APPENDIX

Experimental Methods for Detection of DNA Base Stacking Perturbations by DNA-Mediated Charge Transport Chemistry

Adapted in part from Boon, E.M., Kisko, J.L., Barton, J.K. (2002) *Methods in Enzymology 353*, 506.

INTRODUCTION

Detailed experimental procedures for the electrochemical analysis of DNA structure based on charge transport through DNA-modified surfaces using intercalators as redox probes, as used throughout this thesis, are described here (Figure A.1). Included are procedures for the synthesis, modification and purification of thiol-derivatized oligonucleotides. Hybridization of these derivatized oligonucleotides and self-assembly onto electrode surfaces as well as techniques for electrochemical analysis of the resulting DNA films are also explained. This technology should be generally applicable as a tool for directly probing DNA base pair stacking.

STRATEGY FOR THE CONSTRUCTION OF THIOL-MODIFIED DNA

All reagents and solvents are purchased in their highest available purity and used without further purification. Millipore milliQ (18 MΩ cm) water is used in all experiments. All glassware and plasticware is DNase, RNase, and metal free.

Oligonucleotides are derivatized with alkane thiol linkers for self-assembly on gold surfaces (Figure A.2). The length of the oligonucleotide can be varied. The oligonucleotides are synthesized (trityl off) on a 1 μmol scale on a DNA synthesizer using standard solid phase phosphoramidite chemistry (1000 Å CPG). After the synthesis the DNA is still on the resin and fully

Figure A.1. Schematic representation of a DNA-modified gold electrode with a bound redox active DNA intercalator for use in electrochemical assays.

