CHAPTER 4

Intercalative Stacking as a Critical Feature of DNA Charge Transport Electrochemistry

This work was completed in collaboration with Mike Hill in the Chemistry Department at Occidental College. Manuscript in preparation.

INTRODUCTION

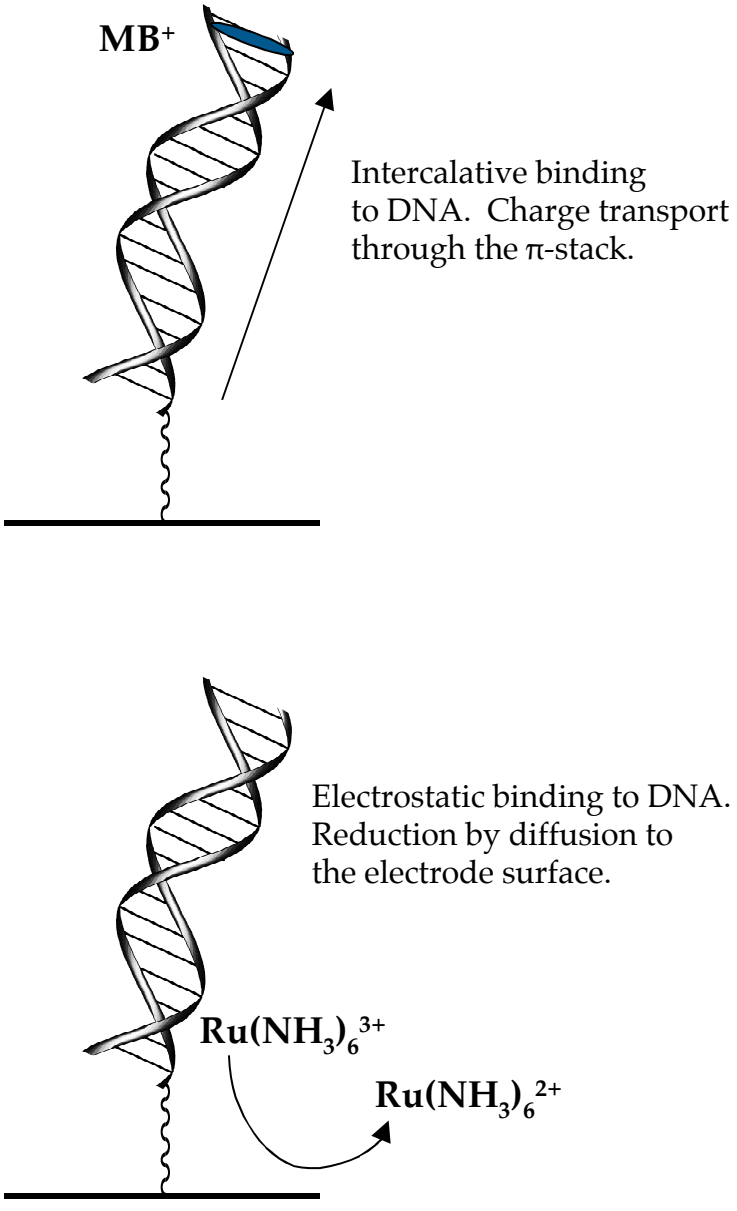
DNA has been extensively studied as a medium for charge transport (1-18). The DNA π-stack has been found capable of mediating oxidative DNA damage over long molecular distances in a reaction that is sensitive to DNA sequence dependent conformation and dynamics (19-31). A mixture of tunneling and hopping mechanisms has been proposed to account for this long range chemistry, which is gated by dynamical variations within the stack. DNA charge transport may play a role within the cell, and, indeed, oxidative damage to DNA from a distance has been demonstrated in the cell nucleus.

We have developed an electrochemical assay to study charge transport through double stranded DNA-modified gold electrodes (32-38). The electrochemistry, binding affinity, and charge transport dynamics of intercalated organic dyes, such as methylene blue (MB^+) and daunomycin (DM), at these DNA-modified electrodes has been examined (32,34,35). Binding by small intercalating molecules such as methylene blue (MB^+) appears to be primarily constrained to the top of the densely packed DNA monolayer, as established previously with daunomycin (34,35) and methylene blue (32). Electrochemical studies involving daunomycin site specifically bound at various positions within DNA monolayers reveal that charge transport can proceed over significant distances in immobilized oligonucleotides (> 35 Å) (34). While this reaction is not sensitive to distance, it is markedly attenuated by the presence of an intervening CA mismatch. In

fact, we have found reduction of intercalators bound to DNA-modified electrodes to be exceptionally sensitive to stacking of the DNA base pairs (34-36,38). Even very small perturbations in DNA base stacking and structure, such as single base mismatches, disrupt charge transport through these surfaces enough that we have been able to develop assays for routine mismatch detection based on this technology (36). We have also applied electrochemistry at DNA-modified electrode surfaces to probe DNA base stacking perturbations associated with DNA-protein binding and kinetics (38). Electrochemistry at DNA-modified surfaces provides an excellent tool for directly probing the base pair stack of DNA.

