CHAPTER 3

Mutation Detection by Electrocatalysis at DNA-Modified Electrodes

Adapted from Boon, E.M., Ceres, D.M., Drummond, T.G., Hill, M.G., Barton, J.K. (2000) *Nature Biotechnology 18*, 1096.

D.M.C. designed and fabricated the DNA chip used in these experiments and mismatch detection experiments on chips were carried out in collaboration with him (Figures 3.8-3.9). T.G.D. assisted in experiments to detect all the possible single base mismatches in DNA (Figure 3.3).

INTRODUCTION

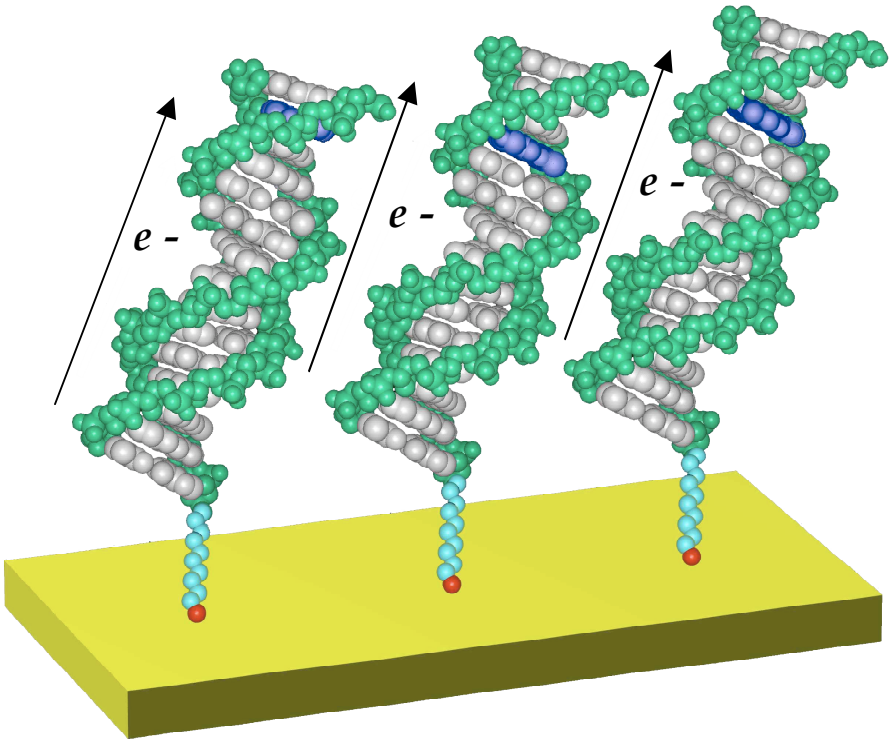
Reliable detection of mismatched base pairs is critical for the study, diagnosis, and treatment of genetic disease, as well as the identification of genomic sequence variations (single nucleotide polymorphisms, SNPs). Of the many assays that have been proposed for large scale mutational analysis (1), those employing "DNA chips" (addressable arrays of immobilized DNA probe sequences) are the most widely studied (2-4). To date, most chip assays are based, directly or indirectly, on differential hybridization: as the binding energy of a mutated test sequence to the single stranded probe is less than the binding energy of a completely complementary strand, the mutation is identified by its diminished association to the chip. While hybridization methods have been used for highly parallel sequencing and mutation detection (2), they are necessarily limited in sensitivity to the difference in melting temperature between a perfect DNA sequence and one with a single defect; this difference can be very small and can vary based on sequence context.

Numerous studies of electron transfer through the π-stack of DNA have demonstrated that long range charge transport is exceptionally sensitive to base stacking perturbations such as mismatches (5-9). In particular, in a double stranded DNA film containing a covalently bound intercalator, daunomycin, reduction of daunomycin was accomplished with a well stacked DNA intervening, but was shut off in the presence of an intervening mismatch. This sensitivity depends upon electronic coupling within the base

pair stack rather than upon the thermodynamics of base pairing. In order to exploit this property in practical mutation detection assays, we have developed a method for electrochemical detection of mismatches based on charge transport through double stranded DNA monolayers on gold electrodes (9-12). Modified electrodes are prepared by self-assembling pre-hybridized duplexes on gold (or alternatively, by in situ hybridization of a surface bound single stranded probe with a test sequence). If the duplexes are completely Watson-Crick base paired, redox active intercalators such as methylene blue (MB^+) yield well resolved electrochemical signals (Figure 3.1). However, the presence of a mispaired base (within the fully hybridized duplex) between the electrode and the site of intercalation switches off the electrochemical response (10). Importantly, in order to detect a mutation, the probes must be intercalators, which are themselves well stacked and thus electronically coupled into the base stack of DNA.

By coupling charge transport through duplex-modified films to an electrocatalytic cycle involving freely diffusing ferricyanide, we can now achieve greatly enhanced selectivity and sensitivity, as described in Chapter 2. Electrocatalysis has been exploited previously in electrochemical assays of DNA hybridization (13), but here we exploit electrocatalysis to amplify DNA-mediated charge transport and hence our ability to detect small perturbations in stacking. In the electrocatalytic process (Figure 3.2), electrons flow from the electrode surface to intercalated MB^+ in a DNA-mediated reaction. The reduced form of MB^+, leucomethylene blue (LB^+), in turn reduces solution-borne ferricyanide, so that more electrons can flow to MB^+ and the catalytic cycle continues. Thus in one experiment the surface-bound DNA is

