

Single-base mismatch detection based on charge transduction through DNA

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ABSTRACT

High-throughput DNA sensors capable of detecting single-base mismatches are required for the routine screening of genetic mutations and disease. A new strategy for the electrochemical detection of single-base mismatches in DNA has been developed based upon charge transport through DNA films. Double-helical DNA films on gold surfaces have been prepared and used to detect DNA mismatches electrochemically. The signals obtained from redox-active intercalators bound to DNA-modified gold surfaces display a marked sensitivity to the presence of base mismatches within the immobilized duplexes. Differential mismatch detection was accomplished irrespective of DNA sequence composition and mismatch identity. Single-base changes in sequences hybridized at the electrode surface are also detected accurately. Coupling the redox reactions of intercalated species to electrocatalytic processes in solution considerably increases the sensitivity of this assay. Reporting on the electronic structure of DNA, as opposed to the hybridization energetics of single-stranded oligonucleotides, electrochemical sensors based on charge transport may offer fundamental advantages in both scope and sensitivity.

INTRODUCTION

DNA-based sensors have potential applications that range from genomic sequencing to mutation detection and pathogen identification (1–4). Biochemical assays (5–10), traditional separation methods (11), gravimetric analyses (12–15) and spectroscopic probes (16–23) have all been employed in the construction of DNA biosensors. Indeed, sophisticated analytical schemes employing high-resolution microscopy to assay the hybridization of DNA target sequences with arrays of immobilized single-stranded oligonucleotides have been developed for highly parallel genomic sequencing and the detection of mutations (16,17). Simpler electrochemical schemes have also been explored (24–35). In a typical assay, single-stranded probe sequences are immobilized on an

electrode, then treated with test DNA samples. If hybridization occurs, the electrochemistry of a positively charged redox-active reporter molecule [e.g. $\text{Co}(\text{phen})_3^{2+}$] added to the solution shows an enhanced response owing to its increased attraction to the more negatively charged duplex-modified surface.

All of these assays rely ultimately on molecular recognition events associated with DNA hybridization to catalog sequence information. Applied to base-mismatch detection, hybridization assays are inherently limited in sensitivity: detection of a point mutation in the test sequence (e.g. a small segment of genomic DNA) requires a distinguishable difference in pairing energies between the probe sequence and a completely complementary versus mutated target strand. With only a single mutation in an extended oligonucleotide, these differences can be very small. Moreover, duplex stabilities for oligonucleotides of a fixed length can vary considerably as a function of base content, with GC-rich sequences significantly more stable than AT-rich analogs. As a consequence, detection of point mutations within libraries of immobilized oligonucleotides (where duplex-binding energies for adjacent probe sequences may vary significantly more than the differential binding energies of a particular probe with its complementary versus mutated test sequences) requires extensive manipulation of hybridization conditions as well as sophisticated deconvolution algorithms.

Monitoring charge transport through double-stranded DNA offers an alternative approach to the detection of point mutations. Photoinduced electron transfer through donor/acceptor-labeled duplexes has been observed in a variety of systems (36–39), and efficient electrochemical reduction of redox-active molecules intercalated into the individual helices of double-stranded DNA films has been reported (40,41). Significantly, DNA-mediated reactions show a weak dependence on distance but are exceptionally sensitive to perturbations in the base stack: intervening bulges inhibit long-range photochemical guanine oxidation (42), and single-base mismatches markedly reduce photoinduced electron-transfer yields (37). Thus, while single-base mismatches may cause only subtle changes in duplex stability and structure (43,44), they appear to induce significant perturbations in the electronic structure of the base-pair stack. DNA-mediated charge transfer may therefore provide a complementary signaling mechanism for DNA-based sensors.

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The properties of DNA films and the electron-transfer reactions of intercalators bound to these monolayers have been explored previously (41–46). In studies of daunomycin (DM, a redox-active antitumor agent) site-specifically bound to immobilized DNA duplexes, efficient electron transfer was observed over DM/electrode separations up to 35 Å (41). Indeed, while not sensitive to distance, this reaction was dramatically attenuated by the presence of an intervening CA mismatch. This observation demonstrated that mismatches could be detected at a DNA-modified electrode, using the efficiency of DNA-mediated charge transport as a signaling device.

In these first studies, the intercalator binding site was controlled by site-specifically crosslinking DM to guanine residues in the duplex. Both to eliminate the time-consuming chemical crosslinking, and to avoid multiple labeling at GC base steps that are possible at any site along a potential genomic test sequence, a practical adaptation of this system would require (i) probes that were non-covalently bound to the DNA films; (ii) *in situ* hybridization of test sequences at the electrode surface; and (iii) large differences in the electrochemical responses of intercalators at electrodes featuring fully base-paired versus mismatched duplexes.

