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COMMUNICATION

An electrochemical biosensor architecture leveraging protein folding and supporting real-time measurements directly in whole blood

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Abstract: The ability to monitor drug and biomarker concentrations in the body with high frequency and in real time would revolutionize our understanding of biology and our capacity to personalize medicine. The few in-vivo molecular sensors that currently exist, however, all rely on the specific chemical or enzymatic reactivity of their targets and thus are not generalizable. In response, we demonstrate here an electrochemical sensing architecture based on binding-induced protein folding that is (1) independent of the reactivity of its targets, (2) reagentless, real-time, and seconds-resolved, and (3) selective enough to deploy in undiluted bodily fluids. As proof-of-principle we use the SH3 domain from human Fyn kinase to build a sensor that discriminates between the protein's peptide targets and responds rapidly and quantitatively even in when challenged in whole blood. The resulting sensor architecture could drastically expand the chemical space accessible to continuous, real-time biosensors.

The ability to measure the concentration of specific molecules in a complex sample stream continuously and in real-time would be of significant value in both the laboratory and the clinic. Few current molecular sensors, however, support such measurements and those few are limited in scope because they function only due to the specific chemical or enzymatic reactivity of their targets. For example, although it has revolutionized the treatment of diabetes, the continuous glucose monitor relies on the specific enzymatic oxidation of glucose and thus is not generalizable to any other analyte. This limitation leaves us without similarly time-resolved sensors for many clinically important targets, including protein biomarkers indicative of disease and effectively all drugs. In short, there exists a critical need for a sensing architecture capable of monitoring the fluctuating concentrations of specific molecules in complex clinical samples irrespective of their chemical reactivity.

Working towards generalizable, real-time molecular measurements in complex samples we have developed electrochemical, aptamer-based (EAB) sensors[1]. As their recognition element, these employ an aptamer, a short oligonucleotide selected from a random sequence library for its ability to bind a specific target molecule (see e.g., ref 2). To construct an EAB sensor such an aptamer is site-specifically modified with a redox reporter and covalently tethered to the surface of an interrogating electrode. Target binding alters the probability with which the reporter approaches the electrode, thus inducing a change in electron transfer rate that is easily measurable when the sensor is interrogated electrochemically. The resulting seconds-resolved molecular measurements can be performed in complex clinical samples and even in situ in the living body[3,4], providing a convenient, highly-time-resolved window in into physiological status or treatment state. Indeed, using their real-time output it has even proven possible to perform closed loop feedback control over drug deliver, providing unprecedentedly precision in the maintenance of plasma drug levels in the middle of the therapeutic window[4,5].

The promise of EAB sensors notwithstanding, the restricted chemical complexity of nucleic acids nevertheless limits the range of targets they are amenable to detecting. Given this, EAB-type sensors could be expanded to a broader range of analytes if we could replace their target-recognizing aptamers with proteins, which benefit from far richer combinatorial chemistry. Achieving this would open the door, for example, to employing the naturally occurring proteins (many of which bind clinically important analytes), single-chain antibodies, and in vitro selected or de novo designed proteins as recognition elements. Previous progress towards this end, however, had been limited to a small number of studies employing either proteins that naturally undergo a binding-induced conformational change (see e.g., ref 6) or short peptide antigens (see e.g., ref 7). Here, in contrast, we demonstrate the rational re-engineering of a single domain *protein* so as to adapt it into a sensing architecture that is directly analogous to EAB sensors (which instead employ *DNA* as their recognition element) and that, like EAB sensors, supports continuous, real-time molecular measurements in sample matrices as complex as undiluted whole blood.

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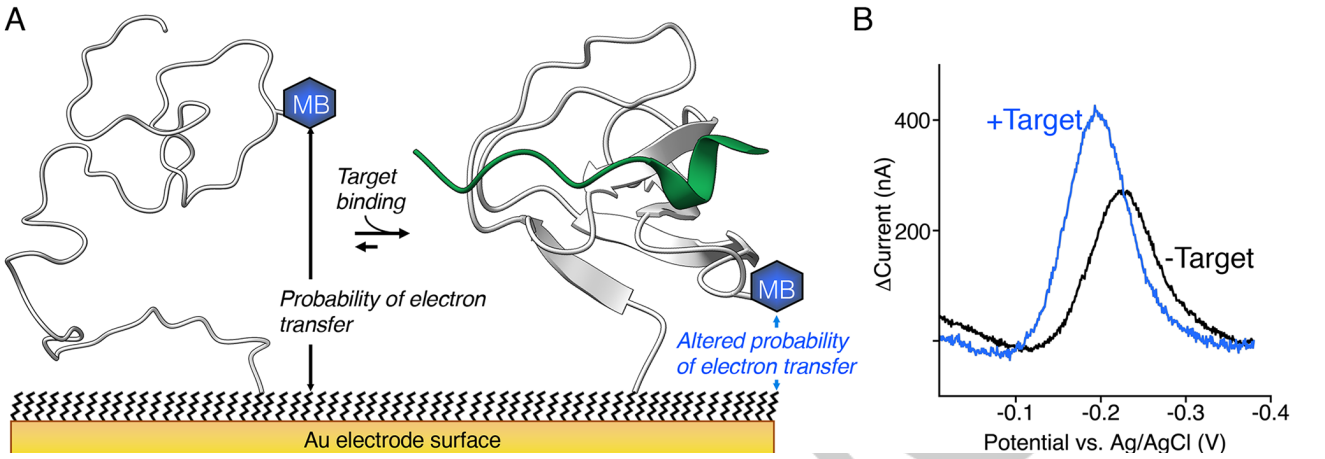

