDH and a modified version of CaM-BP in fusion with camelid VHH antibody domains targeting two different epitopes of α -amylase¹⁸. Addition of α -amylase to the solution of these fusion proteins led to a dose-dependent increase in GDH activity, indicating that the architecture is generic and can be re-targeted to new analytes by simply exchanging the binding domains (Fig. S2A-C). In order to ascertain the general applicability of the approach we screened the PDB databank for structures of proteins with two VHH domains bound to two distinct sites of a protein molecule. We identified complexes of thrombin-activatable fibrinolysis inhibitor, clostridium TcdA toxin and interleukin-23 with two non-identical VHH domains each. Using the VHH sequences extracted from the PDB files

we constructed efficient biosensors to all proteins (Fig. S2 D-F), thereby validating the generalizable nature of the developed sensory architecture.

Optimization of biosensor's performance: The developed biosensor architecture is generalizable as it composed of two exchangeable modules: the binders responsible for the capture of the analyte and the allosteric reporter that converts the concurrent binding events into an electrochemical signal. However, carryover of functions associated with the individual modules into the final assembly constitutes a potential weakness of this design. While the ligand binding domains and the GDH reporter are not known to possess multiple activities or functions, calmodulin is often referred to as a “molecular jack-knife” due to the plethora of functions and interactions it is known to engage in¹⁹. Clearly, Ca^{2+} -binding is one of the most prominent activities of calmodulin, and this activity is known to regulate interactions with the majority of its putative 300 peptide ligands. The influence of Ca^{2+} on biosensor performance would limit their utility as it would require precise control of Ca^{2+} concentrations that vary rapidly and significantly inside and outside of the cell. To analyze the effect of Ca^{2+} experimentally we tested the performance of our biosensors at different Ca^{2+} concentration.

