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## DNA-Mediated Electrochemistry

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### Abstract

The base pair stack of DNA has been demonstrated as a medium for long range charge transport chemistry both in solution and at DNA-modified surfaces. This chemistry is exquisitely sensitive to structural perturbations in the base pair stack as occur with lesions, single base mismatches, and protein binding. We have exploited this sensitivity for the development of reliable electrochemical assays based on DNA charge transport at self-assembled DNA monolayers. Here we discuss the characteristic features, applications, and advantages of DNA-mediated electrochemistry.

### 1. Introduction

Since the elucidation of the double helical structure of DNA, scientists have been fascinated by the possibility that the stacked aromatic base pairs of the duplex may promote charge transport (CT) over significant distances (1,2). Consequently, the nature of the conductive properties of duplex DNA has attracted substantial interest (3–7). Over the past two decades, a wide-ranging collection of experiments has both revealed the fundamental details of DNA-mediated CT and illustrated its potential for sensing applications.

Initial solution experiments featured photoinduced DNA-mediated CT between well defined donor and acceptor sites (8,9). While long-range CT has been shown to yield oxidative damage in DNA up to 200 Å away from the bound oxidant (10,11), DNA CT has also been found to be exquisitely sensitive to the integrity of the base pair stack (9,12) and to the coupling of the donors and acceptors with the DNA (13). Indeed, this sensitivity has prompted both the consideration of biological roles for DNA CT (14) and the construction of electrochemical DNA-based sensors for mutations, base lesions, and protein-binding (15).

Electrochemical techniques provide a particularly convenient means for the study of heterogeneous electron transfer at solid surfaces (16). Typically, redox-active molecules are modified with thiol-terminated alkyl chains and self assembled as well ordered monolayers on metal surfaces (17). Under an applied potential, electrons or holes are then transferred to pendant, redox-active head groups with the rates and yield of charge transfer (as measured electrochemically) providing information on the structure of the thiol terminated linker. In our laboratory, we have extended this methodology to the study and application of DNA-modified surfaces where charge transfer is *mediated* by the DNA monolayers, which are self-assembled onto conductive substrates.

Before embarking on a general discussion of the application of this methodology, it is important to have a clear understanding of the architecture of DNA-modified surfaces. Independent of the material employed, duplex DNA is modified at the 5' end of a single strand with a linker,

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which allows it to interact with the electrode surface (Figure 1). In the case of gold, this tether is an alkane thiol; for graphitic surfaces, it is pyrene. Electrodes are then incubated with modified duplexes and an organized monolayer is allowed to self-assemble. A redox-active molecule is introduced either by exposure to a solution or through covalent attachment to the 5' terminus of the single strand lacking the linker. Subsequent addition of an inert backfilling agent, such as mercaptohexanol, promotes the removal of non-specifically adsorbed duplexes and single strands.

We have extensively characterized these DNA monolayers on both gold and graphite via electrochemical and physical techniques (15). Structural characterization has confirmed that the DNA duplexes within a DNA monolayer adopt an upright orientation, at an angle relative to the surface. The uniformity and reliability of this structure enables the duplexes to serve as an extension of the active electrode surface, thus allowing DNA-bound, redox-active molecules to be reduced via the  $\pi$ -stack. Importantly, fabrication of the surface with rigid, structurally defined DNA duplexes, rather than with single stranded DNAs that can stick to the surface in ill-defined conformations, provides an enormous level of control and reliability in the construction of the monolayers. Moreover, since the electrochemistry of the DNA probe is remarkably sensitive to the integrity of the base pair stack, single base mismatches and lesions, located between the surface and the probe dramatically attenuate the electrochemical yield of CT. These findings have been extended to general assays for the study of DNA/RNA hybrids and RNA transcripts. Furthermore, we have been able to detect a wide variety of DNA-binding proteins based on their association and electronic communication with the base pair stack. These data have underscored the strength and versatility of this methodology.

It is noteworthy that the charge transport properties of DNA monolayers are not simply of utility in the context of electrochemistry. The base pair stack of each individual duplex serves as a conduit for charge transfer, as recently confirmed by single molecule experiments within our laboratory (18). In fact, when considered in the context of an electrical circuit, the DNA duplexes and bound redox-active probes act as transducer elements with extraordinary gain; biochemical binding events such as hybridization are sensitively transduced into electrical signals. This emphasizes the role of DNA as a remarkably unique medium that provides unprecedented opportunities for studies and applications of long-range CT.

Finally, this review is by no means exhaustive but rather seeks to provide an overview of DNA-mediated electrochemical studies undertaken within our group. Here, we present our explorations of features characteristic of DNA-mediated CT that have been critical for applications of DNA monolayers as biosensors. In fact, several other reviews have detailed the use of electrochemical DNA sensors (19–21), and where appropriate, examples from other authors are presented.

## 2. Surface Characterization

For proper interpretation of experimental observations, extensive physical characterization of these self-assembled DNA monolayers is crucial. A gamut of experimental techniques can be utilized for investigating the structure and characteristics of DNA monolayers including (but not limited to) radioactive labeling (22–25), fluorescence self-interference (26–28), scanning tunneling microscopy (29–31), atomic force microscopy (23,25,32–36), scanning electrochemical microscopy (37–39), and surface plasmon resonance (40,41). When dense DNA monolayers are desired, the monolayers are assembled in the presence of  $Mg^{2+}$  to allow for close packing of the duplexes (schematically illustrated in Figure 1). Our group, in particular, has focused on surface characterization via radioactive labeling and scanning probe techniques. These investigations have indicated that DNA monolayers adopt an upright

orientation and have predictable surface coverages, thereby allowing us to develop a detailed picture of DNA monolayer morphology.

### Quantification of Surface Coverage Via Radioactive Labeling

Radioactive labeling of the duplexes provides a reliable measure of surface coverage for DNA monolayers. In a typical radioactive labeling experiment, the 5' end of the DNA complementary to the thiolated strand is labeled with polynucleotide kinase, and the amount of DNA is quantified directly via scintillation counting after self-assembly. On gold and graphite, these measurements indicated surface coverages of ~40 pmol/cm<sup>2</sup> for well packed DNA monolayers self assembled in the presence of Mg<sup>2+</sup> (22,25) and coverages of ~12 pmol/cm<sup>2</sup> for loosely-packed DNA monolayers in the absence of Mg<sup>2+</sup> (23). Interestingly, the well packed monolayer coverages obtained via radioactive labeling correspond to only ~75–85 % of the theoretical coverages of ~60 pmol/cm<sup>2</sup> with DNA helices packed perpendicular to the surface. This fact strongly hinted that the DNA is oriented at an angle with respect to the surface.

The reliability and sensitivity of radioactive labeling relative to electrochemical assays cannot be understated. For example, monolayer formation can be *qualitatively* monitored via attenuation of an anionic probe such as ferri/ferrocyanide, Fe(CN)<sub>6</sub><sup>3-/4-</sup> (22). In addition, a cationic probe such as ruthenium hexamine can be utilized to quantify DNA coverages semi-quantitatively (42,43), but this common electrochemical assay of surface coverage can lead to errors of up to 65 % within each experiment (44). Accordingly, in our laboratory, radioactive labeling studies have proven to be particularly illuminating.

### Scanning Tunneling Microscopy of DNA Monolayers

While radioactive labeling affords a quantitative measurement of the overall surface coverage, the electronic structure of local areas of a DNA monolayer can be probed via scanning tunneling microscopy (STM), providing atomic level information (29). Indeed, STM allows for the investigation of the electronic properties of a DNA as a function of duplex orientation. Therefore, the images obtained yield information on not only the morphology but also the local density of states of the sample. At negative potentials, where the DNA adopts an upright orientation, agglomerates of duplexes are visible because the tip can electronically access the oriented DNA in an effective metal-molecule-metal junction. However, at positive potentials, the DNA is encouraged to lie down and the DNA film is effectively not visible to the STM tip. These data indicated that the DNA duplexes comprising a DNA monolayer aggregate in small hexagonal groups of ~10 nm diameter. Furthermore, the inclusion of a mismatch within the monolayer dramatically attenuates communication between the tip and the surface, demonstrating that the STM is accessing the local density of states of the full DNA duplex.

### Atomic Force Microscopy of DNA Monolayers

The morphology of self-assembled DNA monolayers can be further explored at the nanometer scale with atomic force microscopy (AFM). These investigations have indicated that 5' tethered films are essentially smooth and featureless within the resolution of the AFM tip with the DNA dispersed in a uniform manner (32). AFM measurements also provide important information about the morphology and depth of the DNA monolayer at various substrate biases. By applying a large downward force to the monolayer with the AFM tip, a bare spot can be uncovered on the DNA-modified surface. Height contrast measurements between this bare spot and the surrounding DNA monolayer indicate a film depth which ranges from 4.2 to 4.6 nm for 15 mer duplexes at open circuit on both gold and graphite. In fact, the height of these films can be reversibly modulated from 2 nm (the diameter of the duplex) at positive potentials to 5.5 nm (the full length of the duplex when normal to the surface) at negative potentials (Figure 2). These data, in conjunction with the above experiments, indicate that the duplexes adopt an

upright orientation at negative potentials and are oriented at a ~45° angle with respect to the surface in the absence of an applied bias 25,32).

In a complementary study, we also investigated alternative linkages of the thiol to the DNA. Interestingly, 3' rather than 5' grafted DNA monolayers did not adopt an upright orientation relative to the gold surface, as confirmed by their corresponding film depth of only ~2 nm. Since vertical orientation of the monolayer is absolutely essential for studies based on DNA-mediated CT, this work underscored the importance of considering linker placement when designing self-assembled DNA monolayers (33).

