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An Allosteric Transcription Factor-DNA Binding Electrochemical Biosensor for Progesterone

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ABSTRACT: We describe an electrochemical strategy to transduce allosteric transcription factor (aTF) binding affinity to sense steroid hormones. Our approach utilizes square wave voltammetry (SWV) to monitor changes in current output as a progesterone (PRG) specific aTF (SRTF1) unbinds from the cognate DNA sequence in the presence of PRG. The sensor detects PRG in artificial urine samples with sufficient sensitivity suitable for clinical applications. Our results highlight the capability of using aTFs as the biorecognition elements to develop electrochemical point-of-care biosensors for detection of small molecule biomarkers and analytes.

Biosensors play a pivotal role in medicine as well as the food, environmental, and agricultural industries, and their potential to positively impact society is increasing as our world becomes more digital.¹⁻³ Analytes for detection widely vary based on application, and our particular interest is in hormones. Progesterone (PRG) is a steroid hormone secreted by the corpus luteum and is central to fertility planning, assisted reproductive technologies, and endocrine disorders. Among women, one million in the US suffer from infertility, approximately 7.5 million suffer from reduced fertility, and about 15 million miscarry, with similar numbers in the EU. In developing countries, the statistics are even higher and the rates of infertility approach 30%, with only half of the people in developed and developing countries seeking infertility care.⁴ As simple as tracking PRG levels during an ovulation cycle increases fertility odds. Furthermore, during pregnancy, PRG balance helps nurture and develop the fetus. Determining ovulation windows is challenging due to fluctuating menstrual cycles and inaccurate interpretations of hormone levels. Currently PRG is measured from serum via blood collection at the clinic/hospital or at home, followed by analysis at a commercial laboratory with results provided in a few days to a week.

Today, at-home urine tests measure pregnanediol glucuronide (PDG), a metabolic product of PRG. However, PDG levels are more variable than PRG levels, and up to 12% of women do not metabolize PRG sufficiently to produce detectable PDG.⁵ A rapid, facile at-home fertility planning and monitoring point-of-care (POC) device will alleviate the apprehensiveness associated with routine screenings and give couples the privacy desired during fertility planning. With a mindset towards this goal and recognizing that conventional antibody⁶⁻¹⁰- or aptamer¹¹⁻¹⁵-based sensors (summarized in Table S1) have not yet provided a solution tailored towards POC approach, we are evaluating transcription factors as the biorecognition elements for sensors. Recently our group success-

fully mined the microbial microverse for a bacterial allosteric transcription factor (aTF), SRTF1, responsive to PRG.¹⁶ Bacteria have evolved over 3 billion years to respond to virtually all classes of stimuli, including chemical analytes of interest and represent an almost limitless database to search for unique aTFs. Like antibodies, aTFs recognize analytes with high sensitivity and specificity. In contrast to antibodies, aTFs respond to analyte binding by changing their affinity for a cognate DNA sequence. This change in DNA binding affinity provides an intrinsic transduction mechanism that we capture, electrochemically, for the development of a novel aTF based PRG biosensor.

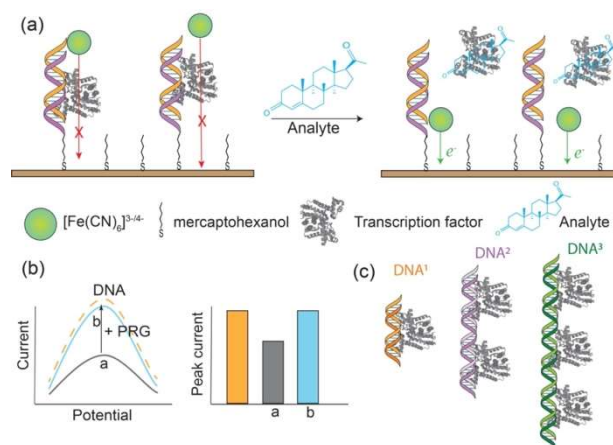


Figure 1. (a) Overview of the electrochemical biosensor: DNA-TF complex on gold electrode blocks mediator access giving low current output. (b) Presence of hormone is detected by a higher current output using square wave voltammetry. (c) DNA with 3 (DNA³), 2 (DNA²), and 1 (DNA¹) binding pockets.

Specifically, our biosensor comprises a gold electrode with a surface immobilized DNA-aTF complex (Figure 1). In the absence of analyte (e.g., progesterone), the overall negative charge of the DNA-aTF complex blocks the negatively charged mediator ($\text{Fe}(\text{CN})_6^{3-/4-}$) approaching the gold electrode and affords a low current output. Addition of the analyte binds the aTF, releasing it from the DNA strand on the surface and decreasing the barrier for the mediator to reach the electrode, thereby resulting in an amplified current output. As binding of analyte to the DNA-aTF complex is a dose-dependent reaction, the difference in the current output before and after addition of analyte correlates to analyte concentration. Herein, we demonstrate the above biosensor concept with a working prototype. We further explore the effect of increasing the number of aTF binding pockets on DNA to improve sensor output.

