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Comparing the Properties of Electrochemical-Based DNA Sensors Employing Different Redox Tags

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Many electrochemical biosensor approaches developed in recent years utilize redox-labeled (most commonly methylene blue or ferrocene) oligonucleotide probes site-specifically attached to an interrogating electrode. Sensors in this class have been reported that employ a range of probe architectures, including single- and double-stranded DNA, more complex DNA structures, DNA and RNA aptamers, and, most recently, DNA−small molecule chimeras. Signaling in this class of sensors is generally predicated on binding-induced changes in the efficiency with which the covalently attached redox label transfers electrons with the interrogating electrode. Here we have investigated how the properties of the redox tag affect the performance of such sensors. Specifically, we compare the differences in signaling and stability of electrochemical DNA sensors (E-DNA sensors) fabricated using either ferrocene or methylene blue as the signaling redox moiety. We find that while both tags support efficient E-DNA signaling, ferrocene produces slightly improved signal gain and target affinity. These small advantages, however, come at a potentially significant price: the ferrocene-based sensors are far less stable than their methylene blue counterparts, particularly with regards to stability to long-term storage, repeated electrochemical interrogations, repeated sensing/regeneration iterations, and employment in complex sample matrices such as blood serum.

Electrochemical sensors that utilize oligonucleotide probes covalently linked with a redox active reporter have become a mainstay technique in the biosensing field.[1−10] Examples include electrochemical sandwich assays,[11,12] competition-type assays,[10,13] the recently described electrochemical DNA (E-DNA) sensors,[2−4] electrochemical aptamer-based sensors (E-AB),[1,7,14,15] and small molecule−DNA chimeras.[16] In each of these techniques, signal transduction is predicated on changes in the efficiency with which a covalently attached redox label is able to transfer electrons to or from the electrode surface. This efficiency is altered either via binding-induced changes in the conformation (or dynamics)[17] of the probe DNA or, in the case of sandwich and competition assays, by forcing redox-labeled oligonucleotides into (or out of) proximity of the electrode surface. Using a redox-labeled DNA strand affords extremely specific and selective detection by combining the specificity of DNA hybridization (e.g., E-DNA sensors) or the specificity of aptamer−ligand interactions (e.g., E-AB) with the specific redox chemistry of the electroactive probe. Given the general paucity of electrochemically active interferants, such sensors have been demonstrated to perform even when challenged with complex, clinically relevant media such as blood serum,[4] crude cellular extracts,[12] and urine and saliva.[4]

To date, the majority of the electrochemical, oligonucleotide-based sensors in this class utilize either the electrochemically active methylene blue (MB)[1,3,4,7−9,18,19] or ferrocene (Fc)[2,10,14,15,20] as the signaling moiety. The popularity of these tags stems from their reversible redox chemistries, convenient redox potentials, and commercial availability in forms that can readily be conjugated

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to chemically synthesized oligonucleotides. Consistent with these attributes, both tags have seen widespread success in this field. Nevertheless, a recent report by Ferapontova et al.[14] indicates that the performance of RNA-aptamer-based sensors fabricated with either methylene blue or ferrocene differ significantly when the sensors are challenged in complex media such as blood serum; specifically, ferrocene-based sensors fail when tested in this complex media. The sensor failure is attributed to the observation that at the positive potentials employed to monitor the oxidation of ferrocene the sensor surfaces are compromised by nonspecific adsorption of serum proteins, thus precluding measurements in this sample matrix. More broadly, the different redox chemistry of these two tags, including differences in their redox potentials and intrinsic electron transfer rates, raises questions regarding how their chemistry might affect sensor performance. To date, however, no systematic study of the differences between sensors fabricated with these different redox active tags has been reported. In response, we detail the differences in signaling properties of a representative E-DNA sensor fabricated using either MB or Fc as the covalently attached signaling redox tag. Thus, we report on the effects of redox tag chemistry on overall E-DNA sensor performance by investigating effects on sensor gain, specificity, target affinity, and stability.

EXPERIMENTAL METHODS

Reagents. The following reagents were used as received: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma Aldrich), fetal calf serum (Sigma Aldrich), ferrocenecarboxylic acid (Fc-COOH), *N*-hydroxysuccinimide (Sigma Aldrich), *N*-hydroxysulfosuccinimide sodium salt (NHS), *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (Fluka), and 3,7-bis(*N*-(3-carboxypropyl)-*N*-methylamino)phenothiazin-5-ium perchlorate (MB-NHS, empBiotech GmbH, Berlin, Germany). The probe and target DNA sequences (Biosearch Technologies, Novato, CA) were as follows: probe, 5′-HS-C6-AGACAAGGAAAATCCTTCAATGAAGTGGGTCG-C7-NH_2-3′; target, 5′-CGACCCACTTCATTGAAGGATTTTCCTTGTCT-3′.