Here, we report the electrochemical detection of DNA mismatches using different redox-active intercalators non-covalently bound to DNA-modified surfaces. The response of intercalating probes associated with the films effectively reports the presence of a wide variety of mismatches. Moreover, single-base changes in sequences of varied base composition are detected with equal sensitivity, demonstrating an advantage of this detection approach over hybridization-based methods. Mismatch detection can also be performed at films formed by the reversible *in situ* hybridization of oligonucleotides to probe sequences immobilized on the surfaces, an important feature for the implementation of a practical assay. Furthermore, we have coupled direct electrode-intercalator electron transfer to an electrocatalytic cycle involving a non-intercalating substrate in solution. The resulting assay exhibits greatly enhanced differentiation between complementary versus mismatched duplexes, and allows the ready detection of point mutations in DNA oligonucleotides.

MATERIALS AND METHODS

Materials

All DNA synthesis reagents were obtained from Glen Research. DM was obtained from Fluka; methylene blue, potassium ferricyanide and ruthenium pentamine chloride were purchased from Aldrich and used as received.

Preparation of DNA-modified surfaces

Thiol-modified oligonucleotides were prepared as previously described (40,41,45); thiol-terminated linkers were attached to single-stranded oligonucleotides (47), which after stringent purification were hybridized to unmodified complements. The resultant duplexes were deposited on polycrystalline gold electrodes for 24 h. Before electrochemical measurements, the electrodes were rinsed thoroughly with 5 mM phosphate, 50 mM NaCl buffer (pH 7). As electrodes containing a high surface coverage of DNA were most useful for our experiments, surfaces were routinely assayed for coverage by

monitoring the attenuation of the oxidation of ferrocyanide (40). Comparable results were obtained with commercial polycrystalline electrodes (BAS) or Au(III) films vapor deposited on mica substrates (Molecular Imaging).

Electrochemical measurements

Cyclic voltammetry and chronocoulometry were carried out on 0.02 cm² gold electrodes using a Bioanalytical Systems (BAS) Model CV-50W electrochemical analyzer. A normal three-electrode configuration consisting of a modified gold-disk working electrode, a saturated calomel reference electrode (SCE, Fisher Scientific) and a platinum wire auxiliary electrode was used. The working compartment of the electrochemical cell was separated from the reference compartment by a modified Luggin capillary. Potentials are reported versus SCE. Volumes of 2.5 ml were typically employed. Unless specifically noted, all measurements were recorded at 20 ± 2°C in 5 mM sodium phosphate buffer containing 50 mM NaCl, pH 7 that had been thoroughly degassed with Ar.

RESULTS AND DISCUSSION

Electrochemical detection of single-base mismatches

As previously described (40,41,45), DNA-modified surfaces are prepared by the self-assembly of 15-bp duplexes derivatized at the 5'-end with a thiol-terminated aliphatic linker. Atomic-force microscopy studies have shown that the duplexes form densely-packed monolayers with the individual helices in an upright orientation with respect to the gold surface (45) (Fig. 1). Redox-active cations [e.g. Ru(NH₃)₆³⁺] and DNA intercalators bind strongly to the modified surfaces and yield well-behaved electrochemical signals; anions [e.g. Fe(CN)₆⁴⁻] and non-binding neutral species (e.g. dimethylaminoferrrocene) do not associate with the electrodes and are electrochemically silent (40).

Detection of mismatches using non-covalently bound DM. The electrochemistry of DM at electrodes modified with the duplex 5'-AGTACAGTCATCGCG [where C indicates the location of a CA mismatch; all sequences are labeled at the 5' end of sequence with the linker of the formula SH-(CH₂)₂CO-NH(CH₂)₆NHCO-] is shown in Figure 2. The integrated charge due to the reduction of DM at fully base-paired films indicates an intercalator-to-DNA binding stoichiometry of ~1:1 (40,45). Because this ratio is far smaller than that predicted by neighbor exclusion, it is likely that DM binds predominantly near the solvent-exposed terminus of the film, with diffusion into the monolayer inhibited by the tight packing of the DNA helices. Similar results were obtained in a previous study where methylene blue was non-covalently bound to DNA monolayers (40). Here methylene blue showed the same binding affinity but lower stoichiometry at saturation when compared with neighbor excluded binding to the same DNA duplexes in solution. When films with lower surface coverage are used, a larger signal is obtained for the redox-active DNA intercalator, which is likely due to increased access to the interior of the film. Thus, even without covalent crosslinking, intercalators appear to be somewhat constrained to the top of densely-packed monolayers.