Although the sensitivity of electrochemistry at DNA-modified films to small changes in the intervening structure of the π-stack suggests that the path of charge transport to DNA bound intercalators in these electrochemical assays is indeed through the base pair stack, the critical feature of intercalative binding has never been conclusively established. Here we fundamentally explore the parameters affecting efficient electrochemistry at DNA-modified surfaces by altering the donor and acceptor partners in the charge transport reaction and focusing especially on coupling to the π-stack by intercalation (Figure 4.1). The results establish a charge transport pathway for reduction of intercalators that is mediated by the π-stack of DNA base pairs.

Figure 4.1. MB^+ and $Ru(NH_3)_6^{3+}$ bound to a DNA modified electrode. Likely modes of binding and electrochemical reduction are indicated.



MATERIALS AND METHODS

Materials

Unless otherwise indicated, all reagents and solvents were purchased in their highest available purity and used without further purification. All DNA synthesis reagents were obtained from Glen Research. Millipore milliQ (18 M cm) water was used in all experiments. All plasticware was purchased DNase, RNase, and metal free (Sorenson Bioscience, Inc.).

Preparation of DNA-modified surfaces

Thiol-modified single stranded oligonucleotides were prepared as described in the Appendix. After stringent purification, the thiol-modified single strand was hybridized with its unmodified complement by combining equimolar amounts of each strand (in 5 mM phosphate, 50 mM NaCl buffer, pH 7) for a final solution of 0.1 mM duplex. Just before deposition on the gold surfaces, 0.1 M $MgCl_2$ was added to each sample. 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. These surfaces have been characterized by cyclic voltammetry, ellipsommetry, radiolabelling of the duplexes, and AFM (32,33,37). We have found the DNA films to be densely packed monolayers with the DNA helical axis oriented in an upright position at approximately 45° with respect to the gold surface (33).

Electrochemical measurements

Cyclic voltammetry was 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. Potentials are reported versus SCE.

Passivation of the electrode surface by electropolymerization of 2-napthol

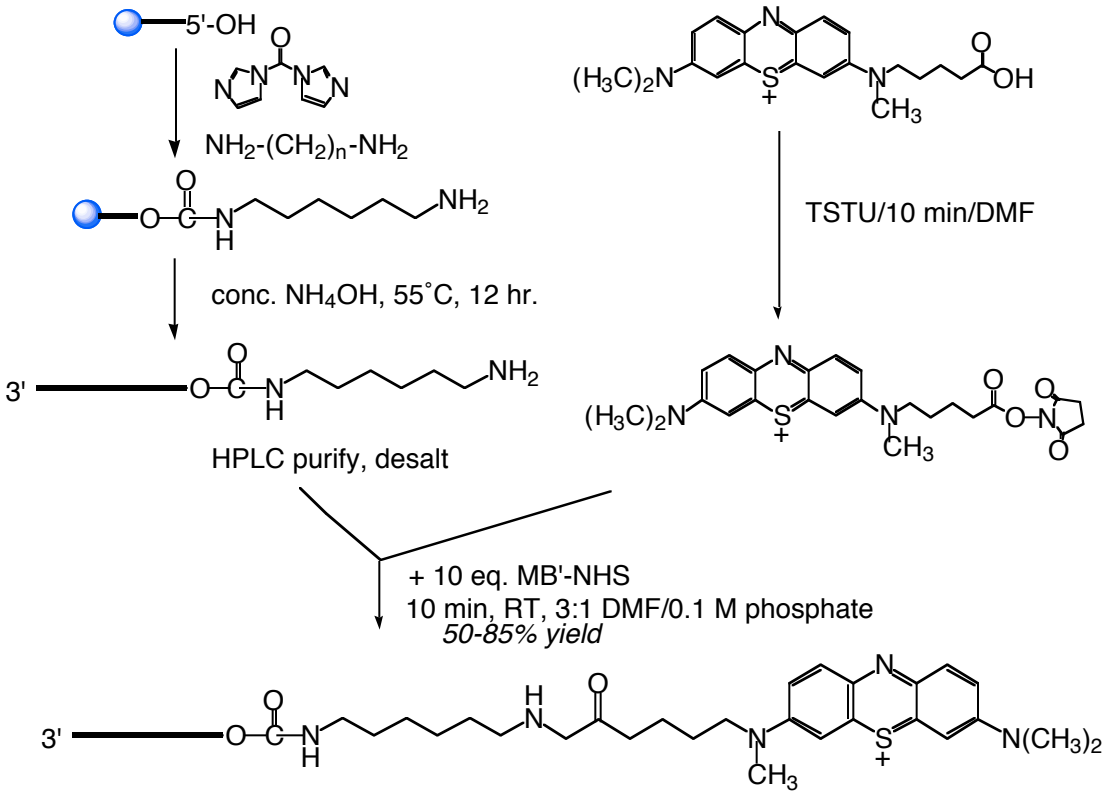
After self-assembly, DNA-modified electrodes were rinsed and then immersed in a 100 mM solution of 2-napthol in 50% acetonitrile, 50% buffer (5 mM phosphate, 50 mM NaCl, pH 7). 2-Napthol was then electropolymerized onto the electrode surface by cycling the potential from 0 mV to 600 mV for several minutes until the cyclic voltammogram was flat. This procedure was then repeated cycling from 0 to 700 mV until the background was flat. Following this electropolymerization, the electrodes were thoroughly rinsed in 50% acetonitrile, 50% buffer, then 100% buffer, and then used in voltammetry studies.