The probe DNA was modified at the 5′-terminus with a C6-disulfide [$HO(CH_2)_6-S-S-(CH_2)_6-$] linker and at the 3′-end with an amine group for redox probe conjugation.

Synthesis of Ferrocence Succinimidyl Ester (Fc-NHS) and Labeling of DNA Probes. Conversion of the Fc-COOH to a ferrocene succinimidyl ester (Fc-NHS) was achieved using a previously described procedure.[15] In brief, NHS and EDC were added to a 25 mM solution of Fc-COOH in dichloromethane to a final concentration of 60 mM for both NHS and EDC. The solution was stored under argon and stirred overnight (~12 h) at room temperature. The resulting solution was then washed three times with water and the organic phase was collected. The organic phase was then dried with $MgSO_4$, filtered, and evaporated. Column chromatography (silica gel column with diethyl ether) was used to purify the Fc−NHS product.

Conjugation of the redox labels to the probe DNA was achieved via the coupling of the NHS ester ferrocene conjugate with the single-stranded DNA terminated with an amine at the 3′-terminus. Fc-NHS (or MB-NHS) (1 μmol) was dissolved in 50 μL of a 0.5 M sodium bicarbonate solution (pH 8.5) and 10 μL dimethylformamide to a final volume of 60 μL. Following this, 10 μL of 200 μM DNA was added, stirred, and allowed to incubate for 4 h protected from light. After successful modification of the DNA probe, the sample was desalted using a G25 spin column (EMP-Biotech) and then further purified by RP-HPLC (C18 column). Methylene blue conjugation was achieved in the same manner, and both stock DNA solutions were stored at −20 °C for future use. While we have not rigorously determined the yield of final product, using mass spectrometry data we estimate a yield of ~20% for both the ferrocene- and methylene-blue-labeled DNA.

Electrode Preparation and Sensor Fabrication. E-DNA sensors were prepared using a well-established procedure described by Xiao et al.[21] In brief, prior to sensor fabrication, gold disk electrodes (2 mm diameter, CH Instruments, Austin, TX) were cleaned both mechanically (by polishing with diamond and alumina oxide slurries successively) and electrochemically (through successive scans in sulfuric acid solutions) as previously described.[21] The linear probe DNA was reduced for 1 h at room temperature in the dark in 10 mM tris(2-carboxyethyl)phosphine hydrochloride (Molecular Probes, Carlsbad, CA) and then diluted to a final concentration of 1.0 μM in HEPES/$NaClO_4$ buffer (10 mM HEPES and 0.5 M $NaClO_4$, pH 7.0, as was used in all the experiments to follow unless otherwise noted). The gold electrodes were incubated in this solution for 1 h at room temperature in the dark, rinsed with distilled, deionized (DI) water, and then incubated in 3 mM 6-mercapto-1-hexanol in DI water for 30 min. Following this, the electrodes were rinsed in DI water and stored in HEPES/$NaClO_4$ buffer for future use.

Sensor Measurements. Fabricated sensors were interrogated using square wave voltammetry (SWV) with a 50 mV amplitude signal at a frequency of 60 Hz, in the absence and presence of fully complementary target (2 μM). For titration measurements and measurements at saturated target concentrations (1 μM), the electrodes were incubated for 30 min with the appropriate concentration of target DNA in HEPES/$NaClO_4$ buffer or 20% fetal calf serum, mixed with HEPES/$NaClO_4$ buffer. Values with reported error bars represent the average and standard deviations of measurements performed on at least four independently fabricated electrodes. Signal gain was computed by the relative change in SWV peak currents with respect to background current (SWV peak current in the absence of target).

To test stability, sensors were stored in the dark, at room temperature in HEPES/$NaClO_4$ buffer in sealed, but air-filled, containers. This allowed long-term stability testing for up to 180 h. In addition to long-term stability, sensor robustness to multiple testing cycles was assessed by subjecting the sensors to a repeated cycle of testing in buffer (no target), testing in saturating target, and regeneration with a 30 s deionized water rinse. These cycles were repeated 15 times in buffer and 5 times in 20% serum. Finally, to test sensor robustness to repeated electrochemical interrogations, sensors were subjected to multiple SWV scans without target using a scan interval of 15 s.

