Electrochemical Detection of Single-Stranded DNA Using Polymer-Modified Electrodes

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Glassy carbon electrodes modified by reductive electropolymerization of a thin film of poly[$Ru(vbpy)_3^{2+}$] or poly[$Ru(vbpy)_3^{2+}$/vba] (vbpy = 4-vinyl-4′-methyl-2,2′-bipyridine and vba = *p*-vinylbenzoic acid) were prepared. The Ru(III/II) couples for the polymer films were reversible in nonaqueous solution but were irreversible in aqueous media. The films modified with poly[$Ru(vbpy)_3^{2+}$] catalyzed the oxidation of aqueous guanosine 5′-monophosphate (GMP) and poly[G], producing a current enhancement in the Ru(III/II) couple for the polymer film. The catalysis was due both to electrostatic condensation of GMP and poly[G] to the Ru-modified surface and to more facile electron transfer to the Ru(III) centers in the polymer compared to the bare electrode. The presence of GMP in solution decreased the extent of decomposition of Ru(III). When single-stranded DNA containing multiple guanines was attached to the electrode modified with the poly[$Ru(vbpy)_3^{2+}$/vba] copolymer, enhancement of 8−13 μA for the Ru(III/II) couple was observed with 8 pmol of attached DNA. This degree of enhancement corresponds to a current efficiency of 65% based on a one-electron oxidation of guanine.

Recent advances in surface modification techniques have facilitated many new methods for bioassay technology, particularly when coupled with sophisticated fluorescent detection technologies. For example, the application of surface modification and analysis to gene expression profiling,[1] sequencing of genomic DNA on high-density arrays,[2] and the detection of nucleic acids to identify infectious organisms[3] has the potential for superior selectivity and sensitivity when compared to existing methods. While these systems present significant advancements, they still involve extensive pretreatment steps and the use of expensive fluorescent microscopes. The electrochemical detection of nucleic acids provides an alternative to fluorescent techniques that potentially eliminates the need for labeling the target nucleic acid and provides an electronic means for spatial resolution.[4−7] The guanine nucleobases of polymeric DNA produce an array of redox-active labels suitable for ultrasensitive detection that, in conjunction with ultramicroelectrode methods, may provide a method for detecting many physiologically relevant nucleic acids at low copy numbers. Incorporation of individual microelectrodes into an array could potentially produce low-cost, rapid-throughput devices with high-density, multiplexed sensor arrays. As a means of attaining this goal, surface-modified electrodes appear well suited for the detection of electron-transfer events at potentials near those observed for guanine bases in DNA.[8]

We have previously shown that nucleic acids can be detected in solution via catalytic oxidation of guanine bases using $Ru(bpy)_3^{2+}$ as the mediator.[9−12] In solution, $Ru(bpy)_3^{2+}$ exhibits a reversible redox couple at 1.05 V similar to the oxidation potential observed for guanosine. Addition of guanine-containing DNA to a solution of $Ru(bpy)_3^{2+}$ therefore leads to catalytic enhancement in the oxidation current according to a two-step mechanism,

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^- \quad (1)$$

$$Ru(bpy)_3^{3+} + DNA \rightarrow DNA_{ox} + Ru(bpy)_3^{2+} \quad (2)$$

where DNA_{ox} represents a DNA molecule where guanine has undergone a one-electron oxidation. The catalytic current can be detected based on catalytic reactions of DNA in solution or immobilized to the electrode via polymer membranes or chemical linkers. Previous studies have centered mainly on the use of tin-doped indium oxide (ITO) electrodes, which do not adsorb nucleic acid nonspecifically and have high oxidative potential limits in neutral aqueous solution. The previous studies have demonstrated feasibility in configurations where both $Ru(bpy)_3^{2+}$ and DNA are in solution and when $Ru(bpy)_3^{2+}$ is in solution and DNA is immobilized.[13−15] Here we demonstrate effective oxidation of DNA in a more traditional electrocatalysis scenario where the metal complex is immobilized.