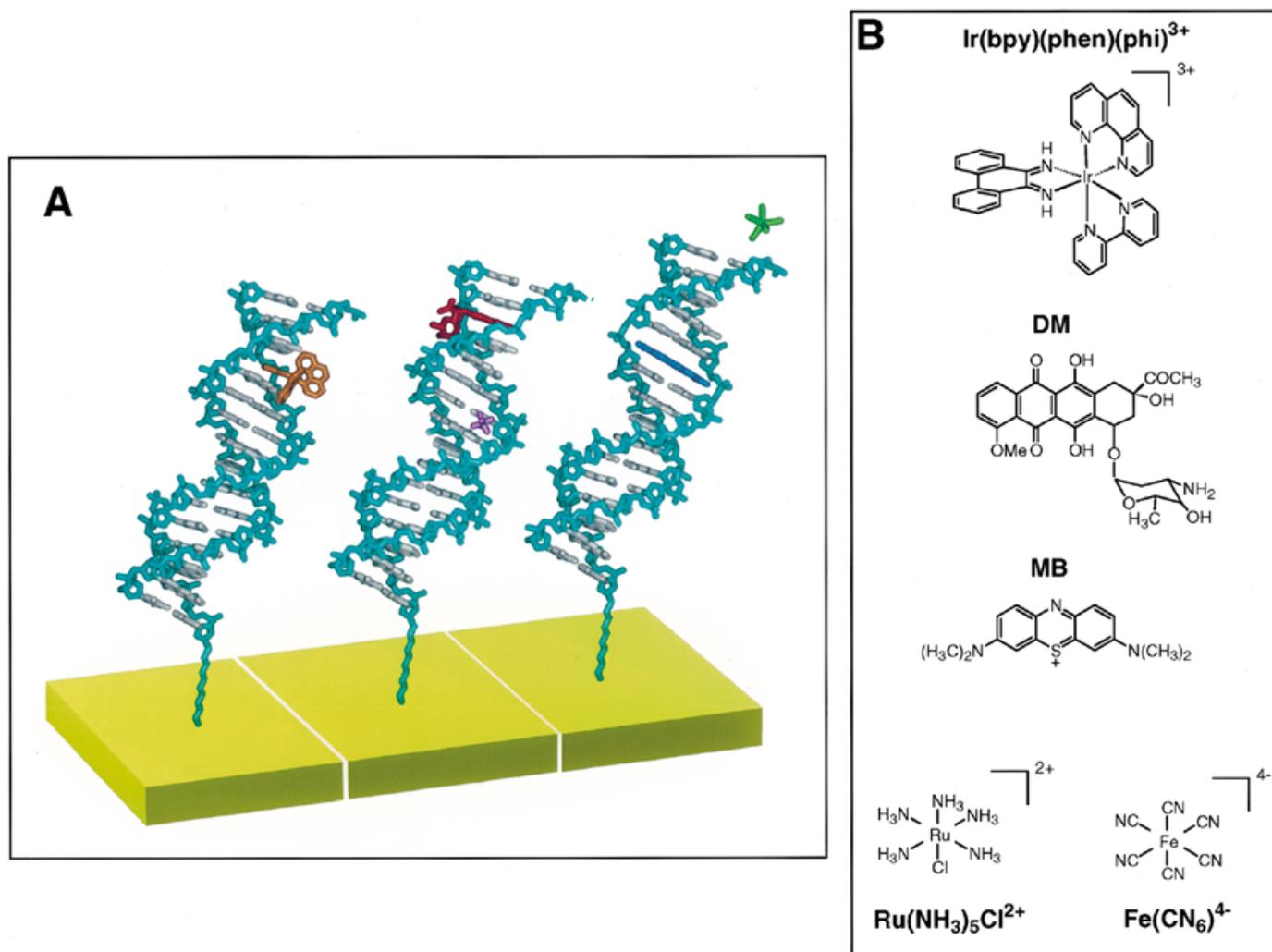


Figure 1. (A) Schematic illustration of DNA duplexes (cyan and white) immobilized on a gold surface. A variety of species are depicted in the vicinity of the monolayer, including the intercalators Ir(bpy)(phen)(phi)³⁺ (orange), DM (red) and methylene blue (blue), the groove-binder Ru(NH₃)₅Cl²⁺ (purple) and Fe(CN)₆⁴⁻ (green), which does not associate with the immobilized helices due to its negative charge. (B) Chemical structures of Ir(bpy)(phen)(phi)³⁺, daunomycin (DM), methylene blue (MB), Ru(NH₃)₅Cl²⁺ and Fe(CN)₆⁴⁻.

The presence of a single mismatch in the DNA duplexes caused a striking decrease in the electrochemical response (Fig. 2). Based on the observation of an almost quantitative decrease in the electrochemical signal with an intervening mismatch using crosslinked DM, it would be expected in the non-covalent system that as long as the base mismatch intervenes between the DM-binding site and the electrode, the charge-transfer event should be inhibited. It is likely that the residual DM response results from a small percentage of intercalator that binds beneath the mismatch. Additionally, some intercalators may adsorb directly to the electrode surface and therefore may be unaffected by perturbations in the DNA monolayer. Notably, the signals at these films correlate with the location of the mismatch along the sequence. Films containing mismatches closest to the electrode surface show the largest attenuation in signal (Table 1). Moreover, the effect of mismatches is most pronounced in densely packed films where larger populations of the intercalating probes appear to bind at the periphery of the monolayer.

Variation of redox-active probe. A range of intercalators and groove-binders (Fig. 1) were examined as probes for the detection of mismatches within DNA films; the results are summarized in Table 2. The efficiencies of mismatch detection using the various reporter molecules reveal several important characteristics of this assay.

