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Exploiting Binding-Induced Changes in Probe Flexibility for the Optimization of Electrochemical Biosensors

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Abstract

Electrochemical sensors employing redox-tagged, electrode-bound oligonucleotides have emerged as a promising new platform for the reagentless detection of molecular analytes. Signal generation in these sensors is linked to specific, binding-induced changes in the efficiency with which an attached redox tag approaches and exchanges electrons with the interrogating electrode. We present here a straightforward means of optimizing the signal gain of these sensors that exploits this mechanism. Specifically, using square-wave voltammetry, which is exquisitely sensitive to electrode reaction rates, we can tune the frequency of the voltammetric measurements to preferentially enhance the signal associated with either the unbound or target-bound conformations of the probe. This allows us to control not only the magnitude of the signal gain associated with target binding, but also the sign of the signal change, generating “signal-on” or “signal-off” sensors. This optimization parameter appears to be quite general: we show here that tuning square-wave frequency can significantly enhance the gain of sensors directed against specific oligonucleotide sequences, small molecules, proteins and protein-small molecule interactions.

Keywords

E-DNA sensors; E-AB sensors; aptamers; scaffold sensors; molecular beacons; antibody detection

A number of reagentless, electrochemical sensors have been reported in recent years that were based, in their earliest implementations, on the target-induced “folding” of electrode-bound oligonucleotides. Examples include sensors for the detection of specific oligonucleotides,^{1–3} proteins,^{4–7} small molecules and ions,^{8, 9} and protein-small molecule interactions¹⁰ (Fig. 1). Comprised of an oligonucleotide probe covalently modified with a redox reporter, such as methylene blue, attached to a gold electrode via a thiol-gold bond, these so-called electrochemical DNA (E-DNA) and electrochemical aptamer-based (EAB) sensors are rapid, specific, and selective enough to deploy directly in complex clinical and environmental samples, including whole blood, soil extracts and foodstuffs.^{1, 8–10, 11} Thanks to these attributes, the E-DNA/E-AB approach shows particular promise for, for example, point-of-care applications.^{12, 13}

Several lines of evidence suggest that this diverse class of sensors share a common signaling mechanism. Specifically, studies of sensor gain (the relative change in signal upon the addition of saturating target) as functions of the density with which the probe oligonucleotides are

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packed on the electrode surface and of the frequency of the alternating current (AC) potential used to interrogate the conformational change (with AC voltammetry) suggest that signaling arises from binding-induced changes in the efficiency with which the attached redox tag approaches the electrode surface.^{5, 14, 15} In other words, target binding results in a change in the “floppiness” of the DNA probe akin to the floppy model first described by Murray.¹⁶ This in turn, alters the efficiency with which the redox probe exchanges electrons with the interrogating electrode to produce the observed faradaic current. Because this mechanism is linked to changes in the movement of the redox tag to the surface, voltammetric signals that are especially sensitive to kinetics could significantly improve the performance of this class of sensors.¹⁴ Consistent with this observation Ikeda and co-workers have shown that, via the use of square wave voltammetry (SWV), it is possible to distinguish between rigid, fully double-stranded DNA probes, more flexible mismatched duplexes, and very flexible, single-stranded DNA probes.¹⁴ With these arguments in mind, we explore here the extent to which varying the frequency of the interrogating square-wave potential alters the signaling of a half dozen sensors representative of this broad class of devices.

While many commonly employed voltammetric methods report on kinetics, SWV is of particular interest as a result of its specific current sampling protocol.^{17, 18} That is, because current is sampled *at the end* of each square-wave pulse (Fig. 2a), the current/frequency relationship in SWV depends on the rate at which electrons are transferred to and from the electrode. Moreover, for a pair of reactions, one rapid and one slow, there will be a frequency at which the two current-versus-frequency curves cross (Fig. 2b). Above this crossover frequency the more rapid reaction produces greater current; below it the situation is reversed (Fig. 2c,d). SWV is thus particularly well suited to distinguish between electron transfer reactions that differ in rate. SWV can also be used to measure the apparent electron transfer rates of surface bound reactions by defining a “critical frequency” which appears as a maxima in the relationship of i_p/f vs. f (where i_p is net peak current and f is SWV frequency - see Fig. SII and refs 19 and 20). Performing such an evaluation on the target-free and target-bound states of the sensor architectures discussed in this paper demonstrates that all six exhibit significant changes in critical frequency upon target binding (Fig. SII).