RESULTS

As our test bed system with which to study the effects of redox label chemistry on electrochemical DNA sensing we selected the well-characterized linear-probe E-DNA sensor.[4,22] This sensor comprises a linear, 32-base DNA probe attached to a gold

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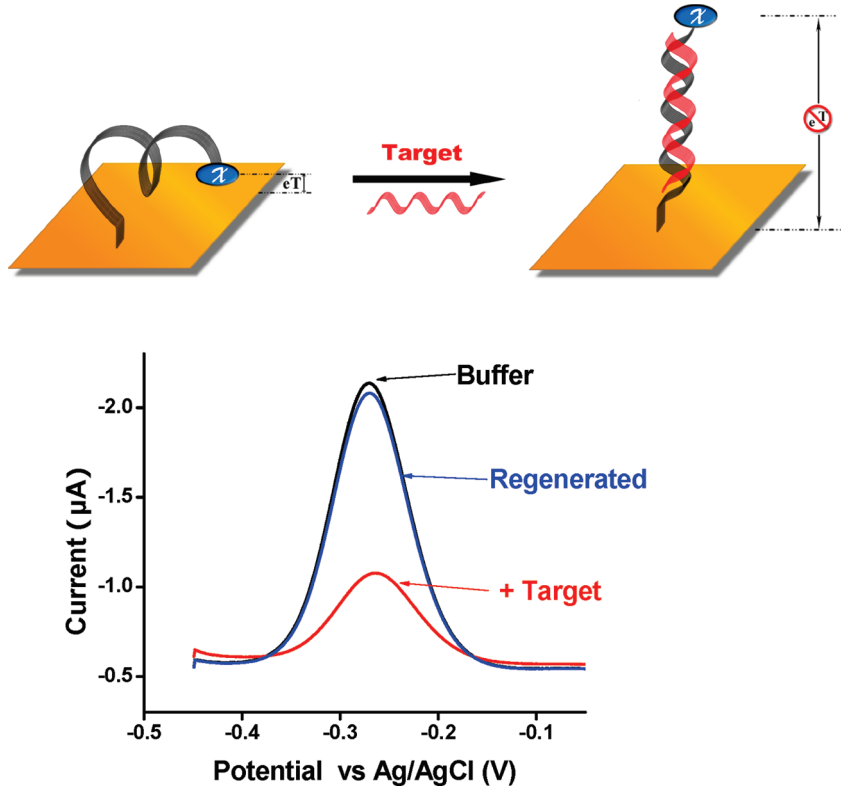

Figure 1. (Top) Signal generation in the electrochemical DNA (E-DNA) sensor occurs when hybridization with a target oligonuceotide reduces the efficiency with which the attached redox probe (χ) transfers electrons to the interrogating electrode, leading (bottom) to a significant decrease in Faradaic current. Because all of the sensor components are strongly attached to the electrode, the sensor is readily regenerated using a 30 s distilled water rinse. Data shown are for a sensor fabricated with methylene blue as the redox tag.

electrode surface at its 5′-terminus (via a six-carbon thiol modification) and covalently linked to either a redox active methylene blue or ferrocene at its 3′-terminus (Figure 1, top). The modified probe is deposited from aqueous solution onto a gold electrode followed by "backfilling" with excess 6-mercapto-1-hexanol as a coadsorbate, which forms a continuous, mixed, self-assembled monolayer.[23] In the absence of target, the flexible single-stranded probe supports efficient collision between the redox tag and the electrode, ensuring a large Faradaic current. Upon hybridization, the formation of a rigid probe−target duplex reduces the collision efficiency leading to a large change in measured signal (Figure 1, bottom).[22]

Both MB and Fc support efficient, sensitive E-DNA sensing. Specifically, at saturating target (1 μM) sensors fabricated with the ferrocene label exhibit 87.2 ± 2.6% signal suppression (calculated as the percent change in signal with respect to signal without target; this and all other reported error bars reflect the standard deviation across multiple, independently fabricated sensors), while sensors fabricated with the methylene blue label exhibit 70.2 ± 0.9% signal suppression (Figure 2). This agrees well with previous reports of E-DNA sensors.[19] The affinities of each type of sensor for the complementary target are likewise similar, with the dissociation constant of MB-based sensors (44 ± 18 nM) being slightly poorer than that of the Fc-based one (25 ± 4 nM).

