Conformationally Gated Electrochemical Gene Detection

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The synthesis and characterization of a 26-base DNA hairpin containing both a redox-active reporter (ferrocene) and terminal thiol functionality for electrochemical gene detection is described. This electrochemical DNA sensor exploits electron-transfer dynamics that alter as a consequence of a large structural rearrangement (hairpin-to-duplex) induced by hybridization of the target DNA sequence. Melting temperature and circular dichroism studies confirm that the 26-mer DNA forms a hairpin structure in the absence of target DNA. The loop region of the DNA hairpin is shown to form a stable duplex in the presence of complementary single-stranded DNA. Atomic force microscopy and ellipsometry experiments of immobilized self-assembled DNA monolayers suggest that hybridization with complementary DNA affords a conformational change that alters the electrochemical response.

Introduction

Identifying specific nucleic acid sequences of viral or bacterial pathogens, hereditary diseases, or genetic abnormalities is of widespread interest in the areas of medicine, biotechnology, and homeland security. Current methods for DNA detection include both sequencing techniques and hybridization assays. The advantages of hybridization assays over sequencing techniques include rapid detection of the analyte and the elimination of sample amplification and purification steps. Electrochemical detection schemes possess several advantages including sensitive electrochemical transducers, single amplification, low cost, rapid detection, minimal power requirements, and compatibility with microfabrication techniques.[1–3] Several methods for sequence-specific DNA detection based on electrochemical methods have been reported. The groups of Barton and Thorp describe the detection of DNA using an electrocatalytic scheme for signal amplification.[4–6] Electrochemiluminescence assays have also been reported for the detection of specific DNA sequences.[7] Another common assay design is a sandwich-type assay possessing three components: a capture strand, a target strand, and a probe strand containing an electroactive reporter group.[8–10] Very recently, the electrochemical detection of DNA by using immobilized hairpins[11,12] and single-stranded DNA[13] has been reported. Such electrochemical DNA sensors, in which electron-transfer dynamics are altered as a consequence of a structural rearrangement induced by hybridization, are of interest. Herein, we report the synthesis and characterization of ferrocene-labeled DNA hairpins, and the use of a solid-phase hybridization assay on a gold-ball electrode to electrochemically detect DNA.

The design criteria for this conformationally gated DNA-detection device are: 1) an easily addressable redox probe and 2) a large structural/conformational change upon hybridization of the target DNA strand. Ferrocene was chosen as the electroactive probe in these studies because of its proven utility in biological diagnostics,[9,14,15] its stability during DNA synthesis,[16,17] and its accessible redox potential under physiological conditions. To afford significant structural rearrangement of the electroactive DNA, we selected a DNA stem-loop sequence, with the loop DNA sequence complementary to a specific DNA target. Binding of target DNA induces a hairpin-to-duplex transition. This structural rearrangement has been previously characterized by using fluorescence resonance energy transfer (FRET) techniques with fluorophore-labeled hairpins.[18] Such fluorophore-labeled hairpins, termed "molecular beacons", are of interest for diagnostic applications.[19] For our studies, when the DNA hairpin is immobilized on the electrode, the five 3′- and 5′-terminal nucleobases form a stem that places the 5′-terminal redox probe in close proximity to the electrode surface, thereby making it electrochemically accessible. Upon binding of the complementary DNA strand to the exposed loop portion of the immobilized sequence, the hairpin will open to the extended-duplex form; this increases the distance between the 5′-terminal ferrocene and the electrode surface. The increased distance between the redox probe and the electrode is manifested as a loss of the electrochemical signal (Scheme 1).