The relationship between faradaic current, electrode reaction kinetics and square wave frequency can significantly impact the signaling of electrochemical biosensors such as the E-DNA/E-AB platform. Specifically, because the critical frequencies of the bound and unbound states of their probes differ significantly, we can tune the SWV frequency to not only optimize the signal gain of these sensors, but often to invert the sign of the observed signal change. An illustrative example of this is provided by the linear probe E-DNA sensor, which comprises a short (17-base) linear DNA probe attached at its 5'-terminus to a gold electrode and modified at its 3'-terminus with a redox-active methylene blue (Fig. 1a).²¹ In its target-free, single-stranded state the linear probe is quite flexible and thus supports the relatively rapid transfer of electrons to and from the electrode. Because it is relatively rigid, the duplex DNA formed upon target binding is less likely to approach the surface and thus target binding reduces the apparent electron transfer rate (Fig. SII). [Note: our probe DNAs are at relatively low packing densities, conditions under which the “diffusion” of the end of even the rigid, double-stranded probe to the electrode surface is thought to dominate over through-DNA electron tunneling (Anne et al., ref 23).] Consistent with this, when we employ AC voltammetry (ACV) to monitor binding, we find that the target *reduces* the observed current, typically by about -70% at saturating concentrations.^{21, 22} The currents observed from the target-free and target-bound states in a SWV scan, however, respond very differently as the frequency of the square-wave pulse is altered. So differently, in fact, that there are frequencies at which the rigid, target-bound state produces *more* current than the flexible, target-free state (Fig. 2 b,c,d). That is, at SWV frequencies below ~20 Hz the signal from the target-bound state is enhanced and the signal from the unbound state is suppressed (as a result of the rapid current decay of the latter,

faster reaction). This results in a *signal-on* sensor (*i.e.*, the presence of target increases the observed current –Fig. 2d). If, instead, we tune the frequency to higher values the opposite becomes true and a *signal-off* sensor is realized (Fig. 2c).

The gain of the linear probe sensor can be increased significantly by changing the SWV frequency: the gain of this sensor increases monotonically as the frequency is reduced from 2500 Hz, peaking at +1140% at the lowest frequencies (~1 Hz) we can investigate with our equipment. Several issues, however, limit the range over which we can usefully vary the SWV frequency. For example, at SWV frequencies below ~10 Hz the current produced by the more dynamic, unbound probe drops relative to the background current. Because signal gain is calculated relative to the background current, the consequence of this is significant sensor-to-sensor variability at low frequencies (see *e.g.*, Fig. 3a and Fig SI2). Likewise the signal-to-noise ratio observed at high frequencies *also* decreases, here as a result of instrument noise. Finally, the shape of the voltammetric response itself can change when the SWV frequency is far from the apparent reaction rate. For example, at frequencies well below the apparent rate two peaks emerge as the peak splits between the anodic and cathodic currents.¹⁸ These changes in peak shape and signal-to-noise affect the precision of the measurements made at extreme frequencies. When these issues are considered, we find that optimal signaling of our linear probe sensor is achieved at ~10 Hz, where we observe a gain of +260% without notable reduction in sensor-to-sensor reproducibility (Fig. 3a). This represents a significant improvement over an identical sensor employing AC voltammetry as the read-out, which, as noted above, achieved a “signal-off” gain of only –70%.²¹

