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Sub-second-resolved Molecular Measurements Using Electrochemical Phase Interrogation of Aptamer-Based Sensors

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ABSTRACT

Recent years have seen the development of a number of biosensor architectures that rely on target binding-induced changes in the rate of electron transfer from an electrode-bound receptor. Most often, the interrogation of these sensors has relied on voltammetric methods, such as square-wave voltammetry, which limit their time resolution to a few seconds. Here, we describe the use of an impedance-based approach, which we have termed electrochemical phase interrogation, as a means of interrogating sensors in this class with high time resolution. Specifically, using changes in the electrochemical phase to monitor target binding in electrochemical-aptamer based (EAB) sensor, we achieve sub-second temporal resolution and multi-hour stability in measurements performed directly in undiluted whole blood. Electrochemical phase interrogation also offers improved insights into EAB sensors' signaling mechanism. By modeling the interfacial resistance and capacitance using equivalent circuits, we find that the only parameter that is altered by target binding is the charge transfer resistance. This confirms previous claims that binding-induced changes in electron transfer kinetics drive signaling in this class of sensors. Considering that a wide range of electrochemical biosensor architectures rely on this signaling mechanism, we believe that electrochemical phase interrogation may prove generalizable towards sub-second measurements of molecular targets.

KEYWORDS. Electrochemical aptamer-based sensor, electrochemical biosensors, Electrochemical phase interrogation, Electrochemical impedance spectroscopy.

INTRODUCTION

Biological sensors that rely on changes in electron transfer kinetics have emerged as a promising means of monitoring specific molecular targets in complex clinical sample matrices. An illustrative example of sensors of this class are electrochemical aptamer-based (EAB) sensors, which are comprised of a target-specific aptamer covalently modified with a redox reporter and bound to an electrode surface[1,2]. A target binding-induced conformational change in the aptamer alters the reporter's electron transfer kinetics in a manner monotonically related to target concentration[3] without requiring reagent additions, washing steps, or target labeling. EAB sensors thus support continuous, real-time measurements[4] of drugs of abuse[2,5], pharmaceuticals[6,4,7], and proteins biomarkers[8,9,10] directly in complex matrices, such as whole blood.

Prior studies of sensors that employ binding-induced changes in electron transfer rate have almost exclusively used voltammetric approaches, such as square wave voltammetry, to monitor the sensor's response to target. In square wave voltammetry, a ramping staircase square wave potential scans across the redox reporter's potential window, where changes in electron transfer lead to corresponding changes in the peak height of the voltammogram[1]. Because its waveform reduces the contribution of the electrical double layer to the current, this approach provides superior measurement sensitivity[11]. Due to the time required to perform a sufficiently well-resolved square-wave scan at the relevant square-wave frequencies, however, the time resolution of this approach is often limited to several seconds. The technique is thus too slow to resolve biological processes that occur on sub-second time scales, such as individual neurotransmission events.

In contrast to voltammetric approaches, electrochemical impedance measurements can achieve sub-second interrogation times. This is because the approach directly measures changes

in interfacial surface properties (i.e., charge transfer and electric double layer formation) rapidly via an applied sinusoidally oscillating (alternating current; AC) potential pulse. Changes in interfacial surface properties have been either measured: 1) "faradaically" using a free solution redox reporter (typically $Fe(CN)_6^{3-/4-}$) that changes its charge transfer ability due to steric or electrostatic blocking of a functionalized electrode[12], or 2) "non-faradaically" by detecting target binding via changes in the electrode's electrical double layer[12]. Despite the popularity of these approaches in the scientific literature, their target quantification fails in realistically complex sample matrices (i.e., undiluted serum or blood samples), presumably because the signal change generated by fouling mimics the change produced by target binding[12,13].

Here, we demonstrate a method of impedimetrically interrogating EAB sensors (**Figure 1A**) that is remarkably insensitive to non-specific adsorption, allowing for real-time, sub-second-resolved, measurements for long durations directly in undiluted whole blood. To achieve this, our approach monitors the phase shift of the current response produced by a redox (faradaic) reporter that is covalently attached to the receptor rather than free in solution. A binding-induced change in the conformation of the receptor alters the rate of electron transfer from the reporter, producing an easily measurable change in phase shift that appears unaffected by non-specific adsorption.

MATERIALS AND METHODS

Aptamer preparation

We first thaw a 2 μL, aliquot of 100 μM aptamer (sequences below) pre-modified with a 6-carbon thiol linker on the 5' end and methylene blue on the 3' end (Biosearch Technologies, Novato, CA, dual HPLC purification, stored at -20°C).

Aminoglycoside-binding aptamer:

5'-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-GGGACTTGGTTTAGGTAATGAGTCCC-O-CH_2-$CHCH_2OH$-$(CH_2)_4$-NH-CO-$(CH_2)_2$-Methylene Blue-3'

Cocaine-binding aptamer:

5'-HO-$(CH_2)_6$-S-S-$(CH_2)_6$-AAGGAAAATCCTTCAACGAAGTGGGTC-O-CH_2-$CHCH_2OH$-$(CH_2)_4$-NH-CO-$(CH_2)_2$ -Methylene Blue-3'

Prior to conjugation, we reduce the disulfide bond by combining 2 μL of 10 mM Tris (2-carboxyethyl) phosphine (TCEP, Sigma-Aldrich, St. Louis, MO) with the aptamer sample for a one-hour thiol reduction in dark conditions at room temperature. We then dilute the sample with 96 μL 1X phosphate buffered saline and quantify the concentration of the aptamer using the molar absorption coefficient at 260 nm provided by the supplier with UV-vis spectroscopy (DU800, Beckman Coulter, Brea, CA). Using 1X phosphate buffered saline, we further dilute the aptamer sample to 500 nM.

Sensor Fabrication

We fabricate each sensor by cutting a 5 cm long, 200 μm diameter bare gold wire (Alfa Aesar, Haverhill, MA) and soldering it to a gold-plated pin connector (CH Instruments, Inc., Austin, TX) with 60/40 lead-selenium solder (Digikey, Thief River Falls, MN) without any polishing done. To isolate the working electrode surface, we insulate the bare gold wire with two layers of polytetrafluorethylene (PTFE) heat-shrink tubing (HS Sub-Lite-Wall PTFE, ZEUS, Orangeburg, SC) using an industrial hot air blower (Master Appliance Corp., Racine, WI), leaving

approximately 3.5 mm of bare gold surface exposed. We then trim the exposed working electrode surface to 3 mm.

