Electrochemical DNA Hybridization Sensor Using Poly[viny1pyridine Os(bipyridine)2Cl]-co-Ethylamine Redox Polymer

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Abstract
DNA hybridization sensors are important for diseases diagnosis. Until now, many approaches have been proposed for the DNA hybridization examination. These include fluorescence based, electrochemical and colorimetric based. Electrochemical hybridization for detection of DNA sequences requires the development of easy to-use, fast, inexpensive, miniaturized analytical devices. In this work, a simple electrochemical DNA hybridization sensor that uses poly[viny1pyridine Os(bipyridine)2Cl]-co-ethylamine redox polymer as the indicator was assembled. The electrochemical behavior of poly[viny1pyridine Os(bipyridine)2Cl]-co-ethylamine redox polymer polymer with both single stranded DNA (ssDNA-SH) probe and double stranded DNA (dsDNA) was determined. Both cyclic voltammetry and impedance spectroscopy results indicated strong binding of the new redox polymer onto DNA. A hybridization process involving a monolayer of a 24-mer thiol-tethered DNA capture probe on gold electrode and the corresponding complementary strand were clearly demonstrated. DNA double strand after disruption with free radicals could also be detected. The biosensor system has potential for use to recognize of damaged DNA sequences.

Keywords: Cyclic voltammetry; Hybridization; Poly[viny1pyridine Os(bipyridine)2Cl]-co-ethylamine.

1. Introduction
Biological analysis is integral in biomedical research, involving medical diagnostics, forensic analysis among other areas. Development of simple chip-based DNA sensors capable of sensitive and selective detection of DNA sequences will have a great impact in clinical diagnosis (Sassolas, et al., 2007; Wang, et al., 2011; Zhi, et al., 2012). Due to the low-abundant DNA biomarkers linked to the diseases in biological samples, it is desirable to enhance the sensitivity and specificity of methods for DNA detection (Kong, et al., 2010) Fabrication of DNA microarrays or DNA chips has enabled parallel detection of DNA sequences (Pheeney, et al., 2013). In particular, DNA microarrays allow studies involving a large number of genes to be conducted. Many gene-specific sequences immobilized on platforms such as glass or other conductive material can be interrogated using labeled biological samples (Zheng and He, 2009). Detection of DNA sequences relies on the formation of Watson–Crick duplexes between immobilized strands (probes) and the target strands in solution (Lubin and Plaxco, 2010). Electrochemical detection platforms for DNA hybridization detection have been examined recently and are finding favor due to low cost as opposed to fluorescence-based optical readouts (Fan, et al., 2010; Liu, et al., 2008). DNA electrochemical biosensor discriminate hybridized, double-stranded (ds-) DNA from single-stranded (Steichen, et al.) using exogenous, redox-active hybridization indicators (double-helix intercalators) (Liu, et al., 2013; Mugweru and Rusling, 2001; Shamsi and Kraatz, 2013). Intercalation of the indicator with DNA strands is simple and convenient for detection method but can also suffer from background signals associated with nonspecific binding of intercalators due to unhybridized ssDNA. Intercalation or electrostatic interaction of indicator molecules to the target DNA strands affects the net charge on the DNA modified surface (Laopa, et al., 2013; Sun, et al., 2007). In order to detect a hybridization event, proper selection of probe is very important in order to get a good current contrast between probe and hybridized events (Turcu, et al., 2004). For proper hybridization event, the probe and target strands should be of equal lengths to avoid overhangs either in the probe or target strand (Wang, et al., 2013). Most common electrochemical DNA sensors, utilize methylene blue or ferrocene as indicators (Baker, et al., 2006; Kang, et al., 2009; Lai, et al., 2006; Xu, et al., 2013). The electrochemical hybridization detection based on these redox indicators present different affinities to both dsDNA and ssDNA. Other redox probes such as [Ru(NH3)6]3+ (Wang, et al., 2013; Zhang, et al., 2006) use electrostatic interaction while ferrocenium (Fe3+), form metal chelates (Ju, et al., 2004). Non specific interaction using these indicators can sometimes generate high background signals during hybridization. These redox active indicators produce the expected electrochemical signals after DNA-intercalation. Some indicators are also known to bind at σ-enantiomer and minor groove of the complementary Λ-enantiomer (Liu, et al., 2006). Groove binding is a specific interaction that only occurs for dsDNA as compared to electrostatic indicators. Groove binding has an advantage of higher
The recognition ability of dsDNA (Ihara, et al., 1996). The combination of chemical stability, redox properties, and sensitivity of redox indicators is essential for future DNA hybridization sensors. Polymeric materials are novel materials with good electrical properties for label-free biosensing applications (Hu, et al., 2013; Zheng and He, 2009). In the present work, DNA hybridization sensor utilizing poly[4-vinylpyridine Os(bipyridine)Cl]-co-ethylamine (osmium based redox polymer) was evaluated as an indicator in DNA hybridization assay. This indicator showed clear signals when a 24 base pair oligonucleotide was coupled to its corresponding complementary strand.