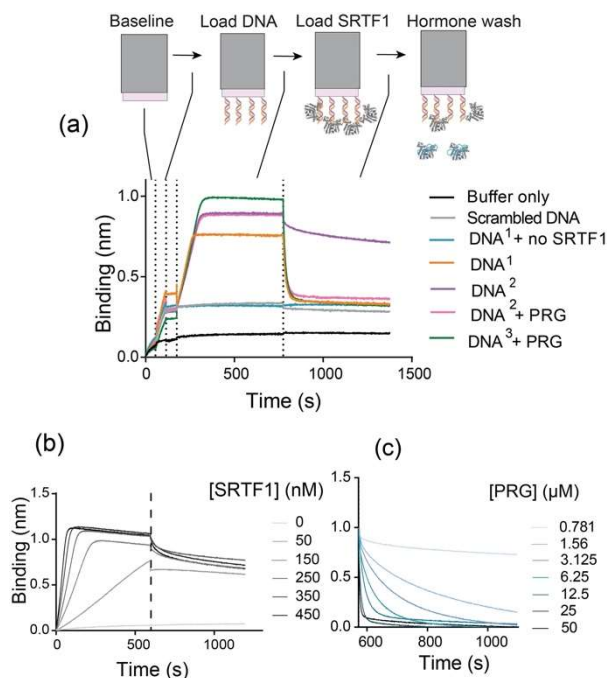


Figure 2. Bio-layer interferometry (a) of DNA¹, DNA², DNA³ (1, 2, 3 binding sites respectively). (b) to calculate K_D , different [SRTF1] were added to DNA² to measure the association constant and thereafter dipped in buffer solution for determination of the dissociation constant. (c) the DNA²-SRTF1 system responds to PRG in a dose-dependent manner. Please see SI page 3 for additional details.

First, we performed bio-layer interferometry (BLI) experiments using synthetic DNAs containing SRTF1 binding site(s), SRTF1, and PRG (Table S2). SRTF1 rapidly binds to the DNA, conjugated to the probe via streptavidin-biotin interactions, and we observe an increase in the BLI binding signal that corresponds to an increase in the thickness from the sensing tip. (Figure 2a). The DNA strand possessing two or three SRTF1 binding sites (DNA² and DNA³, respectively) affords a larger BLI signal in the presence of SRTF1, consistent with greater binding signal with no significant change in the binding constant ($K_D \sim 4$ nM) between DNA and SRTF1 (Figure 2b, S1; Table S3). The scrambled DNA, lacking a binding site for SRTF1, shows no change in BLI signal and highlights the importance of aTF recognition and specificity. In the presence of PRG, SRTF1 rapidly dissociates from the DNA-SRTF1 complex in comparison to the buffer control where no significant change in BLI signal occurs. Varying the concentration of PRG yields a dose-

dependent BLI signal (Figure 2c, S1, and S2). Together these data confirm: i) SRTF1 binds to the cognate DNA sequence in the absence of PRG; ii) SRTF1 allosterically unbinds from the DNA in presence of PRG; and, iii) adding an additional SRTF1 binding site to the DNA increases the amount of aTF bound. This is crucial for the electrochemical sensor design as an increase in amount of aTF will result in higher impedance changes.

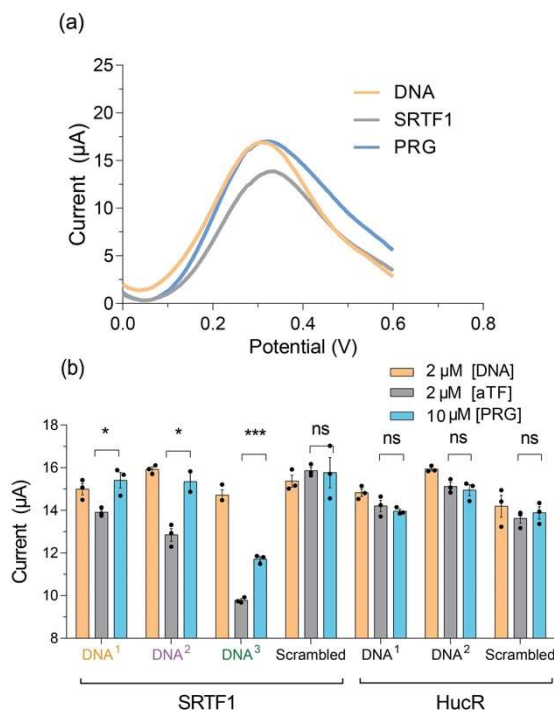


Figure 3. A typical square wave voltammogram (a). Current drops on SRTF1 addition and increases on PRG addition. Peak current of square wave voltammogram plotted (b) for DNA¹, DNA², DNA³, and scrambled DNA with addition of aTF and PRG. The error bars represent the standard deviation for $N = 3$ biological replicates, and statistical significance established according to a t-test. (* $p < 0.05$, *** $p < 0.001$)

