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Sensitization and Inhibition of the Redox Activity of Hybridization Labels by Acridine Orange, Hoechst 33258 and DNA Using a Rapid Electrochemical Method

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We investigated sensitization and inhibition effects of DNA on cyclic voltammetry peak currents of ferricyanide, ferrocene complex, ruthenium complex, acridine orange and Hoechst 33258. We also studied sensitization by acridine orange and Hoechst 33258 of peak CV currents for ferricyanide, ferrocene complex and ruthenium complex in the presence and absence of DNA. This work employed a bare glassy carbon (GC) electrode with double-stranded calf thymus DNA (ctDNA) in solution. Cyclic voltammetry was carried out in 0.1 M KCl at pH 7 with DNA solution (400 ppm) and ligand concentrations in the 10^{-3} molar range. DNA had a negligible effect on the redox activity of ferricyanide. However inhibition was observed in the redox activity of ferrocene and ruthenium complexes. At low concentrations DNA increased the redox activity of the intercalator ligands acridine orange and Hoechst 33258. Acridine orange and Hoechst 33258 influenced the redox activity of different intercalator ligands (ferricyanide, ferrocene complex and ruthenium complex); effects included shifting of anodic and cathodic peaks both in the presence and absence of DNA. This method affords a convenient and sensitive approach for exploring interactions between DNA and electroactive ligands.

Key Words: Acridine orange, Hoechst 33258, Inhibition, Sensitization, DNA, Cyclic voltammetry.

INTRODUCTION

Selective hybridization to sequence-specific single-stranded DNA (ssDNA) is now routinely used to detect DNA sequences associated with particular genetic or pathogenic diseases. Electrochemical detection of DNA hybridization is an area of intense current interest. Whereas conventional methods including fluorimetric detection require labeling of target DNAs, reporter ligands including redox indicator compounds avoid this need by detecting physico-chemical changes brought about by target hybridization on an electrode surface. The nature of the interaction of DNA and reporter ligands therefore plays a crucial role in the detection of specific DNA sequences.

There are three principal types of interaction between DNA and ligands: stacking, hydrogen binding and ionic[1]. The interaction of DNA with ligands such as cationic/anionic metal complexes and organic compounds leads to the formation of DNA-ligand complexes and has a large influence on the physico-chemical properties of the immobilized DNA.

Electrochemical detection methods rely on detection of hybridized DNA *via* a redox-active compound that differentially interacts with double-stranded DNA (dsDNA). Hybridization labels used previously in such systems include daunomycin[2], epirubicin, echinomycin[3], metal complexes such as ruthenium bipyridine[4], cobalt phenanthroline[5] and organic dyes such as methylene blue[6,7]. These compounds are also intercalating agents.

Intercalation occurs when ligands of an appropriate size and chemical nature insert between adjacent base pairs of dsDNA. For insertion to take place the DNA must dynamically open a space between its base pairs by unwinding. The degree of unwinding depends on the intercalating agent. For example, ethidium cation (the ionic form of ethidium bromide found in aqueous solution) unwinds DNA by about 26º while proflavin unwinds it by about 17º. Unwinding causes the base pairs to separate or "rise", creating an opening of about 0.34 nanometers or 3.4 Å[8]. Unwinding also induces local structural changes in the DNA, including lengthening of the DNA duplex and twisting of the base pairs. The mechanism of intercalation is thought to be as follows. In aqueous isotonic solution, the cationic intercalator is attracted electrostatically to the polyanionic DNA. The ligand then displaces charge-balancing sodium and/or magnesium cations that surround the helix to form a weak electrostatic bond with the outer DNA surface. From this initial association, the ligand can slide into the hydrophobic environment between the base pairs and away from the hydrophilic external environment. Base-pairs transiently form such openings due to energy absorbed during collisions with solvent molecules.

The initial interaction may take place at either the major or minor grooves. Recent attention has been given to agents interacting preferentially with the minor groove, generally small crescent-shaped cationic molecules with favourable hydrophobic and electrostatic interactions. Examples of agents binding to the minor groove include Hoechst 33258 and 33342, distamycin, 4',6-diamidino-2-phenylindole (DAPI), acridine orange, netropsin and cyanine dyes. These molecules recognize four AT basepairs and replace a network of well-ordered water molecules with a similar pattern of hydrogen bond interactions. A combination of hydrogen bonding, van der Waals contacts with the walls of the minor groove and electrostatic interactions between the cationic ligands and the DNA is thought to contribute to high affinity ligand binding[9].