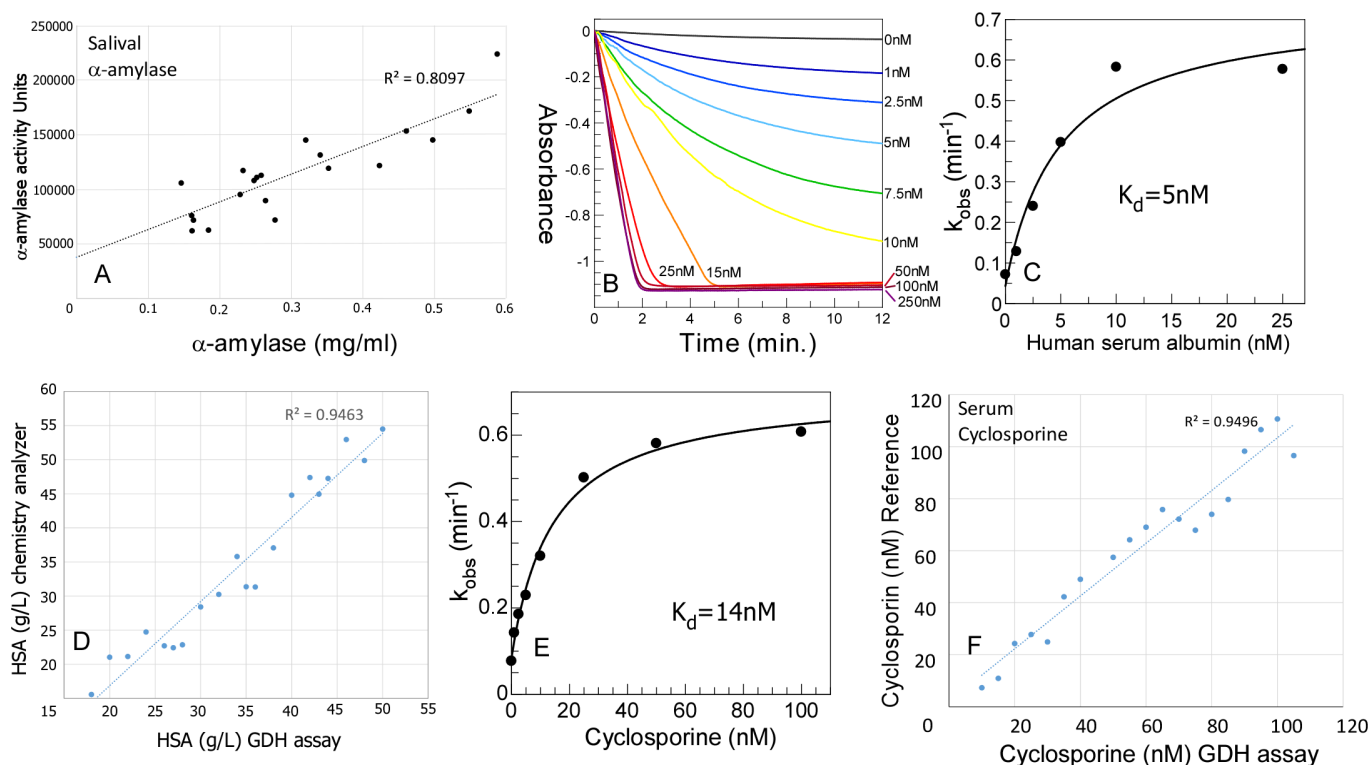


Figure 3 Testing and optimization of two component biosensors . (A) Detection of α -amylase in human saliva using Ca^{2+} tolerant α -amylase biosensors (X-axes) and its comparison with the clinical α -amylase activity assay (Y-axis). (B) GDH activity of 10nM of “ratcheted” CaM-GDH chimera in response to increasing concentration CaM-BP in buffer containing 1mM CaCl_2 . (C) Fit of the activity data of 10nM of cysteine “ratcheted” CaM-GDH-Protein G fusion and 100nM anti-HSA-VHH-CaM-BP in the presence of the indicated concentrations of HSA. The observed rates were fitted leading to a K_d of 5nM. (D) Quantification of HSA concentration in serum of human donors using HSA biosensor (X-axes) or clinical chemistry analyzer (Y-axes). (E) Fit of the titration data of 5nM of “ratcheted” GDH-CaM-Cyclophilin and 30nM of CalA/CalB-CaM-BP with increasing concentration of cyclosporine in the presence of 25% human serum. The fit of the data led to a K_d value of 14nM. (F) Analysis of human serum samples spiked with different concentrations of cyclosporine and analyzed using the cyclosporine biosensor from (E).

Systematic activity analysis of the tacrolimus biosensor at different Ca^{2+} concentrations demonstrated that, as expected, the latter was required for obtaining the response to the ligand, but

the optimal signal-to-noise ratio of the biosensor was between 50 and 100 μM of Ca^{2+} . Below this concentration the tacrolimus

stimulated little or no GDH activity while the higher concentrations of Ca^{2+} led to progressively increasing background activation and resulted in a constitutive activation of the biosensor at concentrations above 1mM (Fig.S3A,B). The utility of the developed systems for diagnostics or bioengineering would increase significantly if the reporter switch could be adopted to be tolerant to Ca^{2+} fluctuations in the physiological range, i.e. between 0.5 and 5 mM ²⁰.

The traditional way of optimizing performance of biosensors based on domain insertion is variation of linker sequences that either reduce or enhance conformational coupling between domains⁴. We decided to test an alternative strategy and explore whether the insertion of calmodulin into a different site of GDH would result in chimeras with a different conformation-activity coupling and therefore, different Ca^{2+} sensitivity profile. The added benefit of this approach is in further testing the success of CaM insertion as way of constructing CaM-BP-operated synthetic allosteric switches. To this end, we reanalyzed the structure of PQQ-GDH and identified a loop connecting β -sheets 5 and 6 as an alternative insertion site (Fig.S4A,B). This loop carries Arg406 and Arg 408 that make ion-pair interactions with C9 and C2 carboxyl groups of PQQ (Fig.S4C). We assumed that regulated displacement of these residues will impact the ability of PQQ to engage in glucose catalysis and create a reversible OFF state.

We constructed a chimeric protein where CaM was inserted between the amino acids S403 and N405 of the GDH molecule (named $\beta 5/6$ -CaM-GDH). The recombinant chimeric protein showed CaM-BP-dependent CaM-GDH activation that was only marginally influenced by the changes in Ca^{2+} concentration once a threshold concentration of 1mM of Ca^{2+} is reached (Fig.S4F). Encouraged by these results, we constructed a two component biosensor for rapamycin using the above described approach. As in the initial testing of the biosensor showed only modest rapamycin-mediated activation, we decided to use a higher affinity version of CaM-BP (KRRWKKNFIAVSAANR) than was used to construct the earlier version of the switch (KRRWKKNFIAVASASA). As shown in figure S4G, this biosensor also showed Ca^{2+} dependence, but increase in its concentrations beyond 1mM resulted in little further change in biosensor's activity. We further tested the performance of the biosensor in 50% bovine serum spiked with 5mM Ca^{2+} and confirmed that this had no impact on observed rates or the amplitude. Similar results were obtained with tacrolimus biosensor constructed using the same approach (Fig. S4I).