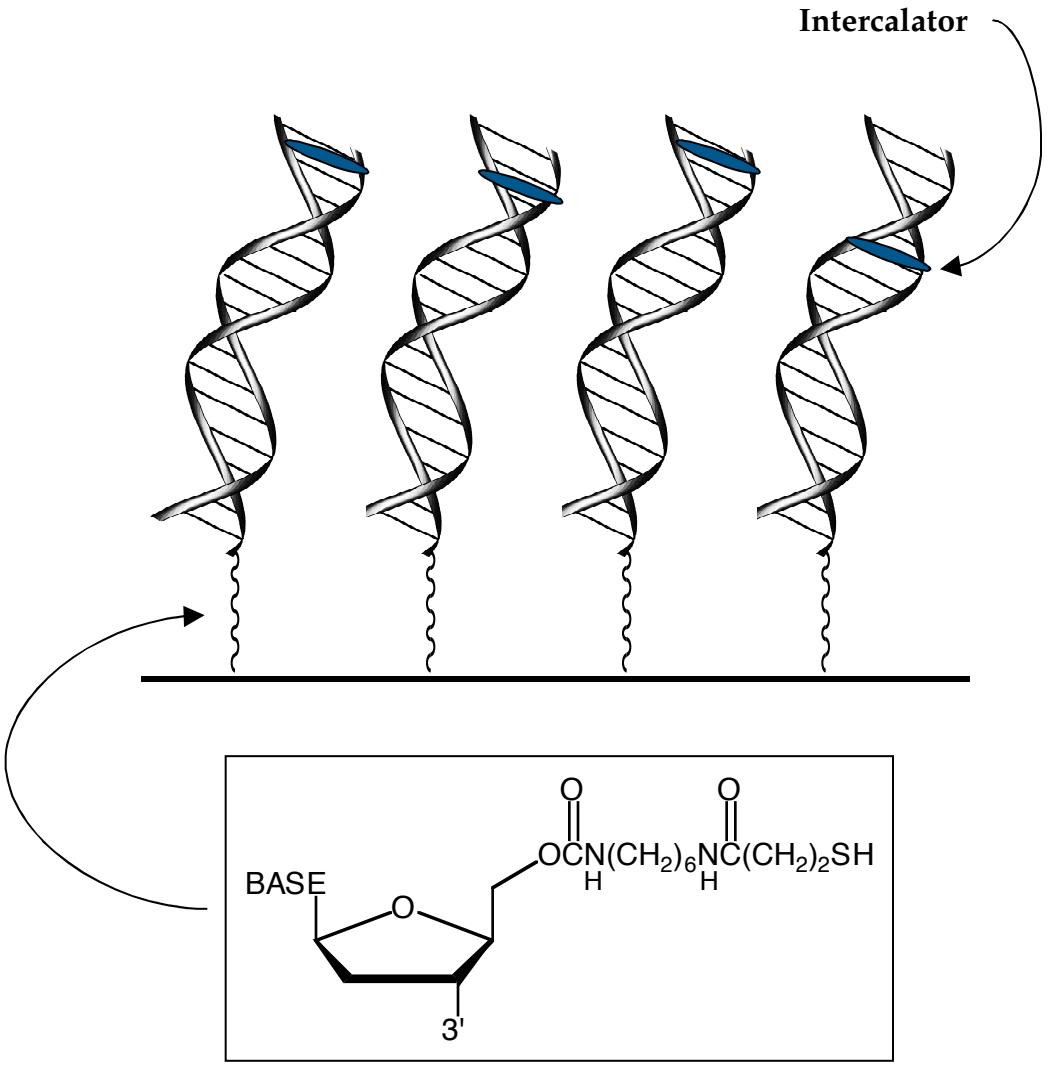
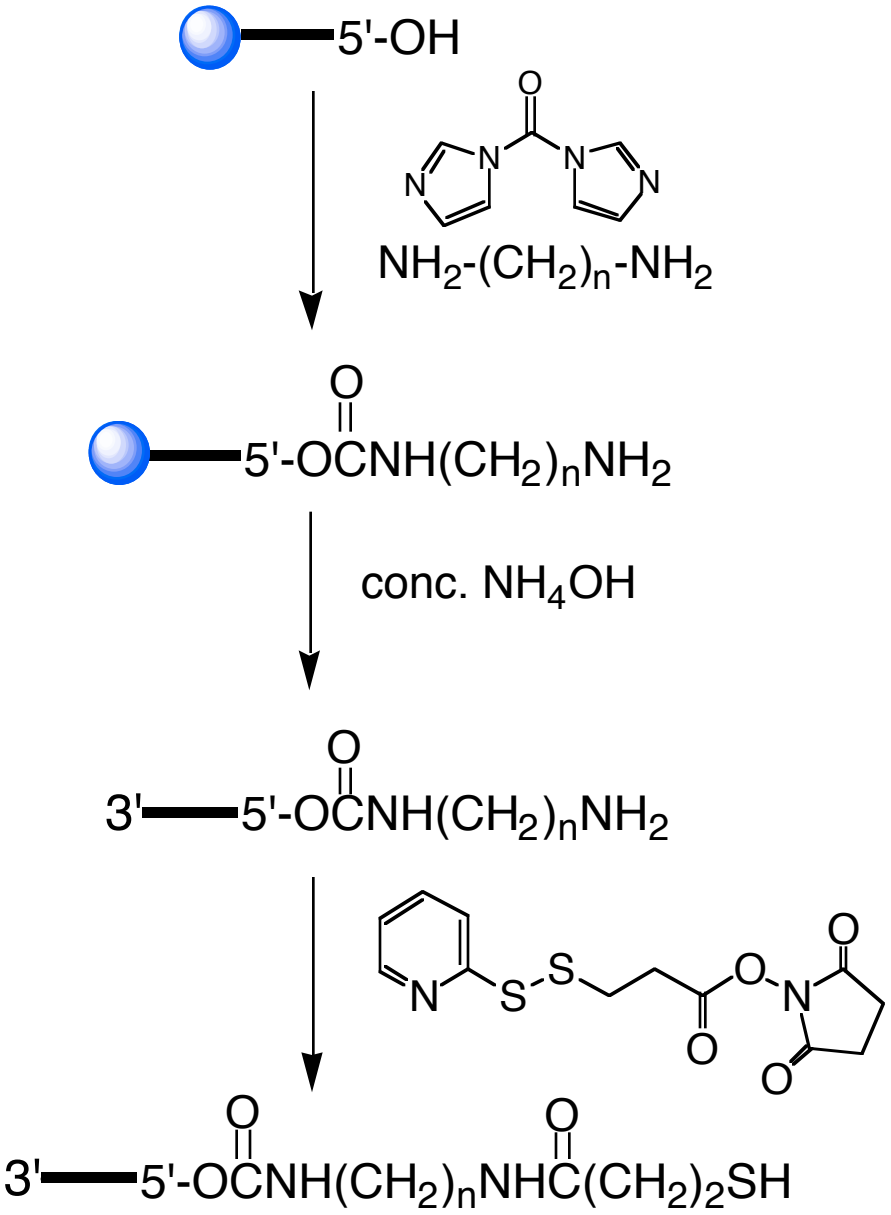