Interactions between minor and major groove binding occur through conformational conversion of the double helix and afford a pathway for non-competitive processes. It is thought that adsorbed DNA sterically excludes redox anions from the electrode surface. At high DNA coverage of the electrode, barriers against anion migration to the electrode surface include channel size and the hydrophobicity of the inner surface of the double helix[10]. Ligand binding may open 'pores' in the DNA adsorption layer so as to permit anion diffusion to the electrode surface[11]. DNA is nucleophilic due to the phosphate exoskeleton backbone of the double helix associated with cationic counter ions. Anions compete with this association by loose binding and counter ion sharing with DNA. Cations, in the case the minor groove binding agents, may sensitize the redox activity of anions bound to DNA by direct interaction. Sensitization and inhibition in the presence or absence of DNA can reveal whether the DNA and intercalators (anions) interact with the sensitizer (cations).

We report a study on the interaction between DNA and electroactive ligands (intercalators and non-intercalators). The influence of DNA on the sensitization and inhibition of the redox activity of several different ligands was studied. We investigated the ability of minor groove binding agents to sensitize or inhibit the redox activity of intercalators (ferricyanide, ruthenium complexes and ferrocene complexes) in the presence and absence of DNA. Electrochemical parameters of the DNA-ligand interaction were calculated and compared.

EXPERIMENTAL

Stock solutions of $K_4[Fe(CN)_6]$ (Sigma), 1,1'-ferrocenedicarboxylic acid (Fluka), $[Ru(NH_3)^6]^{3+}$ (Sigma), acridine orange (Acros) and Hoechst 33258 (Sigma) were prepared in 0.1 M KCl. ctDNA (Sigma) was prepared in phosphate pH 7 buffer at 400 ppm (0.4 mg/mL) with 0.1 M KCl as the supporting electrolyte. All stocks solutions were refrigerated when not in use.

Electrochemical measurements were performed using a Solartron 1286 meter (Solartron Analytical) an Ag/AgCl electrode was the reference electrode and a platinum wire was the counter-electrode: a glassy carbon electrode provided the working electrode. Before each experiment the working electrode was polished and washed with 0.1 M NaOH, 0.1 M HCl and then rinsed with distilled water.

Procedures: Cyclic voltammogram measurements were performed at room temperature under nitrogen. Cyclic voltammogram scan rates of 5, 10, 50 and 100 mV/s were used in a 7 mL vial; interaction solutions were prepared by mixing a fixed amount of redox molecules (ferrocyanide, ferrocene complex, ruthenium complex, acridine orange and Hoechst 33258) with different ratios of supporting electrolyte and dsDNA solutions to maintain the concentration of the redox solute. Tests were run sequentially commencing with the redox solution alone, followed by addition of different amounts of dsDNA solution. To investigate the effects of acridine orange (AO) and Hoechst 33258 on intercalator redox activity, interaction solutions were prepared by mixing the intercalators (ferrocyanide, ferrocene complex and ruthenium complex) with different ratios of acridine orange and Hoechst 33258 with and without added dsDNA solution.

RESULTS AND DISCUSSION

$[Fe(CN)_6]^{4-}$ (anionic): $[Fe(CN)_6]^{4-}$ does not interact with DNA in solution because of coulombic repulsion between negative charges. In the absence of DNA, a standard voltammetric peak of $[Fe(CN)_6]^{4-}$ was observed (E_{pa} = 0.41). On addition of calf thymus DNA (ctDNA) the anodic and cathodic peaks were both slightly enhanced (Fig. 1). However, peak current decreased on further DNA addition. This may be explained by steric hindrance due to increasing viscosity at higher DNA concentrations. No significant shifting of the redox peak was observed, indicating that there was no significant interaction between DNA and ferricyanide.

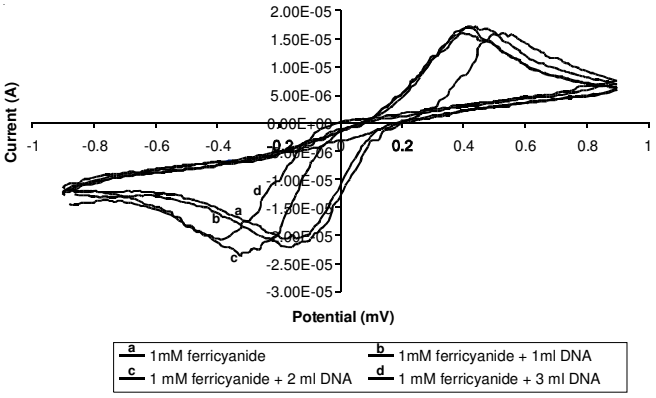

Fig. 1. Cyclic voltammogram (100 mV/s) of 1 mM ferricyanide alone and in the presence of increasing amounts of ctDNA solution (400 ppm) using a glassy carbon electrode in 0.1 M KCl