2. Experimental

2.1. Chemicals Reagents and Instruments

Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), ammonium acetate, ethyl alcohol, poly(4-vinyl) pyridine and poly(ethylene glycol) diacrylate (MW 575), were purchased from Sigma-Aldrich (St Louis, MO, USA). Hexafluorophosphate, sodium dithionite, ether, \(N,N\)-dimethylformamide, and hydrochloric acid were from VWR. Ammonium hexachloroosmate (IV) and 2-bromoethylamine hydrobromide, were from Alfa Aesar. 2,2'-Bipyridyl (bpy) and \([Fe(CN)_{6}]^{3-}\) and \([Fe(CN)_{6}]^{4-}\) were obtained from VWR. The stock solution of the oligonucleotides (4 mM) was prepared with PBS buffer solution and kept frozen at negative 20 °C. In this study, DNA oligonucleotides of the following sequence 5'TC TTATCATCCATGAGTTTTCTCT-SH) and 5'AGAATAGTAGTGATCTCAAGAGA-3' were obtained from integrated DNA technologies (IDT).

Electrochemical measurements were carried out with a computer-controlled electrochemical workstation (CHI 660c, USA) with ohmic drop 98% compensated. A three electrode cell was employed with a Ag/AgCl electrode as a reference electrode, gold electrode working electrode and Pt wire as counter electrode all from Bioanalytical Systems Inc. Phosphate buffer had a pH of 7.0 and a concentration of 20 mM. The polycationic redox polymer, poly[4-vinylpyridine Os(bipyridine)Cl]-co-ethylamine (noted as Osmium based redox polymer) was synthesized according to a procedure described previously with some modification (Rajagopalan, et al., 1996). Briefly, Os(bpy)Cl was prepared by mixing two equivalents of bipyridine with one equivalent of ammonium hexa chloro osmate in 50 mL of ethylene glycol. This was heated to reflux for one hour and precipitated using saturated sodium dithionite.

The oxidation with dithionate was essential in this polymer. The precipitate was filtered and washed repeatedly with water and ether. To prepare the redox polymer, 1:3 ratio of Os(bpy)Cl to poly(4-vinyl-pyridine) were refluxed under nitrogen atmosphere in approximately 50 mL of ethylene glycol for 2.5 h. The solution was cooled to room temperature followed by the addition of 100 mL of DMF and 2-bromoethylamine hydrobromide. The solution was left stirring overnight at room temperature. The crude polymer was precipitated by pouring the solution into rapidly stirred acetone. The hygroscopic precipitate was collected and dissolved in water. This was then filtered and dried under vacuum.

Figure 1. Redox polymer synthesized: The molecule contains osmium redox polymer backbone (where \(n = 1, m = 4, p = 1.2\)).

Oligonucleotide Preparation

Approximately 400 µL of 3% TCEP solution and 20 µL of 0.4 mM ssDNA/SH solution were placed in a vial and mixed thoroughly for about one hour on rocker. About 150 µL of 9.5 M ammonium acetate and 1.5 mL of ethyl alcohol were added to the solution. The mixture was placed in an negative 20 °C freezer for 20 min and the solution later spun in a micro-centrifuge at 13,000 rpm for 15 min. The supernatant solution was poured out and the rest of the sample allowed dried in a speed vacuum at 47 °C. The dried oligonucleotide was re-dissolved in 30 µL of phosphate buffer pH 7. The DNA sample solution was used in the next set of experiments.