A redox probe that intercalates into the DNA base stack appears to be a necessary component for mismatch detection. Probes that associate with DNA through purely electrostatic interactions (48) do not yield measurable differences in the electrochemical response in the presence of base mismatches. Thus, while the electrochemical signals obtained from the intercalators DM (49), methylene blue (50) and Ir(bpy)(phen)(phi)³⁺ (C.S. Stinner, S.O. Kelley, M.G. Hill and J.K. Barton, unpublished results), are all affected by the presence of the mismatch, the response of a groove-binding agent, Ru(NH₃)₅Cl²⁺ [or Ru(NH₃)₆³⁺], is essentially identical at fully-base paired or mismatched films. The reduction of Ru(NH₃)₅Cl²⁺ likely proceeds through the facilitated diffusion

Table 1. Electrochemical response obtained from DM non-covalently bound to DNA-modified electrodes: dependence on mismatch location^a

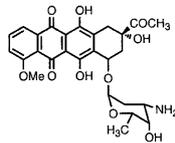
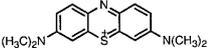
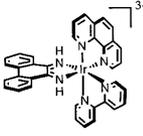
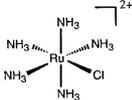
	$Q_{c(int)}$ (nC) ^b	Q_{CA}/Q_{TA}	T_m (°C) ^c
SH-5'-AGTACAGTCATCGCG TCATGTCAGTAGCGC	165(37)	-	68
SH-5'-AGTACAGTCATCGCG TCACGTCAGTAGCGC	47(12)	0.28	54
SH-5'-AGTACAGTCATCGCG TCATGTCAGCAGCGC	57(17)	0.34	56
SH-5'-AGTACAGTCATCGCG TCATGTCAGTACCGC	69(10)	0.42	55

^aBased on cyclic voltammograms measured for 1.0 μ M DM non-covalently bound to duplex-modified electrodes. Values are based on more than five trials each, and results were comparable for experiments run side-by-side, or from different sample preparations.

^bIntegrated background-subtracted cathodic charge.

^cDetermined by thermal denaturation measurements obtained by monitoring hypochromicity at 260 nm in duplex solutions containing 10 μ M duplex, 5 mM sodium phosphate, 50 mM NaCl and 100 mM MgCl₂ in the absence of DM.

Table 2. Electrochemical mismatch detection: dependence on DNA-binding probe^a

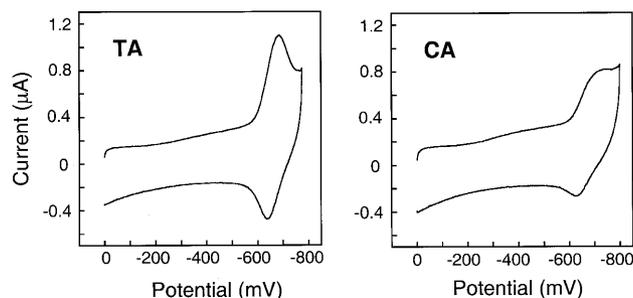
Probe	DNA-binding mode	Q_{CA}/Q_{TA}
	intercalation ^b	0.30(5)
	intercalation ^c	0.52(3)
	intercalation ^d	0.35(6)
	groove-binding ^e	1.0(1)

^aSequence: SH-5'-AGTACAGTCATCGCG (CA mismatch located at bold C). Based on cyclic voltammograms measured for various probes non-covalently bound to duplex-modified electrodes. Values are based on more than three trials each, and results were comparable for experiments run side-by-side, or from different sample preparation.

^bSee (49). ^cSee (50). ^dSee (Stinner *et al.*, unpublished results). ^eSee (48).

of the ruthenium complex along the grooves of the immobilized helices, while the intercalated species may participate in electron transfer mediated by the stacked bases. Therefore, because single-base mismatches do not affect the overall structure of the DNA helix, but rather subtly perturb the electronics of the DNA base stack, intercalated probes are needed for detection schemes based on DNA-mediated charge transport.

Among the intercalators, the bulkier probes exhibit smaller CA/TA charge ratios. This observation suggests that the smaller intercalators more readily diffuse into the monolayer and bind beneath the mismatch. All of the intercalators exhibit linear current versus scan rate relationships (51), however,

**Figure 2.** Cyclic voltammetry of 1.0 μ M DM at a gold electrode modified with SH-5'-AGTACAGTCATCGCG hybridized to its fully base-paired complement (TA) and a complement that features a C opposite the bold A (CA). Voltammograms were obtained with scan rate (v) = 100 mV/s, electrode area (A) = 0.02 cm².

indicating that the reactive species are strongly bound to the surface and thus diffusion based reactivity is minimal. Nevertheless, for the detection of base mismatches using the direct electrochemistry of molecules bound to DNA films, bulky intercalators occupying sites close to the periphery of the well-packed monolayer provide the most sensitive differentiation. By eliminating a step in the preparation of samples, non-covalently bound probes provide an important advance over fluorescence-based sensors currently in use that require the covalent attachment of a reporter group.