Anodic currents in the cyclic voltammogram (CV) of ferricyanide were enhanced by acridine orange in the presence of DNA (Fig. 2). The shift in peak potential was indicative of AO-ferricyanide ion-pair formation:ferricyanide may diffuse for oxidation from either the paired or unpaired form.

$$Fe(CN)_6^{4-} \longrightarrow Fe(CN)_6^{3-} \quad (1)$$

$$AO\text{-}Fe(CN)_6^{4-} \longrightarrow AO + Fe(CN)_6^{3-} \quad (2)$$

In the presence of acridine orange, diffusion and oxidation from the paired form predominates. Ionic pairing may enhance anion diffusion to the electrode resulting in a higher anodic current[10]. However, in the presence of DNA, competition for acridine orange can take place.

$$DNA + AO\text{-}Fe(CN)_6^{4-} \rightarrow DNA\text{-}AO + Fe(CN)_6^{4-} \rightarrow Fe(CN)_6^{3-}$$

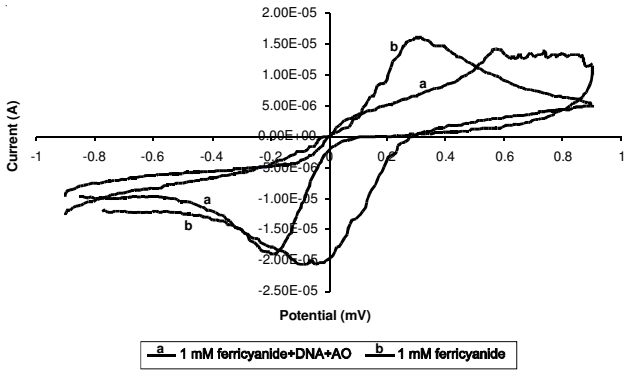

Fig. 2. Cyclic voltammogram (100 mV/s) of 1 mM ferricyanide alone and in the presence of ctDNA and acridine orange

Through mutual repulsion, DNA in the bulk solution can inhibit the diffusion of ferrocyanide ions by competitively binding acridine orange, interfering with enhanced diffusion through ion pairing. Additional ferricyanide diffusing in the paired form will be oxidized from the unpaired form.

The same sensitization effect has been observed upon addition of acridine orange to naphthoquinone-sulfonate (NQS)[10]. Here the anodic peak was enhanced whereas there was no significant change on the cathodic peak. Peak anodic currents in the cyclic voltammogram of naphthoquinone-sulfonate with acridine orange were inhibited at increased DNA concentration.

It is observed that both cathodic and anodic peak currents for ferricyanide were reduced in the presence of Hoechst 33258. This took place both in the presence and absence of DNA (Figs. 3 and 4). Inhibition appears to be due to decreased

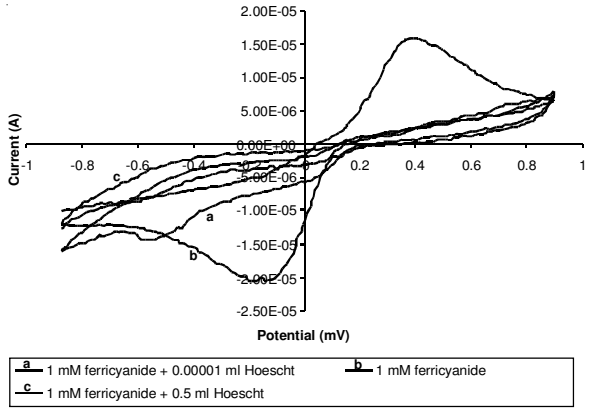

Fig. 3. Cyclic voltammogram (100 mV/s) of 1 mM ferricyanide alone and in the presence of Hoechst 33258

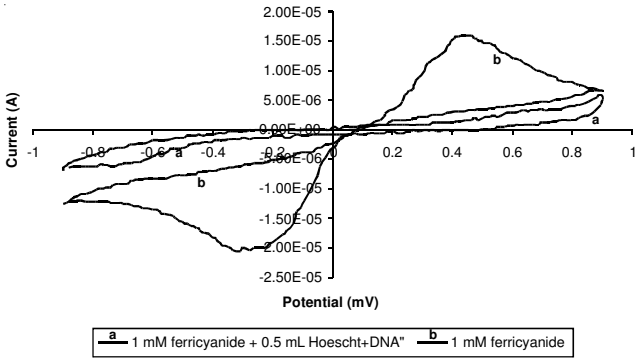

Fig. 4. Cyclic voltammogram (100 mV/s) of 1 mM ferricyanide alone and in the presence of Hoechst 33258 and ctDNA

diffusion of ferricyanide to the electrode, possibly through an interaction with Hoechst 33258.

