Chapter 3

Electrochemical Patterning and Detection of DNA Arrays on a Two-Electrode Platform

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A. Furst synthesized DNA and collected and analyzed all data presented. S. Landefeld and M. G. Hill performed preliminary experiments with the platform.

Introduction

Nucleic acid sensors are critical for the detection of many biological markers of disease. An important diagnostic goal is the development of arrayed, multiplexed sensors, enabling different markers to be tested in parallel with high accuracy. Although fluorescence-based arrays have proven useful for high-throughput screening applications,[1, 2] they generally do not provide the accessibility, ease of use, and unambiguous readout required for clinical diagnostics.

DNA-based electrochemistry offers a promising alternative technology.[3-6] In particular, assays that capitalize on DNA-mediated charge transport (DNA CT) enable selective detection of protein binding events, hybridization, and mismatches and lesions, with near on/off specificity.[7-18] This level of specificity is possible largely because DNA CT-based assays do not rely on the subtle thermodynamic differences of duplex hybridization for the determination of mismatches or lesions, thus eliminating the need for stringent hybridization conditions required for many other DNA-based technologies.[19-21] Instead, DNA CT-based assays depend on the integrity of the π-stack of the nucleobases; disruption of the π-stacking by protein binding or mismatch incorporation attenuates DNA CT.

DNA-modified electrodes are generally prepared by the self-assembly of thiol-labeled duplexes onto gold, followed by passivation of the uncovered electrode surface with an alkylthiol.[22] This method of assembly offers little control over the density and dispersion of the DNA within the film.[23-26] Alternative assembly methods based on the preliminary formation of a mixed alkylthiol monolayer containing active head groups, followed by coupling of the DNA to this monolayer, offer a promising alternative

assembly method that provides more control over the final composition of the DNA monolayer.[27]

Independent of assembly conditions, electrochemical DNA sensors typically rely on electrochemical readout from the surface onto which the monolayers are assembled, providing a measurement of only bulk changes that occur over the entire electrode. Steps toward more complex detection with DNA-modified electrodes have been made through multiplexing, which enables multiple experimental conditions to be run simultaneously with redundancy.[28-30] Yet even these platforms measure average changes over an entire electrode surface. Moreover, comparing individual, isolated electrodes can be misleading, as small variations between monolayer conformations can lead to substantial differences in the electrochemical properties of the resulting DNA monolayer.

A two-electrode detection system enables the determination of more specific spatial information on a single substrate electrode surface. The most widely used two-electrode technique is scanning electrochemical microscopy (SECM).[31-34] This technique has previously been used to detect oligonucleotide hybridization events on DNA-modified surfaces.[35-37] We have previously shown that SECM of high-density DNA-modified electrodes allows for the identification of small imperfections on a surface that can lead to falsely high electrochemical signals when the bulk electrochemical response is measured.[15] Here, we report a simplified, macroscopic two-electrode detection system for analysis of DNA arrays composed of multiple DNA sequences assembled on a single substrate electrode surface.

Our arrays are formed through selective electrochemical patterning of multiple DNA sequences onto a single electrode surface containing a pre-formed mixed

monolayer. Electrochemical readout is then accomplished via amperometric detection at a spatially isolated probe electrode controlled by a bipotentiostat. Because multiple DNA sequences are patterned onto a single substrate, different sequences can be examined under identical experimental conditions. With our assay, we now have the ability to incorporate both redundancy and internal controls onto the same electrode surface.