Several other AFM investigations of DNA monolayers are particularly noteworthy, especially when considering the electrochemical monitoring of DNA with larger macromolecules, such as proteins. Loosely packed DNA monolayers assembled in the absence of Mg<sup>2+</sup> are necessary for improved accessibility of proteins, but such monolayers have a distinct “island” morphology since the DNA has room to lie flat on the gold surface in the absence of an applied potential (23). However, monolayers backfilled with a short chain alkanethiol such as mercaptohexanol were demonstrated to adopt an upright orientation relative to the gold surface even at open circuit (35,36). Furthermore, these studies showed that even loosely packed films formed from longer 24 mer and 30 mer duplexes adopt an upright orientation. This well defined morphology of “deeper” monolayers is a crucial design point to consider in the sequence-specific detection of DNA binding proteins with extensive binding footprints.

### 3. DNA-mediated Electrochemistry of Small Molecules Bound to DNA

We have explored numerous redox-active probes in our investigations of DNA CT, and at first glance, many molecules appear adequate for these studies. The large collection of experiments conducted in our laboratory over the past decade, however, has taught us essential lessons regarding the selection of an appropriate redox probe. Three criteria are paramount: the species must (i) be electronically well coupled to the base pair stack, (ii) be reliably linked to the duplex and (iii) undergo stable reduction or oxidation within a potential window that does not compromise the integrity of the DNA monolayer. Furthermore, in addition to affording insight into the optimum probe design, our systematic studies clearly indicate that no one probe is ideally suited for every experiment. Instead, different markers offer distinct advantages and must be individually evaluated for specific applications.

The behavior of the redox probes employed in our studies do, however, display some common features when monitored electrochemically. When bound to DNA, small molecules appear to act as surface-bound species as indicated by a linear relationship between the peak current and scan rate. This relationship is not strictly linear for DNA-bound redox proteins, where the proteins are not situated in one fixed position or conformation and thus exhibit a slight diffusive component. Nonetheless, covalently probes bound to DNA typically do not display a square-root-dependence of the peak current on the scan rate that is indicative of free diffusion. In addition, DNA binding typically shifts the redox potential of the probe by 20–50 mV from direct reduction at the bare surface and the voltammetry adopts a skewed shape relative to a classically adsorbed couple; the reductive and oxidative peaks are separated slightly due to the intervening DNA/alkanethiol spacer. Electrochemical reversibility and stability are also specific to each probe, but they can be interrogated simply by repetitive cycling over the potential window of interest. Taken together, all of these features allow us to distinguish DNA-mediated processes from ones involving direct electrochemistry.

Early work in our laboratory employed probes that were not covalently attached to DNA but instead interacted with the base pair stack electrostatically or via intercalation (22). Although the majority of our studies have involved the intercalator methylene blue, we have also studied the interaction between DNA and anionic ferricyanide, cationic ruthenium hexamine, the

tris-heteroleptic intercalator Ir(bpy)(phen)(phi)<sup>3+</sup>, and the anti-tumor agent daunomycin (Figure 3). While these non-covalent markers have afforded a significant foundation for the study of DNA-mediated CT, they are intrinsically limited in the mechanistic detail and experimental control they can provide. As a result, more recent efforts have largely focused on developing covalently bound probes, specifically those that are electronically well coupled to the base pair stack. This latter family began with investigations of cross-linked daunomycin, and has been more recently expanded to include other covalently attached molecules such as anthraquinone and phenoxazine derivatives (Figure 3).

### Electrochemistry of Small Molecules Non-covalently Bound to DNA

In our earliest electrochemical experiments, methylene blue (MB), a well studied aromatic intercalator that undergoes a  $2e^-/1H^+$  reduction in aqueous systems, was employed as a reporter of DNA-mediated processes (22). (Figure 4) shows a schematic illustration of the reduction of MB at a DNA monolayer; a reversible redox couple is seen with a midpoint potential centered at -10 to -50 mV vs. NHE, shifted by ~30 mV from bare gold. The measured peak currents scale linearly with scan rate, indicating that the redox molecule is surface-bound, and a combination of electrochemical and spectroscopic measurements give a saturation value of 1.4 (2) molecules per duplex at high MB concentrations. This latter finding suggests that, for well packed monolayers, MB is constrained to the top of the film. Any observed reduction, therefore, can be attributed to a DNA-mediated process, as the surface is inaccessible to the molecule.

To characterize this redox process in greater detail, a number of systematic studies were conducted (44–49). Initially, the behavior of the intercalator MB was compared to the groove-binder ruthenium hexammine (47). At high salt concentrations, where interaction with the charged duplex is inhibited, fewer molecules are reduced, thus illustrating the importance of efficient and tight binding to the monolayer for charge transfer. The reduction of these two probes was also monitored on surfaces that had been passivated with electropolymerized 2-naphthol. If the DNA were truly serving as a medium for charge transport, passivation would not affect the process being measured electrochemically. The reduction of ruthenium hexammine, which is thought to proceed via facilitated diffusion along the duplex, decreased by 70 % while reduction of MB was relatively unaffected. Although this study does not elucidate the mechanism of MB reduction, it supports the notion of a DNA-mediated process.

To further prove that the reduction of distally bound probes proceeds in a DNA-mediated fashion, we explored the electrochemistry of small molecules at DNA monolayers containing single base mismatches positioned between the electrode surface and the probe (45). It was theorized that if the DNA base pairs combined to form a continuous,  $\pi$ -stacked conduit for charge transfer, that the disruption of a single base pair, as in a mismatch, could interrupt the CT pathway. The presence of a single base mismatch causes no global change in the duplex structure, and yet, the inclusion of an intervening CA mismatch proved to have a dramatic effect on the efficiency of charge transfer to DM. These results are in excellent agreement with STM studies of matched and mismatched duplexes and with studies of photoinduced DNA CT in solution (9) (*vide supra*). Interestingly, not only could several mismatches be detected but the DNA-mediated chemistry was also found to be independent of sequence context. Furthermore, this exquisite sensitivity to base pair stacking has since been observed for numerous intercalating probes including MB and Ir(bpy)(phen)(phi)<sup>3+</sup> but not for the groove-binding ruthenium hexammine. Thus the observed electrochemistry is DNA-mediated and a general characteristic of the DNA, independent of the redox probe.

To increase the sensitivity of mismatch detection and further improve discrimination, MB was coupled to freely diffusing Fe(CN)<sub>6</sub><sup>3-</sup> in an electrocatalytic cycle (Figure 5). In this catalytic process, MB undergoes a two  $e^-$  reduction via the base pair stack, and upon reduction to leucomethylene blue, loses some affinity for DNA, thus dissociating from the duplex (45,46).

In turn, the cycle continues when leucomethylene blue is reoxidized by  $\text{Fe}(\text{CN})_6^{3-}$  in solution, and binds again to DNA for another cycle of DNA-mediated reduction. It should be noted that  $\text{Fe}(\text{CN})_6^{3-}$  cannot be reduced directly at the negatively charged monolayer owing to repulsion from the polyanionic DNA monolayer. The entire process is effectively governed by the on/off kinetics of the methylene blue/leucomethylene blue redox couple, as confirmed by rotating disk electrode experiments (48). In fact, during the course of a single voltammogram, the DNA is repeatedly sampled, amplifying the absolute signal of MB and increasing the signal attenuation associated with a mismatch.

The detection of all naturally-occurring mismatches and nearly all common lesions was achieved with the MB/ $\text{Fe}(\text{CN})_6^{3-}$  electrocatalytic couple via cyclic voltammetry and chronocoulometry (Figure 6). In addition, low detection limits were achieved in a chip-based format, with mismatch discrimination possible at electrodes as small as 30  $\mu\text{m}$  in diameter (46). It should also be noted that the relatively stable GA mismatch could *not* be detected without the aid of the described catalytic cycle, emphasizing the utility of electrocatalysis as an electrochemical tool. Furthermore, even base lesions that cause minor thermodynamic destabilization to the duplex could be detected with electrocatalysis: a systematic investigation revealed that the efficiency of CT was dramatically decreased even by subtle base modifications that altered base structure, steric bulk, or hydrogen-bonding (49). Perhaps not surprisingly, modifications with methyl groups that do not participate in hydrogen-bonding had little to no effect on the DNA-mediated electrochemistry. The sensitive detection of all single base mismatches independent of sequence context is remarkable and has proven to be valuable in the application of DNA electrochemistry for the diagnosis of single nucleotide polymorphisms.

MB electrochemistry was also extensively explored through self-assembled DNA monolayers on highly ordered pyrolytic graphite (HOPG), onto which the DNA is anchored to the surface via a pyrene moiety (25). As a surface, HOPG provides an attractive alternative since its accessible potential window is no longer limited by the oxidative reduction or desorption of thiols. The behavior of MB on graphite is quite similar to that on gold. Evidence that the charge transfer is DNA-mediated is provided by the facile detection of the CA and GT mismatches. The wider potential window afforded by HOPG allowed for the study of the voltammetry of  $\text{Ru}(\text{bpy})_2\text{dppz}^{2+}$  and  $\text{Os}(\text{phen})_2\text{dppz}^{2+}$ . As expected, the electrochemistry of both intercalators was found to be DNA-mediated.