In contrast to their signaling properties, the stabilities of MB- and Fc-based probes differ dramatically. For example, sensors employing MB are much more robust to repeated testing cycles (hybridization with target followed by regeneration with deionized

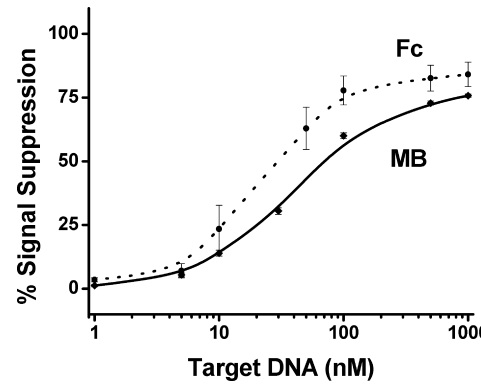

Figure 2. Regardless of which redox tag was employed, both sensor types respond similarly to the addition of a complementary target. The two, for example, exhibit similar dissociation constants (44 ± 18 and 25 ± 4 nM for MB- and Fc-based sensors, respectively). These data and error bars represent the average and standard deviation of measurements taken with four independently fabricated electrodes.

water) than the equivalent Fc-based sensor (Figure 3). Specifically, MB-based sensors are significantly more stable to sensor regeneration measured as the percent recovery of signaling current in the absence of target. While MB-modified probes exhibit an initial drop in signal after the first regeneration cycle (~10% loss, as commonly seen with this class of sensor),[4] the sensors are reproducibly regenerated to within at least 5% of the original signal in buffer in subsequent tests. The Fc-based sensors, however, cannot be acceptably regenerated. In fact, these sensors continue to lose signaling current after each consecutive cycle, ultimately

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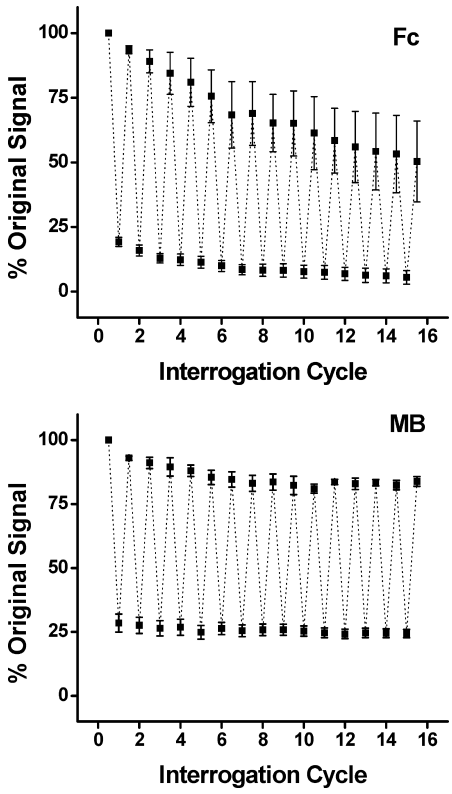

Figure 3. E-DNA sensor regeneration is achieved via a simple 30 s rinse with room temperature deionized water. (Top) Fc-based sensors demonstrate large changes in both signal regeneration (only regenerating to 50% of original signal) and signal suppression after 15 cycles. In addition, the sensor-to-sensor variability increases dramatically, as seen in the error bars. (Bottom) Repeated sensor cycling to hybridization and regeneration of MB-based sensors demonstrates a mean signal regeneration of 90% per use over 15 cycles. Much of the total loss of signal, however, arises during the first three cycles; the sensor exhibits a mean recovery of 98% per iteration after these. The sensor signal is also highly reproducible, producing a mean signal suppression of 74 ± 1.3% when challenged with a fully complementary target.