In order to directly demonstrate that the developed Ca^{2+} -tolerant biosensors could detect biomarkers in human bodily fluids, we constructed an α -amylase biosensor with the above-described VHH domains and used the developed α -amylase biosensor to measure the concentration of the α -amylase in samples of human saliva. In parallel, we assessed the α -amylase concentration using a commercial enzymatic assay. The data from both experiments were compared and showed a correlation very similar to what was reported previously for ELISA vs enzymatic activity assays comparison (Fig.3A)²¹.

Enhancing biosensor's dynamic range. Our engineering efforts that resulted in a CaM-GDH unit tolerant to variations in Ca^{2+} concentrations also led to the reduction of its dynamic range to

less than 6 fold. This complicates the use of the system for low abundant analytes and also increases the technical hurdles for transition from laboratory assays onto miniaturized formats that could be used in Point-of-Care (PoC) applications. The latter route is particularly attractive given the broad use of GDH in PoC glucose biosensors, combined with the highly advanced and commoditized glucose monitor manufacturing processes.

We hypothesized that the performance of the switch module may be enhanced if the system is engineered to operate in the non-equilibrium mode, where the activated state is locked preventing its relaxation. To test this idea, we decided to incorporate cysteine residues in the linkers connecting the CaM domain to GDH. We rationalized that CaM-BP induced conformational change would bring the cysteine residues into close proximity, increasing the chance of disulfide bond formation. These new bonds would function as the molecular ratchet shifting the equilibrium towards activated state. To this end, we designed a variant of the FKBP -R $\beta 5/6$ -CaM-GDH chimera where the protein domains were connected using SGCGG and GGSCG linkers. The protein was produced in the *E.coli* cytosol in order to keep it in the reduced form. While the protein was inactive in the ground state, it dose-dependently responded to the CaM-BP. Analysis of the data revealed that the biosensor displayed a remarkable dynamic range of 104-fold (Fig.3B). This is one of the largest dynamic ranges of a synthetic allosteric switch developed to date⁴. Mass-spectrometric analysis of such "ratcheted" form of CaM-GDH in the absence and presence of CaM-BP detected a mass decrease that matches disulfide bond formation thereby providing strong circumstantial evidence to the proposed mechanism (Fig. S5).

Encouraged by these observations we used the developed switch module to construct a biosensor of human serum albumin (HSA) using Protein G and anti HSA VHH as binding modules. The resulting biosensor displayed increased GDH activity in response to HSA (Fig. 3C and S5A,B). In order to test the practical utility of this biosensor we collected samples of human serum and quantified the concentration of HSA using our biosensor as well as the established clinical diagnostic platform. As can be seen in the Figure 3D both methods showed excellent correlation. We subsequently demonstrated that same assay could be used to quantify HSA in human urine correctly identifying patients with albuminuria (Fig.S6C). Encouraged by these results we constructed a biosensor of another macrocyclic immunosuppressant Cyclosporine A (Fig S7A-D) that displayed a dynamic range close to 10 fold even in the presence of 25% human serum (Fig. 3E). We tested the performance of this biosensor in serum samples containing different amounts of cyclosporine. As shown in the Figure 3F the biosensor-based assay correlated well with the concentration of the drug in the samples and the sensitivity of the assay was at least 10 times higher than required for detection of the clinically relevant concentration of the drug in blood (the clinical reference range of cyclosporine is 100-400nM²²).

In the course of this study we consistently observed that the dynamic range of two component biosensors was smaller than that of its parental switch module. One of the possible explanations lays in the fact that CaM-BP used to design and test the core switch and the CaM-BP used to activate it in the context of a two component system are similar but not identical. This is due to mutations that are introduced to reduce the affinity of peptide