Figure 1. An electrochemical biosensor based on the binding-induced folding of a protein. A. The protein receptor we have employed in our proof-of-principle sensor, a four-residue truncation of FynSH3[8]. Target binding folds the protein, altering the probability (i.e., rate) of electron transfer between an attached redox reporter (MB) and the electrode surface. The figure shows the crystal structure of FynSH3 bound to one of the targets used in this study - the peptide VSL12 (PDB ID 4EIK[9]). B. The binding-mediated shift in the electron transfer rate generates a measurable signal change when monitored using e.g., square-wave voltammetry.

Proteins attached to artificial surfaces have a tendency to irreversibly unfold and adhere[10,11], frustrating our prior efforts to develop protein-based electrochemical biosensors analogous to EAB sensors. Convinced that an improved understanding of how surface confinement affects protein folding physics would help overcome this bottleneck, we have recently explored how and why confinement to chemically well-defined, macroscopic surfaces affects the thermodynamic stability of proteins[12]. This prior work included a demonstration that the SH3 domain from human Fyn kinase (FynSH3) folds reversibly when attached to the surface employed in most EAB sensors. Further building on this work, here we have rationally engineered FynSH3 to function as the receptor for the first protein-folding-based electrochemical biosensor analogous to an EAB sensor.

To adapt FynSH3 into an electrochemical sensor we employed a four-residue carboxy-terminal truncation (FynSH3 CΔ4), leading to destabilization that produces binding-induced folding[8]. We have previously leveraged this binding-induced folding to develop an optical (fluorescent) sensor by adding a BODIPY dye whose fluorescence is enhanced when binding-induced folding sequesters the protein's two tryptophan residues, reducing their ability to quench the dye[8]. Due to the scattering, absorbance and background fluorescence associated with clinical samples, however, this readout fails in undiluted blood serum, much less whole blood. Here we have overcome these limitations by adapting FynSH3 CΔ4 to the electrochemical readout mechanism used in EAB sensors. To do so, we conjugate a methylene blue (MB) redox reporter to the side-chain of the same residue we had labeled with BODIPY in our prior optical sensor, and site-specifically attach this protein via a 7-carbon alkyl-thiol linker on its amino-terminus to a gold electrode surface passivated with a mercaptohexanol self-assembled monolayer (SAM; Figure 1). To ensure that this attachment is site specific, we replaced the protein's two lysine residues with arginine and conjugated the alkyl-thiol linker to the unique remaining primary amine on the amino terminus by NHS-ester chemistry. As we have previously demonstrated, neither of these modifications alter the protein's folding equilibrium constant[12].

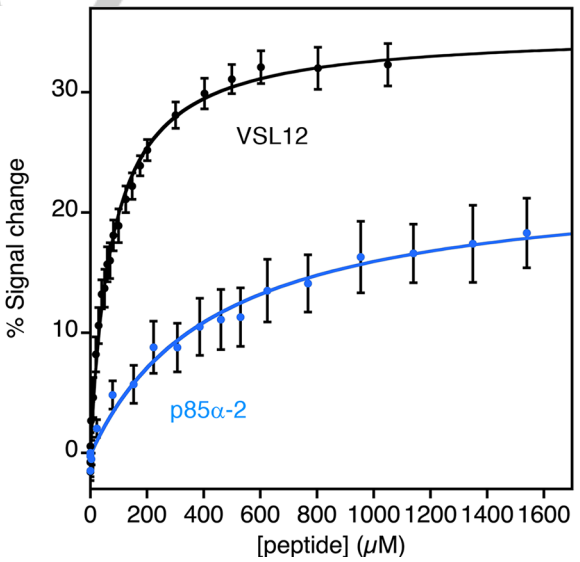

Figure 2. The sensor easily discriminates between known FynSH3 targets differing in affinity. Monitoring binding by square-wave voltammetry, the peptide target VSL12 generates a binding curve with a dissociation constant of 76 ± 4 μM when fitted to a Langmuir isotherm (unless otherwise stated the uncertainties given here and elsewhere in the manuscript represent the standard deviation of six independently fabricated sensors). In contrast, the lower affinity target p85α-2 binds to the sensor with a dissociation constant of 470 ± 50 μM.

