Journal of Fluorescence, Vol. 9, No. 3, 1999

Electrocatalytic Oxidation of Nucleic Acids at Electrodes Modified with Nylon and Nitrocellulose Membranes

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Received July 31, 1998; accepted December 19, 1998

Electrocatalytic oxidation of guanine in DNA was detected at tin-doped indium oxide electrodes modified with nylon and nitrocellulose polymers. The catalytic oxidation occurs via oxidation at the electrode of the complex $Ru(bpy)_3^{2+}$ to the 3+ state, which is then reduced back to the 2+ state by guanine in DNA (bpy = 2,2′-bipyridine). Catalysis is observed as a current enhancement in the cyclic voltammogram of $Ru(bpy)_3^{2+}$ when DNA is immobilized in the film. As seen in solution, the catalytic enhancement in the nitrocellulose film is lower at 800 m*M* NaCl than without added salt because electrostatic binding of the $Ru(bpy)_3^{2+}$ to the DNA at low salt increases the catalytic rate constant. The cyclic voltammogram of $Os(bpy)_3^{2+}$, which does not oxidize guanine, exhibits less current in the presence of DNA because binding to the immobilized DNA precludes communication of the metal complex with the electrode. Electrodes modified with poly[C] gave no enhancement; however, catalytic current was observed upon hybridization to poly[G]. Exposure of the poly[C] electrode to random single-stranded DNA gave no catalytic current. Glassy carbon electrodes modified with the membranes behaved in a manner similar to that of the metal oxide electrodes.

KEY WORDS: DNA biosensor; electrochemistry; ruthenium complexes; hybridization sensor.

INTRODUCTION

The detection of biomolecular interactions at solid surfaces is a key component of microarray technologies for monitoring of gene expression [1], sequencing of genomic DNA [2], and miniaturized immunoassays [3]. The present methods are dominated by techniques where a fluorescent label is attached to the target biomolecule and brought to the surface upon achieving the desired biomolecular recognition [2]. The advantages of the fluorescent methods are that the label can be attached to the target specifically, spatial resolution can be readily obtained using confocal microscopy, and fluorescent methods are sensitive. Disadvantages of the fluorescent methods are that attachment of the label to the target biomolecule is time-consuming and detection of the label requires relatively expensive instrumentation.

A number of research programs are aimed at replacing present fluorescent labeling methods with strategies that detect biomolecules at surfaces via a property that is native to the unlabeled target species. The most widely adopted of these "biosensor" approaches is surface plasmon resonance [4], which involves the modulation of an evanescent wave upon an increase in molecular weight at an appropriate interface. A similar strategy is that used in the quartz crystal microbalance [5], which senses small mass changes at piezoelectric devices. A recent promising strategy involves monitoring changes in the luminescence of porous silicon as a function of adsorbed biomolecules [3].

In addition to these alternatives, a number of electrochemical strategies have been pursued [6]. One approach

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1053-0509/99/0900-0181$16.00/0

to electrochemical sensing of DNA hybridization involves the use of redox-active indicators with a high selectivity for double-stranded DNA over single-stranded DNA [7–10]. When DNA hybridization takes place at an electrode surface, double-stranded DNA is generated that binds more indicator than before hybridization occurs. This binding causes a preconcentration of the indicator at the electrode surface and increases the electrochemical current due to the increase in local indicator concentration. These strategies have been pursued with indicators based on intercalators [11], groove-binding drugs [9], and metal complexes, such as $Os(bpy)_3^{2+}$ and $Co(bpy)_3^{3+}$ [7,8,10]. Additional electrochemical approaches for DNA detection involve monitoring the direct reductive electrochemistry of DNA bases adsorbed on mercury electrodes and the direct oxidative electrochemistry of DNA bases in carbon paste electrodes [12]. DNA hybridization has been followed electrochemically in redox hydrogels, where the molecular recognition brings horseradish peroxidase to the electrode surface and boosts the catalytic current due to hydrogen peroxide [13]. A more recent strategy involves bringing electrochemically labeled signal probes to the electrode surface upon hybridization of the target sequence [14].