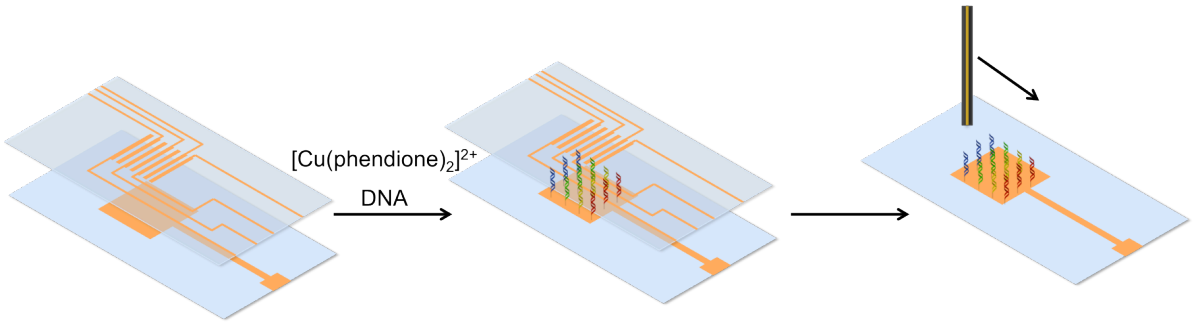

Figure 3.1 Two working electrode patterning and readout platform. DNA arrays are formed electrochemically through the activation of an inert copper complex to an active catalyst from a secondary set of electrodes. Using the current configuration, up to four individual sequences of DNA are patterned onto a single substrate electrode. Subsequent readout of the sequences on the surface is accomplished by manual scanning of a microelectrode across the secondary electrode.

Materials and Methods

DNA Synthesis and Purification

Oligonucleotides were synthesized on an Applied Biosystems 3400 DNA synthesizer. Terminal C6 alkyne moieties were incorporated into the 5' end of one of the strands and were purchased from Glen Research. Complementary unmodified strands were also synthesized. Preparation of all of the oligonucleotides followed a reported protocol. Each oligonucleotide was purified by high-performance liquid chromatography (HPLC) using a gradient of acetonitrile and 50 mM ammonium acetate. Following purification, oligonucleotides were desalted by ethanol precipitation and quantified using ultraviolet-visible spectrophotometry based on their extinction coefficients at 260 nm (IDT Oligo Analyzer). The following sequences were prepared: well matched: 5'-CC-$(CH_2)_6$-GCT CAG TAC GAC GTC GA-3' with its unmodified complement, a mismatch-containing sequence with a CA mismatch at the 9th base pair, and a TBP-binding sequence: 5'-CC-$(CH_2)_6$-GGC GTC TAT AAA GCG ATC GCG A-3' with its unmodified complement.

Preparation of Slides

Gold evaporation was accomplished using aluminum masks and a CVC Metal Physical Vapor Deposition system. Glass slides were coated in MPS ([3-mercaptopropyl]-trimethoxysilane). Slides were cleaned by boiling at 70° C for 10 minutes in Piranha solution (1:4 hydrogen peroxide: sulfuric acid), followed by baking for 10 minutes. Slides were then boiled in a 1:1:40 MPS: water: isopropyl alcohol solution for 10 minutes and then cured at 107° C for 8 minutes. A 150 nm gold

monolayer was formed on the slides with either the patterning or the substrate pattern using aluminum masks and a CVC Metal Physical Vapor Deposition system with 0.5 mm diameter gold wire.

DNA Patterning

Degassed $[Cu(phendione)_2]^{2+}$ was used as the inert catalytic precursor because of its aqueous solubility. $[Cu(phendione)_2]^{2+}$ was prepared by combining 1,10-phenanthroline-5,6-dione (Sigma Aldrich) (294.3 mg, 1.4 mmol) with $CuSO_4$ (Sigma Aldrich) (111.7 mg, 700 μmol) in 5 mL of deionized water. ESI-MS: 580.2 (calc: 580.0). The complex was isolated as the PF_6^- salt or used directly *in situ*. Before application to the electrode surface, the complex was diluted to a final concentration of 50 μM in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) and thoroughly degassed. When a sufficiently negative potential is applied to this compound, Cu(II) is reduced to Cu(I), generating an active catalyst. A BAS Epsilon bipotentiostat was used both to apply potentials and record data. A constant potential was applied to the patterning electrodes that was sufficiently negative to continuously activate the copper at that location. For the constant applied potential, -250 mV v. AgCl/Ag was used, and application was allowed to proceed for 15 minutes. The 100 μM catalyst (20 μL) with 20 μL of 50 μM DNA in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) was used to pattern each strip. Residual catalyst interaction with DNA was a concern, but no change in duplex melting temperatures in the presence of catalyst was observed. Neither protein binding nor hybridization was affected by catalyst.