2.3. ssDNA Probe Immobilization on Gold Surface

A clean gold electrode (area, 0.02 cm²) was immersed in the thiolated single-stranded DNA probe (5'TCTTATCATCCATGAGTTTTCTCT-SH) for at least one hour in solution prepared using the procedure above. The modified electrode was rinsed with distilled water to remove any unbound DNA. The electrode after immobilization with ssDNA was placed in Osmium based redox polymer for about one hour. The electrode was then rinsed with distilled water to remove any polymer not bound onto the electrode. The electrode was then placed in fresh phosphate buffer of pH 7 and was characterized using cyclic voltammetry and impedance.
spectroscopy.

2.4. Hybridization and Hydroxyl Radical Reaction

A gold electrode functionalized with ssDNA using the procedure above was placed in a solution containing a complementary ss-DNA (5’AGAATAGTAGCTAAGAGA-3’). The hybridization reaction was carried out at 37 °C using a water bath. After about one hour, the electrode was removed and rinsed with distilled water to remove any unhybridized DNA. The electrode was then placed in the osmium based redox polymer for one hour. The electrode was rinsed in distilled water before any electrochemical analysis.

Some ds-DNA/Au modified electrodes were incubated in Fenton reagent to induce damage on the hybridized DNA strand. The ds-DNA, and damage ds-DNA modified electrodes were incubated in osmium polymer solution for at least one hour prior to cyclic voltammetry characterization.

3. Results and discussions

3.1. Electrochemical Impedance Spectroscopy

The construction of the DNA biosensor based on DNA-mediated redox polymers as indicators is a good strategy that can enable detection of DNA lesions without using the PCR techniques. In the strategy, DNA can be covalently attached to the indicators. Although covalent attachment has some advantages, it is not possible to detect multiple DNA mismatches at the same time. Here in, hybridization of the ssDNA probe was first investigated using electrochemical impedance spectroscopy (EIS). EIS is a technique, though developed some time back (Metters, et al., 2011; Randviir and Banks, 2013) has recently been gaining attention in electrochemical research, especially in the field of biosensor development. EIS was used in this work to mainly characterize electrodes surfaces modified with ss-DNA and ds DNA. Conducting surfaces including surfaces modified with ssDNA or dsDNA change the double layer capacitance as well as the electron transfer resistance on the electrode. The changes introduced through the surface modification can be monitored by using electrochemical impedance spectroscopy (EIS). (Erdem, et al., 2012) The Electrochemical impedance studies were carried out in 10 mM Fe(CN)\textsubscript{6}\textsuperscript{3−}/Fe(CN)\textsubscript{6}\textsuperscript{4−} in 20 mM phosphate buffer solution. Figure 2 shows the Nyquist plot (Z' versus Z") gold electrode functionalized with ds-DNA and ss DNA. The Randles equivalent circuit model used to fit the data is also shown with Rs being the electrolyte resistance, R\textsubscript{ct} is the charge resistance, C\textsubscript{dl} is the double layer resistance and Z\textsubscript{w} is the Warburg impedance. The semicircle diameter at higher frequencies in the Nyquist diagram reflects the interfacial electron transfer resistance (Rct) which controls the electron transfer kinetics of K\textsubscript{3}[Fe(CN)\textsubscript{6}]/K\textsubscript{4}[Fe(CN)\textsubscript{6}] at the electrode surface. The Nyquist plot obtained by the bare gold electrode has a nearly straight line (data not shown), which is characteristic of a diffusion-limiting process. It can be seen that EIS of the gold functionalized with ss-DNA (figure red) composed of a large semicircle and a straight line featuring a diffusion limiting step of the K\textsubscript{3}[Fe(CN)\textsubscript{6}]/K\textsubscript{4}[Fe(CN)\textsubscript{6}] processes. After the hybridization step (black), the gold electrode (dsDNA/Au) yields larger diameter indicating the hybridization step was successive. The large semicircle indicates that the charge transfer kinetics is slower on the dsDNA/Au electrode compared to that of the ssDNA/Au electrode as would be expected during hybridization step.