Variation in sequence composition. The detection of base mismatches using a charge transport-based assay appears to be independent of DNA sequence context and composition. As shown in Figure 3, the characteristic drop in coulometric signals for DM bound to DNA films containing a single CA mismatch compared to fully-paired films was essentially invariant across AT-rich to GC-rich sequences tested under identical conditions. This sequence-independent response is not achievable based upon differential hybridization, where the detection of mismatches within these different oligonucleotides would require drastically different conditions. The ability to detect a base mispair within a hybridized duplex would therefore allow sequences of different base content to be assayed simultaneously without the need for any manipulation of conditions or readings. Indeed, this feature highlights a unique and valuable aspect of the charge transport methodology.

Variation of mismatch. This assay allows the detection of a series of different mutations as demonstrated by monitoring the electrochemical signals obtained from DM non-covalently bound to films containing a variety of mismatches (Table 3). In general, pyrimidine-pyrimidine and purine-pyrimidine mismatches caused marked decreases in the electrochemical signals; the one purine-purine pair studied, a GA mismatch, did not show a measurable diminution. Interestingly, photo-physical studies of the effects of base mismatches on long-range electron transfer through DNA also revealed insensitivity to GA mispairs (37). This purine-purine pair may be sufficiently well-stacked within the DNA helix to support efficient electron transfer (44).

Surprisingly, a GT pair caused a substantial decrease in current, although it is not highly disruptive to the helix (52).

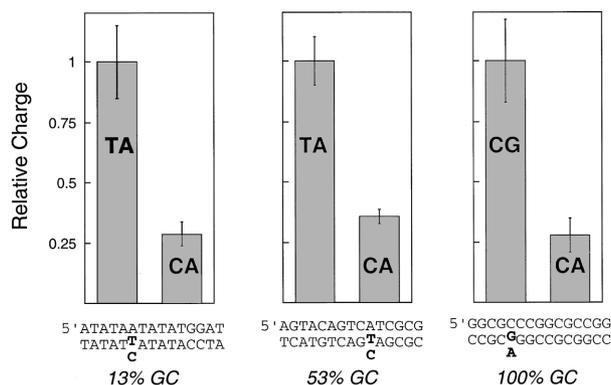


Figure 3. Charge obtained for DNA-modified electrodes in the presence of 1.0 μM DM. These duplexes, featuring varying percentages of GC content, were either fully base paired, or contained a single CA mismatch. Regardless of the sequence composition, and therefore over a wide range of duplex stabilities ($\Delta T_m = 50^\circ\text{C}$), the CA mismatches contained within these duplexes were accurately detected. The melting temperatures for these duplexes, as determined by thermal denaturation measurements obtained by monitoring hypochromicity at 260 nm in duplex solutions containing 10 μM duplex, 5 mM sodium phosphate, 50 mM NaCl and 100 mM MgCl_2 , were (SH-5'-ATATAATATATGGAT): TA = 47°C , CA = 32°C ; (SH-5'-AGTACAGT-CATCGCG): TA = 68°C , CA = 56°C ; (SH-5'-GGCGCCGGCGCCGG): GC = 82°C , CA = 69°C . Charge was quantitated from integrating background-subtracted cyclic voltammograms obtained at $v = 100$ mV/s with $A = 0.02$ cm^2 .

This wobble base pair, although thermodynamically stable, appears to mediate electron transfer poorly. This effect could result from increased base dynamics for the GT pair, or a poorly stacked conformation assumed by this pair that is unfavorable to electron transfer through DNA. Irrespective of the mechanism by which these mismatches attenuate the resultant electrochemical signals, the observation of significant changes in the efficiency of charge transport in the presence of these different pairs indicates that this approach can be generally applied for mismatch detection.

Mismatch detection via *in situ* hybridization

As would be required in an oligonucleotide array, mismatch detection can also be achieved with sequences hybridized reversibly *in situ* at the electrode surface. Thiol-modified duplexes can be deposited on the gold surface, heat denatured, thoroughly rinsed and then rehybridized with the desired target by incubation with ≥ 50 pmol of single-stranded oligonucleotide. This reversible assay is illustrated in Figure 4. Here, two separate duplex-modified electrodes were prepared, each containing the same 15-bp oligonucleotide derivatized with a thiol-terminated linker. One electrode featured this oligonucleotide hybridized to its native complement, while the other was modified with a duplex containing a CA mismatch. Once immersed in DM solution, the electrodes exhibited electrochemical responses characteristic of fully base-paired and CA-mutated films, respectively. The DNA films were stripped of their complements by heat denaturation, yielding single-stranded monolayers of identical sequence. New duplexes were then formed by incubation of the electrodes with the swapped complements (TA \rightarrow CA, CA \rightarrow TA). The electrochemistry of DM at the new films again showed the characteristic behavior expected for fully base-paired and CA-mutated