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Thin polymeric films containing polypyridyl complexes of Ru^{II}, generally based on $Ru(vbpy)_3^{2+}$, can be readily prepared by reductive electropolymerization onto Pt and glassy carbon electrode surfaces from dilute acetonitrile solutions (vbpy = 4-vinyl-4′-methylbipyridine).[16,17] These films exhibit an oxidative redox couple at 1.1 V (all potentials vs Ag/AgCl), slightly above that observed for guanine (1.05 V)[8] in aqueous solution. These films should therefore be active catalysts for the electrooxidation of guanine and DNA, since the Ru(III/II) couple is closely matched to that of guanine.[9] Further, electropolymerization of a mixture of $Ru(vbpy)_3^{2+}$ and *p*-vinylbenzoic acid (vba) should produce films that contain the ruthenium catalyst and to which amine-appended oligonucleotides can be attached via known carbodiimide reactions that label the carboxylate group.[16] In this paper, we describe initial studies in which electrodes modified with films of $Ru(vbpy)_3^{2+}$ are shown to catalyze DNA oxidation and where copolymers of $Ru(vbpy)_3^{2+}$ and vba are used to prepare site specifically assembled loci for DNA detection. The site-specific modification of electrodes with DNA via electropolymerization of modified pyrroles has been pursued previously,[6,7] but not in a system where detection of faradaic current from DNA oxidation was available as a response to DNA immobilization.

Experimental Section

Reagents and DNA. Inorganic reagents used in these experiments were of analytical grade or higher. The inorganic complex, $[Ru(vbpy)_3](PF_6)_2$, was prepared using standard literature procedures.[17] The sensing probe, $CpFe(C_5H_4-C_2H_4NH_2)$ was synthesized by a standard $LiAlH_4$ reduction of the cyano-substituted ferrocene, $CpFe(C_5H_4-CH_2CN)$ followed by workup from diethyl ether. The product gave satisfactory elemental analysis and NMR. Water-soluble carbodiimide (EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride), DCC (dicyclohexylcarbodiimide), TBAH (tetrabutylammonium hexafluorophosphate), NHS (N-hydroxysuccinimide), GMP (guanosine 5′-monophosphate, disodium salt), poly[G], and vba were purchased from Aldrich (Milwaukee, WI) and used as received. Two recrystallizations of vba from 50% ethanol were done prior to electrochemical experiments to ensure purity. MES (2-morpholinoethanesulfonic acid sodium salt and 2-morpholinoethanesulfonic acid monohydrate) were purchased from Fluka (New Ulm, Switzerland). Na_2HPO_4, NaH_2PO_4, NaCl, and acetonitrile were obtained from Mallinckrodt (Phillipsburg, NJ). The acetonitrile was dried over activated molecular sieves prior to use in electropolymerization experiments. Synthetic oligonucleotides were synthesized by the UNC Department of Pathology and purified using Amicon micron 3 concentrators with a cutoff of 3000 molecular weight. Water was obtained from a Milli-Q Plus purification system (Millipore, Bedford, MA). Glassy carbon electrodes (GCE's, 3 mm diameter) were purchased from BAS (West Lafayette, IN) and polished prior to use. Ag/AgCl reference electrodes were purchased from Cypress Systems (Lawrence, KS) and contained 3 M KCl saturated with AgCl.

Electrochemical Analysis. Cyclic voltammograms were collected using a PAR 273A potentiostat/galvanostat. Experiments done in acetonitrile used a two-compartment voltammetric cell equipped with a glassy carbon working, platinum mesh counter, and $Ag/AgNO_3$ reference electrode. Aqueous experiments were carried out using a single-compartment voltammetric cell equipped with a glassy carbon working, platinum wire counter, and a Ag/AgCl reference electrode. Prior to use, all GCEs were thoroughly polished with METADI diamond polishing compound (Buehler, Lake Bluff, IL) and Al_2O_3 (0.5 μm in H_2O) on a felt polishing platform. The electrodes were then rinsed several times with Milli-Q water and dry acetonitrile immediately before use. Electropolymerization reactions were done by filling the working electrode compartment with 3.5 mL of a 0.2 mM $[Ru(vbpy)_3](PF_6)_2$, 0.1 M TBAH acetonitrile solution and scanning reductively 10 times between −0.9 and −2.0 V with a 100 mV/s scan rate. These solutions must be thoroughly dried and degassed prior to reductive scanning, and the reference electrode compartment must be filled with 0.1 M TBAH in acetonitrile. For formation of vba-doped films, a 5:1 $[Ru(vbpy)_3](PF_6)_2$ to vba solution ratio was found to produce films with the greatest reproducibility. Electrochemical oxidation of aqueous GMP, poly[G], and attached DNA probes was done in a 50 mM, pH = 7.0 phosphate buffer, scanning positively from 0.0 to 1.4 V at a 50 mV/s scan rate.