![Figure 2. Nyquist plots in 10mM [Fe(CN)\textsubscript{6}\textsuperscript{3−}] and [Fe(CN)\textsubscript{6}\textsuperscript{4−}] of (a) thiolated ssDNA on gold electrode (b) thiolated dsDNA (after hybridization).](image)

3.2 Electrochemical Characterization of ssDNA Functionalized Electrodes

Electroactive indicators including intercalators have been extensively used for DNA hybridization and labeling. Osmium redox polymers have been exploited in our group for bio-sensing applications owing to their efficient electron shuttling properties combined with the polymeric structure promoting stable adsorption (Amos Mugweru, 2007; Havens, et al., 2010). In the present study, the osmium based polymer was used as an indicator.
in DNA hybridization biosensor fabrication. The redox polymer (about 2mg/mL) was dissolved in 20 mM phosphate buffer of pH 7. This concentration was previously determined as the optimal concentration to generate a significant signal for the hybridization detection. Figure 3(a) shows a cyclic voltammogram obtained using a gold functionalized with ssDNA strand (5'TCTTATCATCCATGAGTTTTCTCT-SH) after adsorbing osmium based polymer indicator.

A pair of redox peaks ascribed to the redox couple of Os(Nath, et al.)/Os(II) ions in the redox polymer moiety appeared at 0.3 V versus Ag/AgCl reference electrode. The polymer film exhibited a high electroactivity in phosphate buffer media with an almost reversible oxidation at 0.3V vs. Ag/AgCl. Figure 3(b) shows a plot of peak current as a function of the scan rate. A linear dependence of peak current of with scan rate was observed. From the voltammograms shown in Figure 3(a), we can deduce that the osmium based redox polymer as an indicator adsorbed quite well onto the ssDNA. The polymer itself has a large hydrophobic backbone that could serve as an anchor for DNA interaction. In this particular experiment, the voltammetric responses of this complex assembly were recorded in phosphate buffer pH 7.0. The anodic (Ia) and cathodic (Ic) peak current responses were equal indicating reversibility. The stability exhibited with this system indicated stronger forces of attraction than merely physical adsorption. The redox polymer itself is positively charged which could play a role in enhancing the stability. A plot of peak current as a function of the scan rate had a linear equation (I(μA) = 0.011 × scan rate + 0.328 mV s⁻¹) with a correlation factor R² = 0.999. It was clear the redox process was non-diffusional and the electroactive polymer adhered quite well to the ssDNA functionalize working electrode surface.

![Cyclic voltammograms of ssDNA/SH after immobilization on Au electrode at increasing scan rate of in 0.2 M phosphate buffer pH 7.](image)

Figure 3. (a) Cyclic voltammograms of ssDNA/SH after immobilization on Au electrode at increasing scan rate of in 0.2 M phosphate buffer pH 7. (b) Linear Plot of ssDNA/SH reduction peak current vs. scan rate after immobilization on Au electrode.

3.3. Target DNA Detection Using Osmium Redox Polymer
A label-free electrochemical detection method based exclusively on the specific signal of hybridized DNA is essential. The analytical performance of the present DNA hybridization detection system was tested after one hour of incubation with target DNA strand (5’AGAATAGTAGGTACTCAAGAGA-3’). The redox polymer can also bind to the hybridized DNA on electrode through electrostatic interactions. The Osmium redox polymer is positively charged as indicated earlier (see the structure of the polymer, Figure 1). The polymer also contains a large organic backbone that can also interact with the DNA as well as groups that can be intercalated onto the hybridized DNA.