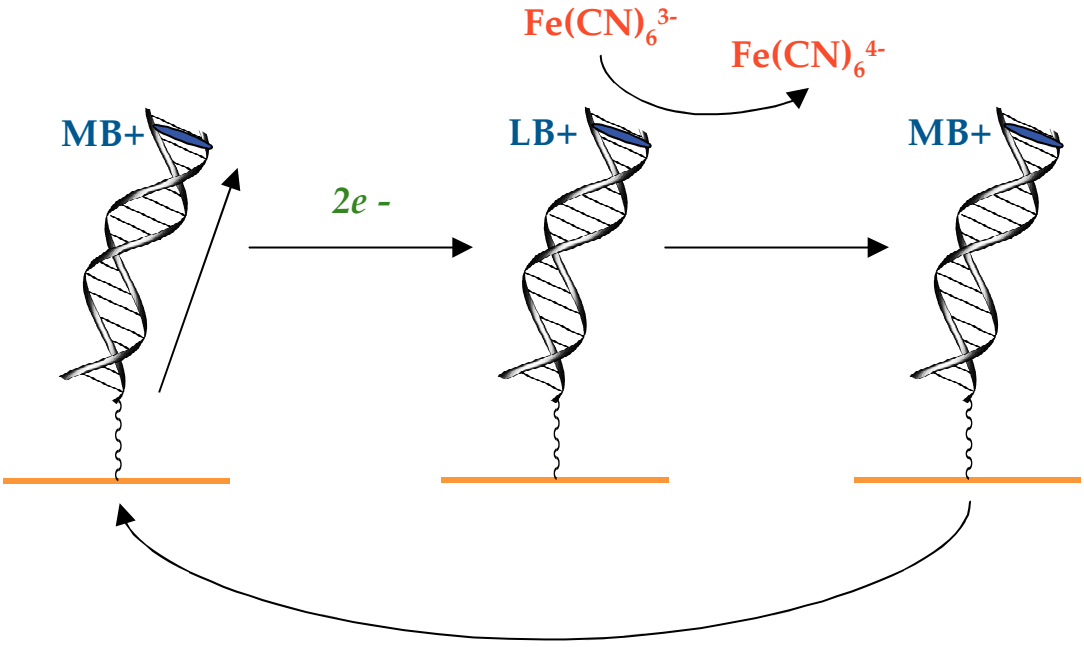
Figure 3.1. Schematic representation of a DNA-modified gold electrode with a bound redox active DNA intercalator for use in electrochemical assays.



repeatedly interrogated. In double stranded duplexes containing one or more mismatches, fewer MB^+ molecules are electrochemically reduced, so the concentration of active catalyst is greatly lowered and the overall electrocatalytic response is diminished. In essence, the catalytic reaction amplifies the absolute signal corresponding to MB^+ in addition to enhancing the inhibitory affect associated with a mismatch. Furthermore, due to its catalytic nature, the measured charge increases with increased sampling times, and in principle is limited by the amount of ferricyanide in solution. As a result, longer integration times provide even greater absolute signals as well as increased differentiation between fully complementary and mismatched DNA.

Here we demonstrate the remarkable sensitivity of this electrocatalytic assay and its potential for use in clinical applications. We apply this technology to detect the eight possible single base DNA mismatches within DNA and DNA/RNA hybrid duplexes, even at very low concentrations within the DNA film. Naturally occurring mutational "hot spots" from the p53 gene are detected as well. Furthermore, we demonstrate the ability to detect common DNA lesions by DNA-mediated charge transport. Finally, we extend this technology to a chip based format for the detection of base pair mismatches at ultramicroelectrodes. The efficient catalytic charge transport through DNA-modified electrode surfaces offers an alternate approach to hybridization based DNA sensors.

Figure 3.2. Schematic representation of electrocatalytic reduction of $Fe(CN)_6^{3-}$ by LB^+ at a DNA-modified electrode. LB^+ is leucomethylene blue, the product of the 2 electron electrochemical reduction of MB^+. Electrons flow from the electrode surface to intercalated MB^+. Once reduced, LB^+ can easily reduce $Fe(CN)_6^{4-}$ (~0.6 eV driving force) and regenerate MB^+ that can continue on in the catalytic cycle. This process can be repeated until the electrode is no longer at a potential to reduce MB^+ or all $Fe(CN)_6^{4-}$ in solution has been reduced, thus repeated interrogation of the DNA monolayer is achieved. MB^+ binding is primarily constrained to the top of the densely packed DNA monolayer, requiring charge transport through the DNA film and electrostatic repulsion keeps $Fe(CN)_6^{3-}$ away from the interior of the anionic DNA film.



MATERIALS AND METHODS

Materials

All DNA synthesis reagents, including all unnatural nucleic acid phosphoramidites, were obtained from Glen Research. Methylene blue and potassium ferricyanide were purchased from Aldrich and used as received. RNA single strands were purchased from Dharmacon Research, Inc.

Preparation of DNA-modified surfaces

Thiol-modified oligonucleotides were prepared using phosphoramidite synthesis as described in the Appendix. Thiol-terminated linkers were attached to single stranded oligonucleotides, HPLC purified, and hybridized to unmodified complements. These duplexes (0.1 mM) were then deposited on polycrystalline gold electrodes for 12-24 hours, thoroughly rinsed with buffer and used for electrochemical experiments. As electrodes containing a high surface coverage of DNA were essential for our experiments, surfaces were routinely assayed for coverage by monitoring the attenuation of the oxidation of ferrocyanide. Comparable results were obtained with commercial polycrystalline electrodes (BAS) or Au(111) films vapor deposited on mica substrates (Molecular Imaging).

DNA chip fabrication

A layer of SiO_2 was thermally grown onto a Si wafer. A chromium adhesion layer of 50 Å was evaporated onto the SiO_2 followed by 3000 Å of gold. Metal lift off was achieved in warm acetone. A 1000 Å Si_3N_4 film was

deposited by low pressure chemical vapor deposition to insulate the gold. By chemical etching, windows of sizes between 2 μm and 500 μm in diameter were then opened over the gold. The die was fixed into the package with a silver epoxy and heated for an hour at 125^{o}C in a convection oven. Contacts between the package leads and the electrodes were made with 25 μm aluminum wires and then insolated with nitrocellulose and polyester resin. The electrodes were modified with DNA and electrochemical measurements were carried out in the same manner as described for the normal electrodes.