The resulting electrochemical sensor responds quantitatively to its target. For example, challenging the sensor with the high-affinity peptide ligand VSL12[13,14] and monitoring the response by square-wave voltammetry we observe the expected hyperbolic binding curve, yielding a signal gain (relative signal change at saturation) of approximately 30% and a dissociation constant of 76 ± 4 μM. We note that the sensor's 2.5 μM limit of detection (defined as 3-sigma above baseline), which is 30-fold below the protein's dissociation constant, is defined by the

naturally evolved binding thermodynamics of the latter and does not reflect any intrinsic limitation of the approach. That is, better limits of detection will come with higher-affinity receptors. To evaluate the sensor's specificity, we challenged it with p85α-2, a peptide known to bind FynSH3 with lower affinity than the peptide employed above[15]. As expected, we find that the 470 ± 50 μM dissociation constant of this peptide is greater than the 76 ± 4 μM dissociation constant seen for the VSL12 peptide (Figure 2). In short, the sensor is as specific as the receptor from which it is fabricated.

Like E-AB sensors, this protein-based sensor is selective enough to operate directly in undiluted whole blood. To achieve this, however, required further engineering of its protein receptor. Specifically, FynSH3 is stabilized at higher ionic strength both in solution[16] and when surface attached[12], causing the variant we employed in our first sensor to fold in whole blood, leaving it unresponsive to the addition of target (Figure 3, black line; see also Figure S1). To counteract this we introduced the destabilizing substitution I50L in the protein's hydrophobic core, shifting the protein's folding equilibrium back towards the unfolded state[12]. As expected, sensors employing this CΔ4 I50L variant respond quantitatively to their target in whole blood. Specifically, the resulting sensor delivers a signal gain of 24.5 ± 1.8 % when challenged with a saturating concentration of the VSL12 target in this complex, clinically relevant, sample matrix, and produces a dissociation constant similar to that seen for the original sensor deployed in buffer (Figure 3).

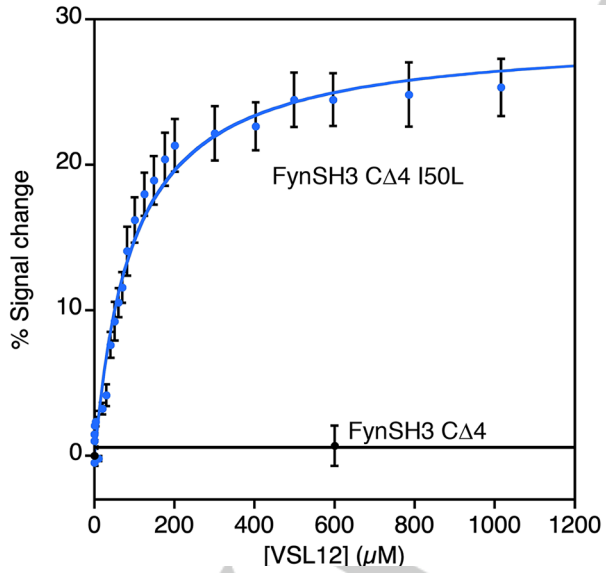

Figure 3. After appropriately tuning the folding equilibrium constant of its receptor (via introduction of a destabilizing core mutation) our sensor responds quantitatively to its target even when challenged directly in whole blood (FynSH3 CΔ4 I50L; blue line). Our original sensor (black line), in contrast, does not respond in this sample matrix, apparently because the higher ionic strength of blood causes its receptor, FynSH3 CΔ4 to fold even in the absence of target (Figure S1).

Because they are reagentless, single-step and rapidly reversible, sensors in the EAB class support high frequency, real-time measurements. To demonstrate this for our protein-based sensor we placed it in whole blood while collecting measurements every 3.5 s (the time required to record the necessary square-wave scans), producing time resolution that is adequate, for example, to resolve pharmacokinetic processes[3]. As expected, upon the addition of 100 μM VSL12 the sensor responds quantitatively and in seconds. Upon subsequent 2x dilution with target-free blood the sensor again responds rapidly and quantitatively to the resulting change in target concentration (Figure 4).