Results and Discussion

The bifunctionalized 26-mer oligodeoxynucleotide was synthesized by using a 3′-hexylthiol-modified controlled-pore glass (CPG) resin and a ferrocene phosphoramidite on an ABI 392 solid-phase DNA synthesizer.[17] The probe sequence contained within this hairpin motif is complementary to a sequence char-

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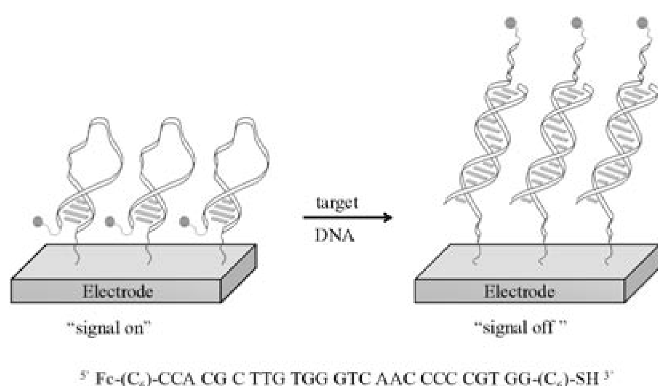

Scheme 1. Ferrocene-labeled hairpin for electrochemical DNA hybridization detection. A Fc-hairpin-SH macromolecule is immobilized on a gold electrode. When a complementary DNA target strand binds to the hairpin, the hairpin opens, and the ferrocene redox probe is separated from the electrode; this leads to a decrease in the observed current. The Fc-hairpin-SH sequence is shown at the bottom. The underlined bases indicate the stem-forming region.

acteristic for *Mycobacterium tuberculosis*.[20] The UV-visible spectrum of the ferrocenyl hairpin possesses a weak absorbance band at ≈450 nm, characteristic for ferrocene compounds ($\varepsilon <$ 1000 $M^{-1}cm^{-1}$).[21] Melting-temperature studies confirm that the 26-mer DNA strand forms a DNA hairpin in the absence of target DNA, with a melting temperature of 45 °C. The Fc-hairpin:target DNA duplex showed a melting temperature of 62 °C. As expected, the extended duplex is more stable than the hairpin. The circular dichroism spectrum of the Fc-hairpin-SH oligonucleotide shows modest transitions at the wavelengths characteristic for B-form DNA, Figure 1; this suggests formation of a stem-loop structure in the DNA hairpin. Upon binding of target DNA, the molar ellipticity increases; this indicates duplex formation.

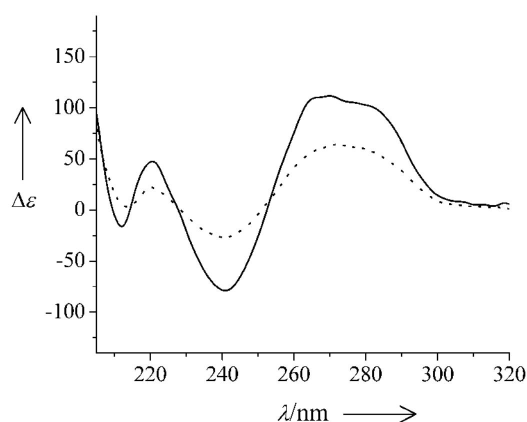

Figure 1. Circular dichroism spectra of Fc-hairpin-SH (····) and Fc-hairpin-SH + target sequence (——).

A gold-ball electrode was coated with the bifunctionalized oligodeoxynucleotide, Fc-hairpin-SH, and β-mercaptoethanol (the β-mercaptoethanol to Fc-hairpin-SH ratio was 10:1). Cyclic voltammograms (CV) of the Fc-hairpin-modified gold electrodes in 1 M $NaClO_4$ show a quasireversible wave at 0.482 V (vs. a normal hydrogen electrode (NHE)). The measured potential of the Fc-hairpin-SH is consistent with previous reports of ferrocene derivatives in well-defined self-assembled monolayers.[22] The electrochemical behavior of the immobilized Fc-hairpin-SH was also investigated by using alternating-current voltammetry (ACV). Of the electrochemical techniques available to study redox events in monolayers, ACV has been particularly effective.[23] Voltammetry with uncoated or β-mercaptoethanol-coated gold-ball electrodes yielded no electrochemical response. Likewise, immobilized hairpins containing no ferrocene also showed no current response within the potential window. The peak potential was determined to be 0.480 V (vs. NHE), in agreement with cyclic-voltammetry experiments. The peak-current-to-baseline ratio decreased with increasing AC frequency (Figure 2a), as previously seen for immobilized ferrocenyl alkanethiol monolayers.[24,25] The greatest current response was observed at a frequency of 1 Hz. From the integrated area of the AC voltammogram, the average surface coverage of electroactive ferrocene-labeled hairpin was calculated to be 2.55×10^{-12} mol cm^{-2}. The majority of the surface is covered with β-mercaptoethanol.