Next, we cleaned the electrode surface to prepare it for modification with the aptamer. For all cleaning procedures and experiments, our electrochemical cell consists of an PTFE-wrapped gold sensor working electrode, a platinum counter electrode (CH Instruments, Inc, Austin, TX), and an Ag|AgCl reference electrode (CH Instruments, Inc., Austin TX). We secure our working, counter, and reference electrodes in a "shot glass" cell vessel with a custom-fabricated Teflon lid fixture. First, we electrochemically clean the electrodes in 0.5 M NaOH (Sigma-Aldrich, St. Louis, MO) by performing repeated cyclic voltammetry scans between -1 to -1.6 V vs. Ag|AgCl at 1 V s^{-1} scan rate for 300 cycles using a CH Multipotentiostat (CHI1040C, CH Instruments, Inc.) to remove any contaminating organics (**Table S1** for complete parameters), followed by a thorough rinsing in deionized water. Then, we increase the surface area of the gold wire electrode by electrochemically roughening in 0.5 M H_2SO_4 (Sigma-Aldrich, St. Louis, MO) using a previously-described procedure[14] that involves repeatedly stepping the potential between 0 and 2.2 V (see **Table S1** for parameter details). This results in a 2 to 5-fold increase in microscopic surface area, attributed to islands of nano-scale dendrites forming on the planar gold surface[14]. Immediately after roughening, we rinse the electrodes thoroughly in deionized water and place them in the prepared aptamer solution for 1 h at room temperature in dark conditions. To passivate the surface, we rinse the electrode with deionized water and immerse them for 10 to 12 h at 4˚C in a 10 mM 6-mercapto-1-hexanol (Sigma-Aldrich, St. Louis, MO) solution suspended in 1X phosphate buffered saline (PBS). Following a final rinse with deionized water, the sensor is ready for use.

Data Collection

We perform electrochemical impedance spectroscopy characterization experiments on a Gamry Reference 600+ potentiostat/galvanostat (Gamry Instruments, Warminster, PA). First, we characterize the modified electrodes at room temperature in 1X PBS. To determine the formal potential, E_0, of the redox reporter, we collect a cyclic voltammogram, sweeping from -0.1 V to -0.45 V at 100 mV s^{-1}, and identify the midpoint between the oxidation and reduction waves of the voltammogram. We then collect a potentiostatic impedimetric measurement by applying an AC potential waveform of 10 mV amplitude centered around the formal potential, measuring the impedance response from 10 kHz to 0.1 Hz. To measure the response to target addition, we collect phase between 10 kHz to 0.1 Hz after adding graduated amounts of stock concentrations of tobramycin sulfate (USP Grade, Gold BioTechnology Inc., St Louis, MO) in 1X PBS. To evaluate the sensor's performance in complex matrices, we also repeated this binding curve experiment in undiluted, heparinized whole bovine blood (Hemostat Laboratories, Dixon, CA).