In all of these electrochemical strategies, the biomolecules must be in direct electrical communication with the electrode. In the double-stranded indicator approach, the indicator must be oxidized or reduced by the electrode while bound to double-stranded DNA; thus, the duplex must be in close proximity to the surface. Similarly, the direct electrochemistry of DNA bases requires adsorption of the DNA to mercury or carbon. The redox hydrogel electrodes are "wired" via a redox polymer that facilitates communication of the redox enzyme with the electrode. We report here on an alternative strategy for detecting redox-active DNA bases where the nucleic acid can be immobilized at a greater distance from the electrode under conditions where direct electrochemistry does not occur. Electrons are then shuttled to the electrode using a soluble mediator.

The electrocatalytic oxidation of guanine in DNA can be accomplished using the metal complex $Ru(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine) via an EC′-type mechanism that is shown minimally in Eqs. (1) and (2) [15–17]:

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

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

where DNA_{ox} has been oxidized by one electron at guanine. The rate constant for Eq. (2) can be assessed from current enhancements in cyclic voltammograms of $Ru(bpy)_3^{2+}$ measured in the presence of DNA [15, 17]. The redox potential of guanine is approximately 1.06 V (all potentials vs Ag/AgCl) [18], which is very similar to that of $Ru(bpy)_3^{3+/2+}$ (1.05 V). When an electrode material is chosen that does not adsorb DNA, such as tin-doped indium oxide (ITO), no direct electrochemistry of DNA is observed in the absence of the mediator, because the guanine base is buried in the macromolecular structure [19]. The advantages of this approach for DNA detection are that the DNA can be immobilized in a manner that maximizes stringency and hybridization kinetics without the constraint of keeping the DNA in electrical communication with the electrode. We have discussed such strategies involving track-etched mylar films and self-assembled monolayers [20–22]. Here we describe a simple approach based on attaching nylon and nitrocellulose membranes to ITO or carbon electrodes.

EXPERIMENTAL

Nitrocellulose–ITO Electrodes. Nitrocellulose paper (Zeta-Probe, Bio-Rad; 80–100 μg/cm^2) was cut into a 5-mm-diameter circle that fit into the electrochemical cell and covered the portion of the ITO electrode exposed to the solution. This procedure was similar to that used for mylar membranes described elsewhere [20]. For all of the experiments with ITO, the electrodes were conditioned with buffer prior to analysis with metal complexes and nucleic acids as described elsewhere [20,21]. Prior to analysis, the membranes were equilibrated with the solution of the metal complex electrochemical analysis for 6 min with $Os(bpy)_3^{2+}$ and 15 min with $Ru(bpy)_3^{2+}$.

The nitrocellulose disks were loaded with DNA by soaking for approximately 5 min in a solution of 5.8 m*M* calf thymus DNA dissolved in water. A variety of soak times was investigated, ranging from 5 min to 18 h. The nitrocellulose paper became saturated with DNA rapidly (within minutes), so short soak times were used for these experiments. The DNA-soaked nitrocellulose disk was inserted into the electrochemical cell, and 200 μl of a 200 μ*M* metal complex solution in the appropriate buffer was pipetted into the cell. Under low-salt conditions, a 50 m*M* Na-phosphate buffer (pH 6.8, $[Na^+]$ = 80 m*M*) was used for the analysis. Under high-salt conditions, a 50 m*M* Na-phosphate buffer and 700 m*M* NaCl (pH 6.8, $[Na^+]$ = 780 m*M*) solution was used. Detection of RNA was identical except that baker's yeast tRNA (Sigma) was used in place of calf thymus DNA. Cyclic voltammograms were collected using an PAR 273A potentiostat at a scan rate of 25 mV/s.