Attachment of DNA to Film-Modified Electrodes. Attachment of the DNA probe was carried out using a standard amidation procedure in which the surface carboxyl groups (present in the film as vba spacers) are activated using well understood carbodiimide chemistry and subsequently undergo amidation reactions with amino-linked single-stranded DNA as described in similar procedures.[18,19] After electropolymerization of poly$[Ru(vbpy)_3^{2+}/vba]$ onto a GCE, the electrode was carefully rinsed with acetonitrile to remove residual $[Ru(vbpy)_3](PF_6)_2$, vba, and TBAH. The electrode was then inverted, and a 50 μL drop of EDC/NHS solution (made by dissolving 10 mg EDC and 1 mg NHS in 1.0 mL Milli-Q water) was carefully placed on the electrode surface. The electrode was covered with an inverted beaker for 30 min. The treated GCE was then rinsed numerous times with water and carefully blotted dry. Again, the electrode was inverted, and a 25 μL drop of a pH buffered, 5 μM DNA probe solution (20-mer, poly[dG] with a 3′-$(CH_2)_6NH_2$ linking group) was placed on the electrode face. The electrode was allowed to rest covered and undisturbed for 90 min before rinsing with an 800 mM NaCl, 50 mM phosphate buffer (pH = 7.0) solution. A high salt solution was necessary for this rinsing step to disrupt any electrostatic interactions between the polymer surface and noncovalently bound DNA.

Quantification of Immobilized Probe. The 20-mer probe was 5′-^{32}P-labeled using T4 polynucleotide kinase and γ-^{32}P-ATP (6000 Ci/mmol) according to standard procedures.[15,20] Unreacted ATP was removed from the labeled probe using a Stratagene NucTrap column. The radiolabeled probe was attached using the identical procedure described for the nonlabeled probe using a stock DNA solution (270 μL total volume) containing 5 pmol labeled probe diluted to 5 μM with nonlabeled probe in either pH 6.5 MES or pH 9.0 carbonate buffer solution. After immobilization, the probe-modified film was mechanically removed from the electrode face by rubbing the polymer onto a piece of filter paper. Control films were obtained in an identical manner by excluding the EDC/NHS amidation step of the reaction. The radioactivity of these samples was then determined in triplicate using both liquid scintillation and phosphorimaging techniques.

Results and Discussion

Preparation of Polymer-Modified Electrodes. Electropolymerization of poly$[Ru(vbpy)_3^{2+}]$ films onto electrode surfaces is well understood, and the resulting films can be fabricated in reproducible thickness using varied scan times and scan rates.[16,17] Parts A and B of Figure 1 show polymer growth for both a simple ruthenium-containing film, poly$[Ru(vbpy)_3^{2+}]$, and a film doped with the carboxyl-containing vba group, poly$[Ru(vbpy)_3^{2+}/vba]$; the copolymer for the latter reaction is shown in Scheme 1. As has been previously demonstrated, poly$[Ru(vbpy)_3^{2+}]$ films can be reversibly oxidized in dry acetonitrile or strong acid solution, where the Ru^{3+} is relatively stable.[16,17] Reversible oxidation of the metal center in the poly$[Ru(vbpy)_3^{2+}/vba]$ copolymer films was also observed in acid or nonaqueous solution. However, when submersed in aqueous solutions at pH > 3.0, the copolymer film decomposes upon electrochemical oxidation of the metal center, producing an