Synthesis and characterization of MB'-5'-DNA

The synthesis of modified MB^+ (MB' = 3-[N-(4-ethoxycarbonylbutyl)-N-methylamino]-7-dimethylaminophenazathionium chloride) was accomplished according to published literature procedures (39). MB' was then coupled to DNA as illustrated in Figure 4.2. MB' (4.71 mmol) was mixed with TSTU (O-(N-succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate) (1 mg) and diisopropylethylamine (1 μL) in dimethyl formamide. The resulting solution was kept at RT for 10 minutes to activate the MB' acid to the N-succinimidyl form (MB'-NHS). This reaction was monitored by thin layer chromatography in a methanol chamber. After activation, amino-modified DNA (NH_2-5'-DNA, 70 nmol in 100 mM phosphate buffer, see below for synthesis) was added to the MB'-NHS solution (final solution 3:1 DMF:H_2O) and the vessel was shaken gently. The reaction proceeded to completion in approximately 15 minutes at RT. The final product was purified by reversed phase HPLC using an acetonitrile and ammonium acetate solvent system. MB'-5'-DNA was characterized by mass spectrometry, electronic spectroscopy, HPLC, and electrochemistry. The UV-vis absorption profiles are exactly the same in the visible region (absorption maxima at 609 and 668 nm, ε_{668} = 81,600 M^{-1} cm^{-1}) for MB^+ and MB'-5'-DNA, yet a change in HPLC retention time indicates the presence of the functional carboxylic acid side chain. A mass of 5112 was found, as expected, by Maldi-TOF mass spectrometry.

NH_2-5'-DNA is made according to published protocol (32-38) and is described in the Appendix. Briefly, oligonucleotides (5'-AGTACAGTCATCGCG-3') are synthesized by standard phosphoramidite

Figure 4.2. Synthesis of MB′-5′-DNA.



chemistry on a controlled pore glass resin. While still attached to the resin, the 5'-OH terminus of the DNA strand is treated in succession with carbonyldiimidazole and 1,6-diaminohexane, cleaved from the resin and HPLC purified.

RESULTS

Ionic strength dependence of MB^+ and $Ru(NH_3)_6^{3+}$ binding to DNA

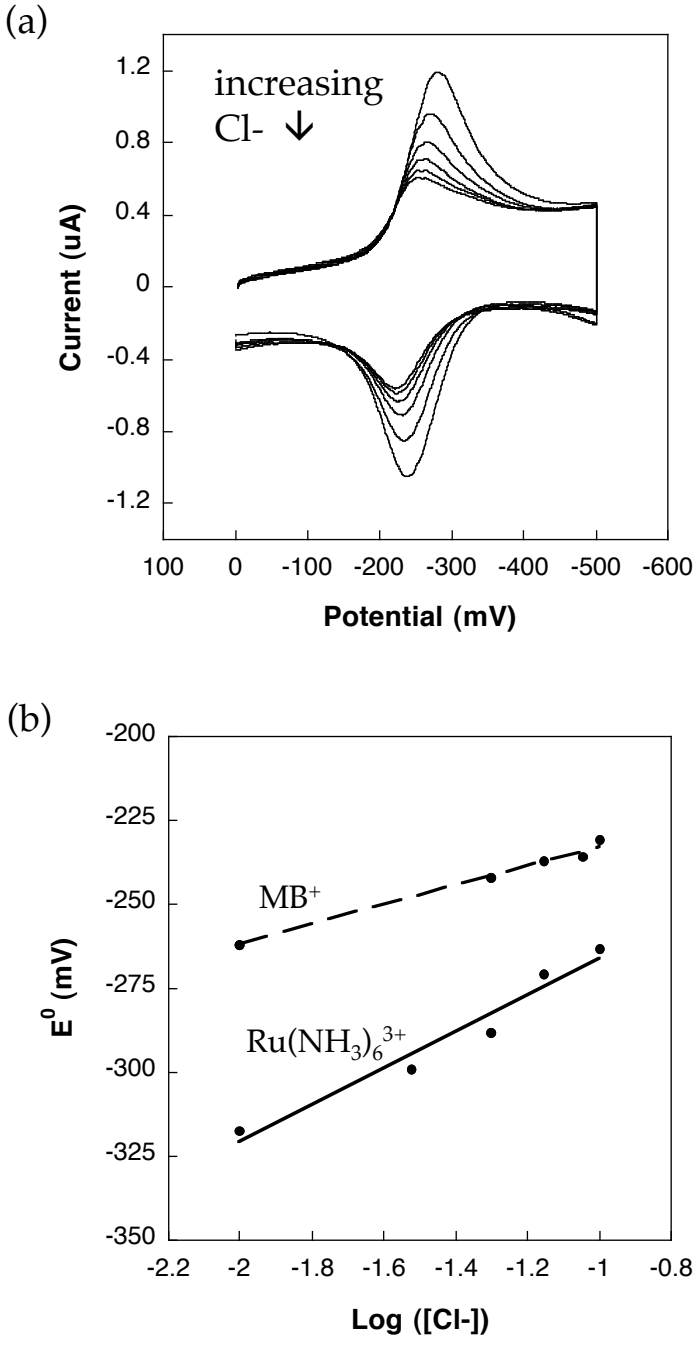
In order to study the importance of the binding of the redox probe to DNA for effective charge transport through DNA-modified electrodes and to further examine the path of charge transport at theses DNA films, we studied the ionic strength dependence on the reduction of MB^+ and $Ru(NH_3)_6^{3+}$ at a DNA-modified electrode. These studies allow us to delineate differences in electrochemistry characteristics associated with intercalation versus electrostatic binding to DNA. Methylene blue (MB^+) binds readily to DNA-modified surfaces by intercalation with an association constant of $3.8(5) \times 10^6$ M^{-1} and is reversibly reduced at DNA-modified surfaces at 250 mV vs. SCE (32). $Ru(NH_3)_6^{3+}$ is a groove binder with approximately the same potential as MB^+ that associates with DNA through electrostatic interactions (40).