RESULTS

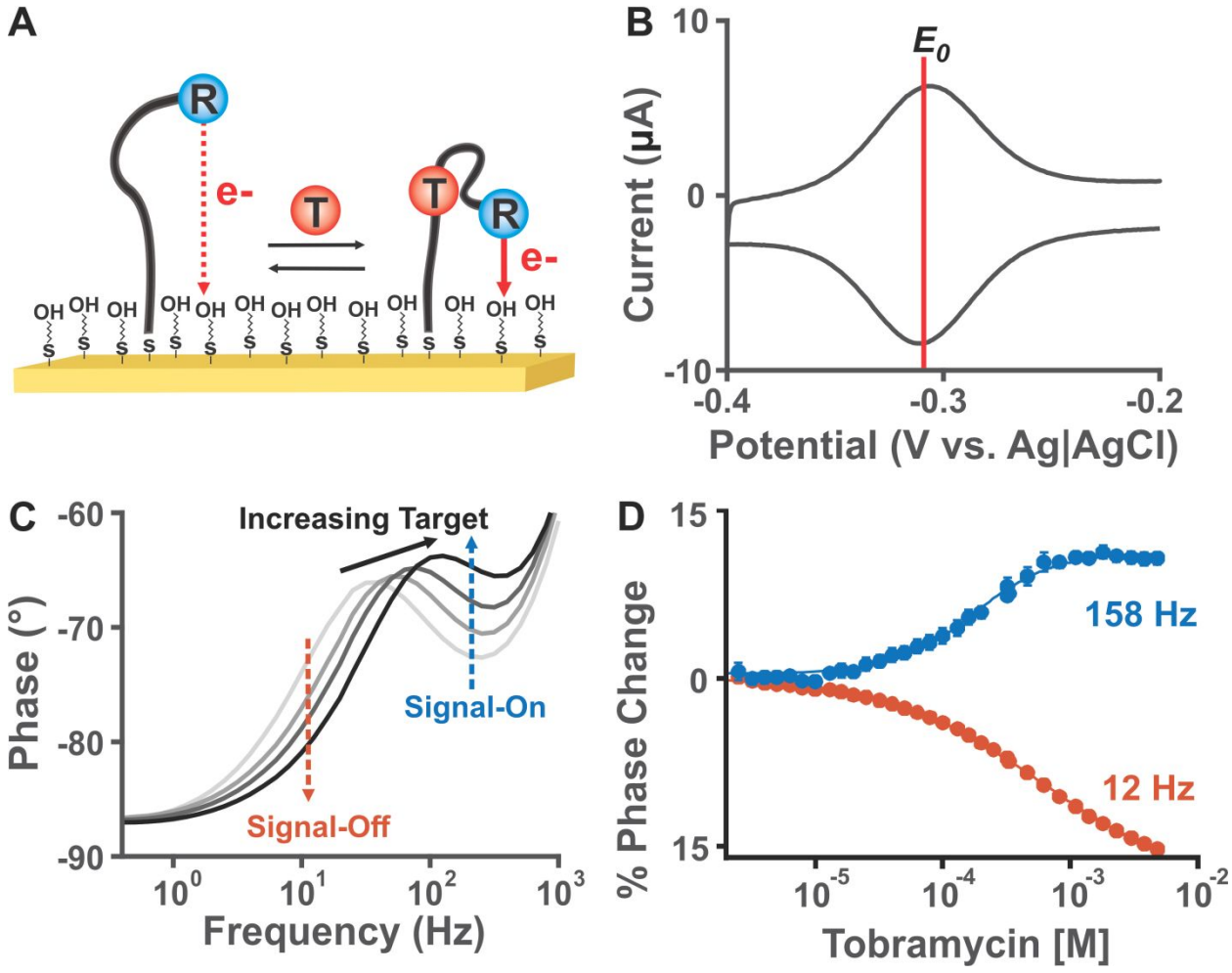

Figure 1. (**A**) EAB sensors consist of a gold electrode coated with a passivating self-assembled monolayer (here 6-mercapto-1-hexanol) and redox reporter-modified (R) aptamers. Signaling in this class of sensors occurs via a binding-induced change in electron transfer kinetics of the redox reporter. In this work, we have employed an aptamer that binds to the aminoglycoside antibiotic, tobramycin. (**B**) Interrogating the EAB sensor using cyclic voltammetry produces the reversible, surface-bound electrochemical reaction expected from the redox reporter, with peaks at the standard reduction potential of methylene blue (E_0). (**C**) We measure changes in electron transfer kinetics by acquiring a phase spectroscopy scan when polarizing the EAB sensor at E_0 and exposing it to increasing amounts of tobramycin in undiluted whole blood. We observe a phase shift towards higher frequencies as the rate of electron transfer from the redox reporter increases upon target binding. This leads to a decrease in phase shift at low frequencies ("signal-off" behavior) and an increase in phase shift at higher frequencies ("signal-on" behavior). (**D**) Correspondingly, when performing alternating electrochemical phase measurements at 12 and 158 Hz (orange and blue traces) in whole blood, we observe binding curves that reach phase changes of up to 15% at saturating target. The error bars in panel D (which are smaller than the marker size) indicate standard error derived from 4 independently fabricated and interrogated sensors.

Electrochemical phase interrogation of EAB sensors reports on target concentration. To demonstrate this, we employed an EAB sensor that detects aminoglycoside antibiotics, such as tobramycin[15]. To measure the full frequency dependence of the sensor's phase response, we applied sinusoidally-oscillating potential wave forms of varying frequencies centered around the formal reduction potential, E_0, (**Figure 1B**) of the methylene blue redox reporter and measured the resulting shift in phase of the current response. In the absence of target, we observe a phase peak at ~25 Hz (**Figure 1C**, lightest grey curve). The addition of tobramycin causes this peak to shift to higher frequencies (**Figure 1C**, progressively darker curves) where the measured phase increases toward 0° at higher frequencies ("signal-on") and decreases toward -90° at lower frequencies ("signal-off"). Performing full titrations at both signal-on and off AC frequencies (12 and 158 Hz, respectively), we observe binding curves with dissociation constants of ~380 μM and up to 15% signal change in phase at saturating target (**Figure 1D**). Given the remarkably low noise level of this approach, even this relatively small total gain is sufficient to support precise and sensitive measurements. For example, the standard deviation of the signal observed at 0 M target is just 0.17% (for the signal-off frequency), which corresponds to a precision of ± 4 μM at low target concentrations.

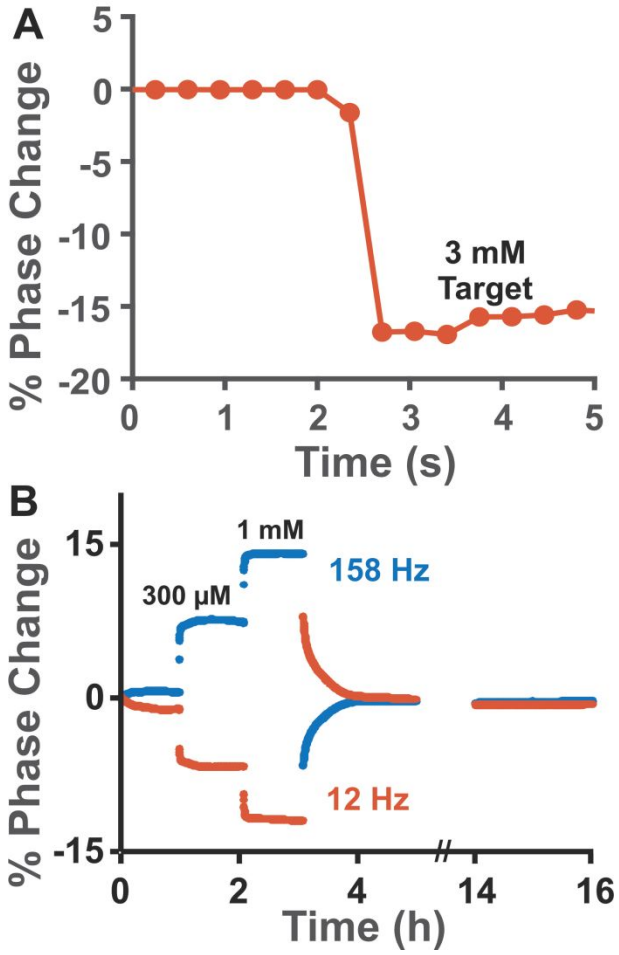

Figure 2. Electrochemical phase interrogation of EAB sensors achieve few hundred milliseconds time resolution and remain stable in whole blood for many hours. (**A**) To illustrate sub-second time resolution, we continuously interrogate the EAB sensor at a signal-off frequency of 12 Hz and spike the phosphate buffer saline solution with saturating amounts of tobramycin (3 mM). (**B**) Electrochemical phase spectroscopy enables measurements in undiluted whole blood for multi-hour time periods. To demonstrate this, here we immersed EAB sensors in undiluted whole blood over 16 h, and sequentially interrogated them at the AC frequencies indicated. As shown, we added 300 µM, then 1 mM tobramycin to the blood before then returning the sensor to target-free blood.