The dramatic effects of altering the frequency of the square-wave pulse hold across every sensor architecture we have investigated. This includes a second E-DNA sensor employing a stem-loop probe² (Fig. 1b) that, like the linear probe architecture, exhibits a shift to a lower critical frequency upon the addition of its complementary target (Fig. SI1). Due to this, the gain of the stem-loop sensor is negative at high frequencies but becomes positive below 20 Hz, achieving gains of up to +140% before significant noise is introduced below 2 Hz (Fig. 3b). Similar results also hold for various electrochemical-aptamer based (E-AB) sensors (Fig. 1c,d). Specifically, our thrombin E-AB sensor, which is intrinsically *signal-off* when probed via ACV,⁵ becomes *signal-on* at SWV frequencies between 4 and 40 Hz (Fig. 3c), and our cocaine E-AB sensor, which is intrinsically *signal-on* when probed with ACV,⁸ becomes *signal-off* at SWV frequencies above 40 Hz (Fig. 3d). Finally, our E-DNA scaffold approach,¹⁰ in which the signal change is predicated on binding-induced changes in the flexibility of receptor-modified, double stranded or single-stranded DNA probes (Fig. 1e,f), follows a similar trend (Fig. 3e,f). The double-stranded scaffold, however, does not switch to *signal-on* behavior even at the lowest frequencies our equipment can achieve (Fig. 3e and Fig. SI1), presumably because the crossover frequency is lower still.

Here we have demonstrated a simple means of optimizing the signal gain of a variety of sensor architectures employing redox-tagged, electrode-bound oligonucleotides as their recognition and signaling elements. Specifically, we find that simply tuning the frequency of the interrogating SWV potential pulse not only affects the magnitude of the signal change observed upon target binding, but even the sign of this signaling, thereby controlling whether the sensor is *signal-on* or *signal-off*. Finally, this effect holds across sensors employing a wide range of probe secondary structures and probe-target interaction mechanisms, suggesting that the observations reported here may serve as guidelines for the enhancement of most, if not all platforms in this broad class of sensors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Recent years have seen the development of a number of reagentless, electrochemical biosensors employing redox-tagged, electrode-bound oligonucleotide probes.^{1–5} Signal generation in these sensors is predicated on binding-induced changes in the flexibility of the probe oligonucleotide, which alters the efficiency with which the covalently attached redox tag can strike, and thus transfer electrons to or from the electrode. Sensors in this class have been reported against a wide variety of targets including, as shown here, DNA (**a** and **b** - linear and stem-loop probe E-DNA sensors), proteins and small molecules (**c** and **d** - thrombin and cocaine E-AB sensors), and protein-small molecule interactions (**e** and **f** - double- and single-stranded E-DNA scaffold sensors).

Figure 2.

The frequency of the interrogating square wave significantly affects the gain of EDNA/E-AB sensors. Specifically, as shown (a) on the example waveform, the current sampling points in SWV are at the end of each potential pulse. Because of this, the observed current will behave very differently depending on whether the electron transfer reaction is rapid or slow relative to the frequency of the square wave pulse. (b) For example, whereas the critical frequency of our target-free linear probe corresponds to a rate of 60 s^{-1} the bound duplex exhibits a much slower rate of 6 s^{-1} (see Fig. SI1). (c) Thus, if the sensor is interrogated at 100 Hz, relatively more current will be observed from the target-free probe and relatively less from the target-bound probe. (d) In contrast, if the interrogation frequency is set to 5 Hz, the more rapid transfer reaction of the free probe will have largely decayed before the current is sampled, thus leading to a much smaller signal than that obtained from the bound probe. Key to these behaviors is

the observation that the integrated area under the current/time plots (**b**) of both the rapid and slow reaction rates are constant (they are proportional to the total number of redox-tags on the electrode, which is fixed).

Figure 3.

Sensor gain varies significantly as a function of the square wave frequency employed. So much so that, for five of the six sensors we have investigated here, the observed signal gain can be either positive or negative depending on the relationship between the critical frequencies of the free and target-bound probes and the interrogation frequency employed. Only the double-stranded scaffold system (e) does not exhibit a change in the sign of its gain, presumably because, limitations in our instrument precluded measurements at frequencies low enough to switch the sensor to “signal-on” behavior.