Gold electrode surfaces modified with the duplex SH-5'-AGTACAGTCATCGCG-3' were fabricated and used in cyclic voltammetry experiments with either 2 μM MB^+ or 20 μM $Ru(NH_3)_6^{3+}$. KCl (10 mM to 100 mM) was then titrated into the electrochemistry cell and the cyclic

voltammogram at each ionic strength was recorded. As the ionic strength is increased at an immobilized polyion, such as DNA, a counterion concentration gradient is set up across the DNA film (in this case, caused by exclusion of Cl^- from the polyanionic DNA film). This concentration gradient essentially sets up a junction potential, similar to that in a Galvanic cell, which causes the observed redox potential of a molecule bound to the film to shift (41). A plot of the observed potential versus the log of the ionic strength yields a straight line with a slope proportional to the number of electrons involved the given electrochemical reaction (expression derived from the Nernst equation) (41). Figure 4.3a illustrates the cyclic voltammograms for MB^+ bound to a DNA-modified electrode in the presence of varying concentrations of Cl^-. Figure 4.3b depicts the analysis of these ionic strength studies for MB^+ and $Ru(NH_3)_6^{3+}$ bound to DNA, revealing that MB^+ has 2e- electrochemistry while $Ru(NH_3)_6^{3+}$ has 1e- chemistry bound to DNA.

Not only does the redox potential of DNA-binding molecules shift as ionic strength is increased, but as illustrated for MB^+ Figure 4.3a, the overall number of molecules that are reduced decreases, as indicated by a lower current in the cyclic voltammogram. High salt concentrations effectively inhibit the DNA binding of both electrostatic molecules and DNA intercalators. Thus, it is apparent that efficient charge transport at DNA-modified surfaces only takes place when the molecules are bound tightly to DNA, either by intercalation (MB^+) or groove binding ($Ru(NH_3)_6^{3+}$).

Figure 4.3. (a) Cyclic voltammetry of 2 μM MB^+ at a gold electrode modified with the thiol terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to its perfect complement in 5 mM phosphate, 50 mM NaCl, pH 7 buffer at KCl concentrations from 10 to 100 mM. (b) Plots of the integrated cyclic voltammetry wave for an electrode identical to that described in (a) as well as the integrated cyclic voltammetry of 28 μM $Ru(NH_3)_6^{3+}$ at an electrode identical that to described in (a) at KCl concentrations from 10 to 100 mM.



This experiment does not reveal whether the charge transport takes place through the π-stacked base pairs of DNA or by some other DNA-mediated fashion, but these data strongly support the conclusion that the observed electrochemistry at DNA-modified electrodes is most efficient when the redox probe is bound to the DNA film.