Ruthenium complex (cationic): A ruthenium complex has been used as a hybridization label by many researchers[12-15]. The complex associates electrostatically with the anionic DNA backbone. A linear decrease in redox current in the presence of added DNA has been observed (Fig. 5). Both anodic and cathodic peaks were shifted towards negative potential, suggesting an interaction between DNA and the ruthenium complex. Ruthenium complex can oxidize guanine bases, while the reduced form of the complex can be oxidized at the electrode, so forming a catalytic cycle. It is possible that high concentrations of DNA decrease the accessibility of the ruthenium complex to both bases and the electrode, thus decreasing the overall redox current.

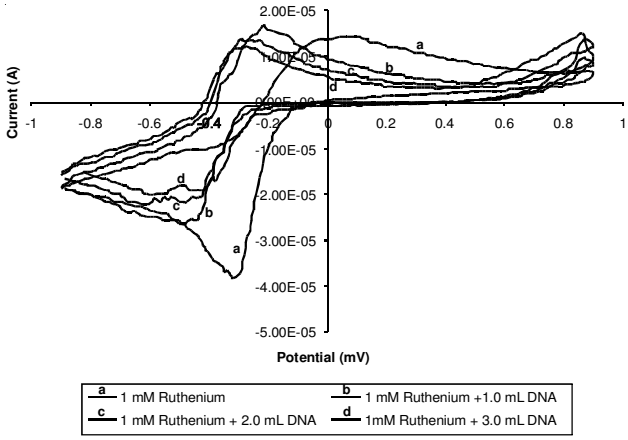

Fig. 5. Cyclic voltammogram (100 mV/s) of 1 mM ruthenium alone and in the presence of increasing amounts of ctDNA solution (400 ppm)

The sensitization effect of Hoechst 33258 on the redox current of the ruthenium complex was also studied. Lee and co-workers suggested that DNA intercalating agents can act as co-reductants of ruthenium complex[16]. They studied electrochemiluminescent and voltammetric signals of $Ru(bpy)_3^{2+}$ when mixed with different DNA intercalators including daunorubicin (DNR), doxorubicin (DOX), ethidium bromide and 4',6-diamino-2-phenylindole (DAPI). DNR, DOX and DAPI showed well-defined redox peaks.

Nevertheless, a decrease was recorded in the redox current of the ruthenium complex when Hoechst 33258 was added to the mixture (Fig. 6). No significant shift of potential was observed, indicating that there is no significant interaction between ruthenium complex and Hoechst 33258.

The same current reduction was observed when DNA solution was added to a mixture of ruthenium complex and Hoechst 33258. An anodic peak appeared, similar to the anodic peak of Hoechst 33258 (0.68 mV) when 0.5 mL of DNA was added, suggesting that DNA and ruthenium form a complex, leaving Hoechst 33258 to be oxidized. However, further increase in DNA concentration reduced the redox current.

Ferrocene complex: Ferrocene complex has been widely utilized by researchers in label-free electrochemical detection of DNA[17-20]. When the target hybridizes to both capture and signalling probes, ferrocene moieties are brought into the proximity of the electrode surface and thereby become electro-

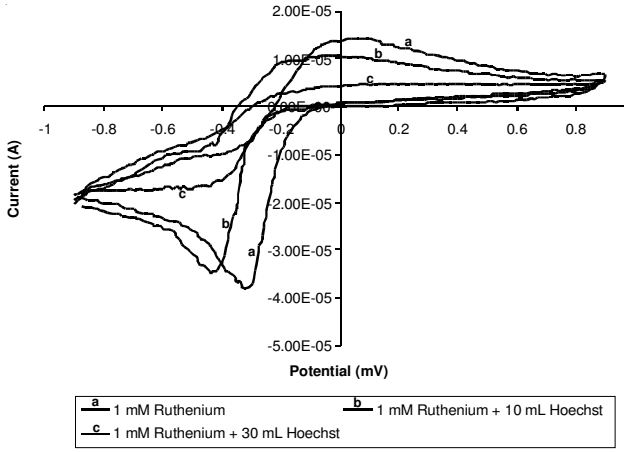

Fig. 6. Cyclic voltammogram (100 mV/s) of 1 mM ruthenium alone and in the presence of Hoechst 33258