Figure A.2. Synthesis of a thiol-modified single stranded oligonucleotide.



protected with the exception of the 5′-OH terminus. This solid phase DNA is transferred to a peptide reaction vessel (coarse frit). The 5′-OH is aminoacylated by reaction with carbonyldiimidazole (CDI) in dioxane (25 mg CDI in 1 mL dioxane for 45 minutes). Following this activation reaction, a six carbon amine terminated linker is added by reaction with 1,6-hexanediamine (32 mg linker in 1 mL dioxane, filter out undissolved oxidized starting material using a 25 mm x 0.45 mm Gleman Acrodisk syringe filter, react for 25 minutes). This product is transferred to a 1.5 mL eppendorf tube. The DNA-5'-NH_2 is cleaved from the CPG resin and all of the bases are deprotected by incubation in 1 mL of concentrated NH_4OH at 55°C for 8 hours. The DNA-5'-NH_2 product is then cooled, decanted and evaporated to dryness *in vacuo*. This product is purified by reverse phase HPLC on a C-18 300 Å column with a gradient of 0-13% CH_3CN in 35 minutes, 13-50% CH_3CN in 50 minutes with NH_4OAc, pH 7 as the aqueous phase (monitored at 260 and 290 nm) (Figure A.3). This purified DNA-5'-NH_2 product is dried *in vacuo* and its purity can be confirmed by mass spectrometry (expected mass = 4717 amu).

The next step of the reaction is another aminoacylation to form the DNA-5'-SS product which is then deprotected to form the final product, DNA-5'-SH. DNA-5'-NH_2 product is dissolved in 200 μL of 0.2 M, pH 8 HEPES buffer and added to 15 mg of 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester in 100 μL CH_3CN. This reaction proceeds at room temperature. After 1 hour the reaction is quenched by the addition of 700 μL of 5 mM phosphate, 50 mM NaCl, pH7 buffer (here after referred to simply as PBS). Upon this addition the solution turns very cloudy due to insoluble side

products. This mixture is centrifuged for 5 minutes and the DNA solution is decanted from the solid side products and gel filtered on a NAP 10 column (Sephadex G-25, DNA grade) with PBS. The DNA-5'-SS elutant is collected, the volume reduced and purified by HPLC (analogously to DNA-5'-NH_2) and dried *in vacuo* (Figure A.3; expected mass = 4914 amu).

Next, the DNA-5′-SS product is resuspended in 200 μL PBS. Dithiothreitol (DTT; 3mg) is then added and the reduction reaction proceeds at ambient temperature for 40 minutes to form the final product, DNA-5'-SH. This is gel filtered on a NAP 5 column (Sephadex G-25, DNA grade) in PBS then HPLC purified and dried *in vacuo* (Figure A.3; expected mass = 4805 amu)

The thiol-modified single strand (DNA-5′-SH) can be tested for the presence of free thiol via HPLC by Ellman's test as follows (Figure A.4). A small aliquot (~100 μL) of the DNA-5′-SH HPLC fraction is reinjected onto the HPLC for an analytical run followed by a second 100 μL aliquot to which 1 μL of 10 mM dithionitrobenzoic acid (DTNB; Ellman's reagent) had been added. Free thiol is monitored by a shift in the DNA-5′-SH chromatogram peak as well as a new peaks in the UV-vis spectrum at 330 and 410 nm.

The single stranded oligonucleotide complementary to the thiol-modified strand is synthesized (trityl off) on a 1 μmol scale on a DNA synthesizer using standard solid phase phosphoramidite chemistry (1000 Å CPG). The CPG-bound DNA is then transferred to a 1.5 mL eppendorf tube and mixed with 1 mL of concentrated NH_4OH and incubated at 55°C for 8 hours to cleave the oligonucleotide from the CPG resin and deprotect all of

Figure A.3. HPLC and mass spectrometry of all isolated intermediates during the synthesis of thiol-modified single stranded oligonucleotides.