Electrode passivation by 2-napthol

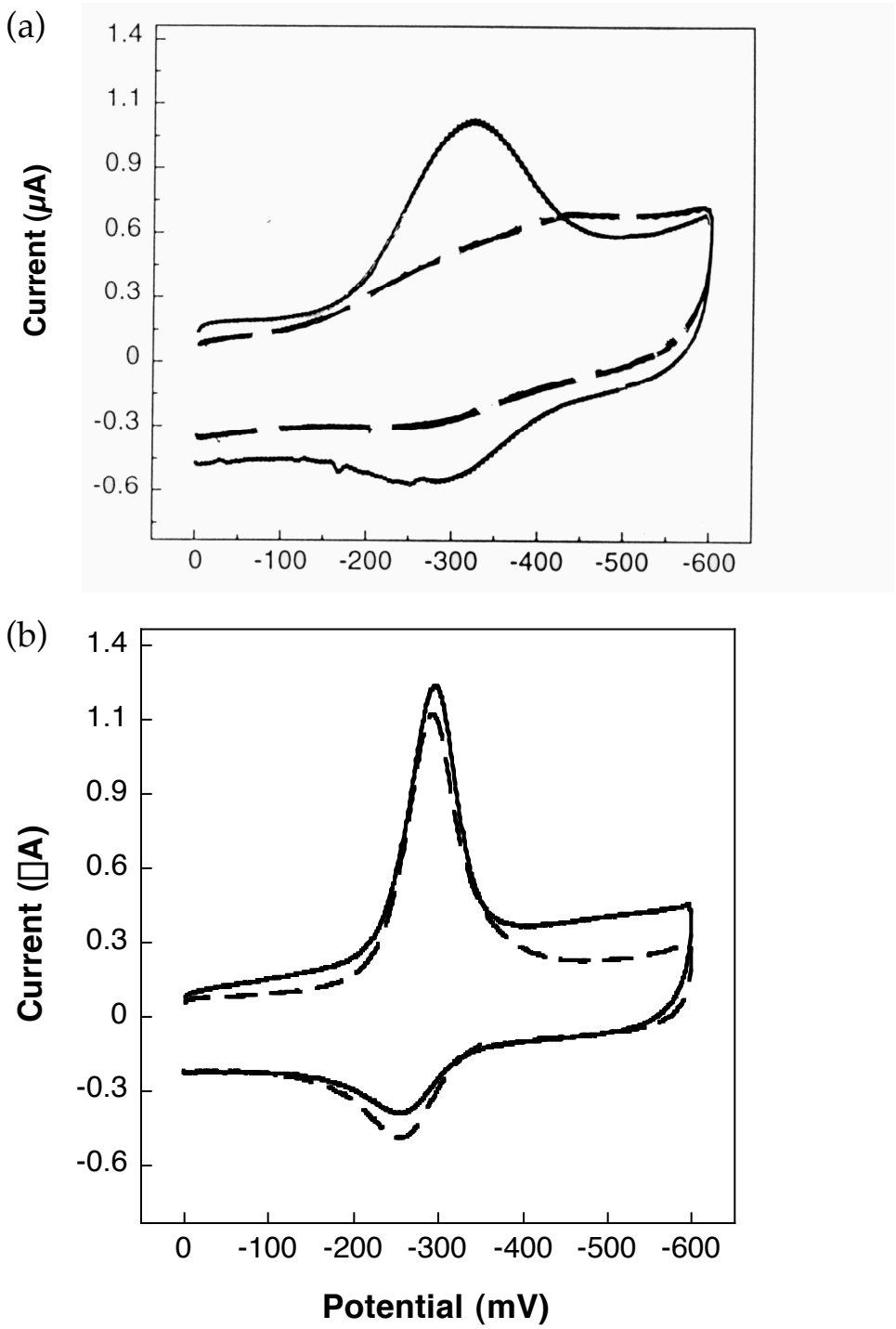
Having established DNA-binding as a critical aspect for efficient reduction at DNA-modified surfaces, we next examine the mode of DNA binding, intercalation (MB^+) and groove binding ($Ru(NH_3)_6^{3+}$). The reduction of $Ru(NH_3)_6^{3+}$ likely proceeds through the facilitated diffusion of the ruthenium complex along the grooves of the immobilized helices. To confirm this as the path of electrochemical reduction of $Ru(NH_3)_6^{3+}$ and to study the difference in electrochemistry between a probe that makes direct contact with the electrode surface and one bound in the DNA film, we performed studies in which the electrode surface was passivated with electropolymerized 2-napthol (42). Electrochemical reduction resulting from direct contact of the acceptor with the electrode surface should be blocked at an electrode surface coated with this polymer. Electrochemical reductions resulting from charge injection into and transport through DNA should not be affected by coating all free gold surface area with 2-napthol.

Gold electrode surfaces modified with the duplex SH-5'-AGTACAGTCATCGCG-3' were fabricated as described previously (32-38). In separate experiments, cyclic voltammograms of either 2 μM MB^+ or 20 μM $Ru(NH_3)_6^{3+}$ were recorded at these DNA-modified electrodes. Separate, but

identical, DNA-modified electrodes were then passivated with 2-napthol (40) and then cyclic voltammograms of 2 μM MB^+ and 20 μM $Ru(NH_3)_6^{3+}$ were recorded again. Figure 4.4 illustrates the electrochemistry of MB^+ and $Ru(NH_3)_6^{3+}$ bound to a DNA-modified electrode before and after passivation with 2-napthol. Electrode passivation with 2-napthol reduces the amount reduced MB^+ by 3%, while it reduces the amount of reduced $Ru(NH_3)_6^{3+}$ by 71%. When the electrode surface is coated with the polymer 2-napthol, this passivation does not affect the electrochemical signal of MB^+ (intercalated in the helix) while the signal of $Ru(NH_3)_6^{3+}$ (facilitated diffusion to the surface) is almost completely eliminated.

Thus, $Ru(NH_3)_6^{3+}$, and likely other molecules that electrostatically groove bind to DNA, are reduced by direct contact with the electrode surface and the electrochemical reduction of these molecules is blocked by electrode passivation with 2-napthol. The DNA film in this case likely facilitates diffusion to the electrode surface in a nonspecific, electrostatic fashion. A molecule such as MB^+, however, binds to DNA by intercalation into the π-stack of DNA. As the reduction MB^+ is not affected by electrode passivation, this result implies that the reduction of MB^+ takes place by charge transport *through* the DNA helix, not by direct contact with the electrode surface, as is likely the case for $Ru(NH_3)_6^{3+}$. These data support a mechanism of charge transport through the DNA base stack to reduce DNA-bound redox active intercalating molecules.

Figure 4.4. Cyclic voltammetry of (a) 28 μM $Ru(NH_3)_6^{3+}$ and (b) 2 μM MB^+ at a gold electrode modified with the thiol terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to its perfect complement in 5 mM phosphate, 50 mM NaCl, pH 7 buffer before and after electrode passivation with 2-napthol.