falling to ~50% of the original signaling current (with a concomitant decrease in sensor-to-sensor reproducibility, as illustrated by the error bars in Figure 3). The signal suppression measured at saturating target concentration is also unstable for Fc-based sensors, increasing with consecutive tests from 80.7% suppression to 90.5% suppression. The MB-based sensors, conversely, exhibit a stable 70% signal suppression over the 15 cycles of regeneration and testing. E-DNA sensors fabricated with Fc-labeled DNA probes are also less stable than MB-based sensors to multiple electrochemical interrogations (Figure 4). After 100 square-wave voltammetric scans (in buffer with no target), Fc-based signal decreases to less than 50% of original signal intensity. The loss of signaling current appears to decay linearly with the number of electrochemical interrogations. In contrast, sensors fabricated with MB-labeled DNA probes exhibit a minimal 2% current loss after 100 interrogations. Finally, sensors fabricated with MB also exhibit significantly improved storage stability (Figure 5). For example, sensors employing the MB redox tag exhibit exceptional solution-storage stability: when stored in buffer at room temperature for 180 h, the MB sensor loses only 38% of its original signaling peak current. In contrast, sensors fabricated using an Fc redox label exhibit a loss of 91% of original signaling peak current over the same time period.

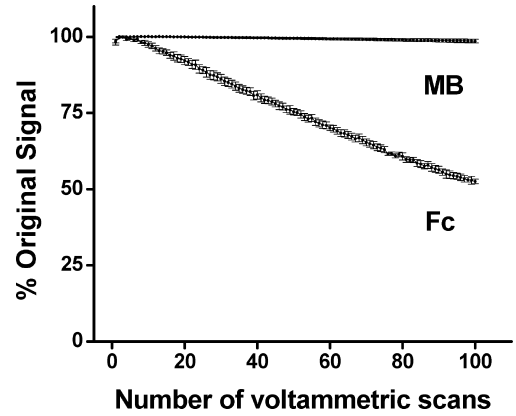

Figure 4. MB-based sensors are much more robust than the Fc-based counterparts to repeated electrochemical interrogations. As demonstrated, after 100 SWV scans (from −0.5 to −0.1 V, 60 Hz, 50 mV amplitude for MB, and from 0 to 0.45 V, 60 Hz, 50 mV amplitude for Fc) sensors fabricated using MB maintain 95% of their original peak current, and sensors fabricated using Fc exhibit an average loss of 50% in peak current.

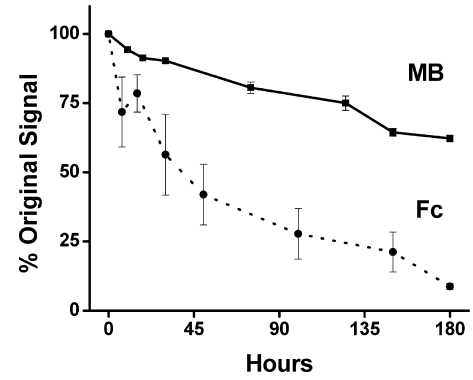

Figure 5. The wet storage stability of sensors fabricated using MB is significantly greater than that of sensors fabricated using Fc. After 180 h, Fc sensors exhibit losses of 90% in peak current (without target), presumably due to loss of probe from the surface. Conversely, sensors fabricated using MB exhibit only a 35% loss of peak current over the same time period.

The improved stability of MB redox tags is still more apparent when the sensors are employed in realistically complex sample matrices (Figure 6). For example, scans of a MB-based sensor over five cycles of hybridization and regeneration in 20% blood serum demonstrated a mean signal regeneration of 85% after the first cycle and 90% for the rest of the following iterations. The Fc-based sensors are only regenerated to 50% of the original signaling current. The decrease in stability is also manifested in the relative error of measurements made with four independently fabricated and tested electrodes. For example, the relative error for Fc-based sensors increases after five cycles to ~25% for Fc sensors compared to the modest (and consistent) ~5% for MB-based sensors. Similar to tests in buffer solution, MB-labeled sensors exhibited excellent reproducibility in signal suppression upon addition of complementary target, showing almost no change in ability during the five cycles (Figure 6). However, signal suppression to complementary target for Fc-based sensors decreases from 75% to ~90%.

DISCUSSION

Here we have shown that, while both Fc and MB support robust E-DNA sensor signaling, the performance attributes of these two