Nylon–ITO Electrodes. Nylon membranes (Hybond N+, Amersham; 480–600 μg/cm^2) were cut into circular shapes, approximately 6 mm in diameter. The nylon disks were soaked for 1 h in a concentrated solution of polycytidylic acid (Sigma) in water. The disks were then removed from the polycytidylic acid (poly[dC]) solution, placed on parafilm, and allowed to dry. As the disks were drying, an additional 15 μl of poly[dC] solution was added to the films in three 5-μl aliquots. The disks were allowed to dry completely. The dried nylon disks were then washed in the low-salt buffer (50 m*M* Na-phosphate, pH 6.8, [Na$^+$] = 80 m*M*) to remove any weakly bound poly[C]. The electrodes that were not hybridized (Figs. 4A and 5A) were subjected to a mock hybridization procedure in which they were not exposed to any additional nucleic acid but were exposed to all other hybridization steps. The disks were then placed in 400 μl of Milli-Q water, heated at 48°C for 1 h, and allowed to cool to room temperature. The disk was removed from the water and washed in low-salt buffer prior to electrochemical analysis. The hybridized electrodes (Figs. 4B and 5B) were treated identically except that a concentrated solution of poly[G] (Sigma) or denatured calf thymus DNA was used. Calf thymus DNA (Sigma) in water was denatured by heating to 90°C for 10 min. As expected, nylon membranes that were not treated with poly[C] adsorbed calf thymus DNA strongly.

Nylon–Glassy Carbon Electrodes. The nylon disks were attached to the glassy carbon electrode surface and held in position by a plastic sleeve. All conditions were the same as for the nitrocellulose-modified ITO electrodes with calf thymus DNA as described above.

RESULTS AND DISCUSSION

Nitrocellulose-Modified ITO Electrodes. The ITO electrodes were modified with filter paper by mechanically affixing a nitrocellulose (0.2-μm) membrane as described previously [20]. The long-dashed line in Fig. 1 shows the cyclic voltammogram of Ru(bpy)$_3^{2+}$ that has been equilibrated into the nitrocellulose membrane. The appearance of the voltammogram is very similar to that of Ru(bpy)$_3^{2+}$ in solution, indicating that the metal complex diffuses freely in the membrane and still undergoes relatively efficient heterogeneous charge transfer with the ITO electrode. The peak-to-peak splitting and absolute current are within experimental error of the same parameters observed in solution.

We next sought to determine whether the complex is able to oxidize DNA that has been immobilized in the film. Without Ru(bpy)$_3^{2+}$, cyclic voltammograms of nitrocellulose-modified electrodes to which DNA was immobilized gave no oxidation current attributable to guanine oxidation and were identical to those without DNA, as we have observed in the case of the mylar films [20]. The dashed and solid lines in Fig. 1 show the voltammograms obtained when the nitrocellulose membrane was soaked in calf thymus DNA and equilibrated with Ru(bpy)$_3^{2+}$ prior to electrochemical analysis. There is a large catalytic current for the DNA-labeled membrane that parallels that seen in solution. Since DNA adsorbed to nitrocellulose membranes is immobilized, this experiment demonstrates that diffusion of the DNA is not required to realize the catalytic current. Further, greater catalytic current is observed at lower salt concentrations. This observation parallels that in solution and arises from a greater rate constant for guanine oxidation by Ru(bpy)$_3^{3+}$ [15]. The rate constant is higher because of the increase in the local concentration of the oxidant on the DNA due to electrostatic condensation of the cationic oxidant on the DNA polyanion. The increase in current due to decreased salt concentration in Fig. 1 is quantitatively similar to that seen in solution.

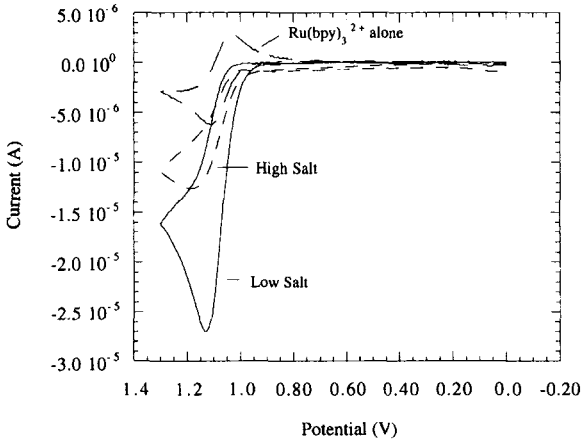

Fig. 1. Cyclic voltammograms at nitrocellulose-modified ITO electrodes showing cyclic voltammograms of Ru(bpy)$_3^{2+}$ (200 μ*M*) at buffer-soaked nitrocellulose, Ru(bpy)$_3^{2+}$ (200 μ*M*) at DNA-soaked nitrocellulose in high-salt (700 m*M* NaCl added) buffer, and Ru(bpy)$_3^{2+}$ (200 μ*M*) at DNA-soaked nitrocellulose in low-salt (no NaCl added) buffer.