Figure 4(a) shows the cyclic voltammograms of a gold electrode with a duplex DNA after hybridization step. The peak current increases of ds DNA also increases with scan rate. The only difference between ds DNA and ssDNA using the Osmium redox polymer is that the peak currents are much higher than those of ssDNA. A plot of peak current as a function of the scan rate had a linear equation (I(μA) = 0.015 × scan rate + 0.088 mV s⁻¹) with a correlation factor R² = 0.998. Again, we observe a non-diffusional process. As observed with ssDNA the Osmium redox polymer adhered firmly onto dsDNA functionalize working electrode surface. Figure 5 shows cyclic voltammogramms of bare gold electrode, gold electrode functionalized with ss-DNA probe, gold electrode with ds-DNA after hybridization experiment. It was observed that the peak currents of electrode functionalized with ss-DNA probe increased after the hybridization experiment. It is likely that the increase in peak current results from the redox polymer being less concentrated on ssDNA/Au than on dsDNA/Au surface. This is because the double helix structure is absent in ssDNA, and accordingly the groove site that usually accommodates some the redox polymer. We also observe a shift to positive potentials from about 0.3 V to about 0.35 V vs. Ag/AgCl.

The shift in peak potential to more positive potential may imply a hydrophobic groove binding mode between the redox polymer and the dsDNA (Millan and Mikkelsen, 1993). We believe the cationic transition metal complex bind to the DNA using electrostatic interactions with the polyanionic backbone of the immobilized dsDNA. The signal characteristic of adsorbed redox polymer has been used to quantify immobilized ssDNA on the surface and to detect dsDNA after hybridization. The fact that the signal is obtained in fresh buffer allows differentiation between the contributions of adsorbed species and that of species diffusing to the electrode. Hybridization step resulted in an increase in peak current, implying the ssDNA molecules used as capture probes formed duplex on the biosensor surface. These results clearly indicate that the new electroactive redox polymer is an excellent electrochemical indicator that differentiates dsDNA from ssDNA.
**Figure 4.** (a) Cyclic voltammograms of dsDNA obtained after hybridization step at increasing scan rate of 10, 20, 30, 40, 50, 60, and 70 mV/s in 0.2M phosphate buffer pH 7 using Osmium based redox polymer. (b) Linear Plot of peak current vs. scan rate for dsDNA probe after hybridization using Osmium based redox polymer.

**Figure 5.** Shows the cyclic voltammograms of bare Au electrode, ssDNA-SH/Au and dsDNA/Au modified electrodes.

Oxidation and reduction of the polymer on the electrode surface may affect the association of both the dsDNA and ssDNA to the redox polymer. Using the redox potentials observed, we can estimate the relative binding constants of redox polymer and the dsDNA and ssDNA. The ratio of the equilibrium constant between the oxidized and the reduced forms (K_red/K_Ox) when bound to the both dsDNA and ssDNA can be estimated according to the equation below.

\[
E_b - E_f = \frac{RT}{nF} \ln \frac{K_{red}}{K_{Ox}}
\]  

where \(E_b\) is the formal potentials of the Osmium polymer redox couple in the DNA free form while \(E_f\) is the formal potentials of the osmium polymer redox couple in the DNA bound form. \(K_{red}\) and \(K_{Ox}\) are the corresponding binding constants for the reduction and oxidation species to DNA. In our case the \((E_b - E_f)\) is approximately 30 mV for ssDNA and this translates to about a binding constant of 1.2 while for dsDNA with \((E_b - E_f)\) has a calculated binding constant of about 1.5. The result suggests that binding affinity of the redox polymer to the dsDNA is much stronger than the redox polymer and ssDNA. This is could be attributed to
presence of groove in ds DNA providing another binding pocket to the osmium redox polymer (Selvi and Palaniandavar, 2002). There are other factors that are known to influence the overall binding of the redox polymers to DNA. These include the variation in the geometry, size and hydrophobicity of the ligands and their substituent’s. Figure 6 shows the pictoriarl representation of the DNA biosensor for hybridization detection.

Figure 6. Pictorial representation of DNA biosensor.