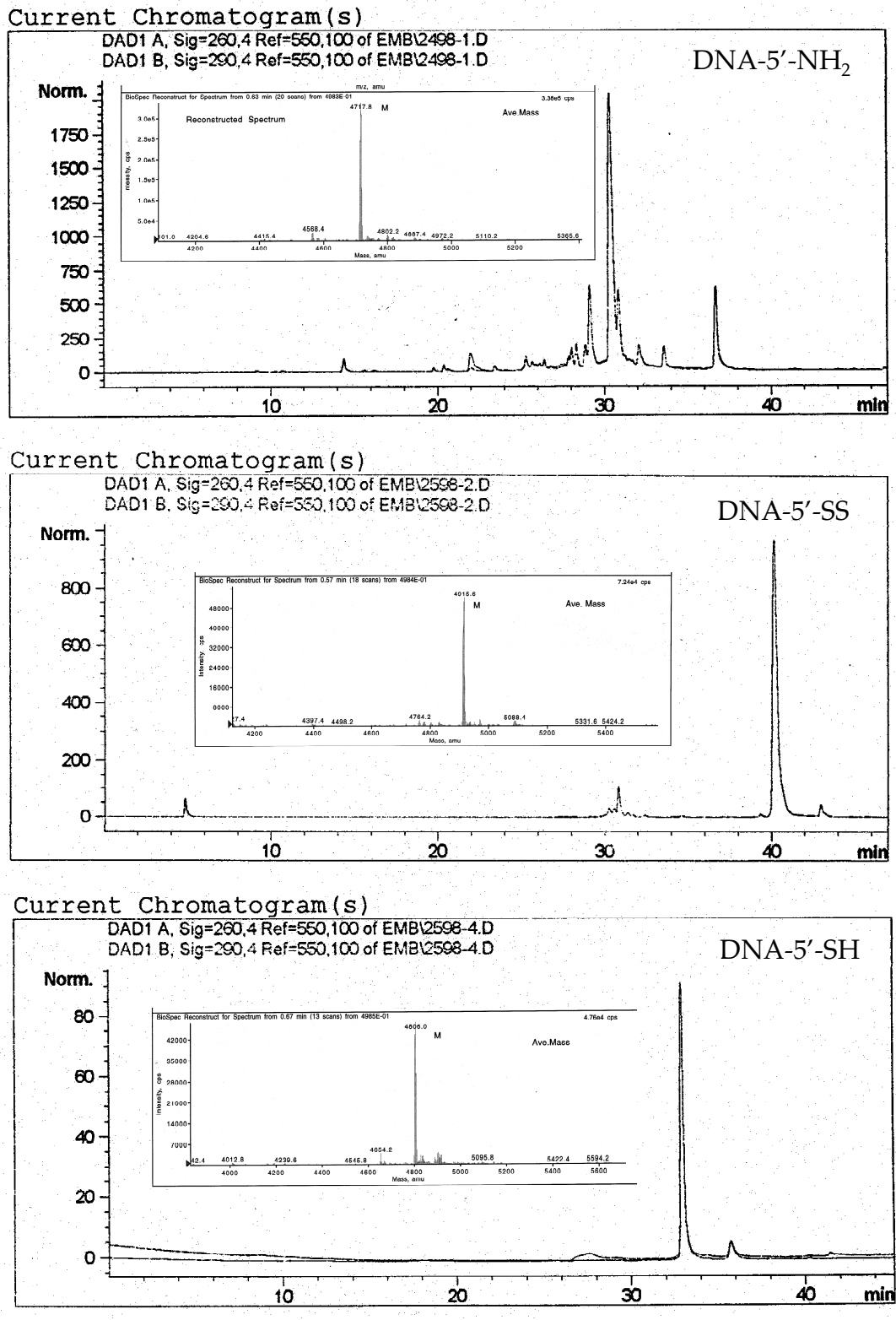
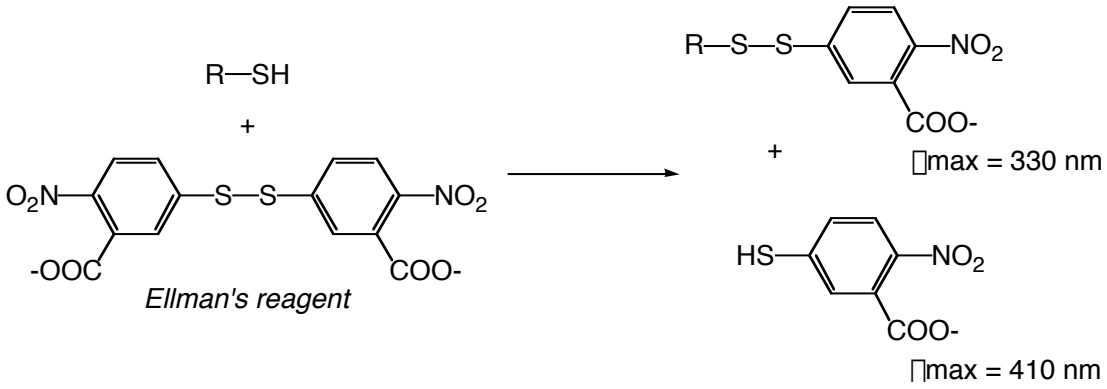