The electrochemical biosensor utilizes ferrocyanide/ferricyanide as the mobile electrochemically active mediator, and, thereby, avoids the requirement to covalently conjugate a redox probe to the protein or DNA. The biosensor consists of three electrodes: gold working (diameter 1.6 mm) and counter electrodes along with a Ag/AgCl reference electrode. Following the electrode cleaning by cyclic voltammetry in 0.5 M H_2SO_4 (Figure S3), we immobilized the 5'-thiol modified DNA and 1 mM mercapto-6-hexanol, to prevent non-specific interactions, on the working electrode following published procedures.^{17,18} The DNA surface coverage is $\approx 3 \times 10^{-11}$ mol/cm² (Figure S4 and S5), as determined by cyclic voltammetry measurements with tris-ruthenium hexamine, and the value is in agreement with previous reports.⁵ Electrochemical and AFM studies support the DNA immobilization and SRTF1 binding. Upon immobilization of the DNA and protein to the electrode, the resistance increases as does the height from the electrode surface from ~ 700 pm to 8 nm (Figure S6, S7, and S8). DNA derivatized electrodes are widely used in biosensors with elegant strategies employing DNA-mediated charge transport,^{19,20} conformational changes²¹⁻²⁴ during binding events, and electrocatalysis events by covalent and non-covalent association of redox mediators^{18,25}. These strategies are used to detect target molecules²⁶⁻²⁸, hybridization events^{29,30},

single base mismatches³¹ as well as for catalytic oxidation of guanine.³²

Following the DNA immobilization, we measured the current by square wave voltammetry (0-0.6 V, step size: 4 mV, amplitude: 50 mV, frequency: 50 Hz) in the absence and presence of aTF. The current is greatest for the DNA modified sensor and decreases upon addition and formation of the DNA-SRTF1 complex (Figure 3b). Upon addition of PRG, the current increases and returns to its DNA baseline. Use of the scrambled/non-specific DNA results in no change in the current in the presence of SRTF1 or PRG (Figure 3b). Similarly, use of DNA with a non-specific aTF (HucR, a uric acid transcription factor), which does not recognize the DNA binding site, affords no change in the current in the presence of this aTF or PRG. Further, in the absence of SRTF1, no significant change in current is observed on addition of PRG to DNA functionalized electrodes (Figure S9). Increasing the number of SRTF1 binding sites on the DNA from one to two and three increases the magnitude of the current change upon addition of SRTF1. The biosensors with DNA² and DNA³ exhibit a 20% and 35% decrease in current upon aTF complexation, respectively. This result is consistent with greater protein surface coverage impeding ferro/ferricyanide mobility to the electrode surface for electron transfer, and a lower background signal. However, only in the DNA² system, does the current return to a baseline level in the presence of PRG. We suspect that the larger DNA³-SRTF1 complex on the surface inhibits PRG binding, and thus this reaction does not readily go to completion.

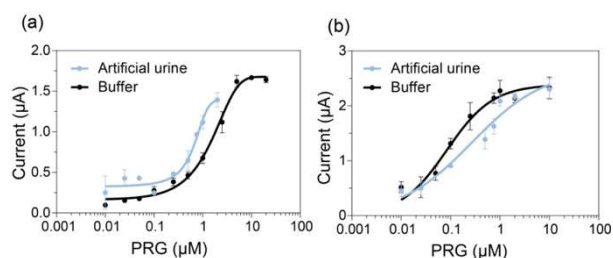


Figure 4. Calibration curve of the electrochemical sensor system. DNA¹ (a) and DNA² (b). Difference in current output before and after addition of PRG is plotted against concentrations of PRG in artificial urine and buffer. The error bars represent the standard deviation for N = 3 biological replicates.