Scanning Across Surface

The height of the probe electrode (z) was adjusted manually by lowering the electrode onto a 100-um teflon spacer placed on the corner of the substrate pad. No attempt was made to control for drift; while the measured currents were remarkably consistent for each substrate pad, the absolute signals varied somewhat from substrate to substrate. Electrochemical images of substrate surface are presented as scans from left to right. In all cases, the scanning origin is indicated by a distance of 0 on the x-axis, and the distance increases positively in the direction of scanning. Additionally, multiple scans can be obtained at different locations (y) on the substrate pad. Variation in current between different locations on the substrate pad enabled calculation of standard deviation.

TATA Binding Protein Experiments

TATA-Binding Protein (TBP) was purchased from ProteinOne and stored at -80° C until use. MicroBiospin 6 columns (BioRad) were used to exchange the shipping buffer for Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). Prior to electrochemical measurements with TBP, electrodes were incubated with 1 μM Bovine serum albumin (BSA) for 30 min, followed by rinsing with Tris buffer. TBP was titrated onto the surface in a range of 1 μM to 25 μM protein, with each concentration allowed to incubate for 15 minutes prior to scanning.

Hybridization Experiments

After a preliminary scan of duplex DNA on the electrode surface, dehybridization was induced through the heating of the surface in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) to 65°C for fifteen minutes. Subsequently, the surface was rinsed with 65°C phosphate buffer (pH 7.0). The complementary strand (25 μL of 50 μM strand) was then added to the surface and incubated for one hour while the surface cooled to ambient temperature. The surface was subsequently scanned again.

Results

Both substrate and patterning surfaces were prepared from glass microscope slides coated with MPS ([3-mercaptopropyl]-trimethoxysilane) using an established protocol.[38] MPS acts as a molecular adhesive between the glass microscope slide and a gold monolayer, forming an attachment to the glass through silanization and a bond to the gold through a thiol-metal bond. A gold layer (150 nm thick) was then deposited onto the slides in either a series of lines (the patterning surface) or a square (the substrate electrode; Figure 3.2), using aluminum masks and a CVC Metal Physical Vapor Deposition system. Mixed monolayers were formed on the substrate surfaces using an ethanolic solution of 1M 12-azidododecane-1-thiol (C_{12} thiol azide) and 1M 11-mercaptoundecylphosphoric acid, which forms a monolayer capable of passivation against ferricyanide and methylene blue, electrochemical reporters of DNA CT. Alkynyl-labeled DNA was then patterned onto the substrate by sandwiching 0.1 mM $[Cu(phendione)_2]^{2+}$ (phendione=1,10-phenanthroline-5,6-dione) and the DNA solution of choice between the substrate and patterning pads separated by a thin (200 μm) Teflon spacer (unpublished results). Subsequent electrochemical reduction of $[Cu(phendione)_2]^{2+}$ at specific working electrodes on the patterning pad results in the covalent attachment of DNA sequences to specific locations on the substrate pad *via* Cu(I)-catalyzed azide/alkyne coupling. Multiple DNA sequences are patterned on the same substrate electrode through sequential washings of the surface and the addition of an alternate sequence of DNA with copper catalyst between the substrate and patterning pads. The strategy for the spatially resolved electrochemical activation of the catalyst is shown in Figure 3.3. While the substrate electrode was washed after patterning, residual

catalyst interacting with DNA was a concern. However, no change in DNA duplex melting temperatures in the presence of catalyst was observed (data not shown). Moreover, protein binding and hybridization were unaffected by catalyst, indicating that residual copper catalyst is not of great concern.

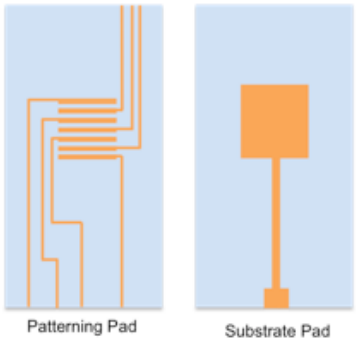

Figure 3.2 Design for patterning electrodes and substrate electrode. The patterning electrodes pad *(left)* contains four working electrodes that are individually-addressable interspersed with three reference electrodes. The substrate electrode pad *(right)* contains a single, large gold pad and a working electrode contact to the pad.