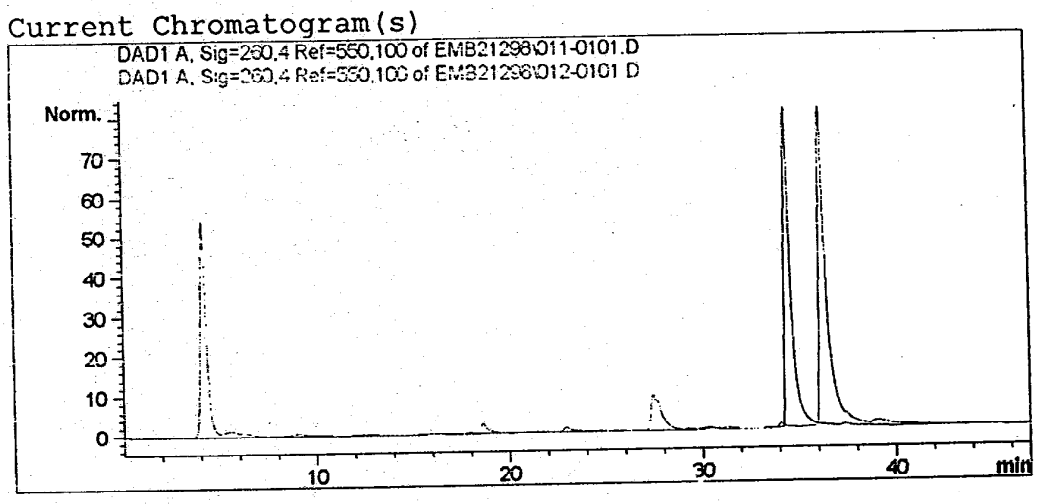
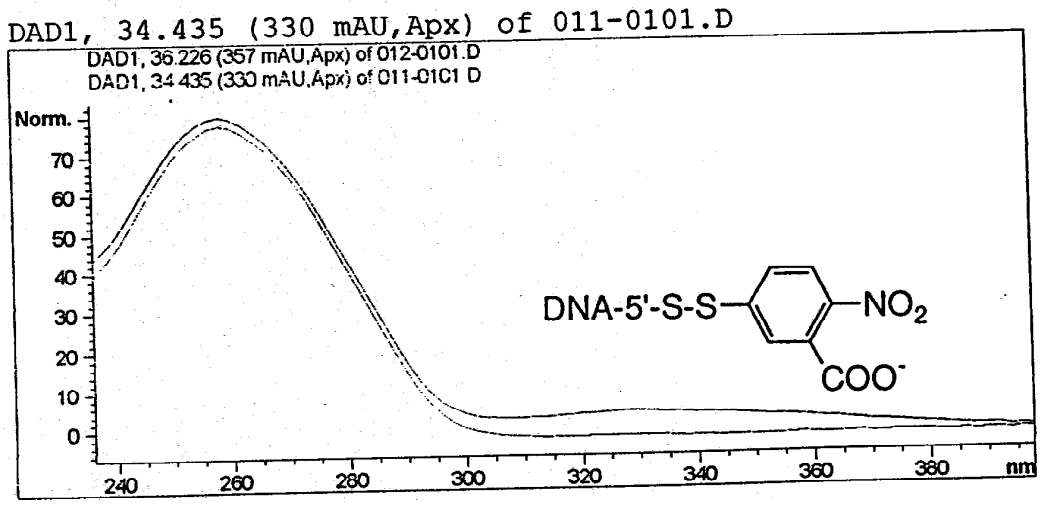