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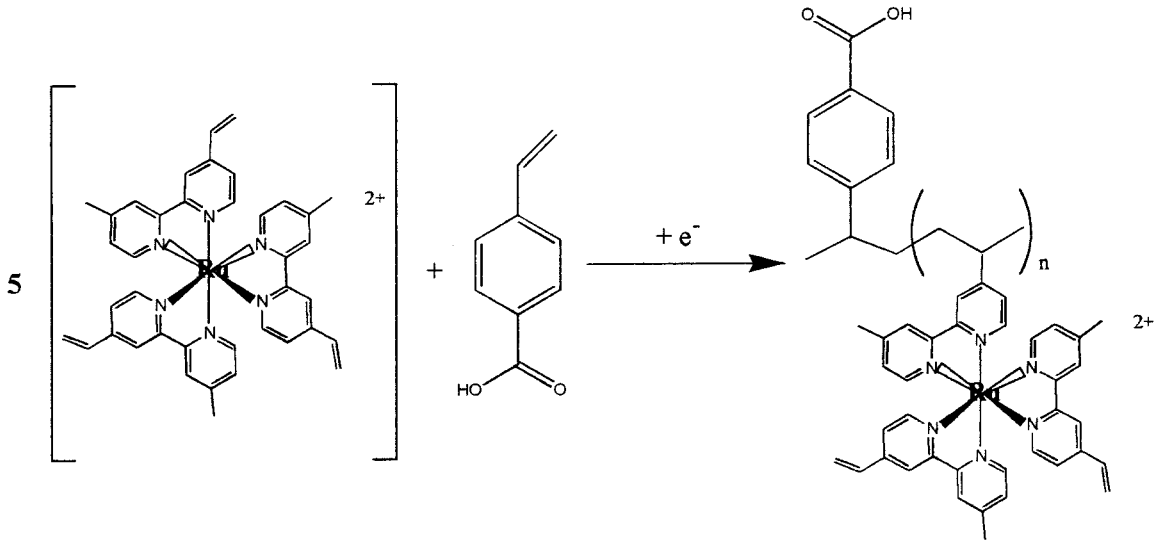
Scheme 1



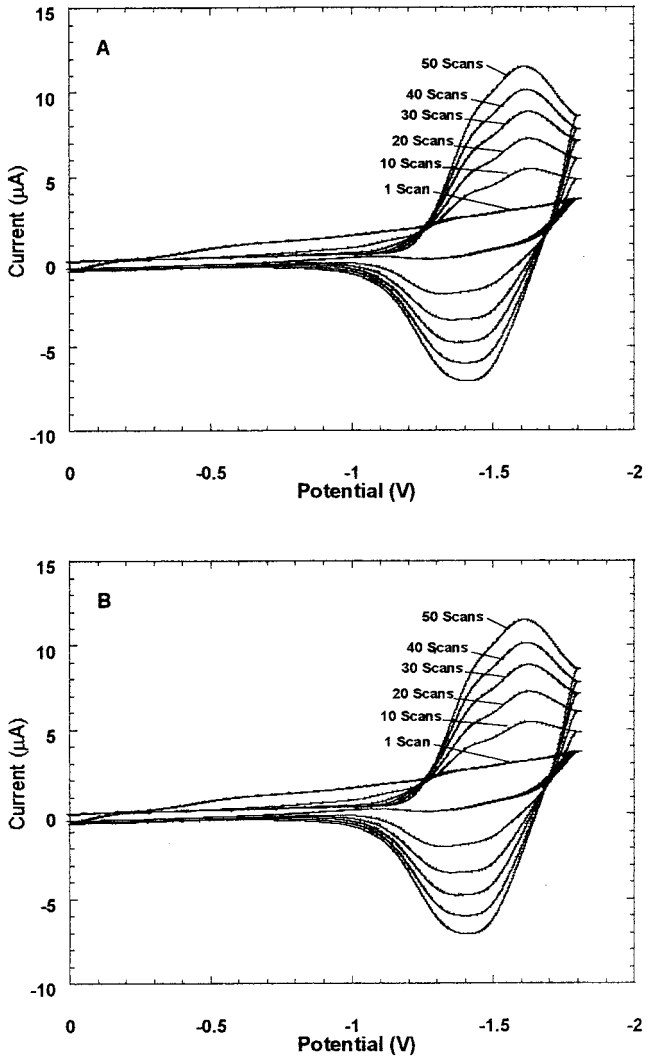


Figure 1. Cyclic voltammograms showing the electropolymerization of (A) poly[$Ru(vbpy)_3^{2+}$] and (B) 5:1 poly[$Ru(vbpy)_3^{2+}$/vba] from an acetonitrile solution containing 0.1 M TBAH onto a glassy carbon electrode (100 mV/s scan rate, $Ag/AgNO_3$ reference). Concentration of $Ru(vbpy)_3^{2+}$ in solution was 0.2 mM.

irreversible wave. Thus, rapid rereduction of the oxidized Ru center by DNA will be required to regenerate Ru^{2+} prior to decomposition.