### Electrochemistry of Small Molecules Covalently Bound to DNA

To facilitate the systematic investigation of charge transduction of DNA, it became necessary to employ redox-active probes that are covalently conjugated to the base pair stack. Our initial efforts focused on covalently attached daunomycin (DM), which can be site-specifically cross-linked to the exocyclic amine of guanine, forming a covalent bond, as shown in (Figure 7) (50–52). Most importantly, this allows for controlled placement of the probe within the sequence. Since functionalization with DM occurs after the synthesis of the DNA, it does, however, necessitate that all guanines other than the one to be linked are replaced by inosine, a close base analogue. For the purpose of electrochemical measurements, this substitution has no influence on the results, but this requirement does have to be considered when developing sensing applications. Importantly, covalent attachment of this probe for DNA electrochemistry has made possible several seminal conclusions about DNA-mediated CT involving *ground-state* reactants.

In initial experiments, the separation between the gold surface and DM adduct was varied from 15 Å to 45 Å, and no effect on the yield or rate of electron transfer was observed (50). The inclusion of a mismatch in the intervening DNA, however, completely shuts off the electrochemistry of DM, conclusively demonstrating that CT is DNA-mediated. Subsequently,

with DM held in a fixed position in the duplex, the length of the thiolated alkyl tether was varied to probe the effect of tether length on the rate of charge transfer (51). While the yield of CT remained the same regardless of chain length (the number of intervening methylene units was increased from 4 to 9), significantly, the rate of electron transfer decreased with increased tether length with a  $\beta_n$  of 1.0 per  $-\text{CH}_2-$ unit, as expected for the variation in coupling with  $\sigma$ -bonded systems. Therefore, CT through the alkyl tether, not through 30 Å of  $\pi$ -stacked DNA, was the rate-limiting step of the DNA-mediated reduction. Taken together, these findings illustrate that the DNA-DM construct behaves as a single redox-active entity with dramatically different rates of CT through the stacked DNA and the  $\sigma$ -bonded alkyl chain.

In a later study, DM was employed to prove that DNA-mediated electrochemistry occurs via the base pair stack and not the sugar-phosphate backbone (52). With two DM moieties crosslinked to guanine residues, voltammograms of duplexes containing a nick in the backbone, a nick and a mismatch, a nick on both strands, and no modifications were compared. Nicks in the phosphate backbone did not attenuate CT, but, consistent with previous results, mismatches significantly attenuate the electrochemistry of DM. Interestingly, a later study on HOPG showed that the electrochemistry of thiols incorporated within the sugar-phosphate backbone was DNA-mediated but proceeded at low yield (53). Therefore, while DNA electrochemistry proceeds through the base pair stack rather than through the sugar-phosphate backbone, it can promote reactions on the DNA backbone.

While DM afforded several key findings for our applications, it does possess some inherent limitations. As mentioned above, all guanine residues, other than the one to be linked, have to be replaced by inosines, limiting its applicability in biosensors. Moreover, the stability of the linkage between DM and DNA is heat-sensitive. As a result, we have been focused on developing a new generation of probes that can be covalently attached to DNA with synthetic flexibility, are stable, and are electronically well coupled to the base pair stack.

Through a variety of experiments conducted both in our laboratory and others, it has become apparent that efficient coupling of the probe to the base stack is critical for efficient DNA CT. Recently, the groups of Saito and Gooding introduced thymines modified with anthraquinone as effective, covalently attached probes of charge transfer at DNA modified surfaces (54–57). Using this technology, Gooding and coworkers demonstrated the application of these modified probes to the detection of primer extension reactions (54,55), and Saito and coworkers demonstrated the photostimulated detection of mismatches with applications to genotyping of single nucleotide polymorphisms (56,57). However, the low current densities obtained in these studies led us to conduct a comparative study of anthraquinone linked to DNA through conjugated and saturated tethers (58). Importantly, we found efficient redox chemistry and mismatch sensitivity for anthraquinone only when linked through an alkyne and not when tethered by an alkyl chain (Figure 8). Thus not only the choice of probe but also how the probe is coupled to the base pair stack are key to effective DNA-mediated CT.

More recently, we have investigated two phenoxazine-based probes, Redmond Red and Nile Blue, as covalent reporters of DNA CT (Figure 3). Redmond Red is commercially available, affording facile incorporation into DNA with no sequence restrictions (59), and Nile Blue can be attached in high yield to DNA by reacting its exocyclic amine with a carboxy-NHS-ester-thymine (60). These probes can be incorporated via standard phosphoramidite chemistry, have virtually no sequence restrictions, are stable to light/heat, and support DNA-mediated electrochemistry. Accordingly, they are preferable to DM for routine use and high throughput experiments; both of these probes have already been applied for electrochemical monitoring of protein/DNA interactions (*vide infra*). Furthermore, Nile Blue has proven effective for detection of hybridization and has displayed electrocatalytic activity (unpublished results).

Redmond Red, moreover, has proven to be a particularly sensitive reporter of abasic sites in DNA. The development of these probes holds great promise for future experiments.

#### 4. Electrochemical Detection of Hybridization and Transcription Products

The studies described thus far report of findings achieved with pre-hybridized duplexes on gold or graphite; the monolayers were formed exclusively via self-assembly of duplex DNA prior to electrochemical interrogation. It is of tremendous importance, from both a fundamental and an applications perspective, to demonstrate similar behavior of DNA-mediated electrochemistry when duplexes are instead formed *in situ*. This approach allows for repeated, internally normalized investigations of a single surface, thereby laying the groundwork for the detection of lesions, mutations, and mismatches in chip-based genetic analysis.

Initial promising results have led current efforts to focus on the design and development of an electrochemical assay for the products of transcription. Consequently, we first explored DNA-mediated CT in DNA/RNA hybrids. Given the known correlation between several RNA sequences and the development of disease, the ability to simply and quantitatively detect the concentration of particular RNA sequences could ultimately be exploited for both disease diagnosis and chemotherapeutic design.

##### Electrochemical Detection of Mismatches In Situ

A comparative *in situ* experiment on modified gold surfaces allowed for a CA mismatch to be reversibly detected via DNA-mediated CT (45). DNA was immobilized on two separate electrodes, one bearing duplexes with well matched complements, the second containing duplexes with a single base mismatch. Following exposure to a solution of DM, the electrodes exhibited the behavior expected for their respective complements, namely the signals were much larger for the well matched duplex. The duplexes were then dehybridized by heat denaturation and re-incubated with the opposite complement; the resulting well matched duplex exhibited an increased signal while the response from the mismatched duplex displayed significant attenuation. Importantly, the signals observed for single-stranded DNA were qualitatively different from those observed for either mismatched or well matched DNA, confirming duplex formation.

By utilizing the MB/Fe(CN)<sub>6</sub><sup>3-</sup> electrocatalytic couple, highly sensitive detection limits for hybridization have been achieved (46). Monolayers containing well matched DNA were first self-assembled at gold microelectrodes manufactured in a chip-based format and investigated with chronocoulometry. The DNA was subsequently rehybridized with 100 pM of mismatched complement before chronocoulometry was performed again (Figure 9). Dramatic signal attenuation was observed *in situ* in the presence of even a single mismatch. Controls performed with single stranded DNA again yielded voltammetry which was broad, weak, irreproducible, and qualitatively different, again confirming duplex formation.

In addition to cyclic voltammetry and chronocoloumetry, an alternative electrochemical technique, electrochemical impedance spectroscopy, can also report on duplex formation *in situ*. Differences in the surface coverage are reflected in the electron-transfer resistance, ( $R_{ET}$ ), as determined by this measurement. Using Fe(CN)<sub>6</sub><sup>3-</sup> as an electrochemical reporter, electrode surfaces were taken through several dehybridization/rehybridization cycles (using simple heat denaturation and room-temperature annealing), where the impedance at the surface was monitored at each step. The sensitivity of this technique allowed for excellent discrimination between dehybridized and rehybridized surfaces, allowing for label-free detection of duplex formation (44).

## Electrochemistry Through DNA/RNA Hybrids

As a complement to the hybridization studies described above, our laboratory has also investigated DNA-mediated charge transport through DNA/RNA hybrids directly. It is known that these naturally-occurring intermediates adopt a conformation that more closely resembles A-form DNA, whereby the helix is significantly wider and more compact than canonical B-form. While these structural differences could potentially interfere with a charge transport pathway down the helix, electrochemical studies within our laboratory have demonstrated that DNA/RNA hybrids are indeed capable of conducting charge in a manner similar to DNA/DNA duplexes (61,62). Non-covalent MB reduced via a DNA/RNA hybrid exhibits nearly identical voltammetric behavior to that observed with DNA. Moreover, electrocatalytically coupling MB with  $\text{Fe}(\text{CN})_6^{3-}$  affords discrimination of all possible mismatches in DNA/RNA hybrids, as was monitored by chronocoulometry. These studies reveal that DNA-mediated CT can serve as an effective methodology for the detection of transcription products. Current efforts are now focused on the development of a sensitive and quantitative electrochemical hybridization assay for mRNA using covalently attached anthraquinone.

## 5. Electrochemical Monitoring of Protein/DNA Interactions

Self-assembled DNA monolayers also provide particularly convenient platforms for electrical monitoring of protein/DNA interactions. These interactions play crucial roles in many cellular processes such as transcription, repair, and replication. In particular, the association of transcription factors with DNA is an important area for exploration in proteomics and genomics; it is these interactions that control the developmental and regulatory responses of the cell, often in a complicated fashion. Therefore, the development of convenient and inexpensive methodologies for monitoring protein-DNA interactions remains of critical importance.