Figure A.4. Ellman's test for the detection of free thiols via HPLC analysis.







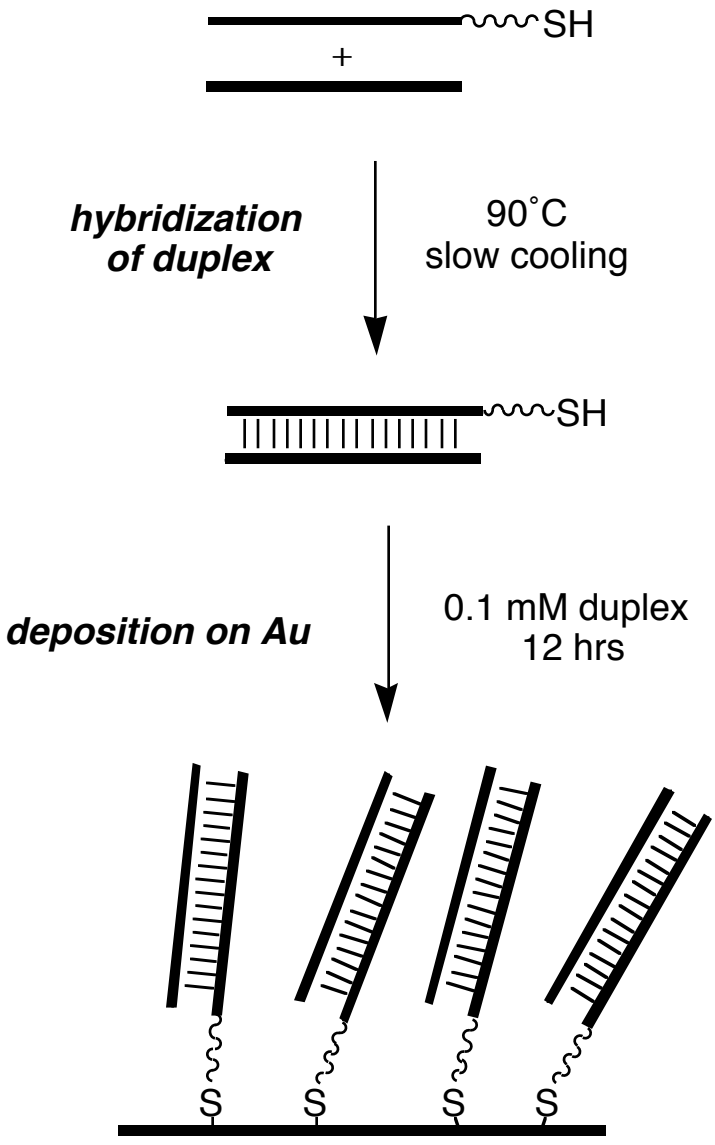
the bases. This product is then cooled, decanted, and evaporated to dryness *in vacuo*. This product is purified by reverse phase HPLC.

The two purified, complementary single stranded oligonucleotides are then quantitated and hybridized to make thiol-modified duplexes (Figure A.5). Oligonucleotide stock solutions are prepared and quantitated by UV-vis spectroscopy (λ_{max} = 260 nm, ε($M^{-1}cm^{-1}$). The extinction coefficients of single strands are calculated by the sum of the extinction coefficients of the individual bases: ε(dA) = 15,400, ε(dG) = 11,500, ε(dC) = 7,400, ε(dT) = 8,700). Duplexes are formed by combining equimolar amounts of each strand in PBS for a final solution of 100 μM duplex. This solution is degassed and blanketed with Ar, heated to 90°C for 5 minutes and then cooled slowly to room temperature (1-2 hours). Just before deposition on the clean gold electrode, 100 mM $MgCl_2$ is added to each sample.