Electrochemical phase interrogation supports sub-second-resolved, real-time read-out of EAB measurements. To demonstrate this, we interrogated the EAB sensor using a 12 Hz AC pulse during the addition of 3 mM tobramycin. The read-out timescale of the resulting measurements, which is defined here by the data acquisition software rather than the physics of the system, is 350 ms (**Figure 2A**). This already represents up to two orders of magnitude improvement in measurement frequency over EAB sensors interrogated using square-wave voltammetry, but could

be improved further with a faster processing electronics than what we have employed. For example, if a pulse of a single period is collected, the time resolution achievable using a pulse of frequency 158 Hz should approach 6 ms. In addition to read-out timescale, several factors can limit time resolution. These include mixing time to achieve sample homogeneity and diffusion of target to the sensor surface. Thus, while in PBS the sensor's signal rapidly stabilizes upon a change in target concentration (**Figure 2A**), it requires more time in whole blood (**Figure 2B**), which is far more viscous. When whole blood is rapidly homogenized, in contrast, our sensor's signal stabilizes faster than shown in **Figure 2B** (see **Figure S3**).

In contrast to traditional impedimetric approaches to biosensor interrogation, electrochemical phase interrogation of EAB sensors produces stable baseline signals even in highly complex sample matrices. For example, we see only 0.5 to 1% baseline drift over the course of 16 h of continuous measurements directly in undiluted whole blood (**Figure 2B**). We presume that this derives from the EAB signaling mechanism, which relies on binding-induced change in the rate of electron transfer from the surface-bound redox reporter rather than adsorption of the target to a surface, thus rendering it relatively unaffected by non-specific adsorption.

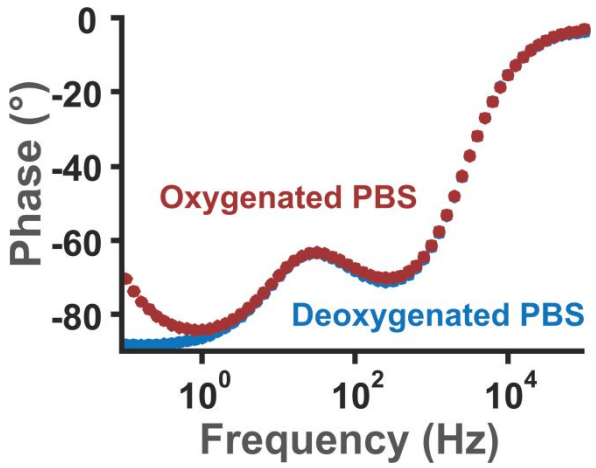

Figure 3. Electrochemical phase interrogation allows deconvolution of the contribution of other interfering electrochemical processes taking place at the EAB sensor interface, such as oxygen reduction. We show this by recording an electrochemical phase spectroscopy scan when our phosphate buffered solution is oxygenated (red trace) and when deoxygenated by bubbling with argon gas (blue trace). In doing so, we find that low frequencies (< 1 Hz) are sensitive to the presence of oxygen and lead to an increase in phase.

Compared to voltammetric approaches, electrochemical phase interrogation minimizes the contribution of interfering surface electrochemical processes because it directly monitors the redox reporter's faradaic charge transfer. This includes oxygen reduction, which, upon degradation of the sensor's passivating monolayer[16], can overlap with the reduction potential of methylene blue[17], reducing measurement precision. Because transfer from the reporter is much more rapid than transfer from oxygen reduction, electrochemical phase interrogation cleanly separates the two processes (**Figure 3**). Thus, interrogation of our EAB sensor at frequencies insensitive to the oxygen reduction (>1 Hz) can minimize any drift arising from monolayer degradation and subsequent oxygen reduction.