Electrochemistry and ionic strength dependence of covalently bound MB^+

MB^+ was covalently tethered to the 5′ end of DNA in order to restrict the intercalation site of MB^+ to the top of the DNA film. Clarification of the binding site allows us to investigate charge transport in DNA over fixed distances. Furthermore, this allows a more detailed study of the effect of intercalation on the electrochemical reduction at DNA-modified surfaces. An analog of MB^+ (MB') was synthesized (Figure 4.2) containing a functional carboxylic acid side chain that allowed the covalent attachment of MB^+ to the 5′ end of a single stranded oligonucleotide. MB'-DNA adducts were characterized by HPLC retention times, mass spectrometry and UV-visible absorption spectrophotometry. The UV-vis absorption profiles are exactly the same, yet a change in HPLC retention time indicates the presence of the functional carboxylic acid side chain.

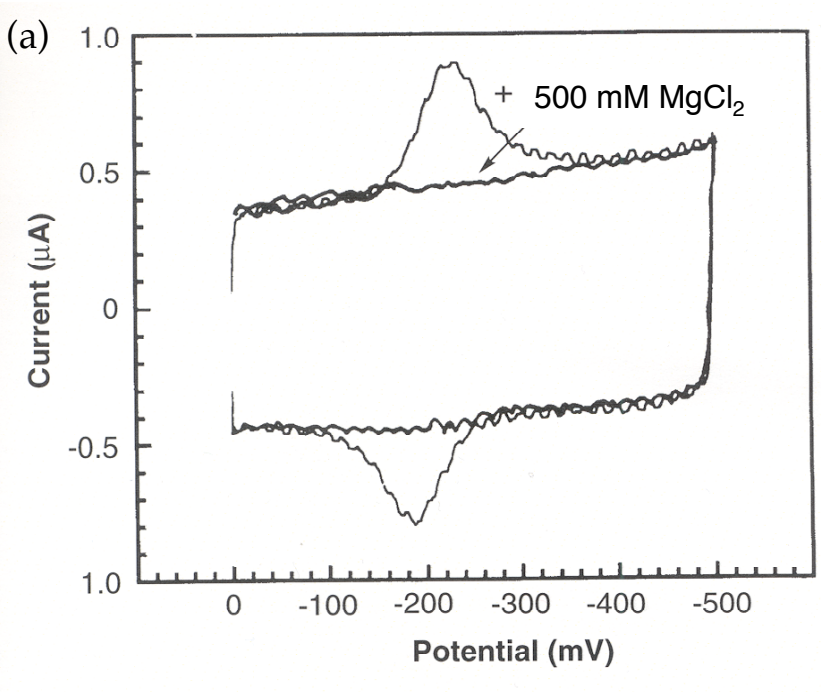
At a gold surface modified with thiol-derivatized SH-5'-AGTACAGTCATCGCG-3' hybridized to 3'-TCATGTCAGTAGCGC-5'-MB' (MB'-DNA-SH), a pronounced electrochemical signal is observed (Figure 4.5). Thus electron transfer proceeds from the electrode to the intercalator. MB' undergoes a reversible reduction near -250 mV that correlates well with noncovalently bound MB^+ indicating that covalent attachment does not affect the inherent redox properties of the molecule. The oxidation of ferrocyanide at these surfaces was effectively blocked indicating that MB'-DNA-SH adducts form complete monolayers on the bulk gold electrode surface.

As demonstrated above, high salt concentrations can effectively inhibit intercalator to DNA binding. To investigate whether the covalently bound MB' could effectively intercalate into DNA even though it was restricted by

the length of its aliphatic linker, we examined the cyclic voltammetry and UV-visible spectroscopy of MB^+ in the presence of high salt concentrations. Upon addition of 500 mM $MgCl_2$ to covalently bound MB'-DNA-SH films, the electrochemical signal for MB' is lost (Figure 4.5). Thus, with increased ionic strength, MB' deintercalates and the charge transport reaction is prohibited. Similarly, when increasing concentrations of $MgCl_2$ were titrated into a solution of 500 nM MB^+ and 500 nM duplex (5'-AGTACAGTCATCGCG-3'), the absorption at 668 nm decreased with increasing ionic contribution, indicating that the intercalative properties of MB' are not restricted by the aliphatic linker (Figure 4.6).

This experiment supports the through-helix model for electron transfer and emphasizes the need for intercalating reactants. When MB' is covalently attached to the 5′ end of DNA, we can force it to deintercalate with high salt concentrations, yet it is clear that MB' is still attached to the 5′ end of the helix. If charge transport involved direct contact between the MB' and gold surface, such direct contact would be facilitated in this deintercalated form. Earlier studies were also consistent with the helices being bound in an upright configuration in the presence of applied potential (33). Hence, at low salt when MB' is intercalated, charge transfer must proceed to the MB' bound over 40 Å from the gold surface. Interestingly, since an electrochemical signal is not observed at higher salt concentrations with MB' deintercalated, but still attached, this indicates that the rate of electron transfer must be much slower through the aliphatic linker to MB' than through the DNA π-stack to π-stacked MB' such that the electrochemical response is too slow to detect within our potential window. These results underscore the need for

Figure 4.5. (a) Cyclic voltammetry at a gold electrode modified with the thiol terminated sequence SH-5'-AGTACAGTCATCGCG hybridized to a complement modified with MB' in 5 mM phosphate, 50 mM NaCl, pH 7 buffer with either 0 mM $MgCl_2$ or 500 mM $MgCl_2$ added. (b) At high ionic strength, MB^+ does not bind to DNA.