Electrochemical measurements

Cyclic voltammetry and chronocoulometry were carried out anaerobically (bubbling argon) on 0.02 cm^2 gold electrodes using a Bioanalytical Systems (BAS) Model CV-50W electrochemical analyzer. Buffer and electrolyte conditions were 5 mM sodium phosphate buffer containing 50 mM NaCl, pH 7, ambient temperature. 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. A modified Luggin capillary separated the working compartment of the electrochemical cell from the reference compartment. For DNA chip measurements, a silver wire was used as a reference electrode and a platinum wire as the auxiliary electrode; a Faraday cage and a low current module (BAS) provided high signal/noise ratios. Potentials are reported versus SCE.

RESULTS AND DISCUSSION

DNA-modified films for electrocatalysis

DNA-modified electrodes are prepared by derivatizing single stranded oligonucleotides (typically 15mers) at the 5′ end with a thiol-terminated alkyl chain [$SH(CH_2)_2CONH(CH_2)_6NHCO$-DNA], herein referred to as SH-5'-DNA (9-12). The derivatized single stranded oligonucleotides are hybridized to their unmodified complements and a drop of the resulting duplex solution is placed on the gold surface. After 12-24 hours, the electrodes are rinsed thoroughly with buffer and used for electrocatalytic studies. Analyses by radioactive labeling (11) and scanning probe microscopy (12) indicate that the duplexes form densely packed monolayers oriented in an upright position with respect to the gold surface. MB^+ binding is primarily constrained to the top of the densely packed DNA monolayer (11), requiring charge transport through the DNA film. Also in the solution for electrocatalysis is $Fe(CN)_6^{3-}$; electrostatic repulsion keeps it away from the interior of the anionic DNA film. In all cases, the duplexes in the monolayer can be reversibly denatured, then rehybridized with single stranded test sequences. These experiments are carried out at ambient temperature under conditions where the DNA is fully hybridized; mismatch discrimination is based on the detection of DNA perturbations in base stacking, not reduced hybridization.

Detection of all single base mismatches by electrocatalytic reduction of MB^+

In separate experiments, all possible single base mismatches were incorporated into DNA duplexes formed using SH-5'-AGTACAGTCATCGCG-3' (Table 1). Electrode surfaces modified with these duplexes were interrogated in chronocoulometric analyses at -350 mV with 0.5 μM methylene blue (MB^+) as the intercalated catalyst and 2.0 mM $Fe(CN)_6^{3-}$ as the solution-borne substrate. The marked sensitivity of this assay is evidenced by the detection of all mismatches, including purine-purine mismatches, without any manipulation of experimental conditions (Figure 3.3). Improved signal differentiation is achieved with increasing sampling time, a result that reflects the catalytic nature of this assay.

Mismatched duplexes are easily distinguished from canonical duplex DNA, but the mismatches cannot be readily distinguished from one another, as most yield approximately the same electrocatalytic response. Although GA and AA do yield electrocatalytic currents slightly higher than the other mismatches, these differences are not great in comparison to the overall attenuation in signal from fully matched sequences. Nonetheless, this methodology provides a clear strategy to detect a GA mismatch. GA mismatches have not been effectively detected using traditional methods owing to their high thermodynamic stability. In fact, even the repair machinery of the cell inefficiently recognizes the GA mismatch (14).

Indeed GA mismatches could not be detected by DNA charge transport without coupling to our electrocatalysis assay (10). Mismatches are generally stacked in a DNA duplex but undergo somewhat greater dynamical

Table 3.1

		Q (□A)[a]
TA/WC	SH[b]-5'AGTACAGTCATCGCG TCATGTCAGTAGCGC	18.7 (± 1.1)
CA	SH-5'AGTACAGTCATCGCG TCATGTCAATAGCGC	3.2 (± 0.6)
GA	SH-5'AGTACAGTCATCGCG TCATGTAAGTAGCGC	6.3 (± 0.2)
TT	SH-5'AGTACAGTCATCGCG TCATGTCTGTAGCGC	3.2 (± 0.8)
GT	SH-5'AGTACAGTCATCGCG TCATGTTAGTAGCGC	4.0 (± 1.4)
CT	SH-5'AGTACAGTCATCGCG TCATGTCATTAGCGC	3.2 (± 0.3)
CC	SH-5'AGTACAGTCATCGCG TCATGTCACTAGCGC	3.3 (± 0.6)
GG	SH-5'AGTACAGTCATCGCG TCATGTGAGTAGCGC	5.2 (± 0.9)
AA	SH-5'AGTACAGTCATCGCG TCATGACAGTAGCGC	6.9 (± 0.8)
Ta/WC	SH-5'AGTACAGTCATCGCG ucaugucaguagcgc	21.8 (± 1.8)
Ca	SH-5'AGTACAGTCATCGCG ucauguca**a**uagcgc	3.4 (± 0.7)
Ga	SH-5'AGTACAGTCATCGCG ucaugu**a**aguagcgc	6.7 (± 1.2)
Tu	SH-5'AGTACAGTCATCGCG ucauguc**u**guagcgc	3.5 (± 1.1)
Gu	SH-5'AGTACAGTCATCGCG ucaugu**u**aguagcgc	2.7 (± 0.9)
Cu	SH-5'AGTACAGTCATCGCG ucauguca**u**uagcgc	1.6 (± 0.7)
Cc	SH-5'AGTACAGTCATCGCG ucauguca**c**uagcgc	4.1 (± 1.1)
Gg	SH-5'AGTACAGTCATCGCG ucaugu**g**aguagcgc	2.4 (± 0.8)
Aa	SH-5'AGTACAGTCATCGCG ucaug**a**caguagcgc	5.5 (± 0.8)
WT	SH-5'ATGGGCCTCCGGTTC TACCCGGAGGCCAAG	21.3 (± 0.7)
248	SH-5'ATGGGCCTCCGGTTC TACCCGGAG**A**CCAAG	8.3 (± 0.5)
249	SH-5'ATGGGCCTCCGGTTC TACCC**T**GAGGCCAAG	9.5 (± 0.2)
dUG	SH-5'AGTACAGTCATCGCG TCATGT**U**AGTAGCGC	3.0 (± 0.4)
OH T	SH-5'AGTACAGTCATCGCG TCATG**R**CAGTAGCGC	1.7 (± 0.2)
Ab	SH-5'AGTACAGTCATCGCG TCAT TCAGTAGCGC	2.2 (± 0.6)
Aox	SH-5'AGTACAGTCATCGCG TCATGTC**O**GTAGCGC	1.1 (± 0.1)