PREPARATION OF THE ELECTRODE SURFACE

The gold electrodes are prepared by standard procedures. They are polished with 0.05 μm alumina, sonicated in distilled H_2O for ~20 minutes, electrochemically etched in 1M H_2SO_4, and rinsed well with distilled H_2O. The electrodes are then inverted and a ~10 μL drop of the thiol-modified DNA duplex solution is deposited onto each electrode surface (Figure A.5). The electrodes are kept in a moist environment at room temperature during the assembly process. To ensure maximum density of the monolayer, self-

Figure A.5. Fabrication of a DNA-modified electrode surface.



assembly is usually allowed to proceed overnight, but assembly does proceed much faster if the thiol and gold surface are perfectly clean. After assembly, the complementary DNA strand can be removed and replaced with test strands by *in situ* hybridization as follows (see also Chapter 3). The DNA electrode is immersed in 90°C PBS for 5 minutes and then rinsed thoroughly in PBS. Next, the electrode is immersed in a solution of 100 pM test strand oligonucleotide in PBS with 100 mM $MgCl_2$ at 90°C and allowed to cool to room temperature. Alternatively, if test strand is limited in quantity, a drop of the test strand in PBS with 100 mM $MgCl_2$ can be placed on the hot electrode surface and allowed to cool. During the cooling process, it is important to prevent evaporation of the solution, so drops of PBS should be placed on the surface as needed.

ELECTROCHEMICAL ASSAYS OF BASE STACKING

Cyclic voltammetry (CV) is carried out in a two compartment cell filled with PBS that is degassed and blanketed with Ar. The DNA-modified Au working electrode and the Pt wire auxiliary electrode are separated from the saturated calomel reference electrode (SCE) by a modified luggin capillary.

Before electrochemical analysis, excess DNA is rinsed away from the electrode with PBS and monolayer coverage is qualitatively checked with 2 mM $Fe(CN)_6^{3-}$ in PBS by scanning the potential from 0 to 600 mV and back at 100 mV/sec. If a CV signal is not observed, it is interpreted that the

monolayer is so dense that $Fe(CN)_6^{3-}$ cannot diffuse to the Au surface and participate in redox chemistry (Figure A.6). It is important to scan each electrode with $Fe(CN)_6^{3-}$ several times, as often a bad monolayer will look covered during the first scan, but DNA that is only electrostatically or hydrophobically bound to the surface will diffuse away upon cycling the potential. A good monolayer is stable to cycling the potential between ~ 600 mV and ~-650 mV repeatedly and can be kept at RT several days to a week, sometimes longer, as long as the monolayer is keep wet. A fully covered monolayer is very important for studies of stacking perturbations as a loosely packed monolayer will provide access of the intercalator to the interior of the monolayer, thus not forcing the charge to pass through the whole DNA helix. With a densely packed surface, in electrochemical studies of DNA intercalators, it is assumed that any CV signal observed is the result of charge transport through the DNA monolayer (detection of mismatches and other perturbations are consistent with this model, Chapters 2,3,7,8, see also Chapter 4). Monolayers can be extensively characterized by AFM, ellipsometry, and radiolabeling of the duplexes, which is described elsewhere (Shana O. Kelley, C.I.T. Thesis 1998, see also Chapter 6).

If the electrode surface is sufficiently covered, a catalyst such as methylene blue is added to the solution for a final concentration of 2.0 μM-0.5 μM. Another catalyst can be used, but it must satisfy these requirements: it must bind to DNA by intercalation, it must not bind either too tightly or too loosely (the catalyst must be able to access the DNA base stack as well as the ferricyanide in solution), and its reduction potential must be around –200 to –600 vs. SCE. After addition of the catalyst and degassing with Ar, the

potential is scanned from 0 to –600 mV (vs. SCE) at 100 mV/sec. If the duplexes on the surface are well packed and contain no mismatches or other π-stack disrupting lesions, a large catalytic wave is observed at ~-350 mV. If there are mismatches, the background will start to rise at about –600 mV, but there will be no distinct peak (Figure A.7; Chapter 2).