In a typical experiment, a loosely packed DNA monolayer is self-assembled with or without a covalently appended probe and the surface is backfilled (Figure 10). Subsequently, voltammetry of the DNA-modified electrode is recorded before and after addition of protein. Detection of protein binding can be achieved in two ways: 1) proteins that perturb the base pair stack will attenuate the yield of DNA CT to a distally-bound electroactive probe and 2) proteins featuring a redox-active cofactor such as an iron-sulfur cluster will cause the appearance of a new DNA-dependent electrochemical signal. These experiments have particularly benefited from the development of covalently attached probes which have allowed for conclusive quantification of the yield of DNA CT before and after protein binding. Furthermore, such electrochemical assays are general and based on exquisitely sensitive DNA-mediated electrochemistry, so a wide range of protein/DNA interactions and binding motifs can be sequence-specifically interrogated in real time.

### Detection of Transcription Factors

Our early studies at DNA-DM monolayers demonstrated that we could easily detect TATA-binding protein (TBP), a ubiquitous transcription factor that bends duplex DNA by  $\sim 90^\circ$  (23). The addition of TBP to a DNA-modified surface results in distortion of the DNA and lowers the yield of DNA CT (Figure 10). Later, we extended this methodology to the detection of TBP at DNA-modified microelectrodes (60). At DNA monolayers modified with Nile Blue, TBP could be readily detected at either the macro- or microscale. However, microelectrodes allowed for the rapid detection of nanomolar concentrations of this transcription factor: 300 nM TBP could be reversibly detected with total signal loss in less than 30 seconds. Furthermore, 30 nM concentrations of TBP were detected even in the presence of micromolar amounts of bovine serum albumin, EndonucleaseIII, or Bam HI methyltransferase. These data hinted at

the potential of assays based on DNA CT for the high throughput, multiplexed electrical monitoring of numerous DNA binding proteins on a single chip.

### Detection of Base Flipping Enzymes

Methyltransferases are base-flipping enzymes that catalyze the transfer of a methyl group from S-adenosyl-methionine to adenine or cytosine within a DNA duplex. Establishing base flipping by a protein was historically quite difficult, usually requiring a crystal structure, but the electrical detection of base flipping using DNA electrochemistry has been found to be a sensitive, rapid, and attractive method for this characterization.

While methyl substitution does not appreciably perturb the base pair stack, methyltransferases, in carrying out the base flip, significantly attenuate DNA-mediated CT. Based upon this attenuation, we have detected base flipping in binding both uracil DNA glycosylase and *HhaI* methyltransferase to their cognate sequences (23). At a surface featuring a DNA-DM monolayer, for example, the addition of *M.HhaI* disrupts the integrity of the base pair stack with the concomitant decrease in the DM redox signal. Based upon the crystal structure of the protein/DNA complex, it is apparent that upon base flipping the internal cytosine of the recognition sequence 5'-GCGC-3', *M.HhaI* also intercalates Gln 237, a non-aromatic residue into the base pair stack, filling the space occupied by the flipped out cytosine, and interrupting the  $\pi$ -stacking within the duplex. Accordingly, electrochemistry experiments were conducted with a Q237W mutant enzyme. In this case, we expected that the mutant protein would insert the aromatic tryptophan residue into the  $\pi$ -stack upon base flipping, so little signal loss would be found; indeed, little attenuation in DM signal upon binding the mutant protein was observed. Moreover, to establish that the lack of attenuation was the result of restoration of the  $\pi$ -stack and not simply poor DNA binding by the mutant, we also examined binding of both wild type and mutant *M.HhaI* to a DNA-DM monolayer containing an abasic site at the position of what would be the flipped out cytosine. On this DNA-DM monolayer, the DM redox signal is only weakly detected both in the absence of protein and in the presence of wild type *M.HhaI*, owing to the poor stacking of the duplex with an intervening abasic site. Nonetheless, in the presence of the Q237W mutant, the DM redox signal is restored. This enhanced signal for DM on binding the mutant reflects insertion of the tryptophan from the mutant protein within the base pair stack, so as to restore proper stacking in the DNA-DM duplex. Hence DNA-binding proteins are seen to modulate DNA CT both positively and negatively, depending upon how they affect the conformation of the DNA.

### Electrical Monitoring of Proteins in Real Time

Another advantage of monitoring protein binding to DNA electrically comes from the fact that this technique allows the sensitive detection of events in real time. This was illustrated first by showing double-stranded cleavage on a DNA-DM monolayer using the *PvuII* restriction enzyme (23). In the presence of magnesium ion, *PvuII* cleaves DNA sequence-specifically. Addition of *PvuII* to a DNA monolayer containing the *PvuII* recognition sequence with DM attached to the top of the film but without magnesium causes little perturbation in the DM redox signal. However, the addition of magnesium ion is seen to trigger loss of the DM signal; as the protein cleaves the DNA, the fragment containing the redox probe is released into solution. Interestingly, the kinetics of this signal loss parallels closely that seen for the protein restricting DNA in solution followed by DNA cleavage analysis using gel electrophoresis. The advantage of the electrochemistry technique, however, is clear: the restriction reaction can be monitored in real time.

Monitoring the repair of thymine dimers in DNA by photolyase provides another illustration of how protein/DNA reactions may be monitored sensitively and in real time using DNA electrochemistry (63). Photolyase is a repair enzyme from *E. coli* containing a flavin cofactor

that binds pyrimidine dimers in duplex DNA and repairs them, upon photoactivation, in a reaction involving electron transfer from the reduced flavin cofactor to the dimer flipped out of the DNA duplex and bound in the pocket of the enzyme near the cofactor. Importantly, in monitoring photolyase reactions electrochemically on DNA monolayers, we did not utilize an externally bound DM redox probe; instead we depended upon the redox signal of the flavin, intimately bound in the protein pocket against the DNA. In binding the photolyase to a DNA monolayer containing the thymine dimer, we found first a weak redox signal arising at 40 mV versus NHE. Through control studies with apoprotein and a mutant, we assigned this redox signal to the flavin and attributed the weak signal to poor electrochemical coupling owing to the intervening destacked thymine dimer. We then carried out the repair reaction by illuminating the electrode surface. With irradiation, we observed an increase of almost an order of magnitude in the integrated flavin signal, and we confirmed repair of the thymine dimer using HPLC analysis. Thus we could monitor *in real time*, as a function of irradiation, the repair of the thymine dimer by photolyase; as the photolyase repairs the thymine dimer, the base pair stack is restored, the photolyase becomes well coupled into the base stack, and the flavin signal is enhanced (Figure 11). Subsequently, we found that once the DNA is repaired, the protein dissociates from the monolayer and the signal intensity is lost.

The ability to monitor, in real time, the binding and chemical reaction of photolyase on DNA highlights the potential of DNA-mediated CT more generally in the analysis of DNA-binding proteins. DNA electrochemistry is unique in its sensitive reporting on DNA conformational changes that may be associated with protein reactions.

### Electrochemistry of Base Excision Repair Enzymes Containing Iron Sulfur Clusters

Our DNA electrochemistry experiments with photolyase provide illustrations not only of how DNA electrochemistry can be used to assess DNA conformational changes associated with protein binding, but also how DNA electrochemistry may be utilized to measure the redox potentials of DNA-binding proteins in their active, DNA-bound form. For these experiments no external redox probe, such as MB or DM, was utilized. Importantly, we could determine the potential of the internally bound flavin cofactor and under conditions where the protein was bound to DNA to carry out its chemistry.

DNA electrochemistry has now been more generally applied in assessing the redox characteristics of DNA-binding proteins in their *active, DNA-bound* form. An illustrative example is found in the characterization of the redox chemistry associated with two base excision repair (BER) enzymes: MutY, a glycosylase that excises adenine from 8-oxoguanine:adenine mispairs, and Endonuclease III, a glycosylase that targets oxidized pyrimidines (64–71). Crystal structures of these enzymes indicated that they both contain an iron-sulfur cluster with the C-X<sub>6</sub>-C-X<sub>2</sub>-C-X<sub>5</sub>-C motif in close proximity to the DNA backbone (65–68). This Fe<sub>4</sub>S<sub>4</sub> motif, moreover, is conserved in base excision repair proteins from bacteria to man. Interestingly, in MutY, this cluster is not necessary for protein folding or stability but is crucial for DNA binding and *in vivo* activity (69). EPR experiments have demonstrated that the cluster is in its [4Fe-4S]<sup>2+</sup> form when isolated from the cell, but the protein cannot be reduced within a physiologically relevant window nor oxidized by ferricyanide without degradation (70,71). These studies, suggesting a lack of redox activity associated with reaction by the repair proteins, were, however, conducted in the absence of DNA. Significantly, we found that EndoIII and MutY display stable, quasi-reversible signals with potentials of ~ 90 mV versus NHE (72,73), at DNA-modified, surfaces, backfilled with mercaptohexanol for passivation; this potential is appropriate for a physiologically relevant redox switch. Importantly, these signals are not observed at surfaces backfilled with mercaptohexanol but lacking DNA. Furthermore, weak signals for the proteins on a surface

containing a DNA duplex with an abasic site demonstrate that the observed redox processes for the proteins are DNA-mediated.

Experiments performed on DNA-modified, highly oriented pyrolytic graphite (HOPG) allowed for the direct comparison of the electrochemistry of EndoIII in the presence and absence of DNA (74). Qualitatively similar results were obtained in the presence of DNA on Au and HOPG. Due to its extended potential window, however, HOPG allows for the study of the electrochemistry of EndoIII also in the absence of DNA, a measurement made impossible by the potential window limit on gold. Oxidation of the  $[4\text{Fe}-4\text{S}]^{2+/3+}$  form of the cluster without DNA led to an anodic signal at 250 mV versus NHE which was followed by rapid decomposition, in confirmation of solution studies; reductive scans revealed a cathodic peak assigned to the  $[4\text{Fe}-4\text{S}]^{2+/1+}$  couple of EndoIII. The ability to measure the redox potential of both free and bound EndoIII on the same surface thus allowed for the convincing demonstration that binding to DNA shifts the redox potential of the cluster by  $-200$  mV and stabilizes its  $[4\text{Fe}-4\text{S}]^{3+}$  state (Figure 12). The cluster therefore is redox-active at ambient temperature in its physiologically relevant, DNA-bound form.