An alternative explanation for the catalytic enhancement observed in Fig. 1 is that binding of Ru(bpy)$_3^{2+}$ to DNA causes a preconcentration of the metal complex at the electrode, which increases the current. This effect is responsible for the increased current observed with Co and Os complexes at electrodes with covalently attached DNA [7,8,10]. Such an origin for the catalytic enhancement is unlikely in Fig. 1 for a number of reasons. First, there is no rereduction wave upon switching the potential,

which is consistent with a failure to accumulate Ru(III) at the electrode because of the electrocatalytic reaction [Eq. (2)]. Sensors that operate by the preconcentration effect show current enhancement in both the forward and the reverse waves. Second, current enhancement is observed at 700 m*M* added NaCl, where DNA binding by $Ru(bpy)_3^{2+}$ is negligible [17]. Third, cyclic voltammograms of the DNA-modified electrode showed no faradaic current due to DNA; if the electrode cannot communicate with guanine directly, then it is difficult to envision how DNA-bound metal complex could be oxidized. Fourth, electrodes modified with nucleic acids that do not contain guanine do not give current enhancements (see below).

We sought to rule out definitively a preconcentration effect by investigating the electrochemistry of $Os(bpy)_3^{2+}$ at the DNA-modified electrode. The osmium complex does not oxidize guanine [23,24], so any current enhancement observed in the presence of DNA would have to arise from preconcentration of the mediator due to DNA binding. Figure 2A shows the cyclic voltammograms of nitrocellulose electrodes equilibrated with $Os(bpy)_3^{2+}$ with and without immobilized DNA. As shown, the current for $Os(bpy)_3^{2+}$ is *lower* in the presence of DNA at the nitrocellulose electrode than in the absence of DNA. This experiment shows that the increased current when DNA is bound to the nitrocellulose electrode is due solely to the catalytic reaction [Eq. (2)] and not due to a binding difference. Further, the effect of salt is small (Fig. 2B) compared to the large salt effect observed for the catalytic reaction.

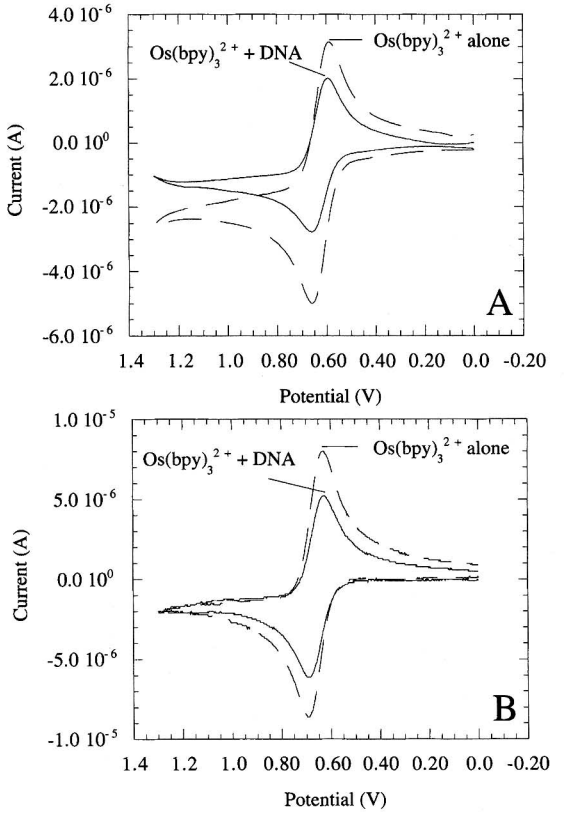

Fig. 2. Cyclic voltammograms of $Os(bpy)_3^{2+}$ (200 μM) at nitrocellulose-modified ITO electrodes soaked in buffer or in DNA: (A) 700 m*M* NaCl added; (B) no NaCl added.