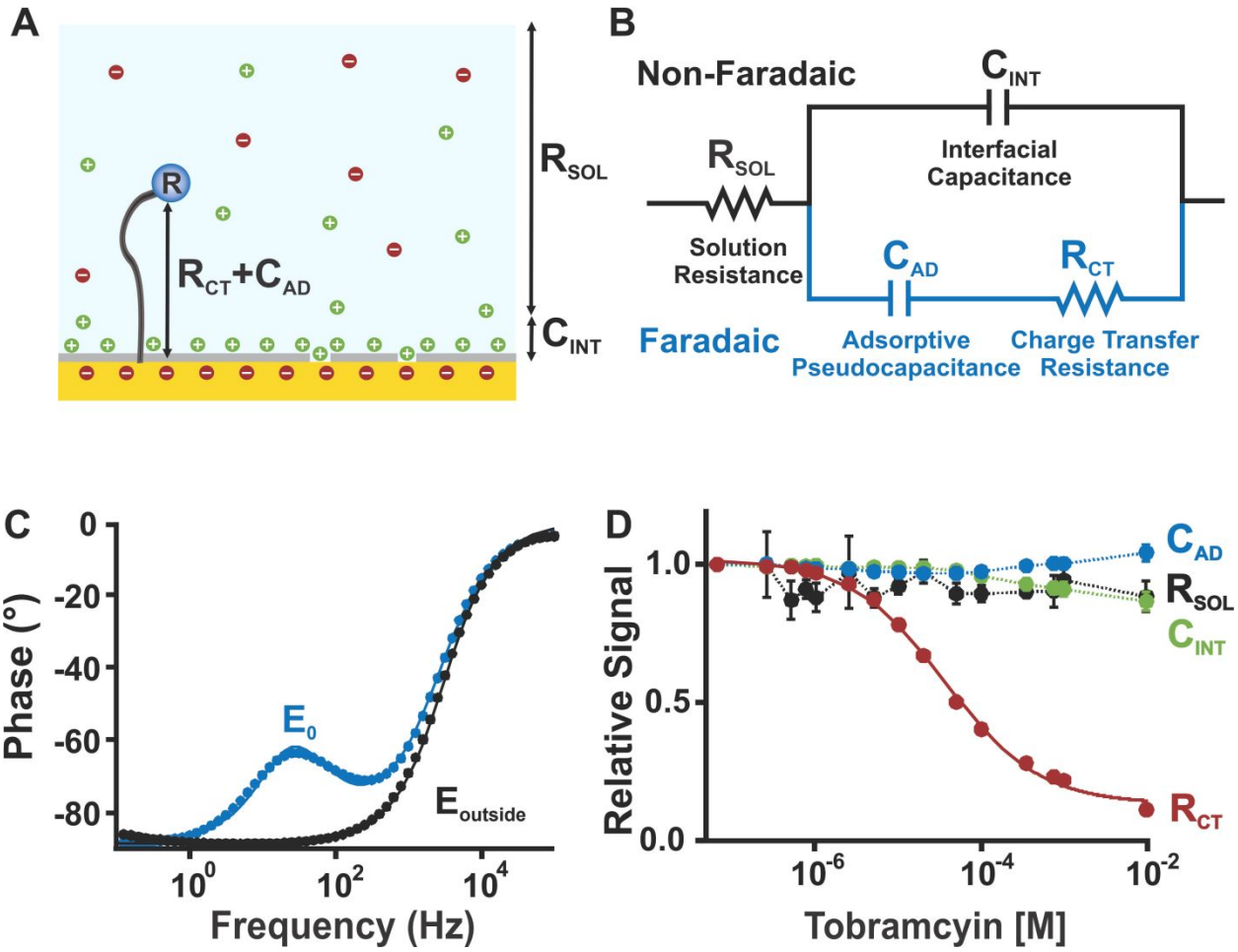

Figure 4. To better understand the signaling mechanism of EAB sensors, we model the electrochemical phase spectroscopy response as a combination of resistor and capacitor circuit elements. (**A**) We represent EAB sensors as a solution-electrode interface separated by a self-assembled alkanethiol dielectric (grey) with defects present. The electrolyte solution separating the working from the counter electrode contributes to a resistance, R_{SOL}. The solution-electrode interface equilibrates via the formation of an electrical double layer that we represent as a non-ideal capacitor (i.e., constant phase element), C_{INT}, to capture the defects within the self-assembled monolayer. Finally, we represent the redox reporter charge transfer with a combination of a charge transfer resistance, R_{CT}, and an adsorptive pseudocapacitance, C_{AD}, which accounts for the presence of a limited number of redox reporters on the electrode surface able to transfer electrons at a given rate[18]. (**B**) When no faradaic charge transfer occurs, our equivalent circuit simply contains the solution resistance, R_{SOL}, in series with the interfacial capacitance, C_{INT} (both in black). When faradaic charge transfer does occur, we add to our equivalent circuit the charge transfer resistance, R_{CT}, and pseudocapacitance, C_{AD} (both in blue), in parallel with the interfacial capacitance. (**C**) We model the EAB sensor interface's impedimetric response when polarized at $E_{outside}$ (no redox reaction taking place, black) with the RC circuit of R_{SOL} and the interfacial capacitance, C_{INT}. We then model the EAB sensor interface when polarized at E_0 (where charge transfer occurs, blue) with the complete equivalent circuit. We find that both our equivalent circuits fittings (solid lines) match well with our experimental results (dotted lines) ($\chi^2 = 0.033$ for $E_{outside}$, $\chi^2 = 0.021$ for E_0, fitting results in **Table S2**, **S3**). Of note, we have eliminated for these measurements the contribution of dissolved oxygen to the current, which appears as a shoulder at the lowest frequencies by bubbling argon gas in the electrochemical cell. (**D**) In fitting our entire equivalent circuit to the electrochemical phase spectroscopy scans of a tobramycin-binding EAB sensor exposed to an increasing amount of target in PBS, we find that target addition mainly affects the charge transfer resistance (R_{CT}). This is because as R_{CT} decreases, the effective resistance of the interface in accepting electrons from the reporter decreases due its increased rate of charge transfer. Fitting the resulting binding curve to a

Hill-Langmuir isotherm returns a K_D of 36 μM (error bars represent standard deviations seen across three independently fabricated and interrogated sensors).

To understand the origins of the target-induced phase response, we modeled the EAB sensor's behavior both at the redox potential of the reporter, E_0, and at potentials far removed, $E_{outside}$. Specifically, we model the experimental data with equivalent circuits using resistors and capacitors to represent each of the interface's components (**Figure 4A**). The phase shift in the phase spectrum approaches 0˚ at frequencies above 10 kHz (**Figure 4C**), indicating that under these conditions, the system behaves as a simple resistor of resistance R_{SOL}. This presumably occurs because these frequencies are faster than both the rate of double layer formation[19,20] and any faradaic processes[21]. At lower frequencies, formation of the electric double layer contributes capacitance to the observed behavior. For example, when the applied potential is far from the redox potential of the reporter (and thus no faradaic processes occur), the phase monotonically decreases towards -90˚ with decreasing frequency ($E_{outside}$, **Figure 4C**). To capture this effect, we include in our model a non-ideal capacitor (of capacitance C_{INT}) placed in series with the solution resistance, R_{SOL} (see black circuit elements in **Figure 4B**). The nonideality of this capacitor, reported as a constant phase element, accounts for surface monolayer defects and electrode roughness (see supporting information for additional details)[22]. Finally, when we interrogate the sensor at the reporter's redox potential, E_0, we observe a peak in the phase spectrum at frequencies corresponding to the electron transfer rate of the aptamer-attached redox reporter (**Figure 4C**)[21]. We modeled this as a resistor and a constant phase element (of resistance R_{CT} and capacitance C_{AD}, respectively) in series with each other and in parallel to the interfacial capacitance, C_{INT}. These elements reflect the resistance associated with faradaic charge transfer and the adsorptive pseudo-capacitance associated with oxidation and reduction of a fixed number of surface-attached, redox-reporter-modified aptamer[18,20,23,24].

By fitting our experimental data sets to the two equivalent circuit models described above, we find that the target-induced phase peak shift arises due to binding-induced changes in charge transfer resistance. To see this, we first fit data collected at $E_{outside}$ to determine R_{SOL} and C_{INT} (**Figure 4C**). Using these values, we then modeled the system's behavior at E_0 (**Figure 4C**). Both models accurately capture our experimental observations, producing estimates of R_{CT} that vary as a function of target concentration with the Hill-Langmuir isotherm (**Figure 4D**). In contrast, R_{SOL}, C_{INT} and C_{AD} are effectively independent of target concentration (**Figure 4D**), indicating that the phase response we observe upon target addition arises due to the binding-induced change in R_{CT}. This is consistent with previous arguments that signaling in this class of sensors is solely associated with binding-induced changes in the electron transfer kinetics of the redox reporter[18,25]. To see this, we note that R_{CT} is inversely proportional to the electron transfer rate (**Eq. 1**) per:

$$R_{CT} = \frac{2RT}{F^2 A k_{et}} \qquad \textbf{Eq. 1}$$

where R is the ideal gas constant, T the temperature, F Faraday's constant, A the quantity of redox reporters (in moles), and k_{et} the electron transfer rate.