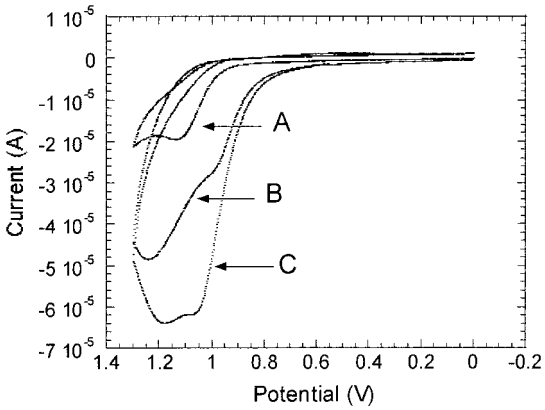


Figure 2. Cyclic voltammograms showing the oxidation of GMP using (A) an unmodified GCE and a poly[$Ru(vbpy)_3^{2+}$] film-modified GCE in the (B) absence and (C) presence of GMP (50 mV/s scan rate, Ag/AgCl reference, 50 mM pH = 7.0 phosphate buffered solution).

Detection of GMP and Poly[G]. Electrocatalytic oxidation of guanine was detected first at electrodes where the ruthenium catalyst was immobilized on the GCE but the guanine was in solution. A GCE modified with poly[$Ru(vbpy)_3^{2+}$] films was scanned oxidatively in the presence and absence of guanosine 5′-monophosphate (GMP) dissolved in a pH 7.0, phosphate buffer solution (Figure 2). Addition of 1.0 mM GMP causes a significant increase in the oxidation current (Figure 2C) compared to that for the film alone in buffer (Figure 2B). Scanning an unmodified GCE in the presence of 1.0 mM GMP solution produces a significantly smaller oxidation current with an onset at close to 1.2 V (Figure 2A). In contrast, the voltammogram of GMP at the modified electrode shows an onset of catalytic enhancement at closer to 1.0 V.

Further support for the proposed catalytic reaction is provided by the observation that the GMP electron donor protects the film from decomposition. In the absence of a suitable concentration of electron donor, complete decomposition of the film occurs such that almost no faradaic oxidation current is observed on a second scan of the modified electrode (Figure 3A). In contrast, when GMP is present in solution, some fraction of the oxidized Ru(III) centers are reduced to the stable Ru(II) form by a GMP molecule prior to rapid decomposition. Thus, there is still significant oxidation current during a second scan (Figure 3B). In an experiment where a poly[$Ru(vbpy)_3^{2+}$] film was oxidized in the absence of GMP solution followed by a