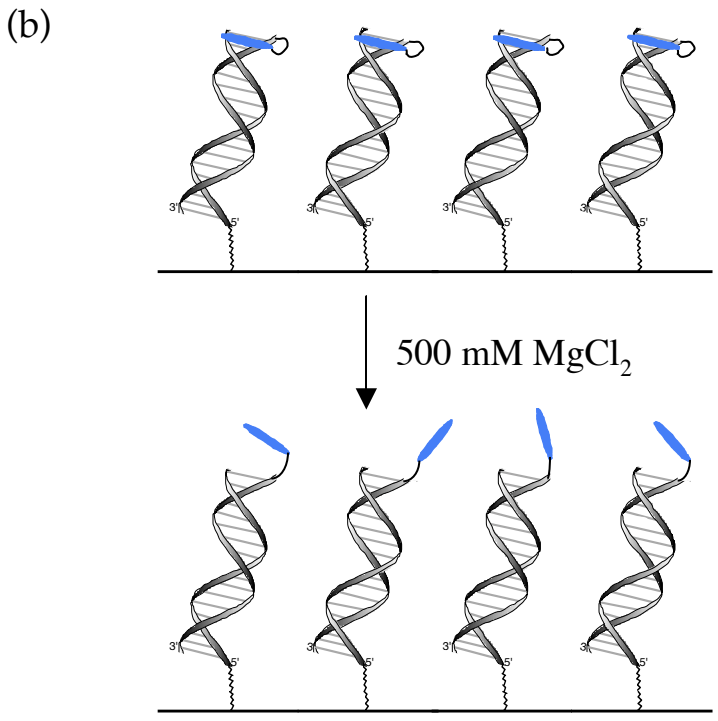
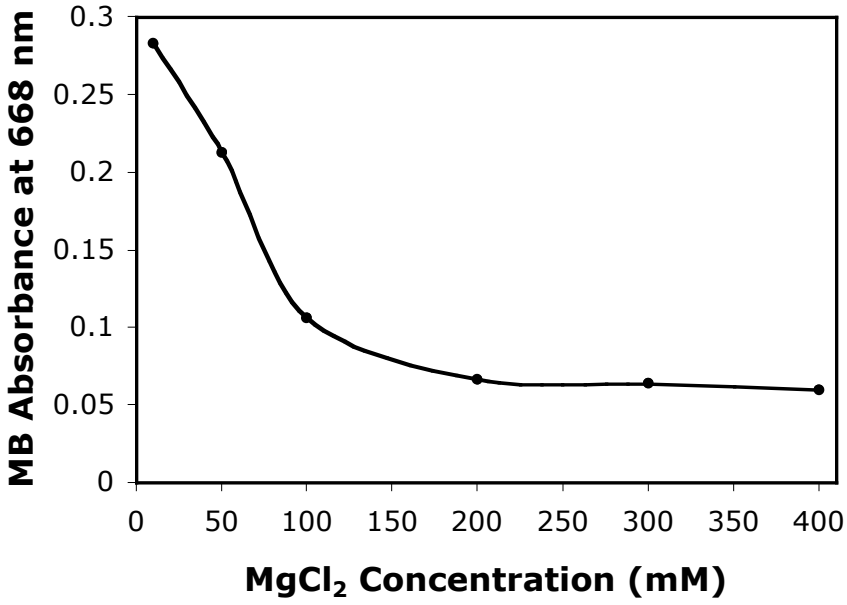


Figure 4.6. Plot of UV-visible absorbance at 668 nM for a solution containing 500 nM MB^+ and 500 nM 5'-AGTACAGTCATCGCG hybridized to its complement in 5 mM phosphate, 50 mM NaCl, pH 7 buffer and titrated with $MgCl_2$ concentrations from 10 to 400 mM.



intercalating probes and confirm that the efficient long range electrochemical reduction of MB^+ is π-stack mediated.

DISCUSSION

In these studies we examined aspects affecting the efficient reduction of molecules at DNA-modified surfaces. We used ionic strength studies to examine the effect of redox probe binding to DNA and established the requirement of tight DNA binding for efficient electrochemical reduction. Furthermore, electrode surface passivation studies establish that the electrochemical reduction of molecules bound to DNA by intercalation does not proceed via direct contact of acceptor molecules to the electrode surface, but is mediated by the DNA molecule itself. This aspect of DNA-mediated electrochemical reduction was made especially clear in studies using covalently bound MB^+ such that the binding site of MB^+ was restricted to the top of the DNA film and intercalation to DNA could be controlled by ionic strength. Despite being covalently attached the DNA surface, at high ionic strength when the MB^+ is not intercalated into the base pairs, reduction of MB^+ is not observed. Electrochemical reduction on these DNA-modified surfaces requires MB^+ intercalation. Taken together, it is clear that charge is transported from the electrode surface and through the base pair stack of DNA where it is accepted by a DNA intercalator, which is itself coupled into the π-stacked interior of the DNA duplex.