The nitrocellulose electrodes were modified with tRNA, which also contains guanine, and interrogated by cyclic voltammetry in the presence of $Ru(bpy)_3^{2+}$ (Fig. 3). As with DNA, catalytic current is observed under both high- and low-salt conditions. The effect of salt is less than in Fig. 1 because cations exhibit a much lower affinity for the heterogeneous tRNA structure than for the regular anionic lattice of the double-stranded DNA used in Figs. 1 and 2 [25].

Nylon-Modified ITO Electrodes. Although nitrocellulose membranes have been used frequently for nucleic acid studies [26], nylon membranes are often preferred because they bind nucleic acids with both higher affinity and selectivity. We used nylon-modified ITO electrodes to show that hybridization can also be detected using the membrane electrodes. Figure 4A shows the cyclic voltammogram of $Ru(bpy)_3^{2+}$ at a Hybond N+ nylon electrode to which poly[C] was immobilized. When the membrane was immersed in a solution of poly[G] and subjected to the hybridization protocol, current enhancement was obtained due to catalytic oxidation of the hybridized poly[G] target (Fig. 4B). The assay is specific for the appropriate sequence as shown in Fig. 5, which

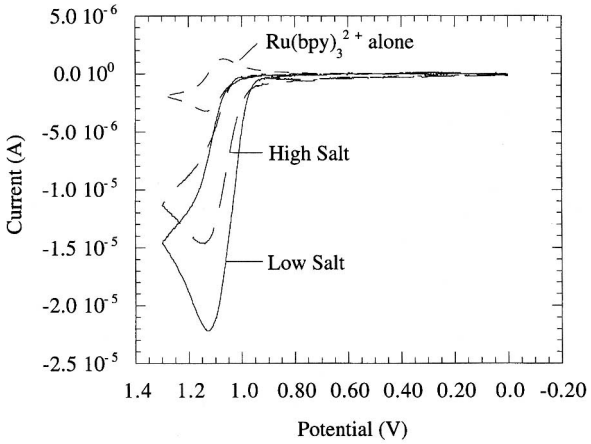

Fig. 3. Cyclic voltammograms at nitrocellulose-modified ITO electrodes showing cyclic voltammograms of $Ru(bpy)_3^{2+}$ (200 μM) at buffer-soaked nitrocellulose, $Ru(bpy)_3^{2+}$ (200 μM) at tRNA-soaked nitrocellulose in high-salt (700 m*M* NaCl added) buffer, and $Ru(bpy)_3^{2+}$ (200 μM) at tRNA-soaked nitrocellulose in low-salt (no NaCl added) buffer.

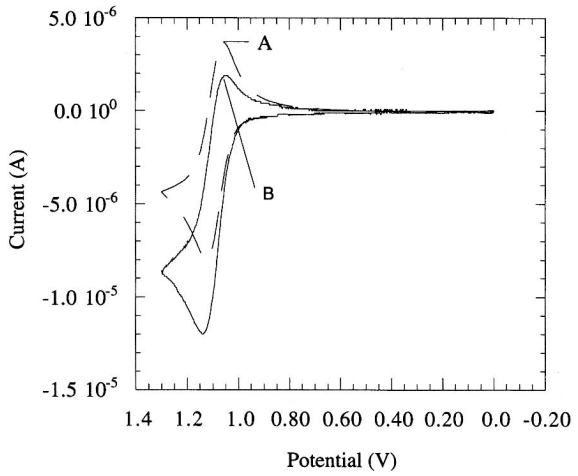

Fig. 4. Cyclic voltammograms of 200 μl of $Ru(bpy)_3^{2+}$ at ITO working electrodes to which a Hybond N+ nylon membrane was attached. Membranes were impregnated with poly[dC] and subjected to the hybridization protocol in (A) buffer and (B) a concentrated solution of poly[G].

compares the voltammetry for the poly[dC] membrane where the hybridization procedure was carried out in buffer (Fig. 5A) or in a solution of single-stranded calf thymus DNA (Fig. 5B). As shown, no catalytic current was observed with single-stranded DNA that did not contain the complementary poly[G] strand.