Next, we evaluated the analytical response of the system towards PRG by quantifying the current changes of the biosensor system in the presence of varying concentrations of PRG (0-10 μM) in Tris buffered saline, pH 7.4 and purchased artificial urine spiked with 2 μM BSA to better mimic the physiological conditions. We plotted the peak currents before and after addition of PRG. The difference in peak currents positively correlates with the PRG concentration. The limit of detection for the DNA¹-SRTF1 biosensor in buffer is 47 nM. Whereas in artificial urine, the LOD increases to 56 nM (Figure 4a; Table S4). There is no significant effect to the addition of BSA and PRG in absence of SRTF1 to DNA functionalized electrode (Figure S9 and S10). A bottleneck for achieving lower limit of detection of sensor is the relatively small change in current signal from the baseline observed at lower PRG concentration. We addressed this challenge using the DNA²-aTF biosensor, which possesses two aTF binding sites per DNA – i.e., increased number of binding sites for the same electrode surface area. At a low concentration of PRG, more SRTF1 unbinds from the DNA² than DNA¹, affording a greater current change from the baseline and lowering the LOD. The LOD for the DNA²-SRTF1 decreases to 17 nM in buffer and to 29 nM in artificial urine (Figure 4b; Table S4). From

a woman's health perspective, the current PRG detection range is suitable for monitoring all trimesters of fetus development which span a urine PRG concentration from 30 nM to 1 μM (Table S5). These results are comparable to our previously developed FRET based sensor that had an LOD of 15 nM in artificial urine (Table S1).¹⁶ Efforts are ongoing to further advance the design and components as well as the type of electrochemical measurement to lower the LOD so as to identify optimal times for conception based on a PRG measurement (Table S5).

Biologically, transcription factors control gene expression and are a mainstay of many synthetic biology experiments performed in whole cell (WCB) and cell-free platforms.³³⁻³⁵ Transformation of these strategies to biosensing of small analytes is of significant interest.³⁶⁻³⁸ Successes in WCB include detection of environmental pollutants^{37,39-41} and diagnostic biomarkers^{33,42} via regulation of a reporter gene followed by subsequent expression of, for example, green fluorescent protein (GFP).^{37,43,44} WCBs offer signal amplification through gene expression, high selectivity, and high reproducibility. A recent study describes a PRG WCB based on transcription of GFP due to a slight conformational change of a non-natural TF upon PRG binding.⁴⁵ However, challenges include maintenance of cells, costs, biocontainment and regulatory concerns, as well as difficulty in detection of analytes that are toxic to cells. Whereas, cell-free systems hold promise of safety, high sensitivity, and fast response time with examples of detecting virus, nucleic acids, antibiotics and biomarkers.^{38,46-48} The majority of the cell-free systems are based on protein synthesis using crude or purified components with optical output signals based on fluorescence, colorimetric, and bioluminescence reporters.^{33,49} On the electrochemical front, Barton et al.⁵⁰ describe a transcriptional activator TATA binding protein (TBP) that binds and kinks a redox molecule labeled DNA, which significantly attenuates charge transport through DNA. Recently, Mousavi et al., report an electrochemical sensor to detect antibiotic genes by using a cell-free expression of restriction enzymes mediated by transcription factor-analyte binding. The restriction enzyme cleaves the reporter genes that binds to capture genes on the electrode producing a detectable electrochemical signal.⁵¹ To date, the work on TFs based electrochemical sensors includes the capture of binding events between DNA and aTFs to detect transcription factors (example, NF- κ B)^{50,52-59} or interaction between analyte and transcription factor that result in conformational changes. Finally, Sode et al., report a glutamine binding protein tagged with a redox probe that undergoes conformational changes in the presence of L-glutamine altering the electrochemical current output.⁶⁰

In summary, we report an electrochemical transcription factor-based biosensor for the detection of small molecule analytes. This aTF-DNA sensor joins the family of electrochemical-based biosensors, which are being increasingly investigated for health monitoring.⁶¹⁻⁶⁷ The advantages of such biosensors, generally, include rapid test-to-results, high selectivity/sensitivity, and low-cost of detection. Our sensor demonstrates clinically relevant sensitivity required for adaptation into a prototype POC biosensor. The key findings of this work are: 1) translation of transcription factor binding affinities (to target DNA and analyte) into an electrical readout; 2) increasing the number of aTF binding sites on DNA improves sensitivity; and, 3) detection of physiological levels of PRG in buffer and artificial urine samples. Our results hold promise for the development of a new suite of electrochemical biosensors based on natural or engineered bacterial aTFs.

ASSOCIATED CONTENT

Supporting Information. is available free of charge on the ACS Publications website at DOI:

Materials and methods, DNA sequences, expression and assay conditions, electrode characterization (AFM, impedance spectroscopy), and binding kinetics.

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Author Contributions

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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CONFLICT OF INTEREST

KS, RB, CG, CMK, JEG, and MWG are inventors on a patent describing this technology filed by Boston University, which is available for license.

ABBREVIATIONS

aTF, allosteric transcription factor; POC, point-of-care; PRG, progesterone; BLL, bio-layer interferometry; WCB, whole cell biosensor; PDG, pregnanediol glucuronide; LOD, limit of detection; SWV, square wave voltammetry.

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