chemically accessible for detection. The use of a voltammetry technique to interrogate the sensors allows for repeated collection of electrons from ferrocene labels. Although this detection method is not truly electrocatalytic, there is a built-in signal-amplification mechanism that results from the interrogation step[21].

The oxidation peak observed in this experiment (Fig. 7) can be attributed to the reversible oxidation of the ferrocene (fc) group to ferrocenium (fc^+). On DNA addition the cyclic voltammogram of ferrocene complex showed a linear decrease in peak currents. This decrease may be ascribed to steric hindrance associated with increased viscocity at elevated DNA concentrations; this may interfere with ferrocene diffusion to the electrode surface. Redox peaks were also shifted towards negative values, suggesting an interaction between ferrocene and DNA.

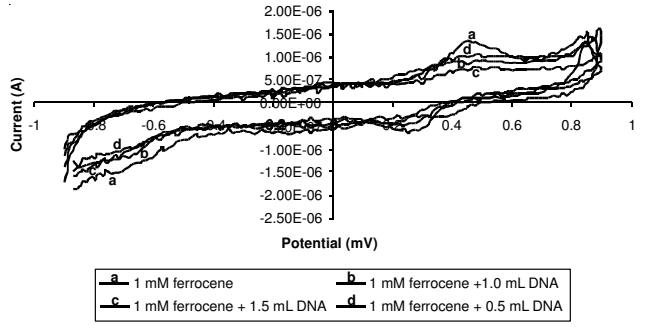

Fig. 7. Cyclic voltammogram (100 mV/s) of 1 mM ferrocene alone and with increasing amounts of ctDNA solution (400 ppm)

Acridine orange reduced the anodic current of ferrocene. No shifting was observed, suggesting that there is no significant interaction between acridine orange and the ferrocene complex. As before, reduced current may be due to interference between acridine orange and ferrocene diffusion.

On addition of DNA to a ferrocene/acridine orange mixture the redox peaks were shifted towards negative values. There was also a slight enhancement of new redox peaks (0.1 and -0.3 V) at higher acridine orange concentrations. These shifts may be ascribed to an interaction between DNA and acridine orange. Intercalated DNA can form a non-aqueous phase or sometimes a visible precipitate which can hinder ferrocene diffusion to the electrode. The ferrocene anodic current was markedly reduced by addition of DNA to the ferrocene/acridine orange mixture.

The possible sensitization or inhibition effects of Hoechst 33258 on ferrocene redox activity was also investigated. In the presence of Hoechst 33258 the ferrocene anodic peak was shifted towards positive values and also increased in magnitude. However, further increases in Hoechst 33258 concentrations produced a linear reduction in anodic current.

Hoechst 33258: Few studies have addressed the interaction between Hoechst 33258 and DNA. Hashimoto *et al.*[22] developed a microfabricated disposable DNA sensor for detection of hepatitis B virus DNA. After hybridization and washing, the sensor was immersed in Hoechst 33258 solution: here target DNA significantly increased the magnitude of anodic currents. Choi *et al.*[23] developed a DNA biochip using Hoechst 33258 as the hybridization label. An increase in current was observed in the presence of target DNA: Hoechst 33258 generated an irreversible oxidation response. The increase in current magnitude was ascribed to Hoechst 33258 being concentrated at the electrode surface as a result of DNA hybridization[23]. Kobayashi *et al.*[24] carried out DNA quantification based on aggregation induced by Hoechst 33258. Here DNA binding of Hoechst 33258 was found to cause a significant change in diffusion coefficient.

We investigated cyclic voltammogram sensitization and inhibition by Hoechst 33258 in the presence of different concentrations of DNA. There was a marked enhancement in the magnitude of the anodic current on DNA addition; the peak shifted to more negative values (Fig. 8). However, further increases in DNA concentration reduced the anodic current. Sufen *et al.*[1] observed the same reduction and shifting of the anodic current; the reduction was attributed to reduced diffusion of Hoechst 33258 bound to high molecular weight DNA.