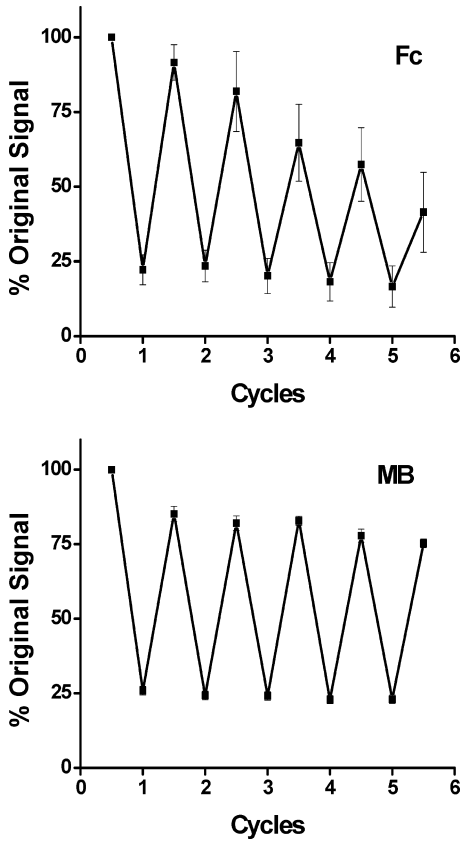

Figure 6. Fc-based sensors are less stable than the MB-based sensors when repeatedly challenged in complex media such as blood serum. (Top) After five cycles of hybridization and regeneration in 20% blood serum, Fc-based sensors lose more than 50% of the original signal (without target). In addition, the sensor-to-sensor reproducibility decreases. (Bottom) MB-based sensors subjected to the same tests maintain at least 85% of original signal, with most of the loss occurring after the first cycle.

chemically distinct redox tags differ significantly. For example, while the gain and target affinity are slightly improved for Fc-based E-DNA sensors, sensors fabricated from MB-labeled DNA dramatically outperform their Fc-based counterparts in terms of their electrochemical and long-term storage stability, sensor-to-sensor reproducibility, and performance in complex, multicomponent samples.

Both MB- and Fc-based sensors exhibit loss of the DNA monolayer over time, which can be caused by desorption of the thiolated oligomer or oxidation of the sulfur on the thiol.[24,25] As described above, Fc- and MB-based sensors exhibit loss of measured peak current over long-term storage in buffer solution (SWV peak current should give a qualitative indication of the number of signaling molecules on the electrode surface given no change in the apparent rate of the reaction as is expected in these measurements).[26] Given the identical chemistry of the DNA attachment to the surface, redox probe attachment, and the exact sequence identity of the DNA itself, we anticipate that the loss should be similar for both sensors. This, however, is not the case since Fc-based sensors exhibit 91% signal loss after 180 h storage in buffer, whereas the MB loses only 38% in the same time frame. Given the overall reproducibility of MB-based sensors, we believe that this signal loss of these sensors is primarily a result of SAM degradation. The loss of signaling ability of Fc-based sensors can occur as a result of the loss of the signaling DNA monolayer, poor electrochemical stability (i.e., reversibility of the redox couple), or a combination of these two effects. In addition, the even poorer reusability in blood serum of the Fc-based sensors could be attributed to the nonspecific adsorption of serum proteins consistent with the recent report by Ferapontova et al.[15] Adsorption of these proteins could impede access to the electrode surface, thus further contributing to poorer signal regeneration of signaling, as seen in Figure 6.

Evidence of the electrochemical instability of the Fc redox tag can be seen in the repeated electrochemical interrogations and the hybridization/regeneration cycles. These experiments were performed on much shorter time scales as to preclude significant SAM degradation.[25] In both experiments, the Fc-labeled E-DNA sensors exhibit much greater loss in signaling current, indicating a loss in Fc activity. This is particularly apparent in the repeated electrochemical interrogations (Figure 5). The MB-based sensors exhibit essentially no loss in signaling current, as shown by the measured peak current of each square-wave voltammetric scan. The Fc-based sensors, however, exhibit significant signal decrease over the same scanning period. Although precautions were taken to avoid this (e.g., using HEPES or sodium perchlorate buffers), the oxidized form of the Fc, or ferricenium ion (Fc^+), is extremely prone to nucleophilic attack, which can lead to ligand exchange or an irreversible change in the electrochemistry.[27] Any strong nucleophile could potentially cause the loss of attached Fc on the E-DNA sensors or irreversible oxidation, which would be manifested as a loss in measured peak current as is clearly seen in the data presented within.

Here we have demonstrated that E-DNA sensors fabricated using MB-labeled oligonucleotides significantly improve the solution-phase storage stability, electrochemical stability, and signal verity of an electrochemical biosensor and, critically, achieves these improvements without sacrificing electron transfer efficiency or otherwise degrading sensor performance. Improved stability coupled with commercial availability point to methylene blue as being a better option for developing electrochemical DNA-based sensors. However, as shown within, choosing the proper redox tag can depend on which sensor attributes are desirable.

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