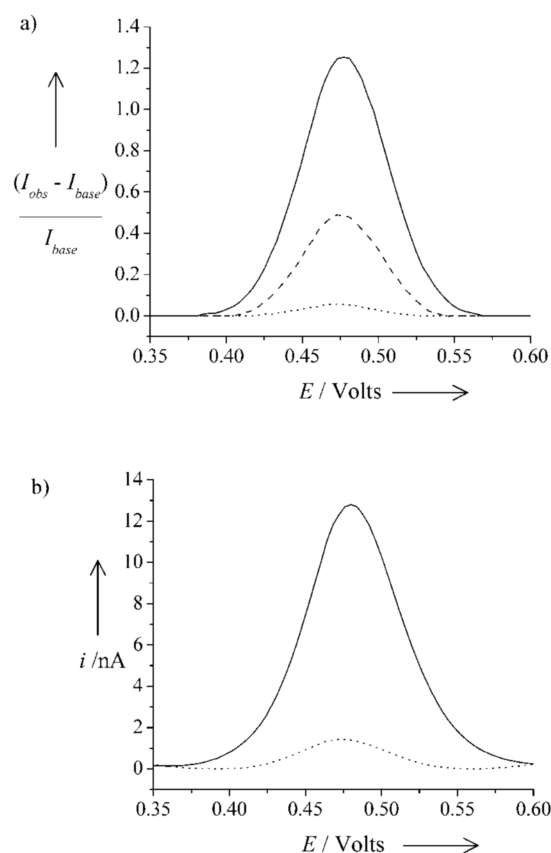

Figure 2. a) AC voltammograms of immobilized Fc-hairpin at 1 Hz (——), 10 Hz (-----), and 100 Hz (····). b) AC voltammograms at 1 Hz before (——) and after (····) addition of 1 μM target DNA. Conditions: 1 M $NaClO_4$, amplitude = 25 mV, sample period = 1 s.

The ACV signal diminishes when target DNA is added to the electrochemical cell. Incubation in the presence of 1 μM target

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DNA affords a loss of >90% of the electrochemical signal after 1 hour (Figure 3b). Probe sensitivity is improved at lower temperatures. For example, at 5°C the electrochemical response decreases by ~70% within 10 minutes following addition of 10 nM target DNA to the electrochemical cell (data not shown). Addition of 10 μM random, noncomplementary DNA sequence has no effect on the current response. When complementary DNA containing a single-base mismatch is added, the probe current decreases by ~30% after 1 hour. These results suggest that single-base-mismatch detection may be possible, since such discrimination is observed with optimized fluorescence-based systems.