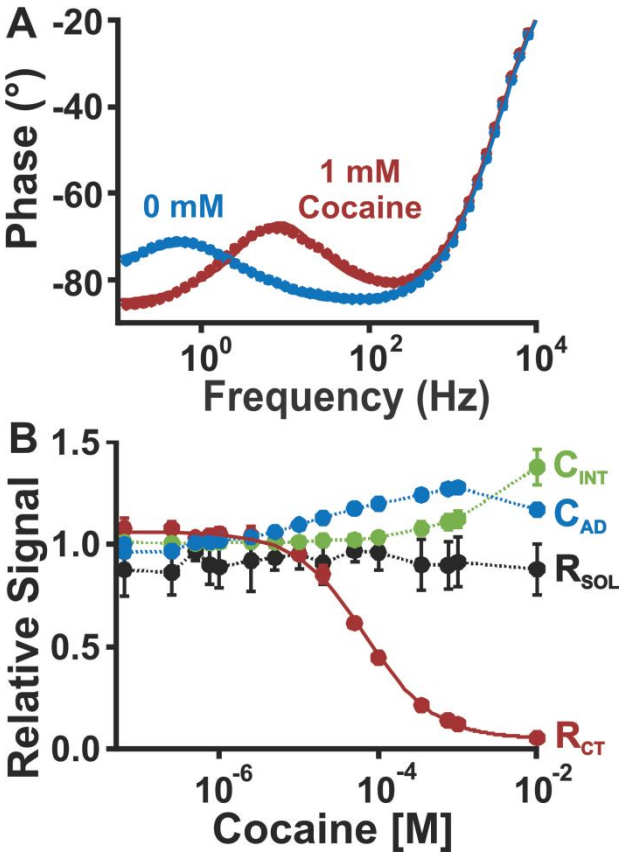

Figure 5. Electrochemical phase interrogation is applicable to other EAB sensors. (**A**) Shown, for example, is the phase response of a cocaine-detecting EAB sensor when challenged with its target (here in PBS). With target addition, we measure a phase peak shift towards higher frequencies. (**B**) Fitting the resulting phase change to our proposed equivalent circuit model in **Figure 4B** for four sensors, we find transfer resistance (R_{CT}) to be the dominant contributor to the measured phase change. This supports that, as was true for the aminoglycoside sensor, signal generation is due to a binding-induced change in electron transfer rate.

Electrochemical phase interrogation appears to be generalizable to other EAB sensors. To see this, we applied it to a sensor against the drug of abuse, cocaine, which employs a previously-described aptamer Neves et al.[26] When we interrogate this sensor at the reduction potential of the redox reporter, we observe a phase peak that shifts to higher frequencies upon the addition of increasing cocaine concentrations both in PBS (**Figure 5A**) and in undiluted whole blood (**Figure S4**). Fitting this to the equivalent circuit we used to characterize the aminoglycoside sensor, we find that the observed change in phase is, once again, largely due to changes in R_{CT} (**Figure 5C**).

Signal generation in this sensor is thus also driven by binding-induced changes in electron transfer kinetics.

DISCUSSION

Electrochemical phase interrogation of EAB sensors provides a sub-second-resolved, real-time approach to measuring specific small molecules with time resolution up to two orders of magnitude better than those typically achieved using voltammetric interrogation of sensors in this class. Likewise, this technique rejects the confounding effects of oxygen reduction, rendering measurements less sensitive to monolayer degradation and enabling long-duration measurements in whole blood without the need to resort to drift correction mechanisms or antifouling coatings. In short, the approach combines the speed of impedimetric measurements and selectivity of EAB sensors, thus supporting high time resolution measurements in complex, unprocessed clinical sample matrices.

Electrochemical phase interrogation is not the first means of collecting sub-second EAB sensor measurements,[27,28] but it does provide potential advantages over prior approaches. For example, chronoamperometry employs a series of oxidizing and reducing potentials to induce exponentially decaying current responses, which can be fitted to determine receptor occupancy with sub-second resolution[27]. In this approach, the temporal resolution is determined by the time required to fully resolve the portion of the current decay that is associated with charge transfer. A limitation of chronoamperometry, however, is the difficulty of accurately fitting exponential decays due to other decays of similar lifetimes that are unrelated to the charge transfer event (e.g., the formation of the electric double layer formation)[27]. To improve temporal resolution further, Santos-Cancel et al.[28] proposed the sampling of currents at tens of microsecond time intervals. The

binding-induced signal change they observed, however, is linked to alteration in the formation of the electrical double layer, which may render the approach sensitive to non-target-driven changes at the interface (ionic strength and biofouling) that invariably occur in realistically-complex clinical samples.

Electrochemical phase interrogation combines the time resolution benefits of impedance-based measurements with the fouling resistance of surface-tethered redox reporters. This superior time resolution stems from the rapid AC potential pulses that cleanly monitor charge transfer, instead of deriving it by scanning across a large potential window (like in voltammetric approaches) or across a large frequency range (like in other impedance-based approaches). We envision that applying the phase interrogation approach to EAB sensors could resolve rapid biological events in other complex matrices or in the living body thanks to their fouling-insensitive signaling mechanism of binding-induced change in electron transfer. Beyond EAB sensors, we expect that electrochemical phase interrogation can be applied to other sensing platforms that rely on binding-induced changes in electron transfer rate. These include sensors for which signal generation is associated with binding-induced changes in (1) a surface-attached redox reporter's reorganizational energy[29-31]; (2) the coupling constant that defines how rapidly electrons transfer between an electrode and the reporter[32-34]; and (3) the diffusion of a surface-tethered redox reporter[35,36]. Thus, we believe electrochemical phase interrogation provides a widely-adaptable platform for sensing directly in complex clinical media because it combines the high time resolution of impedance-based measurements with the fouling resistance of surface-bound redox reporter sensing platforms.