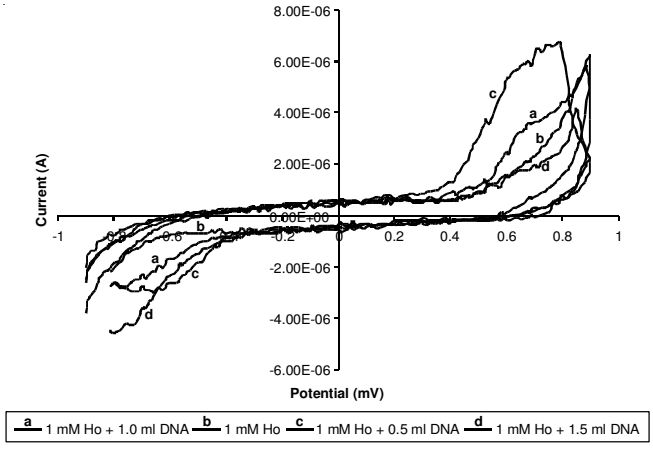

Fig. 8. Cyclic voltammogram (100 mV/s) of 1 mM Hoechst 33258 alone and with increasing amounts of ctDNA solution (400 ppm)

Acridine orange: Sun *et al.*[25] previously employed acridine orange as a bioprobe in linear sweep polarographic determination of nucleic acids. In this study quantitation was based on the decrease in the acridine orange reductive peak.

The sensitization and inhibition effects of different DNA concentrations on acridine orange peak currents was investigated. On DNA addition a new redox peak appeared at 0.1 V (anodic) and -0.3 V (cathodic) (Fig. 9).

A modest increase was also recorded at higher DNA concentrations. However, when further DNA solution was added

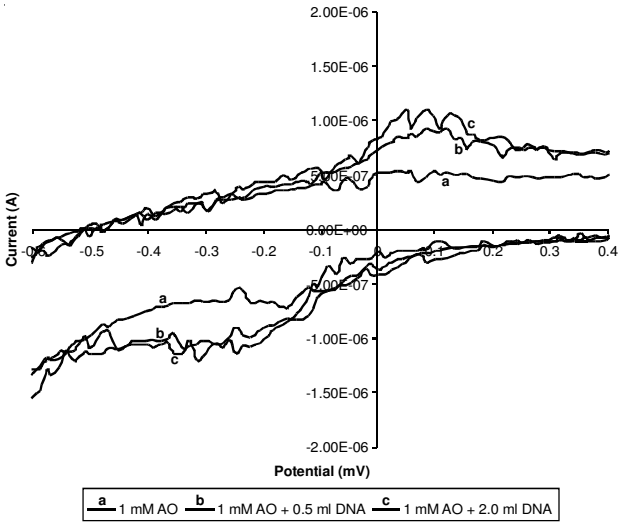

Fig. 9. Cyclic voltammogram (100 mV/s) of 1 mM acridine orange alone and with increasing amounts of ctDNA solution (400 ppm)

(≥ 2.0 mL) a visible DNA precipitate was observed that interfered with the cyclic voltammogram process.

Conclusion

We report that DNA has negligible effects on the redox activity of ferricyanide, but inhibits the redox activity of both ferrocene and ruthenium complexes. At low concentrations DNA increased the redox activity of two intercalator ligands (acridine orange and Hoechst 33258). This enhancement is probably due to the electrocatalytic effect of the DNA. However, further increase in DNA concentration inhibited both anodic and cathodic currents in the cyclic voltammograms of both ligands, possibly by blocking diffusion of acridine orange and Hoechst 33258 to the electrode surface.

Acridine orange and Hoechst 33258 altered the redox activity of ferricyanide, ferrocene complex and ruthenium complex. Effects included shifting of the anodic and cathodic peaks, both in the presence and absence of DNA. Enhanced diffusion of an anionic ligand (ferricyanide) by ionic pairing to acridine orange is suggested because acridine orange and ctDNA, respectively enhanced and inhibited the cyclic voltammogram peak of ferricyanide. In the presence of Hoechst 33258, however, the cyclic voltammogram redox current of ferricyanide was markedly inhibited, possibly due to complex formation between cationic Hoechst 33258 and anionic ferricyanide. This would result in the formation of an electro-neutral species which could inhibit electron transfer to the electrode surface.

In conclusion, it is suggested that mixtures of minor groove binding agents and intercalators can combine the selectivity of minor groove binders and the sensitization effects of intercalating agents, offering a new method for increasing the sensitivity and selectivity of DNA-based biosensors.

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