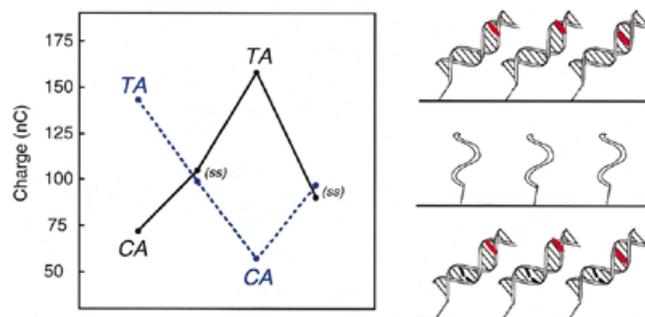


Figure 4. Charges (Q_c) measured during the *in situ* detection of a CA mismatch. Electrodes were derivatized with sequence SH-5'-AGTACAGT-CATCGCG, where either a C or T was incorporated into the complement across from the italicized A. Using cyclic voltammetry, the electrochemical response of 1.0 μM DM non-covalently bound to duplex-modified electrodes was first measured for the intact TA- or CA-containing duplexes (TA/CA), then the electrodes were immersed in 90°C pure buffer for 2 min, rinsed and the charge was remeasured (ss). The electrodes were then incubated with 100 pmol of the opposite complement in the presence of 5 mM phosphate buffer containing 50 mM NaCl and 100 mM MgCl_2 . Upon completion of this hybridization, the electrodes were rinsed and the charge was again remeasured. Finally, electrodes were again heated and the response was quantitated.

duplexes. Electrodes can be cycled through this sequence of events repeatedly.

Table 3. Electrochemical response obtained from DM non-covalently bound to DNA-modified electrodes: dependence on mismatch composition^a

	$Q_{c(\text{int})}$ (nC) ^b	T_m ($^\circ\text{C}$) ^c
SH-5'-AGTACAGTTCATCGCG TCATGTCAITAGCGC	165(37)	68
SH-5'-AGTACAGTTCATCGCG TCATGTCAICAGCGC	57(17)	56
SH-5'-AGTACAGTTCATCGCG TCATGTCTITAGCGC	95(18)	57
SH-5'-AGTACAGTTCATCGCG TCATGTCAICTAGCGC	51(23)	56
SH-5'-AGTACAGTTCATCGCG TCATGTCCITAGCGC	49(30)	62
SH-5'-AGTACAGTTCATCGCG TCATGTAAITAGCGC	153(38)	60
SH-5'-AGTACAGTTCATCGCG TCATGTCCITAGCGC	93(17)	58

^aBased on cyclic voltammograms measured for 1.0 μM DM non-covalently bound to duplex-modified electrodes. Values are based on more than five trials each, and results were comparable for experiments run side-by-side, or from different sample preparation as long as electrodes exhibited high surface coverages. Electrodes with lower surface coverages yielded higher charges (>1 DM/duplex), and decreased attenuations in the presence of mismatches.

^bIntegrated background-subtracted cathodic charge.

^cDetermined by thermal denaturation measurements obtained by monitoring hypochromicity at 260 nm in duplex solutions containing 10 μM duplex, 5 mM sodium phosphate, 50 mM NaCl and 100 mM MgCl_2 in the absence of DM.

It is noteworthy that the cyclic voltammetry of DM at single-stranded films shows an irreversible and broad reduction, which becomes smaller upon subsequent scans. These signals are smaller than those obtained at surfaces modified with fully base-paired duplexes, but larger than those observed at

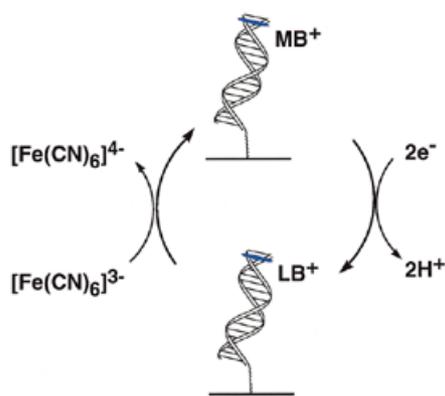


Figure 5. Schematic representation of electrocatalytic reduction of $\text{Fe}(\text{CN})_6^{3-}$ by MB at a DNA-modified electrode. LB^+ is leucomethylene blue, the product of the electrochemical reduction.

analogous mismatched duplexes; electrochemistry can therefore be used to confirm *in situ* hybridization, but mutations are ultimately identified at duplexes that feature single-base mismatches. We believe these broad signals reflect the less dense monolayer of single stranded oligonucleotides, which allows DM access to the gold surface. Assays of genomic DNA with small single stranded regions or multiple mismatches likely would not hinder full monolayer formation and thus should provide full coverage of the gold surface.

Mismatch detection using electrocatalysis

Although duplexes containing mismatches can be distinguished by direct voltammetry of redox-active intercalators, the absolute electrochemical signals are limited by the surface concentration of the intercalator (~ 50 pmol/cm²). In order to increase the inherent sensitivity of this assay, we have coupled the direct electron transfer to an electrocatalytic process involving a species freely diffusing in solution (Fig. 5). This effectively amplifies the intercalator signal and improves the discrimination between signals obtained for mismatched versus base-paired duplex films.