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ASSOCIATED CONTENT

Supporting Information

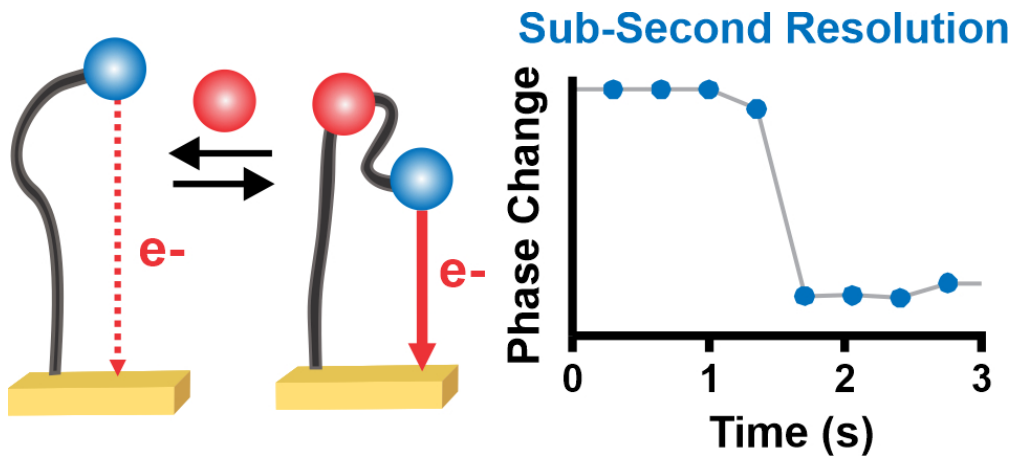
Sensor cleaning parameters, circuit fitting, cocaine-binding EAB sensor results.

REFERENCES

(1) White, R. J.; Plaxco, K. W. Exploiting Binding-Induced Changes in Probe Flexibility for the Optimization of Electrochemical Biosensors. *Anal. Chem.* **2010**, *82*, 73-76, 10.1021/ac902595f.
(2) Baker, B. R.; Lai, R. Y.; Wood, M. S.; Doctor, E. H.; Heeger, A. J.; Plaxco, K. W. An electronic, aptamer-based small-molecule sensor for the rapid, label-free detection of cocaine in adulterated samples and biological fluids. *J. Am. Chem. Soc.* **2006**, *128*, 3138-3139, 10.1021/ja056957p.
(3) White, R. J.; Phares, N.; Lubin, A. A.; Xiao, Y.; Plaxco, K. W. Optimization of electrochemical aptamer-based sensors via optimization of probe packing density and surface chemistry. *Langmuir* **2008**, *24*, 10513-10518, 10.1021/la800801v.
(4) Ferguson, B. S.; Hoggarth, D. A.; Maliniak, D.; Ploense, K.; White, R. J.; Woodward, N.; Hsieh, K.; Bonham, A. J.; Eisenstein, M.; Kippin, T. E.; Plaxco, K. W.; Soh, H. T. Real-Time, Aptamer-Based Tracking of Circulating Therapeutic Agents in Living Animals. *Sci. Transl. Med.* **2013**, *5*, 213ra165-213ra165, 10.1126/scitranslmed.3007095.
(5) Swensen, J. S.; Xiao, Y.; Ferguson, B. S.; Lubin, A. A.; Lai, R. Y.; Heeger, A. J.; Plaxco, K. W.; Soh, H. T. Continuous, Real-Time Monitoring of Cocaine in Undiluted Blood Serum via a Microfluidic, Electrochemical Aptamer-Based Sensor. *J. Am. Chem. Soc.* **2009**, *131*, 4262-4266, 10.1021/ja806531z.
(6) Ferapontova, E. E.; Gothelf, K. V. Optimization of the Electrochemical RNA-Aptamer Based Biosensor for Theophylline by Using a Methylene Blue Redox Label. *Electroanalysis* **2009**, *21*, 1261-1266, 10.1002/elan.200804558.
(7) Arroyo-Currás, N.; Somerson, J.; Vieira, P. A.; Ploense, K. L.; Kippin, T. E.; Plaxco, K. W. Real-time measurement of small molecules directly in awake, ambulatory animals. *Proc. Natl. Acad. Sci.* **2017**, *114*, 645-650, 10.1073/pnas.1613458114.
(8) Lai, R. Y.; Plaxco, K. W.; Heeger, A. J. Aptamer-Based Electrochemical Detection of Picomolar Platelet-Derived Growth Factor Directly in Blood Serum. *Anal. Chem.* **2007**, *79*, 229-233, 10.1021/ac061592s.
(9) Zhao, S.; Yang, W.; Lai, R. Y. A folding-based electrochemical aptasensor for detection of vascular endothelial growth factor in human whole blood. *Biosensors Bioelectron.* **2011**, *26*, 2442-2447, 10.1016/j.bios.2010.10.029.
(10) Liu, Y.; Zhou, Q.; Revzin, A. An aptasensor for electrochemical detection of tumor necrosis factor in human blood. *Analyst* **2013**, *138*, 4321-4326, 10.1039/C3AN00818E.
(11) Dauphin-Ducharme, P.; Arroyo-Currás, N.; Kurnik, M.; Ortega, G.; Li, H.; Plaxco, K. W. Simulation-Based Approach to Determining Electron Transfer Rates Using Square-Wave Voltammetry. *Langmuir* **2017**, *33*, 4407-4413, 10.1021/acs.langmuir.7b00359.
(12) Daniels, J. S.; Pourmand, N. Label-Free Impedance Biosensors: Opportunities and Challenges. *Electroanalysis* **2007**, *19*, 1239-1257, 10.1002/elan.200603855.
(13) Bogomolova, A.; Komarova, E.; Reber, K.; Gerasimov, T.; Yavuz, O.; Bhatt, S.; Aldissi, M. Challenges of Electrochemical Impedance Spectroscopy in Protein Biosensing. *Anal. Chem.* **2009**, *81*, 3944-3949, 10.1021/ac9002358.
(14) Arroyo-Currás, N.; Scida, K.; Ploense, K. L.; Kippin, T. E.; Plaxco, K. W. High Surface Area Electrodes Generated via Electrochemical Roughening Improve the Signaling of Electrochemical Aptamer-Based Biosensors. *Anal. Chem.* **2017**, *89*, 12185-12191, 10.1021/acs.analchem.7b02830.