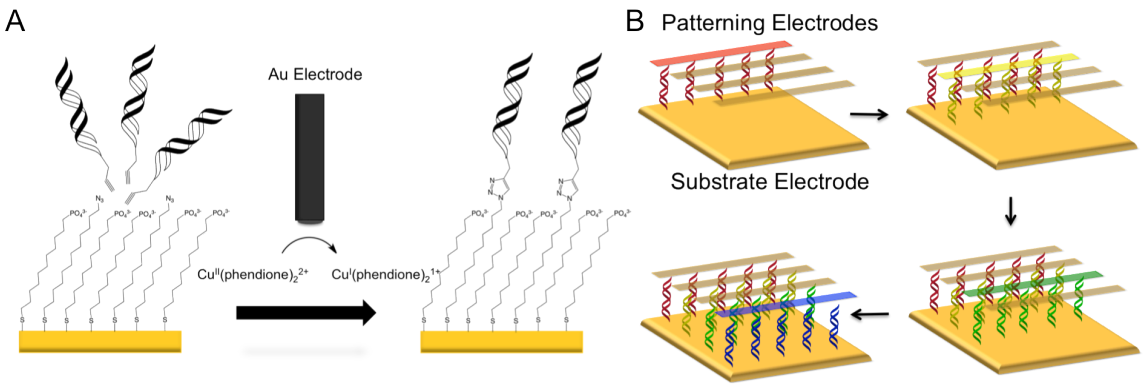

Figure 3.3 Selective activation for specific covalent attachment of DNA to particular locations. (A) An inert Cu(II) catalyst is electrochemically activated to an active Cu(I) species capable of catalyzing the [3+2] azide-alkyne cycloaddition between alkyne-modified DNA and an azide-terminated thiol monolayer. (B) Up to four different sequences of DNA can be patterned onto a single substrate pad through sequential catalyst activations.

Covalent attachment of DNA was confirmed by cyclic voltammetry (CV) at the substrate pad in the presence of 200 μM ferricyanide and 2 μM methylene blue (MB). Cyclic voltammetry at the substrate pad (i.e., from the bottom of the electrode surface) reveals a large, irreversible reduction peak at ~ -0.4 V, characteristic of DNA-mediated electrocatalysis of ferricyanide by methylene blue (Figure 3.4).[39] Although this voltammogram confirms that DNA is present on the surface, it provides no information on either the homogeneity of the DNA on the surface or the sequences of DNA present. Indeed, the bulk electrocatalytic signal in Figure 3.4 was obtained from a pad patterned with two strips of well-matched DNA and two strips of DNA containing a mismatch. Thus, while single-electrode measurements can provide some information about changes on a surface, they report only the bulk response averaged over the entire electrode, giving no information about the types of DNA on the surface, and providing no spatial resolution.