Moiroux and co-workers have reported that immobilized single-stranded DNA containing a terminal ferrocene also gives well-defined electrochemistry, and that the scan rate dependence is consistent with a surface-confined electrochemical reaction.[13] To eliminate the possibility that our electrochemical signals arise from disordered single-stranded DNA and to verify the formation of the immobilized stem-loop structure on the gold surfaces, we characterized self-assembled monolayers of Fc-hairpin-SH on Au(111) surfaces. Studies of DNA duplex monolayers by AFM have been previously reported by Kelley and co-workers.[26] AFM measurements of the Fc-hairpin-SH immobilized on a Au surface showed that the distance from the gold surface to the top of the adsorbed hairpin monolayer was ~28 Å. Incubation of the sample in the presence of target DNA increases the height of the monolayer to ~42 Å, as shown in Figure 3. By assuming that the thiol linker is fully extended and collinear with the DNA, the length of the immobilized DNA hairpin and the extended duplex are estimated to be 54.8 Å and 80.5 Å, respectively.[27] Monolayer heights of 28 and 42 Å correspond to orientations ~32° normal to the gold surface; this is consistent with previous studies of DNA monolayers. Ellipsometric measurements of immobilized Fc-hairpin on a gold surface gave thicknesses of 29.1±0.4 and 41.6±0.5 Å after target binding. These data, combined with the CD spectra and T_m measurements, support the formation of the immobilized stem-loop structure on the Au surface, and the extended duplex upon addition of target DNA.

As mentioned earlier, electrochemical detection systems based on a large structural rearrangement of the probe strand provide a new approach for gene detection. Recently Fan et al. have described immobilized hairpin DNAs on flat Au electrode surfaces for gene detection.[12] They observed, as we have, that the electrochemical signal was strongly dependent upon the presence of target DNA and that the peak currents were directly proportional to the scan rates. Optimization of the system enabled detection at 10 pM; this indicated that the approach has potential real-world applications. In addition, DNA hairpins of varying sizes can be prepared as done previously for fluorescence-based assays with molecular beacons.[28] Our results are consistent with their findings. Importantly, our solution characterization studies of the DNA probe and target sequences as well as the surface characterization studies on the hairpin and duplex strands provide support for the detection mechanism, which entails a target-strand induced structural rearrangement on the electrode surface. These combined detection results and mechanistic data support the development of new approaches, surface immobilization chemistries, and probes for the detection of DNA.

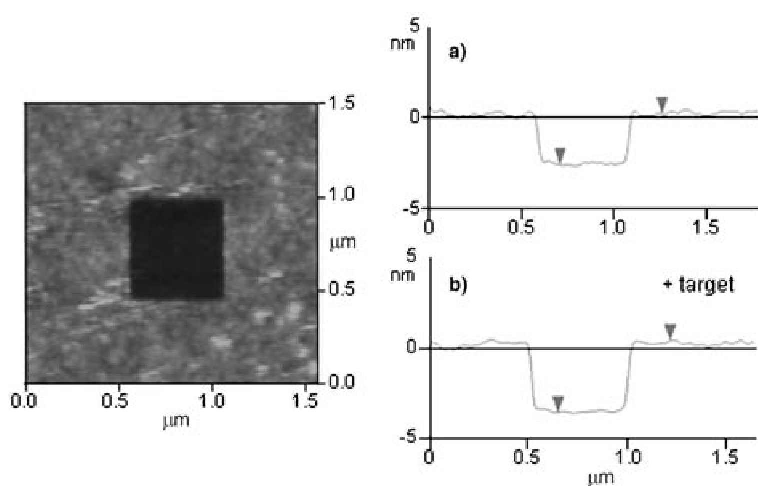

Figure 3. AFM cross sections of immobilized Fc-hairpin-SH monolayers a) before and b) after addition of target DNA.

Conclusion

In summary, the detection signal originates from the electroactive ferrocene probe, which is covalently attached to one stem of the DNA hairpin, and this signal is dependent on the DNA conformation (i.e., hairpin vs. extended duplex). Upon duplex hybridization, the distance between the ferrocene and the electrode surface increases, and the current associated with oxidation of the tethered probe diminishes. This approach does suffer from several limitations including a lack of single-base mismatch discrimination, nontrivial immobilization procedures, and that detection is recorded as a loss of signal (i.e., "off-sensor"). Importantly, however, this design concept does complement existing electrochemical detection methods and provides further motivation for chemical and biological devices based on conformationally gated sensing.