These data underscore the importance of characterizing the redox chemistry of DNA-binding proteins in their biologically relevant, DNA-bound forms. Base excision repair enzymes represented an interesting target for us to study, given the ubiquity of the iron-sulfur clusters in these enzymes with no apparent function. In our laboratory, the finding that these repair proteins become activated to redox chemistry upon binding DNA presents an attractive context for possible DNA-mediated CT *in vivo*. Importantly, this is but one example, and other DNA-binding enzymes that contain a potential redox cofactor may also take advantage of this chemistry. DNA electrochemistry thus provides a first assay to characterize the redox chemistry of DNA-binding, and, significantly, to begin to determine whether these proteins may utilize DNA-mediated redox chemistry within the cell.

### Electrochemistry of a Transcription Bound to DNA

SoxR is a 17 kDa transcription factor that binds DNA as a dimer with a [2Fe-2S] cluster in each monomer (59,75,76). The loss of the cluster does not affect protein folding, DNA binding, or promoter affinity, but oxidation of the cluster triggers the transcription of SoxS and the subsequent expression of over 100 other genes responsible for the oxidative stress response in bacteria. Interestingly, the redox potential of this cluster in the absence of DNA was determined as  $-290$  mV vs. NHE. Importantly, this measurement of the potential of SoxR led to a conundrum: given the reducing environment of the cell, the cluster would always be in its oxidized form thereby making SoxR essentially constitutively activated *in vivo*. How does the cell keep this protein reduced and transcriptionally silent unless required? Since DNA-modified surfaces allow us to determine the redox potential of proteins in their DNA-bound forms, we explored the electrochemistry of the SoxR transcription factor on DNA-modified HOPG to ask what is the redox potential of SoxR in its physiologically relevant DNA-bound form.

To facilitate these experiments, self-assembled DNA monolayers were prepared on graphite with an appended redox-active probe, Redmond Red (59). Redmond Red provided a convenient internal reference for the voltammetry of the SoxR [2Fe-2S] cluster (Figure 13). With this extra probe for calibration, we could conclusively determine that binding to DNA shifts the redox potential of the [2Fe-2S] cluster of SoxR by 490 mV, to  $+200$  mV in its DNA-bound form. Importantly, this potential shift provided a direct rationale for how SoxR stays transcriptionally silent *in vivo* until activated under oxidative conditions: the DNA-bound and physiologically relevant form is always reduced and only conditions of extreme oxidative stress serve to oxidize and thereby activate this transcription factor. We are examining currently whether this activation occurs from a distance, by DNA-mediated CT.

It is interesting to note that the shift seen here on binding to DNA was opposite to that seen for the base excision repair proteins and of a significantly larger magnitude. Thus this large potential shift also provided insight into changes in the structure of SoxR on binding to DNA. Because the binding affinities for DNA of the oxidized and reduced protein are comparable, a significant structural difference between the free (low energy) and DNA-bound (high energy) forms of SoxR was postulated based on the electrochemical data. Later, crystal structures of SoxR with and without DNA (77) validated our proposal based on these electrochemical data. These experiments once again underscore the importance of investigating the redox chemistry of DNA-binding proteins in their biologically relevant forms, bound to DNA.

## 6. Conclusion

The intrinsic ability of DNA to conduct charge along its base pair stack and the remarkable sensitivity of this chemistry to the integrity of the DNA duplex has intrigued us for over two decades. A combination of detailed structural characterization and careful experimental design has allowed the investigation of this chemistry on DNA-modified surfaces to explore the range and applications of DNA-mediated CT. Through a host of electrochemical studies, we have not only uncovered several fundamental properties of this chemistry but have also demonstrated its potential use in a variety of biosensing and biochemical applications.

DNA-modified surfaces offer an ideal platform on which to investigate CT. Structural studies have provided information on the morphology of these self-assembled monolayers, which are found to orient themselves upright and away from the surface, thereby creating an accessible conduit for the transport of charge. We have employed several different redox-active, DNA-bound molecules to serve as sensitive reporters of this process. While earlier work largely involved non-covalent intercalating moieties, subsequent studies have taken advantage of the precise control and improved electronic coupling afforded by covalent attachment.

In all cases, DNA-mediated CT displays an exquisite sensitivity to the stacking of the base pairs that intervene between the electrode surface and the pendant probe, allowing for the electrochemical discrimination of single base mismatches, lesions and conformational perturbations. Our investigations are now being extended to DNA/RNA systems, with particular focus on the ability to detect products of transcription. Initial findings illustrated that DNA/RNA hybrids behave similarly to homologous DNA/DNA duplexes, allowing us to utilize DNA-mediated CT for development of a sensitive and quantitative hybridization assay. Finally, due to the possible DNA conformational changes associated with protein binding, we have also been able to demonstrate the sensitive detection of protein/DNA interactions and redox chemistry in real time and under their active, DNA-bound conditions.

This review is intended to survey the structural and electrochemical studies performed in our research group that have elucidated several key features of DNA-mediated charge transport. Much remains to be discovered with regard to the scope and fundamentals of this process. Can we harness this chemistry in the construction of highly sensitive nanoscale devices to detect proteins and RNA in real time in a multiplexed chip format? Are we, in fact, simply mimicking what Nature already exploits for long range signaling among proteins in the cell that utilize DNA CT? Many more questions remain to be addressed, yet it is abundantly clear that DNA electrochemistry and, more generally, DNA CT, represent a remarkably rich field for study.