This proposed path for charge transport is completely consistent with our previous electrochemical investigations at DNA-modified surfaces (32-38). In previous studies, the electrochemistry of intercalated MB^+ (32) and daunomycin (34,35) was investigated. The electrochemical signals obtained with both probes exhibited a linear current versus scan rate relationships, indicating that the reactive species were strongly bound to the surface. Daunomycin could be covalently attached near the top of the film and the results were equivalent (34). Likewise, the charge due to the reduction of these intercalators at fully basepaired films indicated an intercalator-to-DNA binding stoichiometry of approximately 1:1. Because this ratio is far smaller than predicted by neighbor exclusion, it is likely that intercalating probes bind predominately near the solvent exposed terminus of the film, with diffusion into the monolayer inhibited by tight packing of the DNA helices. Furthermore, binding isotherms were measured and they clearly indicated that these molecules bind more tightly to DNA-modified electrodes than bare gold, thus DNA does not seem to be simply facilitating their diffusion to the electrode surface. The data presented here show strongly that reduction of MB^+ at DNA-modified electrodes does not take place by direct contact of the MB^+ with the gold surface, but that tight binding to DNA by intercalation is required.

The data presented here as well as the proposed path for electrochemistry at DNA films are also in strong agreement with previous studies demonstrating detection of single base pair mismatches and other perturbations to the base pair stack (34-36,38). Mismatches offer only very minor structural changes to a DNA duplex; they are generally stacked and do

not significantly distort the sugar phosphate backbone of DNA, but they do undergo greater dynamical motion than Watson-Crick paired bases (43-48). Thus it follows that charge transport in DNA, if the path of that charge transport is through perfectly π-stacked base pairs, would be sensitive to the enhanced base stack motions associated with mismatches. Furthermore, solution based studies examining charge transport between donor and acceptor molecules bound to DNA, or charge transport resulting in oxidation of the DNA molecule itself, have widely been described as mediated by the base stack of DNA. There is currently an ongoing discussion in the literature about how the base stack of DNA mediates this charge transport chemistry (49-65).

The path of charge transport at DNA-modified surfaces seems best described as mediated by the base pair stack of DNA, however the detailed mechanism of this DNA-mediated charge transport has not been established. Unlike for photooxidation experiments (Ru^{3+}, a typical photooxidant used in DNA charge transport studies, E^o ~ 1.6 V), in these electrochemical experiments, the energetics of the redox probe (MB^+ ~ 0 V vs. NHE) are far away from the DNA bridge orbitals (DNA LUMO ~ -1.1 V, DNA HOMO ~ 1.3 V vs. NHE) and so a mechanism in which the charge is actually occupying the DNA molecular orbitals is hard to understand; however, it is clear that the charge transport reaction is quite sensitive to small perturbations of the DNA bridge. Heller and coworkers have put forth an interesting proposal for the mechanism of charge transport at DNA-modified surfaces (66). According to this model, conduction along the axis of DNA in one dimensionally ordered DNA films is attributed to the reduced difference

between the static and high frequency longitudinal dielectric constants. They argue that the high frequency longitudinal polarizability within a DNA-modified surface is raised due to the concerted movement of cations along the axis of DNA when an electric potential is applied, and that the static dielectric constant is lowered due to reduced hydration of DNA films relative to that in solution. This change in the dielectric constant increases the mobility of electrons in the film and makes DNA a one dimensional semiconductor. Although the data presented here do not speak directly to this as the mechanism, our results are not inconsistent with this proposal. It is possible that DNA intercalators could serve as a dopant, making DNA an effective semiconductor in these systems.

In conclusion, we found that redox active, DNA binding probes that are efficiently reduced at DNA-modified electrodes in a manner that is not dependent on direct contact with the gold surface but is dependent on binding by intercalation to DNA. Together with previous studies of DNA intercalators bound to DNA films, these data indicate that the reduction of redox active intercalating molecules bound to DNA-modified electrodes is through the π-stack of DNA. Intercalators, which are themselves coupled into the π-stack of DNA and thus provide electronic access to the base pairs, are an important feature of the reduction of redox probes at DNA-modified electrodes.

SUMMARY

We have studied the electrochemistry of methylene (MB^+) and $Ru(NH_3)_6^{3+}$ at DNA-modified electrodes in order to explore some of the critical parameters affecting efficient charge transport through DNA-modified surfaces. We examine the effect of electrode surface passivation and the effect of the mode (intercalation or electrostatic) of MB^+ and $Ru(NH_3)_6^{3+}$ binding to DNA to establish the importance of intercalation for reduction by a DNA-mediated charge transport pathway. The electrochemistry of these molecules is studied as a function of ionic strength to determine the importance of tight binding to DNA. Furthermore, MB^+ is covalently linked to DNA through a □-bonded tether and ionic strength is used to study the effect of intercalation on the electrochemistry of this modified MB^+ molecule. The results presented here establish that the reduction of DNA-bound intercalators, but not electrostatically bound molecules, is mediated by charge transport through the π-stack of DNA. Intercalation is a requirement for efficient charge transport through the π-stack of DNA at DNA-modified electrodes.

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