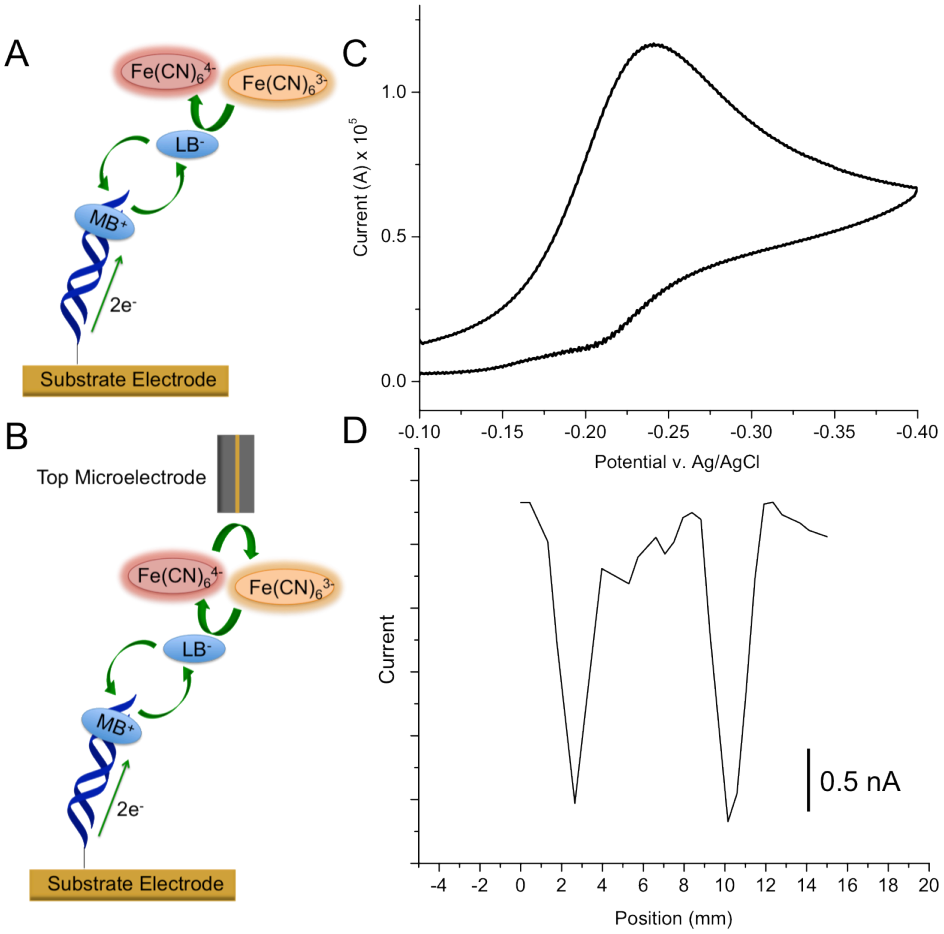

Figure 3.4 Comparison between bulk and spatially-defined electrochemical measurements. (A) One-electrode electrocatalysis. For well-paired DNA, methylene blue (MB) is reduced to leucomethylene blue (LB) through the DNA by the substrate electrode. LB can be reoxidized in solution to MB by ferricyanide, which is thereby reduced to ferrocyanide. (B) Two-electrode electrocatalysis. The second electrode functions both as a detector and a method of reoxidation of ferrocyanide to ferricyanide, thereby accelerating the electrocatalytic process described in (A). (C) Electrochemical data from a patterning pad containing two strips of well-matched DNA and two strips of DNA containing a single base mismatch. Electrochemical signals were obtained from electrocatalysis of 2 μM methylene blue and 200 μM ferricyanide in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). The bulk electrochemical signal from the measurement of the substrate pad shows a classic electrocatalytic peak, indicating the presence of well-matched DNA on the electrode. (D) Electrochemical data from the two-electrode detection method. With this technique, the existence of two different sequences of DNA, one completely matched and one containing a mismatch, becomes apparent. The surface was scanned at ~0.6 mm/sec with a 100 μm gold microelectrode.

A 100-μm gold electrode positioned above the substrate electrode by a simple x,y,z-stage was subsequently employed as a secondary electrode to create a two-electrode detection system with spatial resolution for readout. DNA-mediated reduction of ferricyanide (via methylene blue electrocatalysis) results in the presence of ferrocyanide immediately above areas on the substrate electrode coated with intact DNA duplexes, and this ferrocyanide is readily detected at the probe (Figure 3.4). Thus measuring ferrocyanide oxidation at the microelectrode tip allows for spatial differentiation between the passivating layer and regions containing DNA on the substrate electrode. Notably, this method provides reproducible current outputs for multiple strips of a single DNA sequence (Figure 3.5), demonstrating a high level of reproducibility; the standard deviation for peak currents of DNA of the same sequence is 95 pA, or 1.5%. It should be noted that the full width half max of the DNA peaks is ~1 mm, the same width of the patterning electrodes, indicating minimal diffusive spreading of the catalyst upon activation. It should be noted that the width of the peaks depends on the speed of scanning, which was optimal at ~0.6 mm/sec. Some inconsistencies are observed because the scanning was performed manually. The dramatic attenuation of catalytic turnover at substrate locations featuring a single-base mismatch within the immobilized DNA is characteristic of a DNA-mediated process (Figure 3.4).