for the core switch. While in the context of the ligand – scaffolded biosensor the equilibrium should be shifted to fully bound CaM-BP the structure of the structure of the complex may be different from the native one. To test the possible effect of CaM-BP structure on the activity of the developed CaM-GDH chimeras created in this study we tested a collection of the peptides extracted from the available calmodulin:calmodulin binding peptide complexes (Table S2). As can be seen in the figure S8 there was a significant difference in the activity response of both CaM-GDH variants to the CaM-BPs. In some cases both switches responded similarly to the peptide (peptides 1 and 4) while peptides had dramatically different effects on each chimera. Interestingly, peptides 5, 7, 8, 13 and 12 displayed inhibitory activity towards one of the chimeras. This can potentially indicate that these peptides stabilize the CaM-GDH chimera in the inactive conformation thereby reducing the background activity. Based on this data we concluded that small changes in the structure of CaM-GDH:CaM-BP complex may lead to significant changes in its dynamic range (compare effects of peptides 3 and 4 that differ by one residue). This may provide an explanation for reduction of the dynamic range in the two component systems that are operated by CaM-BPs that structurally differ from the parental peptides that were used identifying best CaM-GDH designs. Therefore, architectures that utilize unmodified peptides may result in higher dynamic ranges. Alternatively, one can envision an integrated engineering approach where modifications of CaM-BP are done in the context of the two component system thereby correlating the structure and function more directly.

The simplicity and low cost of chronoamperometric detection of enzyme-generated electron current on disposable screen printed electrodes makes GDH-based biosensors very attractive for PoC applications. Therefore, we tested whether tacrolimus-induced activity change of the tacrolimus biosensor could be detected using standard electrochemistry measurements. The results described in the supplementary text section and Fig. S8 show that using standard chronoamperometry could be used to monitor activity of the developed GDH biosensors.

As glucose monitoring systems utilize screen printed electrodes layered with a dry mixture containing the biosensor and the electron mediator we wanted to test if the developed biosensors display sufficient stability to be compatible with such formats. To this end we lyophilized CaM-GDH as well as the two component biosensors of α -amylase, cyclosporine, tacrolimus and human serum albumin described above and tested their activity upon re-hydration and seven day storage at room temperature. The data shown in the figure S10 confirms that even without optimization of drying conditions the proteins retained their activity in this procedure.

Calmodulin insertion is a generic strategy for converting proteins into synthetic allosteric switch units. The success of calmodulin insertion experiments prompted us to test whether the developed approach could be used to convert other proteins into allosteric switch units that could be compiled into biosensor architectures. Given their common use as biological reporters, we chose dihydrofolate reductase (DHFR), EGFP and NanoLuciferase (NanoLuc) as examples. We used structural analysis to rationally select the calmodulin insertion sites (see SI). As can be seen in the figure 4A-C all three chimeras demonstrated ON

switch behavior in response to the CaM-BP, albeit with different affinities and dynamic ranges. While Cam-EGFP displayed a modest dynamic range of 1.8 folds (Fig.4B), the CaM-DFFR displayed nearly 200-fold activity change and CaM-NanoLuc displayed the dynamic range of approximately 20-fold (Fig. 4C). We used CaM-NanoLuc module to confirm that, similar to CaM-GDH, this synthetic allosteric switches can be utilized to construct two component signaling system. As depicted in the figure 4D CaM-NanoLuc two component rapamycin biosensor displayed a robust dose-dependent response to the drug with a 4.5 fold dynamic range.

DISCUSSION: The central premise of Synthetic Biology is that biological systems can be built from standard components. While this is well evidenced by synthetic genetic circuits, the idiosyncratic nature of protein interactions has, to a large extent, prevented application of these principles to the design of orthogonal protein-based signaling systems. One of the main difficulties in advancing this direction is lack of generic and robust strategies for the construction of efficient protein switches. Most generic approaches for protein switch construction are based on the reconstitution of split enzymes or proximity induced proteolytic activation²³. While the reconstitution approach has been successfully applied in vivo, split enzymes typically display compromised stability and their use in vitro is problematic. Furthermore, protein reconstitution is a kinetically complex process that in many cases is not truly reversible. Although proteolysis-based systems are more biophysically stable, they are inherently slow and irreversible^{24,25}. Here we present a generalizable approach for the construction of artificial two-domain allosteric switch units (Fig. 4E,F). We demonstrate that introduction of calmodulin into two different sites of GDH results in the formation of efficient peptide-operated allosteric ON switches.