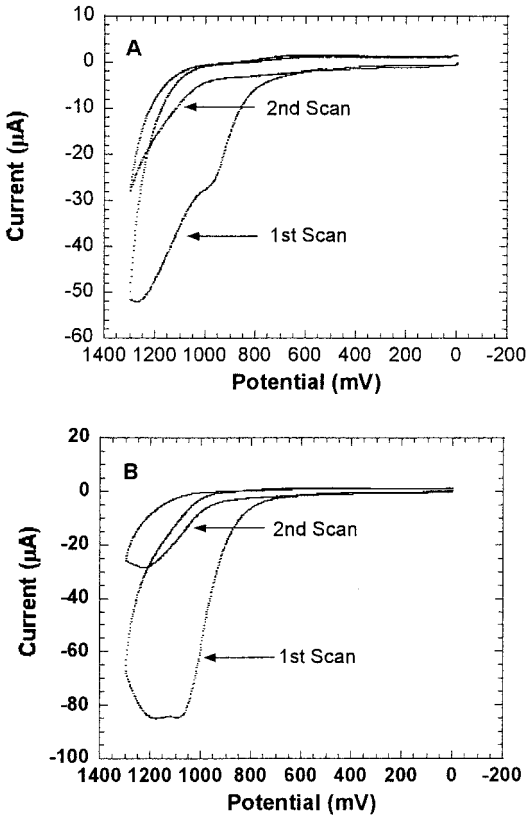


Figure 3. (A) First and second oxidative scans of a poly[$Ru(vbpy)_3^{2+}$] film-modified GCE. (B) First and second scans of a poly[$Ru(vbpy)_3^{2+}$] film-modified GCE in the presence of GMP (50 mV/s scan rate, Ag/AgCl reference, 50 mM pH = 7.0 phosphate buffered solution).

second oxidative scan in the presence of GMP, a voltammogram identical to that for the second scan in Figure 3A was observed. Dilution of GMP solutions by 2 orders of magnitude (0.01 mM) had little effect on the amount of current produced during the first oxidative scan. This effect can be attributed to the electrostatic attraction between the positively charged polymer surface and the anionic GMP, which probably produces a high local concentration of the substrate at the electrode surface. Apparently, 1.0 mM GMP was sufficient to saturate the binding region of the polymer film.

Cyclic voltammograms of the oxidation of poly[G] using unmodified and poly[$Ru(vbpy)_3^{2+}$] electrodes are shown in Figure 4. In this case, no electrochemical oxidation of poly[G] can be detected at the unmodified electrode (Figure 4A). This lack of response is presumably due to a combination of the slow diffusion of the large polymer to the film and steric protection of the oxidizable guanine sites in the three-dimensional structure of the homopolymer. With a poly[$Ru(vbpy)_3^{2+}$]-modified electrode (Figure 4C), a current enhancement over that observed for poly[$Ru(vbpy)_3^{2+}$] oxidation alone (Figure 4B) is seen. The catalysis is probably due to a combination of the binding of the polyanion to the cationic polymer film and better electron transfer kinetics from guanine to the ruthenium catalysis compared to the direct oxidation by the unmodified electrode. The more efficient catalysis observed for poly[G] compared to the GMP is probably due to greater electrostatic attraction of the polyanion for the cationic electrode.

Detection of Immobilized Probes. Because of the irreversible oxidation of the films in nonaqueous solution, we sought to determine whether attachment of the DNA to the polymer could increase the guanine−Ru(III) electron-transfer rate and thereby further stabilize the film to oxidation. As shown in

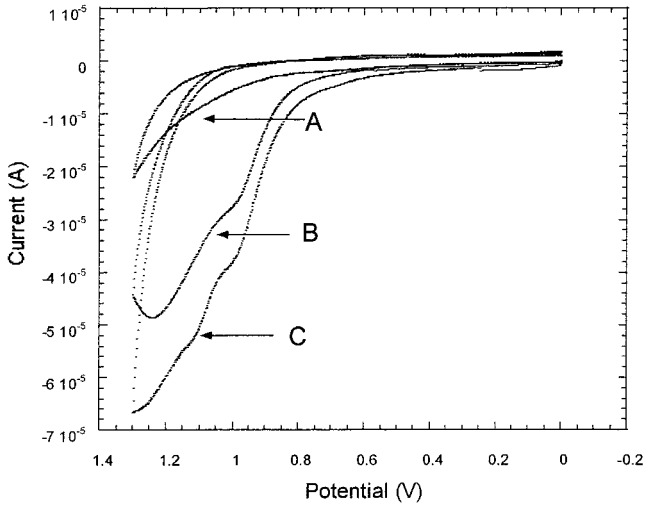


Figure 4. Cyclic voltammograms showing the oxidation of poly[G] using (A) an unmodified GCE and a poly[$Ru(vbpy)_3^{2+}$] film-modified GCE in the (B) absence and (C) presence of poly[G] (50 mV/s scan rate, Ag/AgCl reference, 50 mM pH = 7.0 phosphate buffered solution).