Glassy Carbon Electrodes. The working electrode in all of the foregoing examples was tin-doped indium oxide (ITO). This material was chosen because it does not adsorb DNA, which must be avoided when the target DNA is in solution, as we have discussed in detail elsewhere [16]. However, we suspected that when the DNA was adsorbed to the nylon film, other electrode materials would now allow observation of the catalytic current, as long as DNA binds to the film more strongly than to the electrode. Figure 6 shows the cyclic voltammogram of a glassy carbon electrode with a nylon film attached before (Fig. 6A) and after (Fig. 6B) immobilization of DNA on the nylon film. The catalytic enhancement is similar to that observed at the same salt concentration for the ITO electrodes, showing that a greater range of electrode materials is likely compatible with detecting catalytic currents at the membrane-modified electrodes compared to solution.

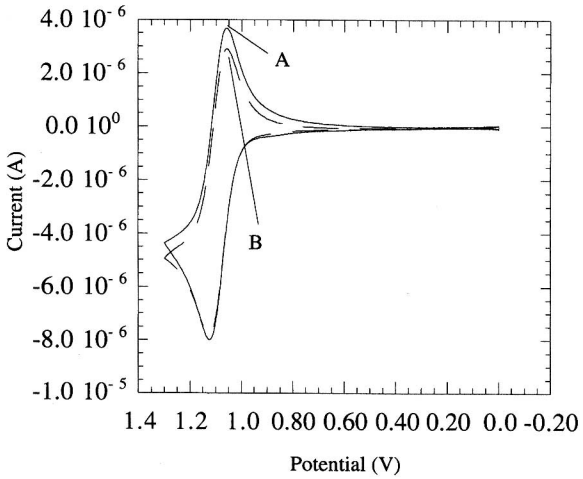

Fig. 5. Cyclic voltammograms of 200 μl of $Ru(bpy)_3^{2+}$ at ITO working electrodes to which a Hybond N+ nylon membrane was attached. Membranes were impregnated with poly[dC] and subjected to the hybridization protocol in (A) buffer and (B) a concentrated solution of denatured calf thymus DNA.

CONCLUSIONS

The voltammetric data show that the catalytic oxidation of guanine in DNA can be detected in nylon and nitrocellulose films and gives results similar to those in solution. These findings demonstrate that the DNA does not need to diffuse to achieve the catalytic reaction and that the biomolecule can be adsorbed in a manner where properties such as the response to cation concentration are similar to the native form. These electrodes join a growing family of interfaces [20,21] where DNA can be immobilized without direct electrical connection to the electrode but where catalytic oxidation of guanine in the biomolecule can be realized with small inorganic catalysts. Nylon membranes have been used to develop novel assays for both gene expression analysis [27] and large-scale sequencing by hybridization [28]. The combination of these approaches with the nonradioactive

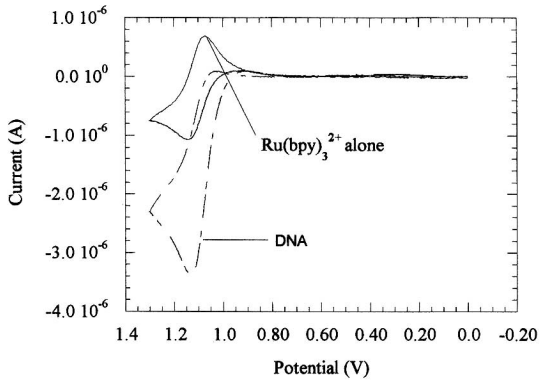

Fig. 6. Cyclic voltammograms (scan rate = 25 mV/s) of 200 μ*M* $Ru(bpy)_3^{2+}$ at a nylon-modified glassy carbon electrode (A) with no DNA or (B) after adsorption of DNA to the nylon film.

approach described here, which requires no labeling, may offer particularly simple direct sensors for sequencing and expression analysis. Independent quantitation of radiolabeled DNA on the nylon electrodes suggests sensitivities in the femtomole per square centimeter range, which is suitable for many of these applications.

ACKNOWLEDGMENTS

This research was supported by the David and Lucile Packard Foundation and Xanthon, Inc. The authors thank Dr. C. R. Loomis for many enlightening discussions concerning these studies and Dr. Carole Golden for insightful comments on the manuscript.

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