Unlike many previous reports, here switch construction required minimal optimization which may be a consequence of relatively large conformational transitions of the regulatory domain. An earlier report where calmodulin was inserted into β -lactomase resulting in its peptide-dependent activity further supports this idea¹³. The fact that all developed chimeras represented ON switches is not unexpected and can be rationalized from the perspective of the known structural transitions of calmodulin. Peptide binding induces structural rearrangement from extended to compact conformation that brings the N- and C- termini closer together by about 10 Å¹⁹. Therefore, it is expected that while the extended conformation would introduce significant distortions into the host structure creating an OFF state, these would be relaxed when the peptide-induced conformational transition of calmodulin brings the linkers connecting the domains together, thereby restoring the near native state of the host protein (Fig. 4E,F). We hypothesize that calmodulin is uniquely suitable for its role as a regulatory domain as its extended apo- form and the compact CaM-BP bound form are highly structured and therefore capable of locking the host protein in two different defined states. Indeed our attempt to replace calmodulin with synthetic peptide receptor known as affinity clamp produced OFF switches but not the desired ON switches, indirectly supporting this notion (Fig.S11).

Unlike split or alternative folding frame systems²⁶ the introduced distortions are much less likely to compromise the overall stability of the chimeric protein, significantly expanding the

number and type of proteins this approach can be used for. Remarkably, our results demonstrate that structurally unrelated reporter domains such as EGFP, DHFR, NanoLuc and GDH can be converted into synthetic allosteric switches using calmodulin

insertion by testing just a small number of rationally designed chimeras. We expect that structure and (or) mutagenesis-based optimization of these switches is likely to significantly improve their dynamic range and response kinetics.