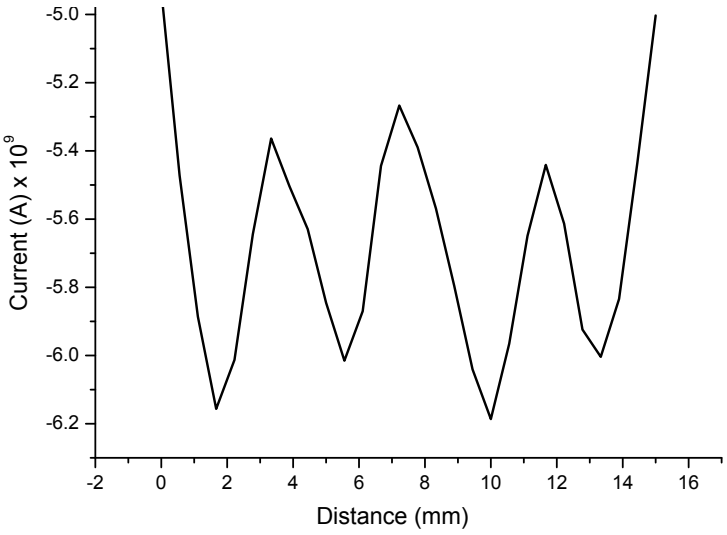

Figure 3.5 Patterning of a pad containing four strips of well-matched DNA. This was measured in 2 uM methylene blue and 200 uM ferricyanide in Tris buffer (10 mM Tris, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6). As can be seen, the size and shape of the signals resulting the patterning of the same sequence four times is exceptionally consistent.

As the ultimate goal of this DNA-array technology is to detect biomolecules (e.g., proteins and nucleic acids), sensitive detection of proteins is essential. Protein binding was tested on this platform using TATA-binding protein (TBP), a subunit of the eukaryotic TFIID transcription factor, which kinks DNA by over 80° when bound to its TATA target sequence.[40] The K_D of this protein is in the nanomolar range (~3.3 nM), and detection limits near this concentration are ideal for TBP detection from biological samples. We have shown previously that TBP binding to duplexes containing a TATA sequence leads to attenuated CT, but does not affect electrocatalysis at duplexes lacking the TBP binding site.[15] Therefore, to test for selective TBP binding, we patterned strips of both TBP-binding sequences and non-binding sequences. Upon addition of 15 nM TBP to a substrate electrode patterned with two strips of well-matched DNA and two strips of TBP DNA, an almost complete loss of electrochemical signal occurs only at the location of the TBP sequences (Figure 3.6).