Experimental Section

All oligodeoxynucleotides were synthesized from 3′- to 5′-end on an ABI 392 solid-phase DNA synthesizer by using previously described methods.[17] The modified DNA (Fc-hairpin-SH) was synthesized by modifying the CPG resin with a C_6-thiol linker (Glen Research). The 5′ end was modified with ferrocenyl phosphoramidite by using the method previously described by Mirkin and co-workers.[16] The 26-mer modified DNA sequence (5′-Fc-C_6-CCACGCTTGTGGGTCAACCCCCGTGG-C_6-SH-3′) was purified by HPLC (Varian, Prostar) with triethylamine acetate (TEA)/CH_3CN and characterized by UV/Vis spectroscopy, MALDI-TOF mass spectrometry, and CD. The target (5′-GGGGTTGACCCACAAG-3′), random sequence (5′-CCCAATCTGGATGTTA-3′), and mismatch DNA (5′-

GGGGTCGACCCACAAG-3′) were synthesized, purified on PolyPak cartridges (Glen Research), and characterized by MALDI-TOF mass spectrometry. Hybridization of complementary strands to form duplexes was accomplished by heating the DNA strands to 90 °C in a heating block and allowing them to cool to room temperature over a period of 1 h. Melting temperatures (T_m) of the duplexes were determined by using a Varian 500 spectrophotometer equipped with a Peltier temperature controller. Duplexes were typically < 2 μM (5 mM sodium phosphate, 50 mM NaCl, pH 7.0 buffer). The temperature was increased at a rate of 0.2 °C min^{-1}, while monitoring the absorbance at 260 nm. The melting temperatures were calculated by using standard procedures.

A CH Instruments 440 electrochemical analyzer was used in a standard three-electrode cell that consisted of a Pt-wire counter electrode, a Ag/AgCl reference electrode, and a gold-ball working electrode. The reference electrode was separated from the working compartment by a modified Luggin capillary filled with the analysis buffer. Gold-ball working electrodes were constructed by melting a gold wire (0.127 mm, Aldrich) in a H_2 flame until a small ball (~0.5 mm) formed on the end. Gold-ball electrodes were cleaned by multiple oxidation–reduction cycles in H_2SO_4 (1 M) at 20 mV s^{-1} until a well-defined voltammogram was obtained. The gold-ball electrodes were thoroughly washed with Milli-Q water, and soaked overnight in a DNA modification solution containing Fc-hairpin-SH (0.1 mM), β-mercaptoethanol (1 mM) in phosphate buffer (50 mM), and NaCl (100 mM). The electrodes were then washed with phosphate buffer (50 mM) and NaCl (100 mM) and transferred to the electrochemical cell. Electrochemical experiments were carried out at room temperature (22 ± 2 °C) in $NaClO_4$ solution (1 M) unless otherwise indicated.

AFM measurements were similar to those described by Kelley and co-workers.[26] They were conducted by using a Nanoscope IIIa multimode atomic force microscope (Digital Instruments) in either tapping or contact mode. Si_3N_4 cantilevers (MikroMasch) were used for all experiments. Au(111) substrates (~500 nm) were created by thermal vapor deposition onto Si(111) substrates by using a 10 nm Cr adhesion layer. Substrates were annealed in a hydrogen flame and then modified by incubation with 0.1 mM solutions of modified DNA in phosphate buffer (pH 7.0, 50 mM), $MgCl_2$ (0.1 M) for 12–24 h. For AFM experiments, β-mercaptoethanol was excluded from the DNA deposition solutions. The DNA-modified surfaces were thoroughly rinsed with incubation buffer prior to AFM measurements. In a typical experiment, a small (500 nm) patch of DNA was removed from the surface by applying a large vertical force and increasing the scan rate to 25 Hz with the AFM tip in contact mode for 1 min. The surface was then imaged (2×2 μm) in contact mode at a scan rate of 2–4 Hz by using the smallest possible vertical force. Samples imaged in tapping mode gave similar results.

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