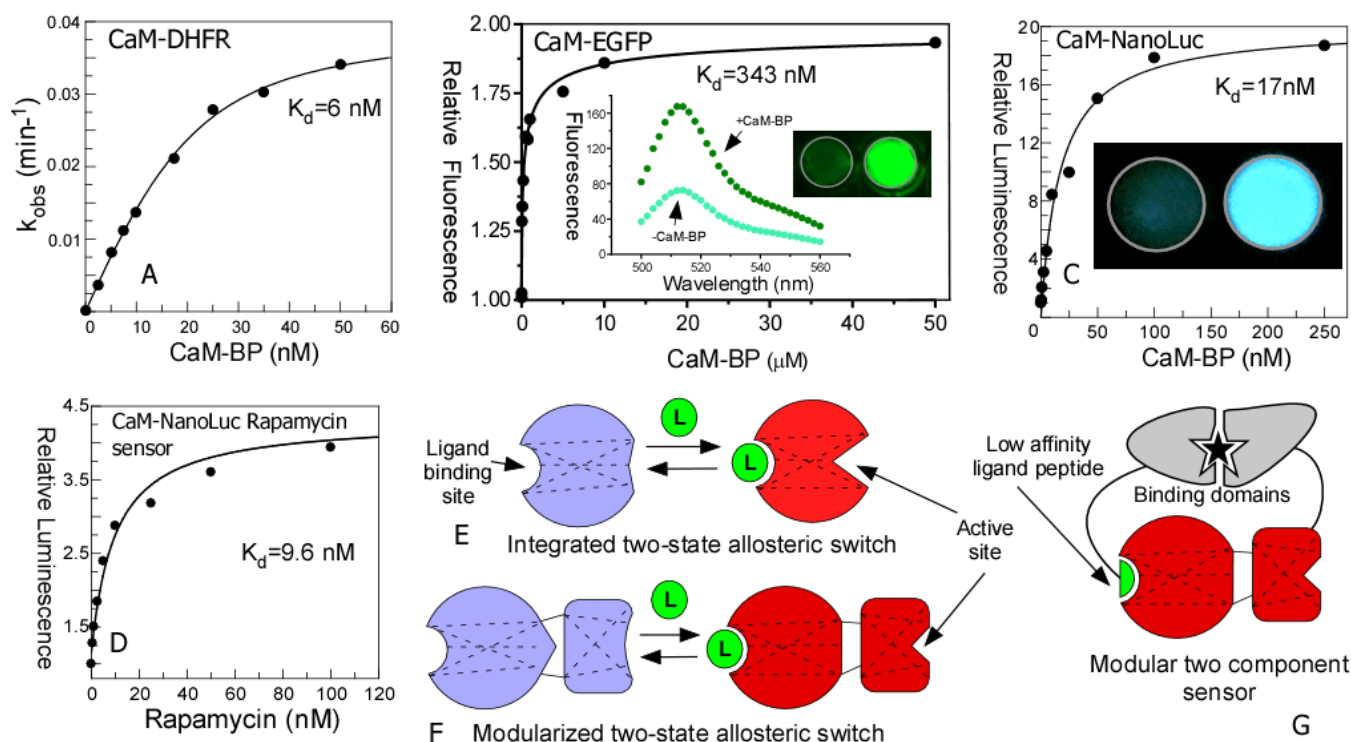


Figure 4. Conversion of constitutively active protein reporters into peptide regulated allosteric modules by calmodulin domain insertion. (A) Activity of 10 nM calmodulin-dehydrofolate reductase (DHFR) chimer as a function of Cam-BP concentration. (B) Titrating 1 μ M of CaM-EGFP chimeric protein with CaM-BP. The $\lambda_{ex/em}$ were 488 and 550nm respectively. The left inset shows the emission scan of CaM-EGFP solution with and without saturating concentrations of CaM-BP. The right inset shows wells of 96 well plate containing CaM-EGFP solution with (right well) and without (left well) saturating concentrations of CaM-BP. (C) Titration of 1nM of CaM-NanoLuc chimeric protein with increasing concentrations of CaM-BP. The inset shows wells of a 96 well plate containing 50 μ L of 1 μ M CaM-NanoLuc and 20 μ M furimazine solution. The right well was supplemented with 5 μ M of CaM-BP. (D) Titration of a mixture of 10nM of FKBP-CaM-NanoLuc and 30nM FRB-mut-CaM-BP with increasing concentration of rapamycin. The luminescence of the resulting mixtures was plotted against rapamycin concentrations and fitted to a quadratic equation leading to a K_d of 9nM. (E) Schematic representation of a single ligand two state allosteric switch. (F) Schematic representation of an allosteric switch based on operably coupled receptor and reporter domains (G) A two component system developed in this study where the system's specificity is "outsourced" to the ligand binding domains while a low affinity allosteric ligand activates a generic allosteric actuator.

One obvious potential pitfall of the presented approach is in the inherent Ca^{2+} dependence of calmodulin where four calcium binding sites with different affinities contribute to complexity of the conformational transitions. Interestingly, this effect was strongly dependent on the insertion site with the later versions showing little effect of Ca^{2+} at concentrations above 1mM indicating that the complex response landscape observed for peptide-induced transitions may also apply to Ca^{2+} induced conformational changes. Moreover, some calmodulin binding peptides are known to induce conformational changes in calmodulin even in the absence of calcium, strongly suggesting that structure-based engineering can resolve the potential limitations of the presented system²⁷. Of a similar concern, is the fact that the developed systems are not orthogonal to the native calmodulin:calmodulin binding peptide systems, however, structural plasticity of calmodulin has been previously explored to create variants with orthogonal peptide specificity^{28;29}. A similar approach can be used to create a potentially unlimited number of orthogonal CaM:CaM-BP pairs that could be used to construct

an array of orthogonal switches and thereon based signaling circuits. Alternatively, analysis of the constructed switches may provide guidance for construction of fully synthetic peptide binding domains with large conformational changes that can replace calmodulin as a regulatory domain.

The synthetic switch modules can be integrated into two component biosensors with tunable selectivity. Here, the analyte drives increase of concentration of low affinity CaM-BP in the vicinity of the CaM-operated switch (Fig.4G). As the system is purely concentration driven, the exact arrangement of the components in space is less important, provided that the linkers connecting the functional elements are sufficiently flexible. This presents a workaround of the central problem of allosteric switch engineering where a sufficiently large conformation change of the receptor domain is required for operation of the reporter domain. The specificity of the biosensor is encoded by the pair of binding domains that are able to bring both subunits of the biosensor into proximity. While we demonstrated

the use of VHH domains and biological targets of macrocyclic compounds any binding domains of sufficient biophysical stability that bind distinct site of the analyte can be used. We demonstrate that such two component biosensors can be used to measure concentrations of xenobiotics and biomarkers in biological fluids, confirming their suitability for their deployment in diagnostic laboratories and for development of the point-of-care applications. The presented architecture delivers a long-awaited tool box for rapid construction of orthogonal protein-based signaling circuits that, with some optimization, can be broadly deployed in vivo and in vitro to construct novel signaling systems of, potentially, unlimited complexity.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Supporting Information: Sequences and expression conditions for biosensors used in this work, assay conditions and data processing methods, supplementary text, figures S1-S11, Table S1,S2 as well as references.

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