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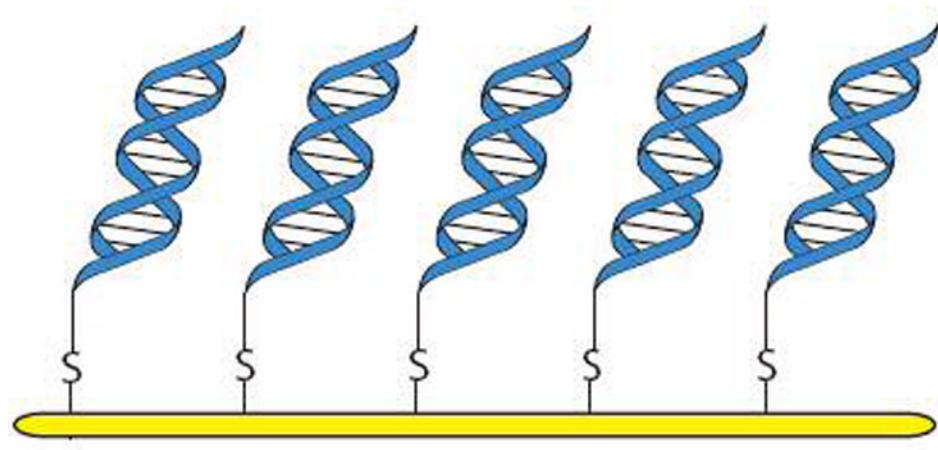
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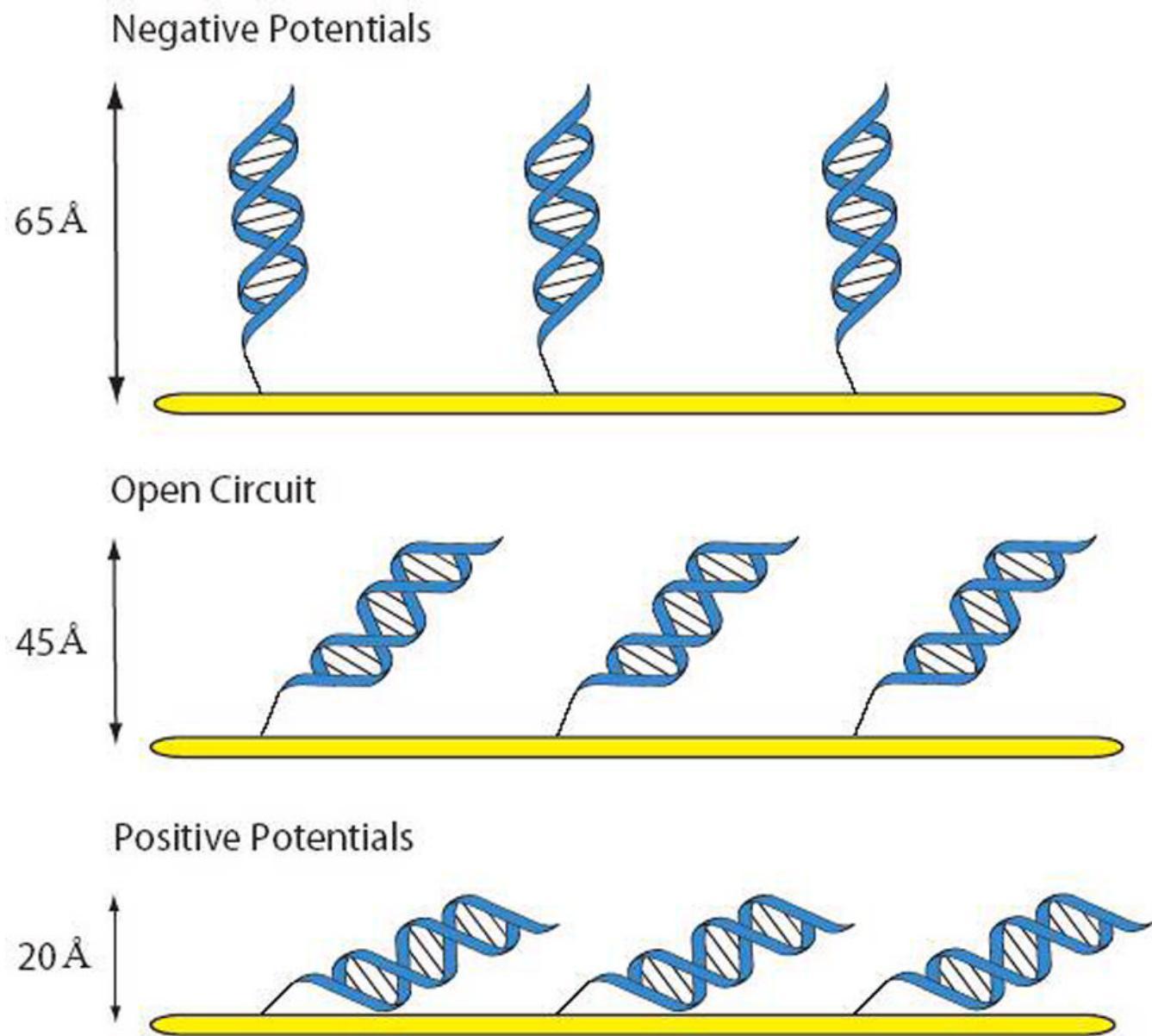
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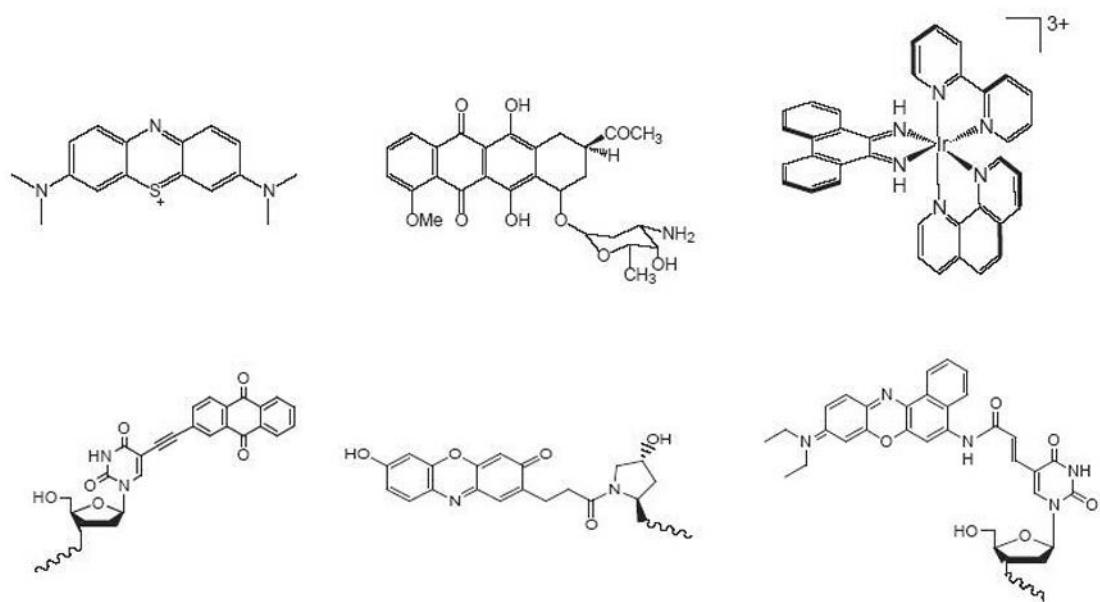
**Figure 1.**

Schematic illustration of DNA duplexes in a close-packed monolayer immobilized on gold via an alkanethiol tether.



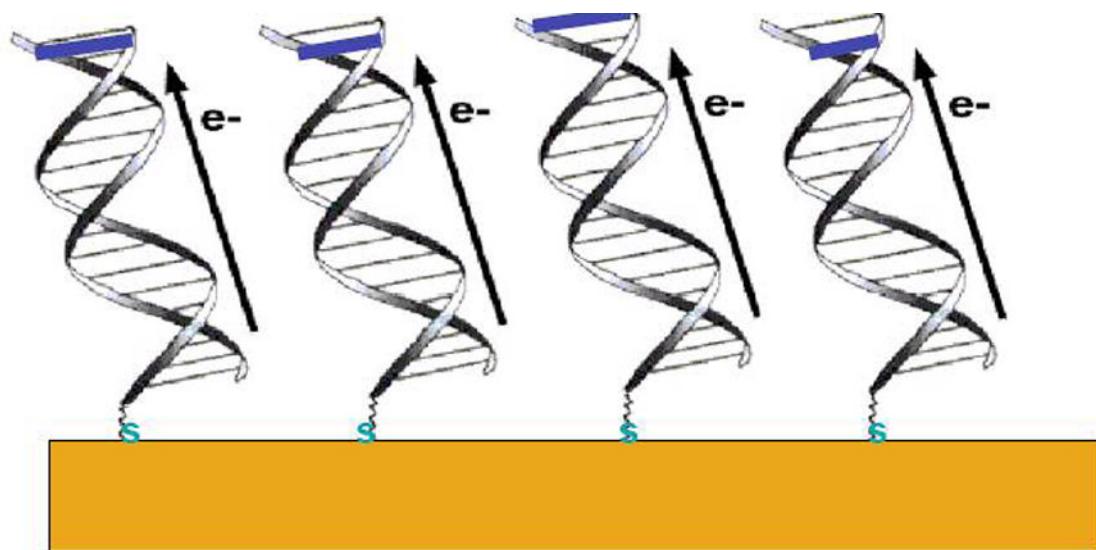
**Figure 2.**

Schematic illustration of the modulation of the height of a DNA monolayer at different substrate potentials. At open circuit, imaging studies have shown that duplexes are oriented upright and at a  $\sim 45^\circ$  angle relative to the surface. At positive potentials, the polyanionic DNA duplexes are attracted to the surface, while at negative potentials (used for most electrochemistry experiments described here) the duplexes are in an upright orientation with the helix axes perpendicular to the electrode surface.



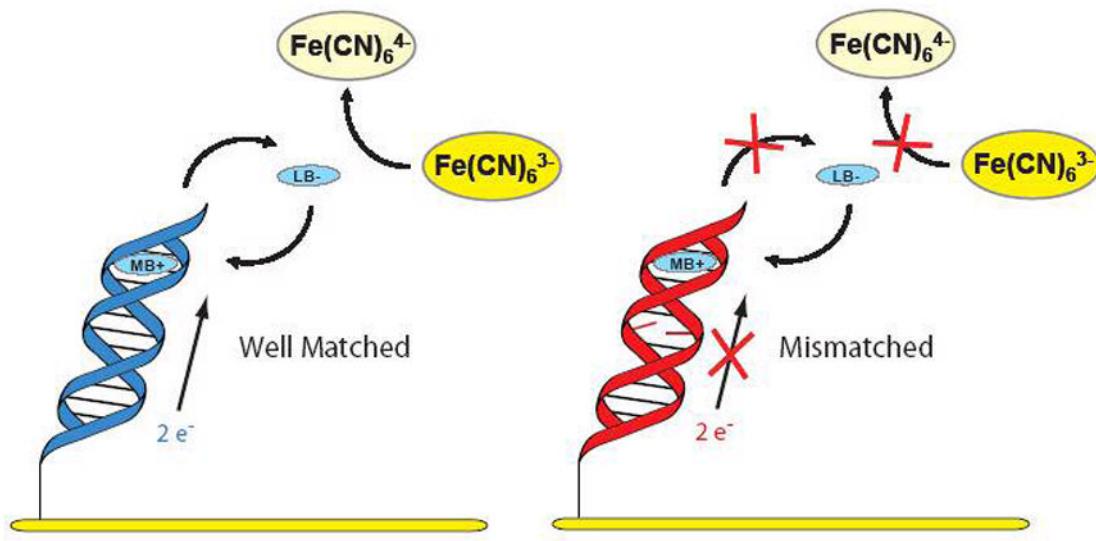
**Figure 3.**

Chemical structures of redox-active probes which have been utilized to explore DNA mediated CT. Non-covalent probes are on the top row (from left to right): methylene blue, daunomycin, and  $\text{Ir}(\text{bpy})(\text{phen})(\text{phi})^{3+}$ . Covalent probes are on the bottom row (from left to right): anthraquinone, Redmond Red, and Nile Blue.