[a]Integrated charge after 5 seconds of electrocatalysis at -350 mV. Values are based on more than five trials each, and results are comparable for experiments run side by side or from different sample preparation. [b]SH-5' stands for $SH(CH_2)_2CONH(CH_2)_6NHCO$ attached to the 5' OH of the DNA single strand.

motion than well paired bases (15-19). DNA charge transport detection, which depends upon the electronic coupling within the base stack, is sensitive to those motions and electrocatalysis allows for the amplification of this sensitivity. As a result, GA mismatches are now detected routinely. Notably, GT wobble base pairs could be detected by DNA charge transport even without electrocatalysis (10).

Therefore, the electrocatalytic detection of mismatches relies not upon their thermodynamic stability but instead upon how well or poorly the mispair is *electronically* coupled into (or stacked with) the base pair stack and thus how efficiently MB^+ can be reduced to generate active catalyst. Our ability to detect single base mismatches generally, irrespective of sequence, and the GA and GT mismatches in particular, underscores the power and novelty of this method for mutational assays as well as in tests of genetic variability (SNPs).

Mismatch detection in DNA/RNA hybrids

In assays for interrogating cellular samples, it may be preferable to test mRNA transcripts rather than genomic DNA. In this scenario, patient mRNA would be hybridized to a chip or electrode modified with single stranded DNA, resulting in DNA/RNA hybrids. The structure of such duplexes is a hybrid between A- and B-form nucleic acid, which results in different base stacking than in pure B-form DNA. Thus we examined all single base mismatches within DNA/RNA hybrids by electrocatalysis to ensure sensitivity to base stacking perturbations in this alternate duplex structure.

Figure 3.3. Chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 □M MB^+ (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired complement (TA) and complements that introduce single base mismatches. Red lines are purine-purine mismatches, blue are pyrimidine-purine mismatches, and green are pyrimidine-pyrimidine mismatches. These experiments are done under completely hybridizing conditions. Mismatch detection is based on reduced DNA-mediated electron transfer efficiency due to the local base stack perturbation cased by a mismatch. The discrimination between Watson-Crick and mismatched sequences increases with sampling time. See Table 1 for error analysis.

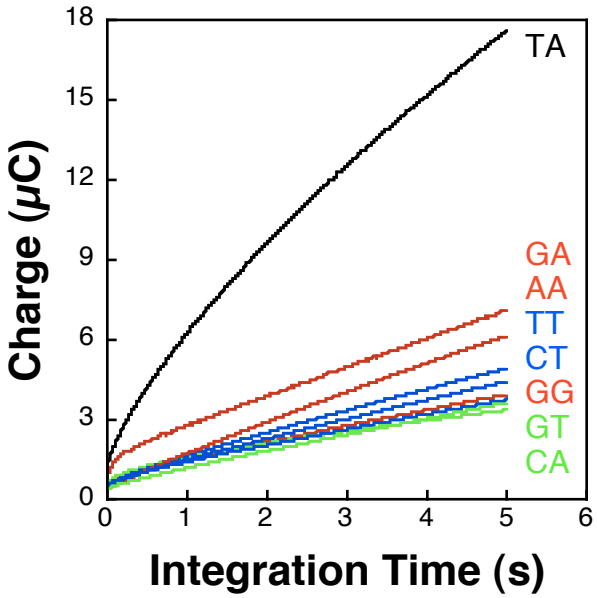
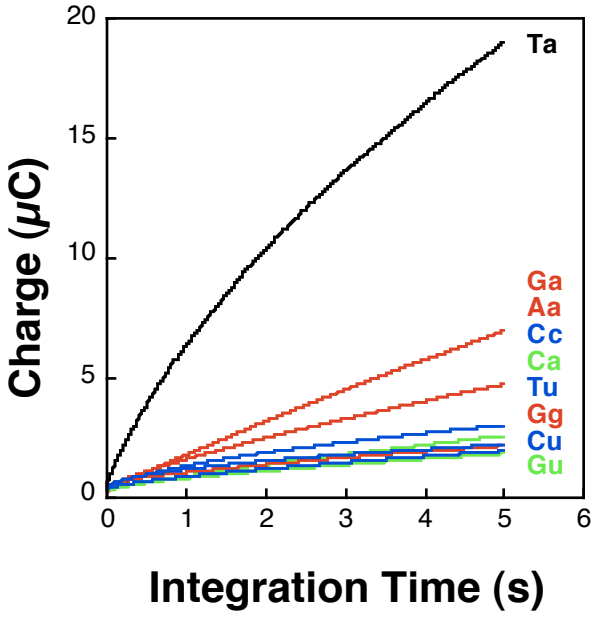


Figure 3.4. Chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 □M MB^+ (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired RNA complement (Ta) and complements that introduce single base mismatches into the DNA/RNA hybrid. Red lines are purine-purine mismatches, blue are pyrimidine-purine mismatches, and green are pyrimidine-pyrimidine mismatches. These experiments are done under completely hybridizing conditions. Mismatch detection is based on reduced DNA-mediated electron transfer efficiency due to the local base stack perturbation cased by a mismatch. The discrimination between Watson-Crick and mismatched sequences increases with sampling time. See Table 1 for error analysis.