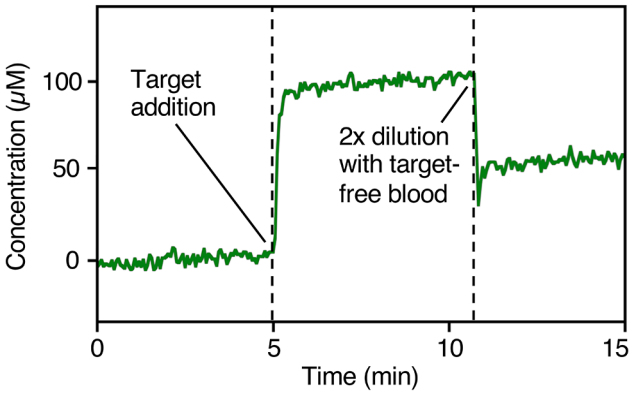

Figure 4. The sensor supports continuous, real-time molecular monitoring with seconds time resolution. Shown are 3.5 s resolved data collected in undiluted whole blood. The figure shows the averaged response from 3 independently fabricated electrodes. The sensor responds rapidly (rate constant ~0.1 s^{-1}), reversibly, and quantitatively to changes in target concentration, demonstrated here by a subsequent two-fold dilution of the sample using whole blood lacking target.

Here we demonstrate the re-engineering of a protein for use in an electrochemical biosensor that, like EAB sensors, leverages binding-induced folding to produce a reversible, reagent-free electrochemical output. The sensor responds rapidly and quantitatively to its target, is capable of specifically discriminating between targets differing in affinity, and can be deployed directly in complex sample matrices typically off-limits to other real-time measurement approaches. It likewise supports continuous, real-time monitoring with time resolution of seconds.

We believe our approach will prove relatively general. Specifically, it should be applicable to all proteins and domains that fold by a two-state mechanism, provided that their folding can be rendered reversible when they are site-specifically attached to an electrode surface. To this end, we believe our recently developed approach for measuring how protein folding thermodynamics are affected by surface-confinement can provide helpful guidance[12]. While no precise estimate exists for the total fraction of the human proteome that fold by a two-state mechanism[17], such proteins are still known to outnumber the number of previously reported aptamer-based biosensors, and we expect advances in in-vitro protein evolution[18] and de novo protein design[19] will further increase the number of receptors amenable to adaptation in sensors of this class.

The design principles described here provide a route by which we can exploit the greater chemical complexity of natural proteins in EAB-type sensors. As the described sensor architecture is analogous to that of DNA-based EAB sensors, we expect the strategies for rendering the latter approach calibration free[20] and drift-corrected[21,22] should likewise be adaptable to sensors in this new class. Our results also provide an example of how quantitative measurements of how a protein's folding

equilibrium responds to its environment can facilitate rational optimization of sensor performance, and that it can be worthwhile to optimize the sensor under physical conditions that mimic, or are identical to, the sample matrix that the sensor ultimately is intended for. Because the pKa of some amino acid side chains are close to physiological pH, protein-based sensors may be more prone to variation with changing pH than DNA-based sensors are. That said, the pH of, for example, blood is under tight physiological regulation and thus we do not expect this to represent a significant problem for in-vivo sensing. Blood is likewise remarkably consistent in terms of ionic strength and ionic composition from day to day and even from person to person. Consistent with this, EAB sensor responses are quite reproducible from one blood sample to the next[20], and we expect protein-based sensors to perform similarly in this regard. Having demonstrated that our terminal truncation approach is compatible with both optical and electrochemical detection, and useable in clinically relevant sampling environments, the next step is to apply this design to generate electrochemical sensors based on both naturally derived and de novo-designed proteins relevant to diagnostic and pharmacodynamic applications.

Acknowledgements

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Keywords: Biosensors • Protein folding • Voltammetry • Protein engineering • Bioelectrochemistry

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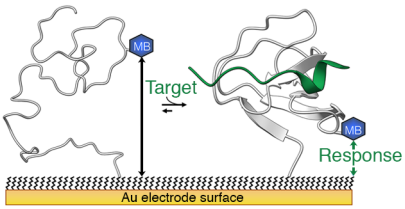

A protein-based biosensor architecture designed to leverage protein folding. The sensor can discriminate between different peptide ligands and responds rapidly and quantitatively to its target even when challenged directly in undiluted whole blood. As this sensor architecture is likely compatible with many proteins, it has the potential to drastically widen the chemical space accessible to reagentless, real-time electrochemical biosensors.

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