Methylene blue was chosen as the intercalated catalyst, with potassium ferricyanide as the solution substrate. Possessing a large negative charge, $\text{Fe}(\text{CN})_6^{3-}$ is electroinactive at the DNA-modified surface even at overpotentials as high as ~ 1 V (Fig. 6), yet its chemical reduction by reduced MB is thermodynamically favored by ~ 0.6 eV. Given the low reorganization energy expected for this process (53), the cross reaction between the electrochemically generated catalyst and substrate should be very rapid. Depending on the rates of the various steps in the overall reaction, the signals may now be limited by the concentration of substrate in solution. Because the presence of mismatches effectively decreases the amount of reduced intercalator bound to the film, the presence of mispairs should also decrease signals obtained from electrocatalytic reactions.

Electrocatalytic reduction of methylene blue. Addition of micromolar MB to a 2.0 mM ferricyanide solution causes a pronounced electrochemical signal at the DNA-modified electrode (Fig. 6). Notably, this signal comes at the reduction potential of MB and is completely irreversible: electrons flow from the Au electrode to intercalated MB and then are accepted by $\text{Fe}(\text{CN})_6^{3-}$ in solution (thus no electrochemical oxidation

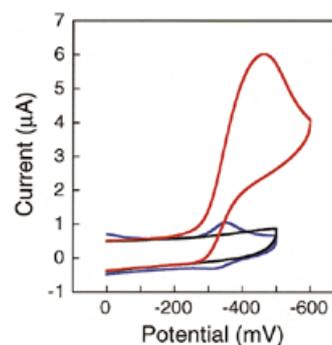


Figure 6. Cyclic voltammetry ($v = 100$ mV/s, $A = 0.02$ cm²) at a gold electrode modified with DNA (sequence: SH-5'-AGTACAGTCATCGCG) of 2.0 mM $\text{Fe}(\text{CN})_6^{3-}$ (black), 2.0 μM MB (blue), 2.0 mM $\text{Fe}(\text{CN})_6^{3-}$ and 2.0 μM MB (red).

peak is observed). Chemically oxidized MB is again available for electrochemical reduction and the catalytic cycle continues as long as the potential of the gold electrode is sufficiently negative to reduce MB.

Although there is no requirement for the redox intercalator to dissociate fully from the monolayer, electrocatalysis involving intercalators bound to DNA-modified electrodes appears to require a catalyst that can dynamically shuttle electrons to solution-borne acceptors. DM is a very poor electrocatalyst (data not shown) and exhibits only very low levels of catalytic reduction in the presence of $\text{Fe}(\text{CN})_6^{3-}$. DM has a stronger affinity for DNA (54) than does methylene blue (55) and may have slower exchange dynamics that would not allow the passage of electrons out to the acceptor.

Electrocatalytic detection of base mismatches. Incorporation of a mismatch into the duplex significantly attenuates the electrocatalytic response obtained with methylene blue (Fig. 7). Fewer MB molecules are reduced at the mismatched-DNA electrode: the steady-state concentration of active catalyst is lower, and a diminished overall catalytic rate results. A range of catalyst and substrate concentrations was investigated to maximize the difference in electrocatalytic response at the fully base-paired (TA) and mismatched (CA) duplexes. Under optimized conditions, the presence of the mismatch causes a 6-fold decrease in the electrocatalytic current, compared to a 2-fold decrease in the peak current obtained when monitoring the direct electrochemistry of methylene blue (at scan rates of 100 mV/s). This enhancement is consistent with the idea that electrocatalysis amplifies only the DNA-mediated charge transport to the catalyst bound near the periphery of the film; MB bound in the interior would not be effective as a catalyst. Hence, coupling direct electrochemistry to a catalytic event both increases the sensitivity of mismatch detection and provides larger absolute signals.

In contrast, $\text{Ru}(\text{NH}_3)_5\text{Cl}^{2+}$ (a groove binder with approximately the same potential as MB) is an effective electrocatalyst for the reduction of ferricyanide at DNA-modified surfaces, but the catalytic signal obtained with this cation is not sensitive to mismatches in the film (Fig. 7). These data are consistent with the results of direct voltammetry of intercalated versus non-intercalated probes (Table 2), and indicate that the