In separate experiments, all possible single base mismatches were incorporated into DNA/RNA hybrids formed using SH-5'-AGTACAGTCATCGCG-3' (the surface-bound strand was DNA and the complements were RNA). Electrode surfaces modified with these duplexes were interrogated using chronocoulometry. As evident in Figure 3.4, all possible mutations were detected readily. In fact, sensitivity to mutations was almost identical to the DNA/DNA results (Figure 3.3). This means that when testing for mutations in clinical samples, genomic DNA, cDNA, or mRNA can be utilized, making electrocatalytic assays flexible and practical for routine use.

Single base mismatch detection in sequences from the human p53 gene

The p53 tumor suppressor gene encodes a multifunctional transcription factor that plays a key role in the prevention of human cancer (20). Although tumor inducing mutations have been observed at more than 100 sites in the p53 gene, more than 90% are found in regions that encode the DNA binding domain (residues 100 – 293). Of these, 25% occur at five "hot spots," in codons 175, 245, 248, 249, and 273 (20). Hot spot mutations are separated into two categories, class I mutations that affect key residues at the DNA binding surface (e.g., Arg 248 and Arg 273) and class II mutations that affect residues responsible for holding the protein in a conformation that readily binds DNA (e.g., Arg 175 and Arg 249) (21).

Two of these mutational hot spots, in codons 248 and 249, were chosen for initial examination using electrocatalysis. The mutation in codon 248 is a class I mutation caused by cigarette smoke that results in lung cancer (22) and

the 249 mutation is a class II mutation caused by Aflatoxin B1 exposure that results in liver cancer (23). The sequence SH-5'-ATGGGCCTCCGGTTC includes both of these hot spots and was synthesized with an alkanethiol linker at the 5' end (Table 1). In separate samples, electrodes were modified with duplexes containing a CA mismatch at the boldface **C** (the common 248 mutation) or a CT mutation at the underlined C (the common 249 mutation). As evident in Figure 3.5, both of them were successfully differentiated from the native duplex with electrocatalytic chronocoulometry at -350 mV with 0.5 μM MB^+ and 2 mM $Fe(CN)_6^{3-}$.

It is therefore possible to detect mutations within natural sequences of double stranded DNA using electrocatalysis. These methods should be fully transferable to other genes and sequences.

Detection of naturally occurring DNA lesions

The DNA in living cells is subject to spontaneous alteration as well as reaction with a variety of chemical compounds and physical agents present in the cell (24). For example, the deamination of cytosine gives rise to a CG to AT transition. Reactive oxygen species are generated through several pathways including mitochondrial leakage and ionizing radiation. 8-oxo-adenine is a possible oxidation product of DNA that results from the addition of a hydroxyl radical to the C8 position of the purine base. Hydroxy pyrimidines, such as 5,6-dihydroxy thymines, are another example of major base damage products formed via the hydroxyl radical during the exposure of DNA to ionizing radiation.

Figure 3.5. Chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 □M MB^+ (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-ATGGGCCTCCGGTTC hybridized to a fully base paired wildtype complement (WT, red) and complements that feature mutations at the boldface **C** and the underlined C. The boldface **C** is a CA mutation at position 248 (green) and the underlined C is a CT mutation at position 249 (blue). See Table 1 for error analysis.

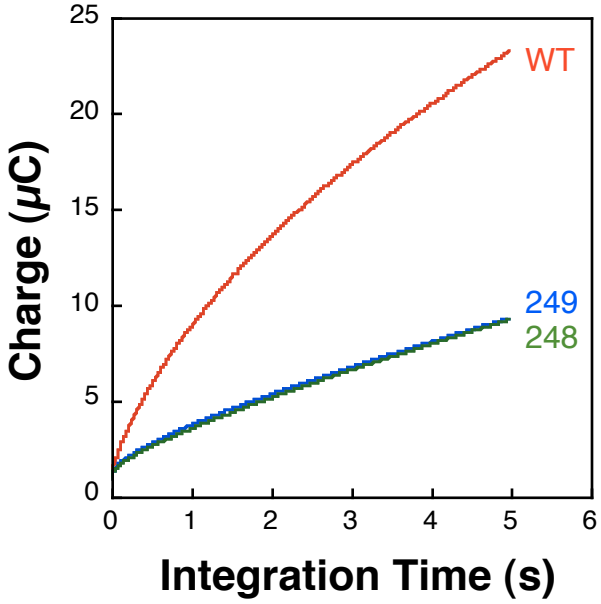
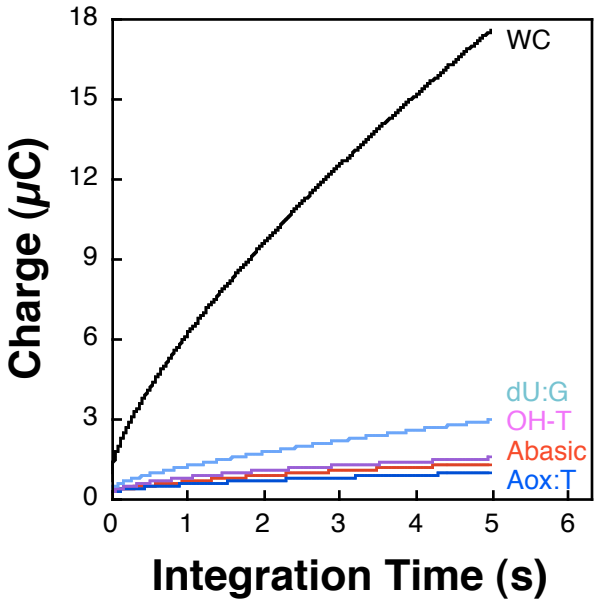


Figure 3.6. Chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 μM MB^+ (pH 7) at a gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired complement (TA) and complements that introduce single base lesions. The cytosine deamination product, deoxy-uracil, paired with guanine is in cyan; the reduction product of thymine, 5,6-hydroxy thymine, is in purple; an abasic site is in red; and oxidized adenine, 8-oxo-adenine, is in blue. See Table 1 for error analysis.