A more sensitive way to check for mismatches is to carry out chronocoulometry. The electrode potential is stepped to –350 mV vs. SCE and allowed to integrate for 5 seconds. If there are no base stack perturbations, the charge should steadily increase over the course of the experiment; the maximum amount of charge accumulated depends on the sequence, but typically, the charge will reach 16 to 25 μC. If there is a mismatch, only 1 to 6 μC will accumulate, depending on the extent of perturbation and the sequence (Figure 2.7; Chapters 2-3,7-8).

Figure A.6. Qualitative test of DNA-monolayer coverage using 2 mM ferrocyanide. (a) Bare Au; surface is not covered. (b) DNA-modified Au; well covered surface. Imperfectly covered surfaces will have smaller but discernable ferrocyanide peaks, and the oxidation and reduction waves will have a larger peak splitting due to sluggish kinetics as a result of the partially blocked gold surface.

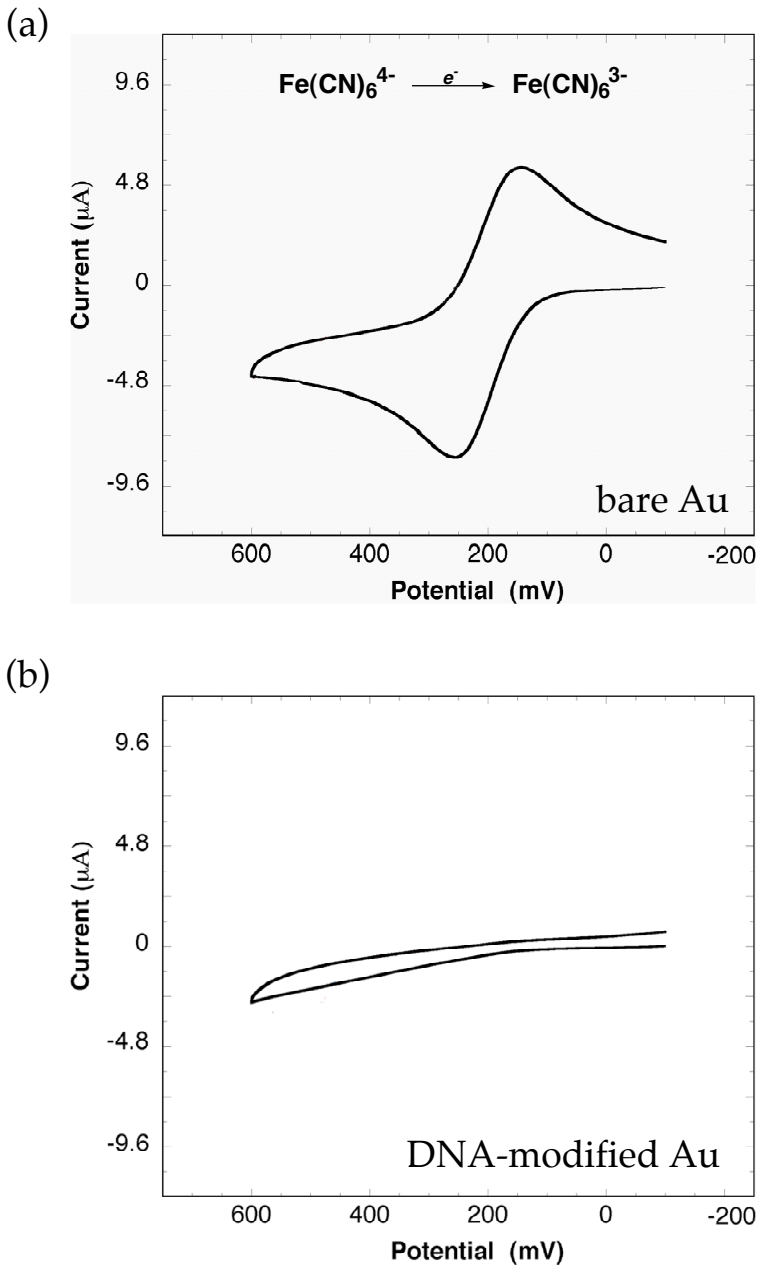


Figure A.7. Electrocatalytic detection of a single CA mismatch using both (a) cyclic voltammetry and (b) chronocoulometry. (c) Electrocatalytic cycle.