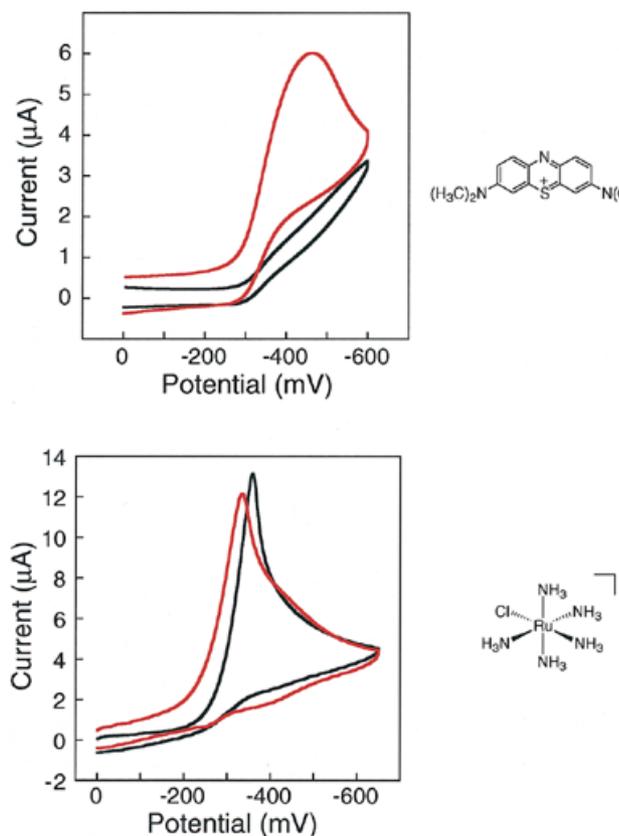


Figure 7. Cyclic voltammograms ($v = 100$ mV/s, $A = 0.02$ cm²) of 2.0 mM $\text{Fe}(\text{CN})_6^{3-}$ plus 2.0 μM MB (top) or 28 μM $\text{Ru}(\text{NH}_3)_6\text{Cl}_2^{2+}$ (bottom) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGT-CATCGCG hybridized to a fully base-paired complement (red) and a complement that features an A opposite the bold C (black).

detection of mismatches based on electrocatalysis also requires a probe that interacts with the DNA through intercalation.

Because the charge transport-based assay features a catalytic reaction whose rate depends on the degree of complementarity within the individual duplexes, the measured charge resulting from the reduction of methylene blue at TA versus CA-containing films increases disproportionately with longer integration times (Fig. 8). Using 0.5 μM MB and 2.0 mM ferricyanide, 10 s potential steps to -350 mV gave faradaic charges of 36 and 6 μC respectively. Increased sampling times continue to increase the differentiation of signals obtained with mismatched versus paired complements. These results highlight the versatility of electrochemical detection methods that are also more amenable to the portability required for a practical device.

Advantages and disadvantages of sensing based on DNA-mediated electron transfer

We have presented results indicating that the sensitivity of electron transfer through DNA films to intervening base mismatches provides a suitable means to probe DNA sequences for the presence of mutations. Several features of this new approach provide important advances over existing technology, including (i) an insensitivity of the detection of

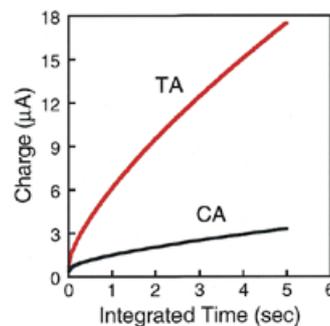


Figure 8. Chronocoulometry at -350 mV of 2.0 mM $\text{Fe}(\text{CN})_6^{3-}$ plus 0.5 μM MB (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base-paired complement (red) and a complement that features an A opposite the bold C (black). The discrimination between base-paired and mismatched sequences increases with increased sampling time.

single-base mismatches to AT or GC sequence content, (ii) the detection of variety of mismatches (including some with comparable thermodynamic stability to Watson-Crick pairs), and (iii) the use of non-covalently bound probes which eliminate the need for chemical modification of biological samples.

The detection of mutations based on DNA-mediated electron transfer therefore provides a complementary method to hybridization-based assays. The ability to detect mutations within intact duplexes would greatly simplify the analysis of multiple test sequences at an addressable array. Analyses could be performed under strongly hybridizing conditions, allowing both native and mutated test strands to bind to the probe sequences, regardless of the overall base composition of the individual addresses in the array. In addition, electrochemical detection methods are better suited for the development of inexpensive, portable devices than the sensors currently available employing high-resolution confocal microscopy (17).

However, electrochemical detection presents new challenges for the fabrication of functional DNA 'chips' on electrode surfaces. The reproducibility and effectiveness of the assay presented here requires tightly packed films that inhibit the diffusion of intercalators into the monolayer. Furthermore, the applicability of our potentiometric and amperometric methods have not been proven with biological samples. While the charge transport-based method described here holds great promise for the detection of known mutations within defined sequences, hybridization based methods combined with algorithmic analyses may be better employed for sequencing assays. We have not yet systematically varied the DNA length on the electrode surface, but an 8×8 array of 15mers should be sufficient to assay for a typical gene with no redundancies. Overall, charge transport and hybridization-based assays may then ultimately provide complementary methods for the analysis of DNA sequence composition and abnormalities.

The efficiency of charge transport through DNA films offers a new approach to DNA-based sensors. Using this methodology, a broad range of point mutations can be detected within heterogeneous DNA sequences, irrespective of base composition. Monitoring electrochemical signals at addressable electrodes, as opposed to detecting fluorescence by high-resolution microscopy or radioactive labelling, may provide a practical detection system for inexpensive devices to search for known

mutations on targeted genes. While others have explored electrochemical schemes for the development of DNA biosensors, the reliance of these schemes on hybridization assays does not offer the same advantages as a charge transport-based approach. The discovery that DNA-mediated electron-transfer reactions are exquisitely sensitive to the stacking of the intervening bases has provided insight into the role of the DNA base stack in modulating this reactivity. As a result, we can now exploit this sensitivity to stacking in the development of a practical assay for single-base changes in DNA sequence.

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