We incorporated several of these possible DNA lesions into the sequence SH-5'-AGTACAGTCATCGCG. Electrodes were modified (in separate samples) with double stranded DNA sequences containing 8-oxo-adenine, 5,6-dihydro thymine, an abasic site, or deoxyuracil paired with guanine (the result of cytosine deamination). The films, once formed, were then examined by electrocatalysis at -350 mV with 0.5 μM MB^+ and 2 mM $Fe(CN)_6^{3-}$ (Table 1). Each lesion was successfully detected within duplex DNA (Figure 3.6).

In general, owing to their relatively high thermodynamic stability and their minor modification to the DNA duplex, these lesions have been labor intensive to detect (29). Postlabeling procedures have been the preferred methods of analysis. These lesions do, however, appear to perturb electronic coupling within the DNA duplex. Remarkably, DNA mediated charge transport coupled to electrocatalysis is sensitive to those perturbations.

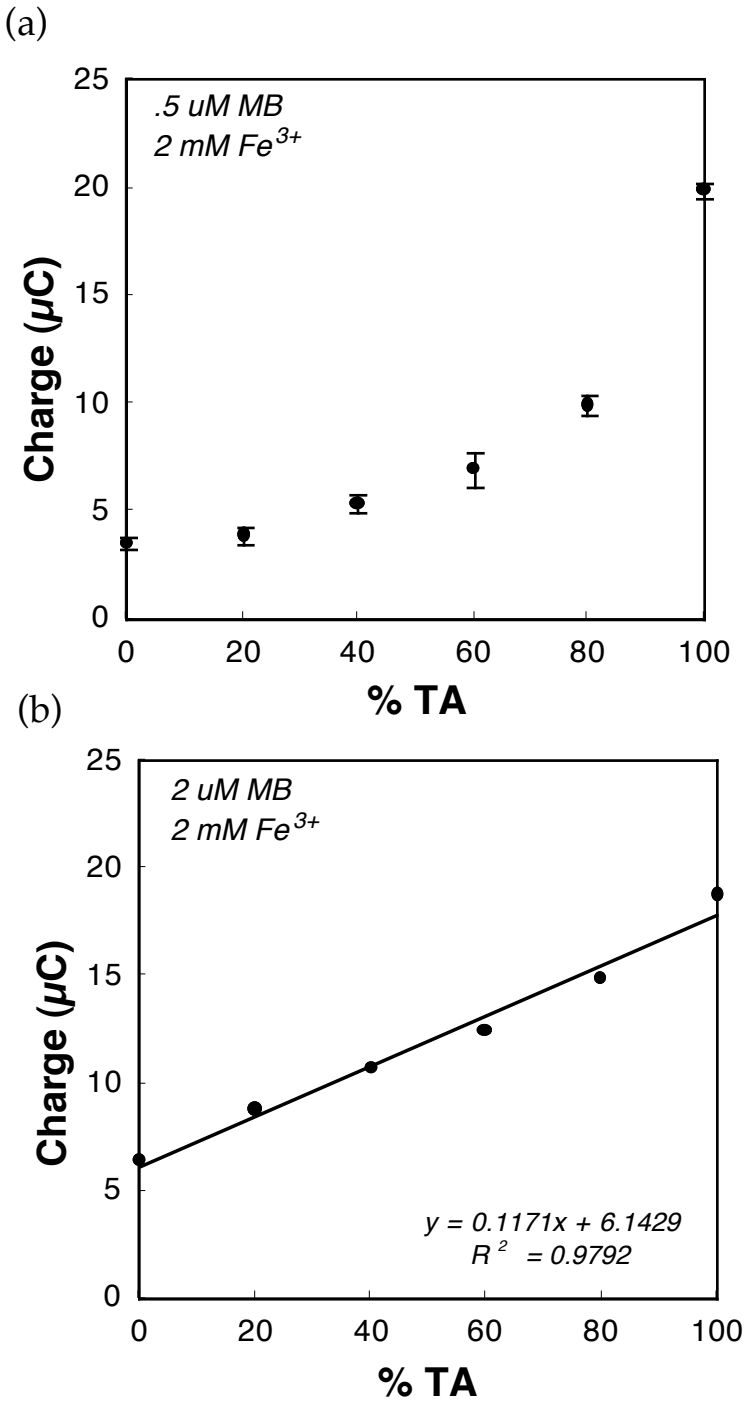
Detection of small percentages of mismatched duplexes within wildtype films

Because somatic mutations associated with genetic disorders may be present in only a small fraction of cells, the ability to detect small numbers of mismatched duplexes within a sea of fully Watson-Crick paired duplexes is of potential great interest. In order to investigate the ability of the electrocatalysis assay to detect such small fractions of mismatched duplexes within a DNA film, monolayers were constructed in which the percentage of helices featuring a CA base step was varied between 0 and 100%. As illustrated in Figure 3.7, using the appropriate experimental conditions (e.g., 0.5 μM MB^+ and 2 mM ferricyanide) the electrocatalytic signal decreases rapidly in a nonlinear fashion as a function of the %CA duplexes in the film.

Consequently, even small amounts of mismatched duplexes in the monolayer result in a pronounced decrease in the electrocatalytic response. Alternatively, using higher concentrations of MB^+ (e.g., 2 μM), a linear response occurs (Figure 3.7).