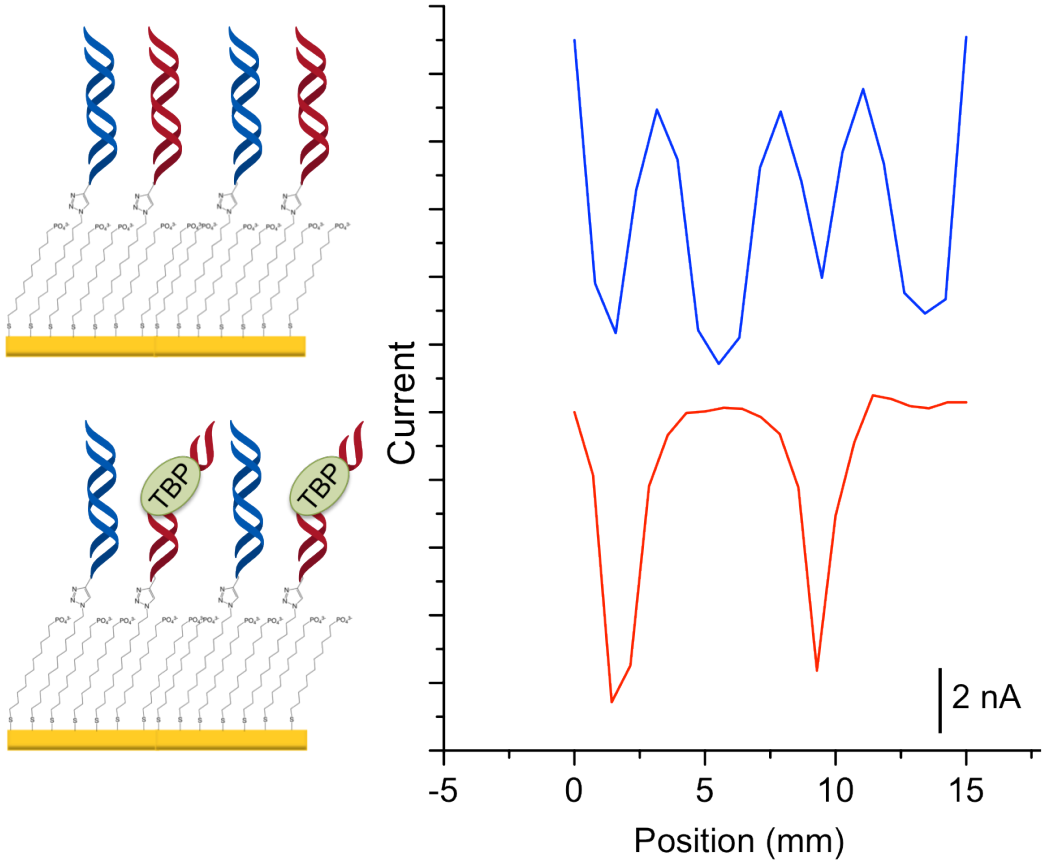

Figure 3.6 TBP detection on a patterned surface. Current increases negatively down the y-axis. The surface was patterned with two strips of well-matched DNA and two strips that contain a TBP binding site. The blue trace is a preliminary scan in 2 μM methylene blue and 200 μM ferricyanide before the addition of TBP but after a 30 minute incubation in 100 μM BSA to control for non-specific protein binding. The red trace is a scan after a 15-minute incubation with 15 nM TBP protein; the current corresponding to the TBP-binding sequences is reduced, while the locations corresponding to the well-matched sequence are unaffected.

This same detection strategy can also be employed to detect selective DNA hybridization. Two strips of well-matched DNA and two strips of DNA containing a CA mismatch were patterned onto an electrode surface. Electrochemically imaging the substrate from the top of the monolayer using the microelectrode via MB-catalyzed ferricyanide reduction yields the expected pattern of alternating high and low currents at the probe tip (Figure 3.7). The electrode was then dehybridized by heating the substrate pad in 65°C buffer for 15 minutes. A strand of DNA fully complementary to the alkynyl strand that was part of the original mismatch-containing duplex was subsequently incubated on the surface for 1 hour, resulting in the formerly mismatched sequences being well-matched and vice-versa. Rescanning the substrate electrode revealed almost complete reversal of signal locations, indicating that the majority of the DNA helices on the surface were dehybridized and rehybridized to an alternate complement (Figure 3.7). The ability to detect both protein-binding and hybridization events on the same platform highlights the utility of this DNA array for broad applications.