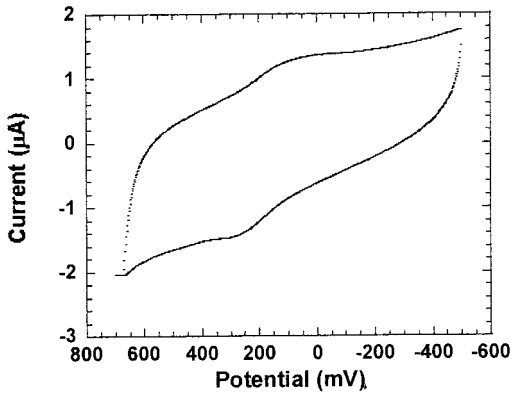
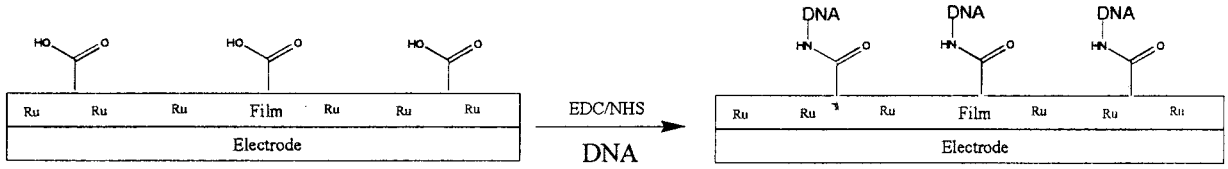


Figure 5. Cyclic voltammogram of a 5:1 poly[$Ru(vbpy)_3^{2+}$/vba] film-modified GCE with immobilized $CpFe(C_5H_4-NH_2)$ (50 mV/s scan rate, $Ag/AgNO_3$ reference, 0.2 mM Ru^{2+}, 0.1 M TBAH solution).

Figure 1B, copolymers of $Ru(vbpy)_3^{2+}$ and vba could be grown on the electrode in a fashion similar to that for the simple polymer. To determine that the carboxyl groups had been incorporated, the amino-modified ferrocene probe ($CpFe(C_5H_4-C_2H_4NH_2)$) was attached to a 10-scan, poly[$Ru(vbpy)_3^{2+}$/vba]-modified electrode, using dicyclohexylcarbodiimide in methylene chloride. The oxidation wave for the Fc^+/Fc couple was observed at 0.2 V upon immersing the film in a 0.1 M TBAH, acetonitrile solution (Figure 5), demonstrating that carboxyl groups that could be modified with primary amines were present in the film.

Having demonstrated that ferrocene containing a pendant primary amine could be attached and detected, we attempted to immobilize a DNA probe bearing a pendant hexylamine (Scheme 2). This strategy is similar to that used with pyrrole films prepared by Korri−Yousoufi et al.[6] Immobilization reactions were performed at two different pH values (6.5 and 9.0) to chemisorb the probe in two distinctly different fashions. At higher pH, amidation preferentially occurs at the primary amine of the attached $-(CH_2)_6NH_2$ group as opposed to any native endogenous amine groups on the purine and pyrimidine rings of DNA.[15] Amidation of these native amine groups would produce electrode surfaces where DNA would be attached not only at the amino-linking group but at several of the native amines to produce a surface where probe molecules are potentially "stapled" to the surface. In contrast, attachment of

Scheme 2



amine-modified DNA at pH 9.0 is generally selective for the primary amine linker,[15] which we suspected would yield films where DNA was attached at a single site.

To confirm attachment of the DNA to the electrode, radiolabeled probes were used in the attachment protocol and then independently quantified. The probes were specifically radiolabeled with ^{32}P prior to labeling, and then the modified electrode was transferred to filter paper and quantified on a phosphorimager screen. Known amounts of radiolabeled probe were transferred directly to filter paper for use as a standard. This procedure gave quantities of attached probe of 8.0×10^{-12} mol and 8.0×10^{-13} mol at pH = 6.5 and 9.0, respectively. Separate experiments using scintillation counting indicated similar extents of modification, although the phosphorimager data are considered more reliable.