This differential response is likely due to the mechanism of electrocatalysis in our system. As suggested by catalyst and scan rate dependencies (Chapter 2), we believe reduced MB^+ diffuses away from the monolayer to reduce $Fe(CN)_6^{3-}$ in solution. In this way, if only catalyst bound to perfectly matched DNA is reduced and thus leaves the monolayer, upon once again binding to DNA, it could bind this time to a mismatched duplex where it would not be efficiently reduced, and therefore will remain. Thus, over the course of the chronocoulometric measurement, at low catalyst concentrations, MB^+ becomes concentrated in mismatched duplexes and is thus taken out of the catalytic cycle. This effect is not observed at high catalyst concentrations because at 2 μM MB^+ the film is saturated with catalyst (11); MB^+ is likely bound in each duplex in the monolayer. But at significantly less than stoichiometric MB^+ concentrations, this nonlinear mismatch detection results. Chapter 5 examines the mechanism of electrocatalysis in greater detail. These low catalyst conditions may be useful in the detection of heterozygous genetic traits, in which half of the genes in a diploid organism carry the potentially harmful variation.

Figure 3.7. Plot of total amount of charge accumulated after 5 seconds of chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus (a) 0.5 □M MB^+ (b) 2.0 μM MB^+ (pH 7) at gold electrodes modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired complement. Charge accumulated is nonlinear with mismatch concentration at low catalyst concentration.



Sensitivity and detection limit

In order to explore the detection limit of this electrocatalytic assay, we fabricated DNA chips for use in electrocatalysis experiments (Figure 3.8). In these experiments we sought to discover whether ultramicroelectrodes were compatible with electrocatalysis. Each chip contained 18 separately addressable gold electrodes ranging in size on a micron scale. The electrodes on the chip were modified with the sequence SH-5'-AGTACAGTCATCGCG and investigated by electrocatalysis at -350 mV with 0.5 μM MB^+ and 2 mM $Fe(CN)_6^{3-}$.

As can be seen in Figure 3.8, the total amount of charge accumulated after 5 seconds of electrocatalysis was found to be strictly proportional to the electrode area. Reproducible results were found down to electrodes as small as 30 μm or less in diameter. For electrodes of smaller dimensions, irreproducible results were obtained which may be a result of electrode fabrication or self-assembly of the DNA film. These small electrode sizes will require optimization. Given a 30 μm electrode, however, it is possible to detect ~10^8 molecules.

Mismatch detection with sequences hybridized at the gold surface on the ultramicroelectrodes is also possible. Here we examined the mismatched sequence after in situ hybridization. As can be seen in Figure 3.9, the electrodes on the chip were first modified with the well matched sequence SH-5'-AGTACAGTCATCGCG and investigated by electrocatalysis at -350 mV with 0.5 μM MB^+ and 2 mM $Fe(CN)_6^{3-}$. Then they were gently washed with 90°C buffer for 2 minutes followed by rinsing and incubation with a complement containing a single mismatch. Once fully hybridized,

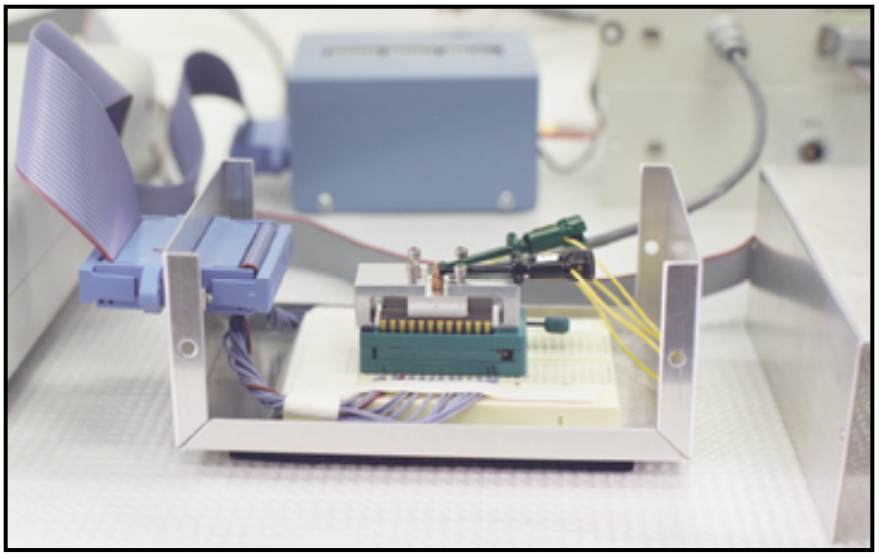
electrocatalysis at -350 mV with 0.5 μM MB^+ and 2 mM $Fe(CN)_6^{3-}$ was then remeasured and the accumulated charge was found to be characteristic of a mismatched DNA monolayer.

We also examined charge accumulated from the single stranded monolayer. It is evident that electrocatalysis on the single stranded monolayer results in charges similar to films with no mismatch. This is attributed to the fact that in a single stranded monolayer (formed by denaturation of the double stranded monolayer) the gold surface is more exposed. Thus MB^+ and ferricyanide are allowed access to the electrode for direct catalysis (not *through* DNA). Furthermore, the line shape for the single stranded monolayer is different from double stranded monolayers, which is consistent with a different mechanism for electrocatalysis (direct verses through DNA). Our observations with single stranded DNA also provide a control demonstrating that detection is not a measure of hybridization. Both double stranded (with no mismatches) and single stranded films yield similar responses. In contrast, the mismatched complement, although fully hybridized, yields an attenuated signal.

Thus mismatches can be detected using ultramicroelectrodes and with very small quantities of test sample. Electrocatalysis is compatible with a chip based assay.

Figure 3.8. (a) Picture of DNA chip apparatus used in these experiments. (b) Plot of total amount of charge accumulated after 5 seconds of chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 □M MB^+ (pH 7) at gold electrodes ranging in size from 30 □m to 500 □m modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired complement. Charge accumulated is proportional to electrode size.