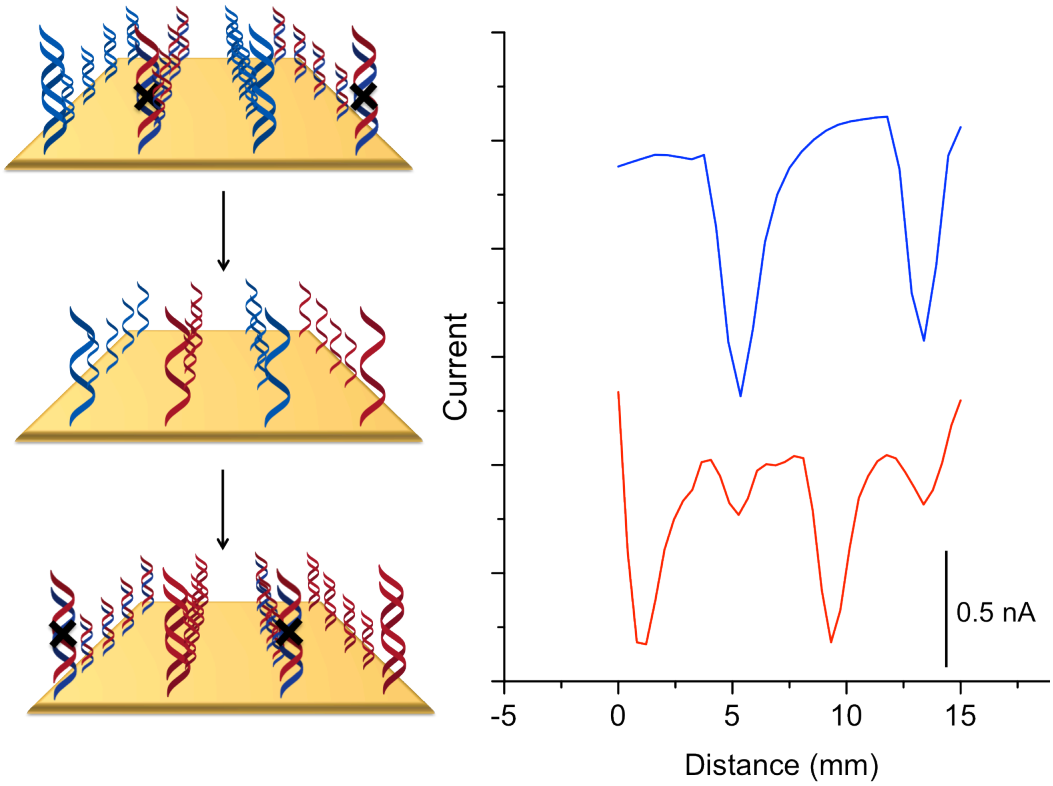

Figure 3.7 Oligonucleotide detection through dehybridization and hybridization. Current increases negatively down the y-axis. The surface was patterned with two strips of well-matched DNA and two strips that contain a mismatch. The blue trace is a preliminary scan in 2 μM methylene blue and 200 μM ferricyanide before dehybridization. The surface was subsequently soaked in phosphate buffer (5 mM phosphate, 50 mM NaCl, pH 7.0) at 65° C for 30 minutes. Single stranded oligonucleotides complementary to the formerly mismatched sequence heated to 65° C were added and allowed to cool to room temperature over 1 hour. The red trace shows the post-rehybridization data, where the mismatched sequences are now well-matched and the formerly well-matched now mismatched.

Discussion

Incorporating low-density DNA monolayers into a two-electrode platform enables sensitive detection of protein binding to DNA, as well as hybridization events, with spatial resolution on a surface. Multiple DNA probe sequences can be accurately grafted onto a single, azide-terminated substrate using readily available alkyne-labeled duplexes and a copper complex that is electrochemically activated to initiate click coupling. Exploiting DNA CT, electrochemical readout is inherently more sensitive and selective compared to assays that rely on electrostatic interactions of probe molecules to the phosphate backbone. Our platform effectively differentiates between fully complementary duplexes versus those that contain single-base mismatches, making it ideally suited for assays based on hybridization events. Using DNA CT, hybridization events are distinguished not based on minor thermodynamic differences of annealing but instead on the existence of a fully formed, entirely complementary duplex.

The ability to graft multiple DNA sequences onto a single substrate enables both incorporation of internal controls and a greatly simplified and standardized experimental platform. Unlike conventional multiplexed systems, our array requires no separate wells on the surface. This translates to the addition of a single sample of interest over the entire array simultaneously, ensuring identical experimental conditions for detection and reducing the necessary sample volume. Moreover, the single substrate electrode enables significantly more control over the incorporation of internal standards and makes comparisons between types of DNA on the array possible. This single-substrate platform also guarantees identical underlying monolayer conformations over the entire surface, leading to comparable DNA monolayers at different patterning sites.

The inherent advantages of DNA CT-based electrochemical readout coupled to a two-electrode fabrication/detection platform have resulted in the successful detection of well-matched and mismatched DNA on the same substrate electrode, as well as the selective binding of a DNA transcription factor. Oligonucleotide detection is easily accomplished with specificity, as illustrated through the switching of the location of mismatched strands on the substrate surface. This represents a completely new, sensitive biosensing platform, taking advantage of a two-electrode setup for both array formation and detection.

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