(15) Rowe, A. A.; Miller, E. A.; Plaxco, K. W. Reagentless measurement of aminoglycoside antibiotics in blood serum via an electrochemical, ribonucleic acid aptamer-based biosensor. *Anal. Chem.* **2010**, *82*, 7090-7095, 10.1021/ac101491d.
(16) Shaver, A.; Curtis, S. D.; Arroyo-Currás, N. Alkanethiol Monolayer End Groups Affect the Long-Term Operational Stability and Signaling of Electrochemical, Aptamer-Based Sensors in Biological Fluids. *ACS Applied Materials & Interfaces* **2020**, *12*, 11214-11223, 10.1021/acsami.9b22385.
(17) Kang, D.; Ricci, F.; White, R. J.; Plaxco, K. W. Survey of Redox-Active Moieties for Application in Multiplexed Electrochemical Biosensors. *Anal. Chem.* **2016**, *88*, 10452-10458, 10.1021/acs.analchem.6b02376.
(18) Creager, S. E.; Wooster, T. T. A New Way of Using ac Voltammetry To Study Redox Kinetics in Electroactive Monolayers. *Anal. Chem.* **1998**, *70*, 4257-4263, 10.1021/ac980482l.
(19) Bertok, T.; Lorencova, L.; Chocholova, E.; Jane, E.; Vikartovska, A.; Kasak, P.; Tkac, J. Electrochemical Impedance Spectroscopy Based Biosensors: Mechanistic Principles, Analytical Examples and Challenges towards Commercialization for Assays of Protein Cancer Biomarkers. *ChemElectroChem* **2019**, *6*, 989-1003, 10.1002/celc.201800848.
(20) Eckermann, A. L.; Feld, D. J.; Shaw, J. A.; Meade, T. J. Electrochemistry of redox-active self-assembled monolayers. *Coord. Chem. Rev.* **2010**, *254*, 1769-1802, 10.1016/j.ccr.2009.12.023.
(21) Dauphin-Ducharme, P.; Arroyo-Currás, N.; Adhikari, R.; Somerson, J.; Ortega, G.; Makarov, D. E.; Plaxco, K. W. Chain Dynamics Limit Electron Transfer from Electrode-Bound, Single-Stranded Oligonucleotides. *The Journal of Physical Chemistry C* **2018**, *122*, 21441-21448, 10.1021/acs.jpcc.8b06111.
(22) Douglass Jr, E. F.; Driscoll, P. F.; Liu, D.; Burnham, N. A.; Lambert, C. R.; McGimpsey, W. G. Effect of Electrode Roughness On the Capacitive Behavior of Self-Assembled Monolayers. *Anal. Chem.* **2008**, *80*, 7670-7677, 10.1021/ac800521z.
(23) Zoltowski, P. On the electrical capacitance of interfaces exhibiting constant phase element behaviour. *J. Electroanal. Chem.* **1998**, *443*, 149-154, 10.1016/S0022-0728(97)00490-7.
(24) Ahmad, S. A. A.; Ciampi, S.; Parker, S. G.; Gonçales, V. R.; Gooding, J. J. Forming Ferrocenyl Self-Assembled Monolayers on Si(100) Electrodes with Different Alkyl Chain Lengths for Electron Transfer Studies. *ChemElectroChem* **2019**, *6*, 211-220, 10.1002/celc.201800717.
(25) Dauphin-Ducharme, P.; Plaxco, K. W. Maximizing the Signal Gain of Electrochemical-DNA Sensors. *Anal. Chem.* **2016**, *88*, 11654-11662, 10.1021/acs.analchem.6b03227.
(26) Neves, M. A. D.; Reinstein, O.; Saad, M.; Johnson, P. E. Defining the secondary structural requirements of a cocaine-binding aptamer by a thermodynamic and mutation study. *Biophys. Chem.* **2010**, *153*, 9-16, 10.1016/j.bpc.2010.09.009.
(27) Arroyo-Currás, N.; Dauphin-Ducharme, P.; Ortega, G.; Ploense, K. L.; Kippin, T. E.; Plaxco, K. W. Subsecond-Resolved Molecular Measurements in the Living Body Using Chronoamperometrically Interrogated Aptamer-Based Sensors. *ACS Sensors* **2018**, *3*, 360-366, 10.1021/acssensors.7b00787.
(28) Santos-Cancel, M.; Lazenby, R. A.; White, R. J. Rapid Two-Millisecond Interrogation of Electrochemical, Aptamer-Based Sensor Response Using Intermittent Pulse Amperometry. *ACS Sensors* **2018**, *3*, 1203-1209, 10.1021/acssensors.8b00278.

(29) Plumb, K.; Kraatz, H.-B. Interaction of a Ferrocenoyl-Modified Peptide with Papain: Toward Protein-Sensitive Electrochemical Probes. *Bioconj. Chem.* **2003**, *14*, 601-606, 10.1021/bc0256446.
(30) Orlowski, G. A.; Chowdhury, S.; Kraatz, H.-B. Reorganization Energies of Ferrocene-Peptide Monolayers. *Langmuir* **2007**, *23*, 12765-12770, 10.1021/la701740q.
(31) Feld, D. J.; Hsu, H.-T.; Eckermann, A. L.; Meade, T. J. Trinuclear Ruthenium Clusters as Bivalent Electrochemical Probes for Ligand–Receptor Binding Interactions. *Langmuir* **2012**, *28*, 939-949, 10.1021/la202882k.
(32) Uzawa, T.; Cheng, R. R.; White, R. J.; Makarov, D. E.; Plaxco, K. W. A Mechanistic Study of Electron Transfer from the Distal Termini of Electrode-Bound, Single-Stranded DNAs. *J. Am. Chem. Soc.* **2010**, *132*, 16120-16126, 10.1021/ja106345d.
(33) Genereux, J. C.; Barton, J. K. Mechanisms for DNA Charge Transport. *Chem. Rev.* **2010**, *110*, 1642-1662, 10.1021/cr900228f.
(34) Boon, E. M.; Ceres, D. M.; Drummond, T. G.; Hill, M. G.; Barton, J. K. Mutation detection by electrocatalysis at DNA-modified electrodes. *Nat. Biotechnol.* **2000**, *18*, 1096-1100, 10.1038/80301.
(35) Katayama, Y.; Ohuchi, Y.; Higashi, H.; Kudo, Y.; Maeda, M. The Design of Cyclic AMP−Recognizing Oligopeptides and Evaluation of Its Capability for Cyclic AMP Recognition Using an Electrochemical System. *Anal. Chem.* **2000**, *72*, 4671-4674, 10.1021/ac990847h.
(36) Le Floch, F.; Ho, H. A.; Leclerc, M. Label-Free Electrochemical Detection of Protein Based on a Ferrocene-Bearing Cationic Polythiophene and Aptamer. *Anal. Chem.* **2006**, *78*, 4727-4731, 10.1021/ac0521955.



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