(a)



(b)

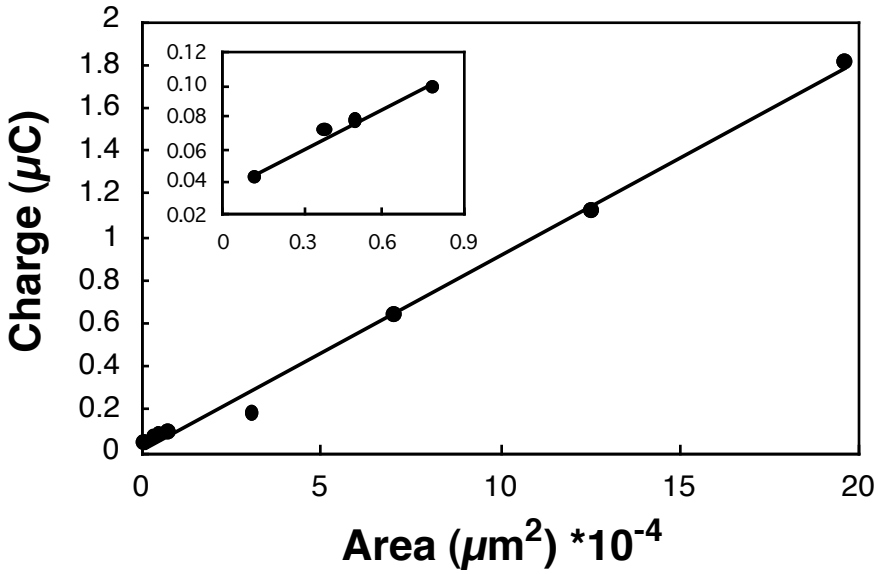
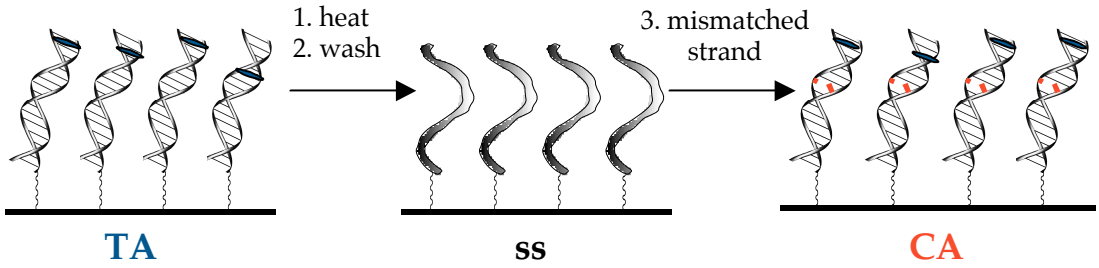
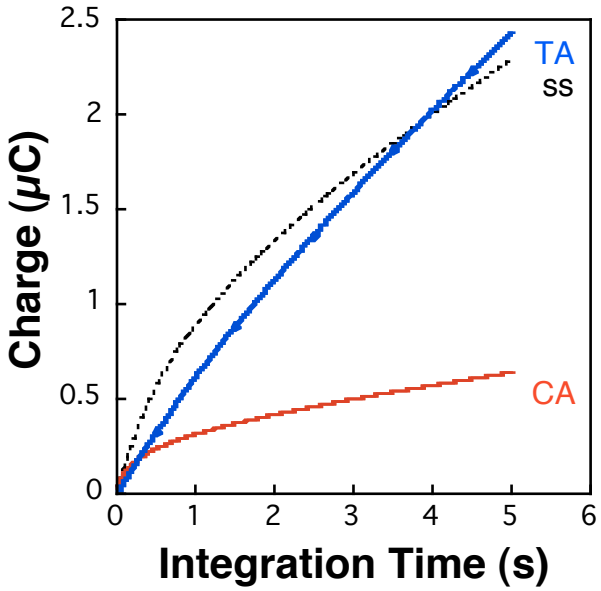


Figure 3.9. Chronocoulometry at –350 mV of 2.0 mM $Fe(CN)_6^{3-}$ plus 0.5 □M MB^+ (pH 7) at a 500 □m gold electrode modified with the thiol-terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a fully base paired complement (TA) and a complement with a single base pair mismatch (CA). The CA complement was hybridized to the chip via in situ hybridization, described as follows. The TA complement was removed by gentle washing of the chip surface with 90°C buffer for 2 minutes. The chip was then rinsed with pure buffer followed by incubation with 100 pM of complement in a solution of buffer containing 100 mM $MgCl_2$. After this, the chip is again rinsed and then used in chronocoulometry experiments. These experiments are carried out under completely hybridizing conditions. Mismatch detection is based on reduced DNA-mediated electron transfer efficiency due to the local base stack perturbation cased by a mismatch.





SUMMARY

A new electrocatalytic method for the detection of single base mismatches as well as DNA base lesions in fully hybridized duplexes has been developed based on charge transport through DNA films. Gold electrodes modified with preassembled DNA duplexes are used to monitor the electrocatalytic signal of methylene blue, a redox active DNA intercalator, coupled to $Fe(CN)_6^{3-}$. The presence of mismatched or damaged DNA bases substantially diminishes the electrocatalytic signal. All single base mismatches, including thermodynamically stable GT and GA mismatches, can be detected within DNA and DNA/RNA hybrid duplexes using this technology without stringent conditions for hybridization since this assay is not a measure of differential hybridization. Furthermore, many common DNA lesions and "hot spot" mutations in the human p53 genome are also discriminated from perfect duplexes. Mismatches can be detected as a small percentage of a perfectly matched film, making it possible to detect mutations associated with genetic disorders in only a small fraction of cells. Finally, the application of this technology into a chip based format is demonstrated. This system provides an extremely sensitive probe for the integrity of DNA sequences and a completely new approach to single base mismatch detection.

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