3.4. Response Characteristics of the DNA Biosensor on Hydroxyl Radical Damaged DNA

Many electrochemical studies involving detection of single base mismatch are being pursued. A single base mismatch can disturbs the p-stack of DNA complementary strand. We have previously carried out research aimed at unraveling the extent of DNA damage by reactive oxygen species (Mugweru and Rusling, 2006; Mugweru, et al., 2004). Reactive oxygen species (ROS), in particular hydroxyl radicals are the most reactive and frequently known to be directly responsible for DNA oxidative damage. In this work, we induced damage to immobilized standard DNA on a gold electrode by the OH• radicals and detected DNA damage using our new osmium based redox polymer. Fenton reagent was used to generate hydroxyl radicals that reacted with DNA on the gold electrode. 4 mM H$_2$O$_2$ and 1 mM Fe$^{2+}$ concentrations for Fenton’s reagent were previously established as optimal conditions for dsDNA incubation. The gold surface with immobilized dsDNA, was exposed to FeSO$_4$ containing hydrogen peroxide for 3 min and later rinsed with distilled water. Hydroxyl radicals are most often created in living organisms by hydrogen peroxide reaction with metals and can be easily realized in vitro by mixing hydrogen peroxide and bivalent iron in acidic medium. The Fe$^{3+}$ ions in this solution react with H$_2$O$_2$ to generate·OH radical according to Equation (2). Figure 7 shows cyclic voltammograms of a gold surface with immobilized dsDNA before incubation with Fenton’s reagent. DNA after incubation in Fenton’s reagent shows a slight shift in peak potential, from 0.3 V to 0.24 V vs. Ag/AgCl electrode. Another smaller peak is also visible at about 0.1 V. The peak current also slightly increases probably due to exposure of some of the redox polymer to the electrode after dsDNA is damaged. Cyclic voltammograms of the Damaged DNA after adsoring Osmium redox polymer clearly show dsDNA modification.

\[ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^- \] (2)

Figure 7. Cyclic voltammogram of dsDNA/Au electrode before and after free radical damage using osmium based polymer as the indicator.
DNA oxidative damage results in modifications observed under natural growth conditions similar to those induced by exposure to peroxides. Hydroxyl radical (OH\textsuperscript{•}) is an aggressive radical which can damage the DNA molecules. OH\textsuperscript{•} can attack every part of DNA (deoxyribose, purine and pyrimidine bases) and modify their structure. The initiation of free radical damage may lead to formation of new free radicals that propagate further damage. The cyclic voltammogram in figure 7 indicates presence of multiple binding locations as a result of interference of the helical structure by the free radicals compromising the integrity of the double helix structure. The chemical damage to dsDNA can result to separation of parts of DNA double strand. Various electrochemical methods with great sensitivity and specificity have previously been used to characterize the nature of OH\textsuperscript{•} interaction with DNA (Zhang, et al., 2008). Interaction mechanism of OH\textsuperscript{•} radicals with dsDNA has been investigated by several authors (Abolfath, et al., 2011). Here we observe that our new osmium redox polymer indicator can be used to distinguish intact dsDNA duplex from other DNA that has been damaged by hydroxyl radicals.

Most hybridization indicators currently used such as ferrocenium ion show about 25% signal change from dsDNA to ssDNA (Ribeiro Teles, et al., 2007). In our new polymer the signal is slightly higher at about 35 which is a great improvement from current literature methods. We are expanding the scope of this work and explore possibility of single base mismatch detection. The new osmium based redox polymer can be used as an efficient DNA hybridization indicator. This new indicator has its redox potential at 0.3 V vs. Ag/AgCl electrode and only small amount of polymer is required. The binding constants of the redox polymer to dsDNA are much higher than that of ssDNA.

4. Conclusion
Ox(bipyridine)\textsubscript{4}Cl\textsubscript{2}]-co-ethylamine redox polymer was evaluated as an indicator for construction of a simple DNA hybridization detection method. The voltammetric response of adsorbed redox polymer on gold functionalized with ssDNA indicated that polymer firmly binds to the DNA. The dsDNA after hybridization shows huge peak current increase due to both electrostatic interaction and intercalation of the redox polymer groups. The approach does not require the labeling of any nucleic acids probes or targets prior to the analysis, making the method advantageous in terms of speed and low costs. The sensor probe has shown to be an effective tool for potential use to detect DNA damage. This hybridization detection method eliminates the need to modify the DNA targets, synthetic oligonucleotides or PCR amplification.

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