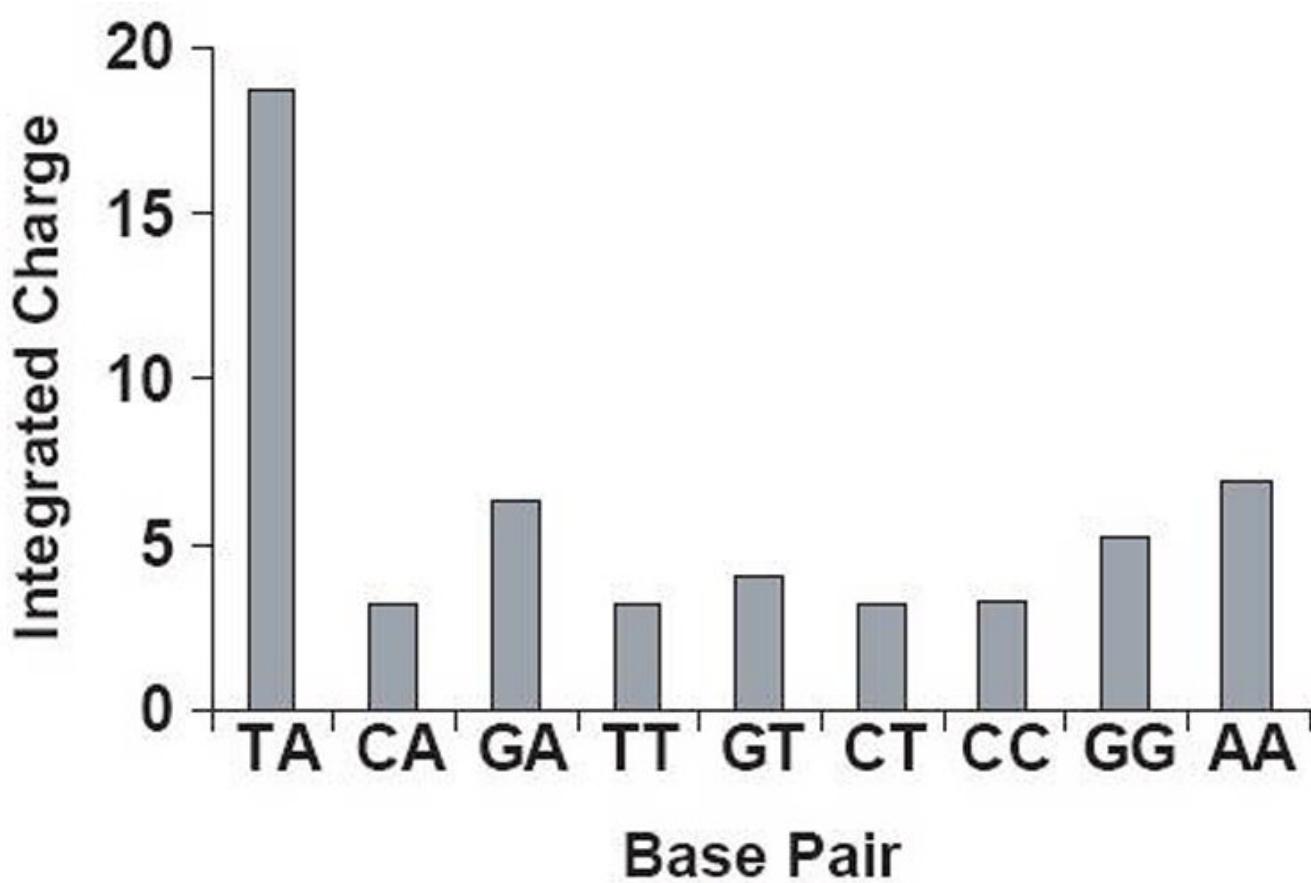


**Figure 4.**

Schematic illustration of the reduction of methylene blue (blue) via DNA-mediated CT at a DNA monolayer. The probe is constrained to intercalate near the top of the monolayer.

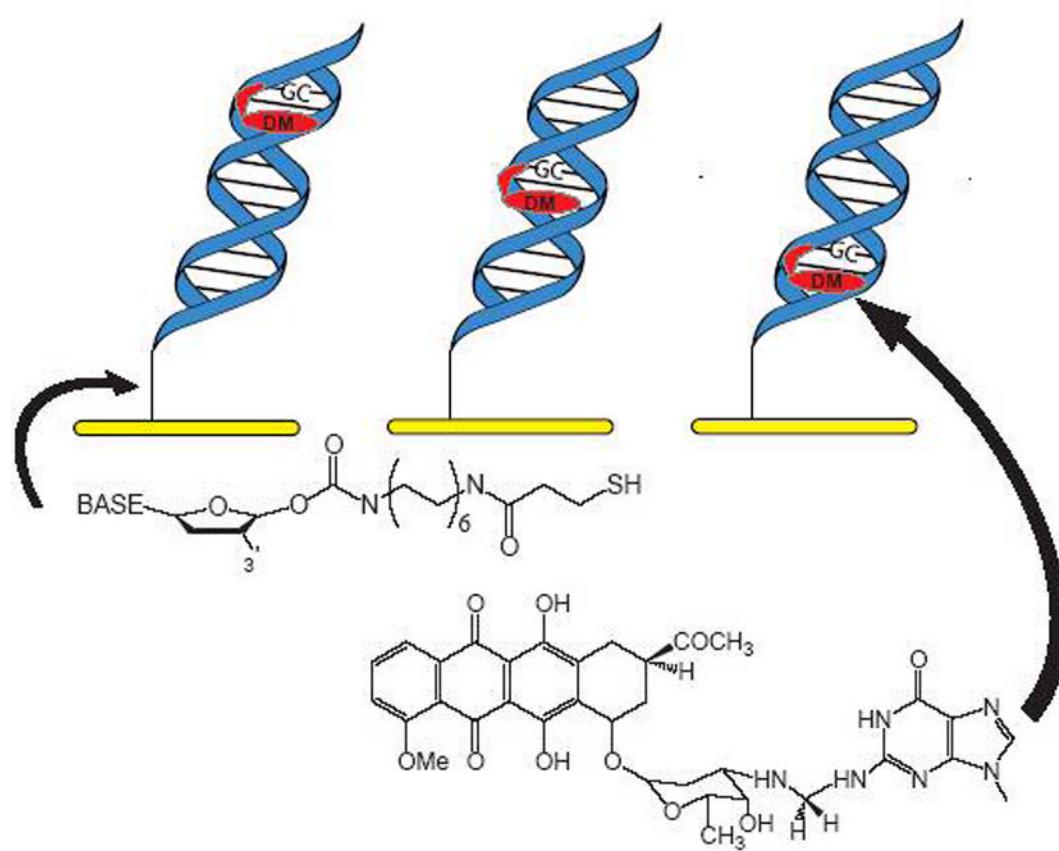
**Figure 5.**

Schematic illustration of the reduction of the methylene blue/ferricyanide electrocatalytic cycle on the DNA monolayer. DNA-bound methylene blue is reduced in a DNA-mediated reaction to leucomethylene blue (LB), which dissociates from the monolayer and is reoxidized by ferricyanide in solution; the reoxidized methylene blue returns to the DNA for another round of DNA-mediated electrochemistry. This electrochemical amplification leads to enhanced discrimination of all single base mismatches in DNA, since the intervening mismatch shuts down the DNA-mediated reaction and thus the electrocatalytic cycle.

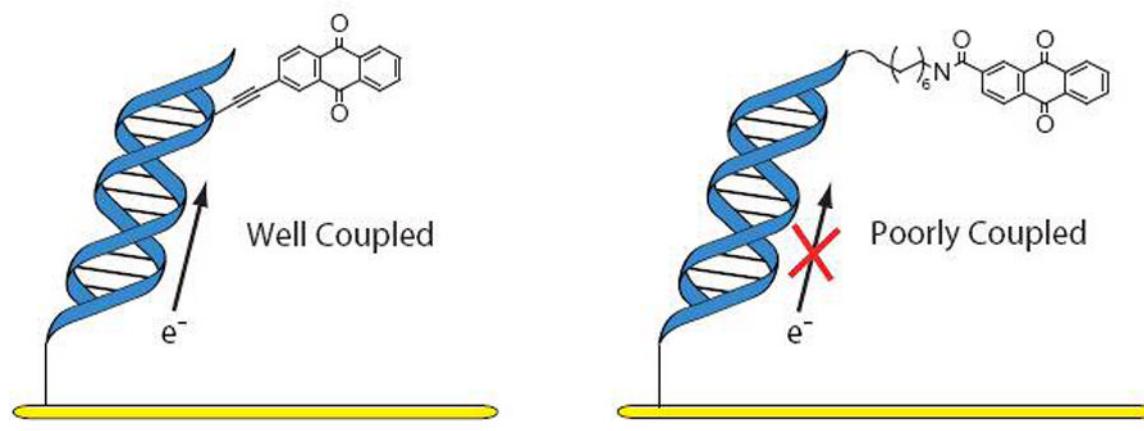


**Figure 6.**

A comparison of the relative integrated charge obtained from chronocoulometry of 2 mM Fe ( $\text{CN}$ )<sub>6</sub><sup>3-</sup> and 0.5  $\mu\text{M}$  MB at well matched DNA monolayers (TA) and in the presence of various mismatches (adapted from reference 46).

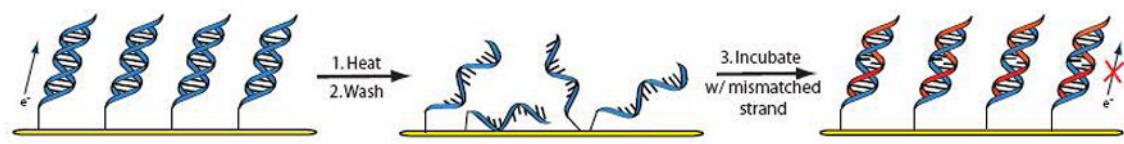
**Figure 7.**

A schematic illustration of DNA duplexes with daunomycin covalently cross-linked at various separations from the electrode surface. Also depicted are the DM-guanine cross-link (right) and the thiol-terminated tether which connects the duplex to the electrode surface (left). This tether provides 16  $\sigma$  bonds between the electrode and the base stack.



**Figure 8.**

A schematic representation of the electrochemistry of anthraquinone derivatives that are well coupled (left) and poorly coupled (right) to the base pair stack.