We next sought to determine whether electron transfer could be detected from the guanine to the Ru(III) at the DNA/Ru-modified electrodes. Films treated with DNA probes at both pH values were oxidized voltammetrically, and the oxidation current was compared to currents produced by film oxidation alone. As shown in Figure 6, only those films treated with DNA probe at pH = 6.5 produced currents with detectable catalytic enhancements. Over a large number of trials at pH 6.5, catalytic enhancements of 8−13 μA compared to the film-only currents could be reproducibly obtained. These voltammograms were integrated and compared to the integrals of the film-only electrodes to obtain the charge collected from guanine. These calculations showed that 10 ± 2 μC of charge were collected from guanine. Since there were 8 pmol of DNA of a probe containing 20 guanines on the film, the current efficiency was approximately 65% based on a one-electron oxidation of guanine. Thus, although the current enhancement does not appear particularly large in Figure 6A, the absolute amount of charge corresponds to fairly extensive oxidation of the immobilized probe.

The immobilization of DNA to the carboxyl-containing polymer was extremely inefficient at high pH. This result arises probably because the reactivity of *both* the terminal alkylamine and the endogenous nucleobase amines at lower pH simply enhances the absolute extent of DNA attachment. So while it is tempting to assign the lack of a current enhancement in the pH = 9 case to a greater Ru−guanine distance due to a single attachment site, the result is more likely due simply to the lower quantity of attached DNA. In fact, if the current efficiency were the same as that seen for the pH = 6.5 case, the quantity of DNA attached at pH 9 would produce <1 μC of increased charge, which would not be detectable. We cannot, however, exclude the possibility that "stapling" of the DNA at pH 6.5 produces a smaller Ru−guanine distance and hence a more efficient electron-transfer reaction.

Conclusions

We have shown that electrodes modified with polymers of $Ru(vbpy)_3^{2+}$ are active toward oxidation of guanine in solution

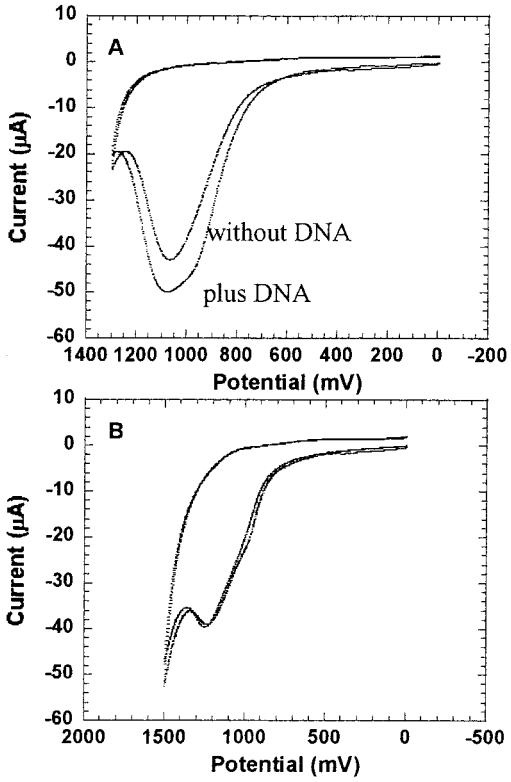


Figure 6. (A) Cyclic voltammograms of a 5:1 poly[$Ru(vbpy)_3^{2+}$/vba] film-modified GCE with 20-mer poly[dG] immobilized at pH = 6.5. (B) Cyclic voltammograms of a 5:1 poly[$Ru(vbpy)_3^{2+}$/vba] film-modified GCE with 20-mer poly[dG] immobilized at pH = 9.0 (50 mV/s scan rate, Ag/AgCl reference, 50 mM pH = 7.0 phosphate buffered solution).

in both a mononucleotide and polymer. Covalent attachment of a guanine-containing oligonucleotide also produces catalytic current with a current efficiency based on one-electron guanine oxidation of 65%. Translation of these promising results to viable sensors for nucleic acids involve two challenges. The first is to identify polymerizable catalysts that are stable in neutral aqueous solution at the potentials needed to realize guanine oxidation. The second challenge is to engineer molecular recognition, presumably through hybridization, into the sensing scheme. We have previously realized that goal with different polymer modifications using probes where inosine is substituted for guanine in the probe strand that is attached to the electrode and therefore produces no catalytic current.[4] When this inosine-modified electrode is hybridized to a guanine-containing target strand, large current enhancements are produced.

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