**Figure 9.**

A schematic representation of the protocol used for DNA electrochemistry before heat denaturation of DNA monolayers (left), after denaturation to yield single stranded DNA (center) and after rehybridization with mismatched DNA (right). A dramatic attenuation in DNA-mediated CT is observed with hybridization of the mismatched strand compared to the well matched DNA. Electrochemistry of the single stranded monolayer gives a broad, weak and irreproducible signal.

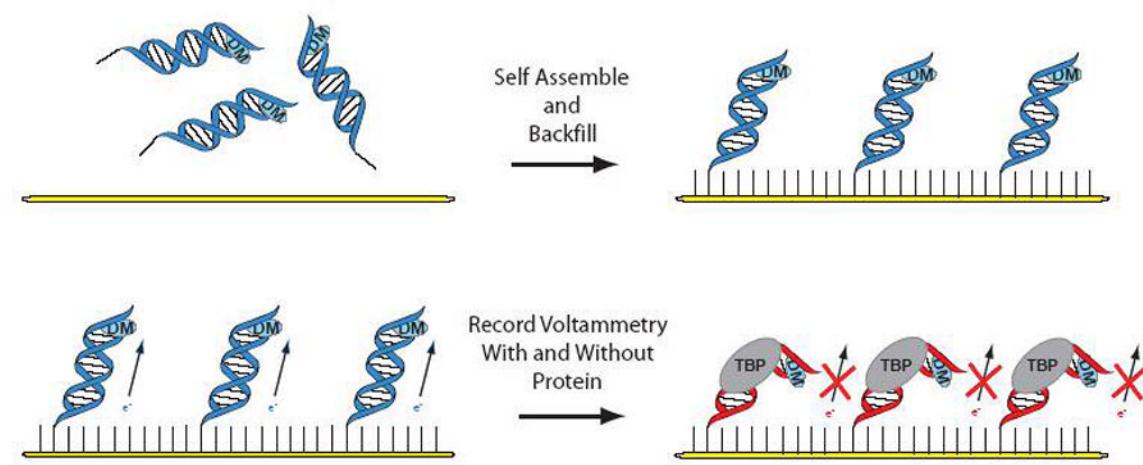
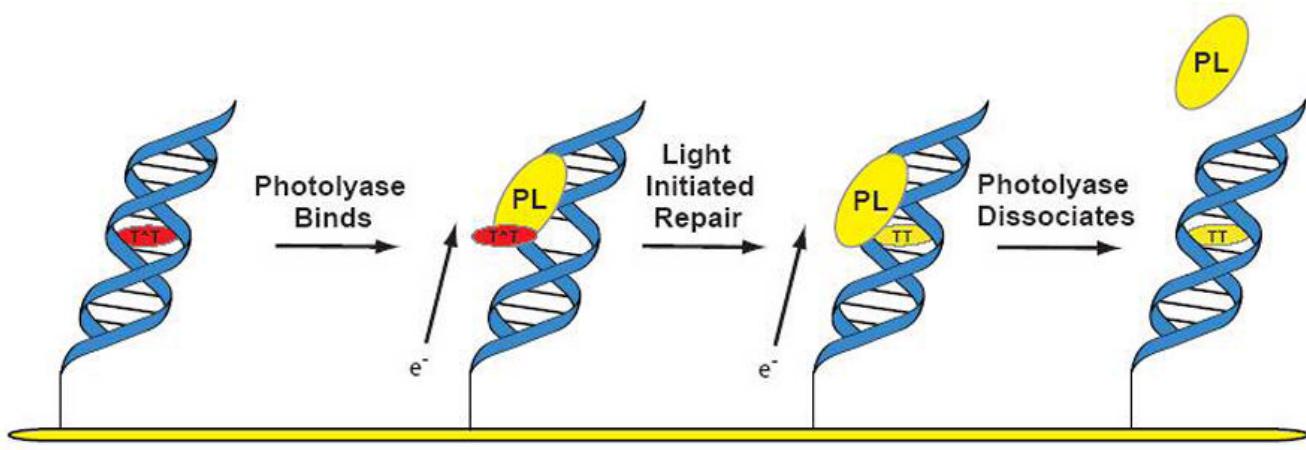
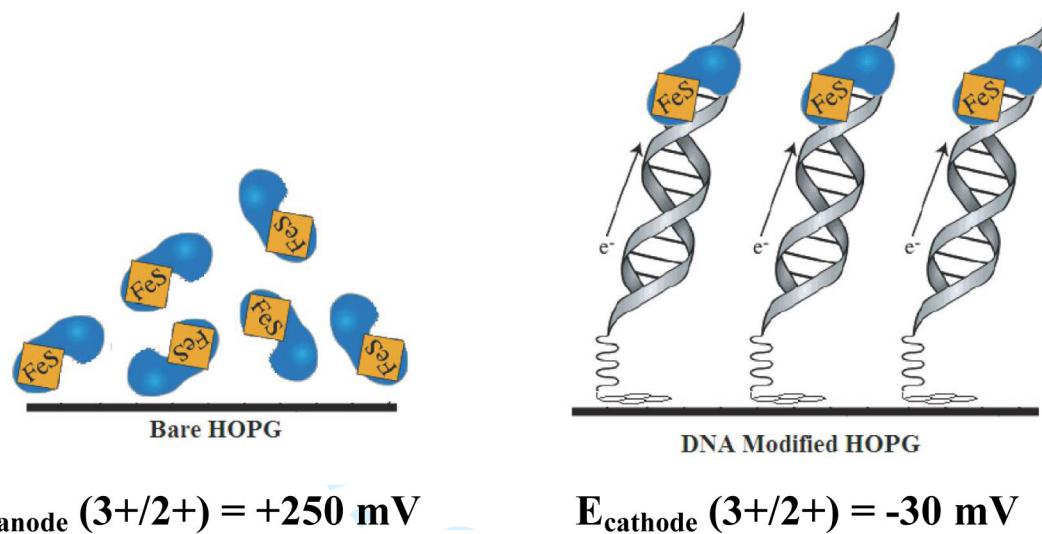
**Figure 10.**

Illustration of the fabrication of DNA-modified gold electrodes for electrochemical analysis of protein binding and reaction. First loosely packed DNA monolayers are formed and the surface is backfilled with mercaptohexanol for passivation. In the absence of protein, DNA-mediated redox chemistry to the DNA-bound probe is observed. Binding a protein that interrupts the base pair stack also interrupts the DNA-mediated redox chemistry, leading to an attenuation in the redox signal.



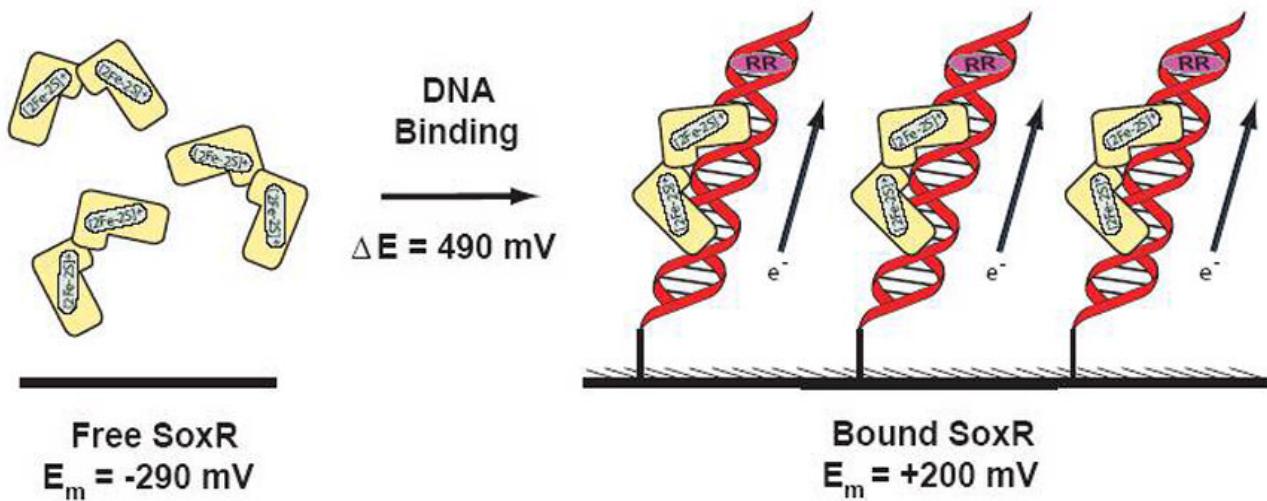
**Figure 11.**

Schematic illustration monitoring, using DNA electrochemistry, the repair of thymine dimers in a DNA monolayer by photolyase at a DNA-modified surface. In the absence of photolyase, no signal is observed, but when the protein is added, a weak signal is observed due to binding of the T<sup>A</sup>T dimer. Control studies show that the signal corresponds to the flavin bound within the photolyase. Upon irradiation, the thymine dimer is repaired, the integrity of the base pair stack is restored, and the signal grows in intensity as more effective coupling of the protein-bound flavin to the DNA base pair stack is restored. Lastly, because the protein has a lower affinity for the undamaged DNA, it dissociates, leading to the slow loss of the protein redox signal.



**Figure 12.**

Schematic illustration of the electrochemistry of Endonuclease III in the presence and absence of DNA on graphite. DNA binding causes a potential shift negative of more than 200 mV.



**Figure 13.**

Schematic representation of the electrochemistry of SoxR on DNA-modified graphite with Redmond Red as an internal standard. Binding to DNA shifts the redox potential of SoxR by approximately 500 mV, and this shift likely reflects a substantial change in conformation on binding.