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A DNA biosensor based on the electrocatalytic oxidation of amine by a threading intercalator

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ABSTRACT

An electrochemical biosensor for the detection of DNA based a peptide nucleic acid (PNA) capture probe (CP) modified indium tin oxide electrode (ITO) is described in this report. After hybridization, a threading intercalator, *N*,*N*′-bis[(3-propyl)-imidazole]-1,4,5,8-naphthalene diimide (PIND) imidazole complexed with $Ru(bpy)_2Cl$ (PIND-Ru, bpy = 2,2′-bipyridine), was introduced to the biosensor. PIND-Ru selectively intercalated to double-stranded DNA (ds-DNA) and became immobilized on the biosensor surface. Voltammetric tests showed highly stable and reversible electrochemical oxidation/reduction processes and the peak currents can directly be utilized for DNA quantification. When the tests were conducted in an amine-containing medium, Tris–HCl buffer for example, a remarkable improvement in the voltammetric response and noticeable enhancements of voltammetric and amperometric sensitivities were observed due to the electrocatalytic activity of the $[Ru(bpy)_2Cl]$ redox moieties. Electrocatalytic current was observed when as little as 3.0 attomoles of DNA was present in the sample solution.

1. Introduction

Optical microarrays are arguably the most widely used type of biosensors in DNA analysis [1] where detection of specific DNA sequences are based on labeling sample DNAs with fluorophores. While fluorescence-based detection technologies have shown tremendous utility, they suffer from the drawbacks of labor-intensive sample preparation, high cost, and complex and bulky fluorescence detection instrumentation. Given the increasing concern that fast-mutating viruses can cause infectious diseases that will lead to pandemics, there is a clear and immediate need for point-of-care detection that can be applied for a large number of subjects. DNA diagnostic systems that are low-cost, easy-to-use, portable, and miniaturizable, while maintaining the level of accuracy and sensitivity of laboratory diagnostics, are highly desirable. Electrochemical biosensors, therefore, are gaining momentum as the method of choice for point-of-care applications [2]. Since electrochemical reactions directly generate electrical signals, there is no need for expensive signal transduction equipment. The electrode fabrication process is highly compatible with advanced microfabrication and semiconductor technology, enabling easy miniaturization and fabrication of high density sensor arrays. In addition, signal amplification through additional chemical/biological means can potentially increase the sensitivity of the biosensor to extraordinarily high levels [3,4]. The drawbacks associated with electrochemical biosensors are the possible electromagnetic interference at very low signal levels, and the limitation of in vivo application due to the need for electrical perturbations to generate signal.

As with other DNA detection techniques, the presence of a target DNA is monitored by its hybridization to surface-immobilized CP. A number of detection strategies for the electrochemical DNA biosensors have been reported [3,5]. The simplest of such strategies would be direct detection (label-free) of DNA involving a solid electrode modified with the CP that produces a measurable electrochemical signal upon hybridization to a specific target DNA. The signal comes from the intrinsic oxidation of nucleobases in the target DNA. Wang et al. successfully detected a 29-nucleotide DNA by observing guanine oxidation through chronopotentiometry on carbon paste electrode with detection limit around 120 ng mL^{-1} [6]. However, it is generally believed that direct oxidation of DNA is irreversible and therefore requires a high overpotential, and often suffers from pronounced fouling effect, which results in poor selectivity and reproducibility. Two approaches have been examined for enhancing the oxidation current of DNA [5,7]. Wang's group pioneered an amplification strategy that entails the adsorptive-accumulation and separation of the hybridized DNA using magnetic beads, followed by a potentiometric stripping detection. A lower detection limit of 40 femtomoles (250 pg) was achieved with this method through adsorptive chronopotentiometric stripping measurements of the free purine nucleobases in the presence of copper ions [8]. Another successful amplification strategy engages the electrocat-

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alytic effect of $Ru(bpy)_3^{2+}$ to amplify the intrinsic signal of guanine oxidation [7]. This biosensor was used for the determination of PCR products of genomic DNA and RNA from viruses as well as genetic abnormalities that cause diseases [9,10] However, since the compounds are unable to interact selectively with ds-DNA, the assay suffers from intrinsically large background current arising from the direct oxidation of $Ru(bpy)_3^{2+}$ and the catalytic oxidation of CP by of $Ru(bpy)_3^{2+}$. Most of the catalytic oxidation current of CP can be eliminated by substituting guanine by electrochemically inactive inosine [11], but little can be done to reduce the direct oxidation of the of $Ru(bpy)_3^{2+}$ at the detection potential.

We are interested in amplified electrochemical detection of nucleic acid using low redox potential biocatalysts/electrocatalysts as sensitivity enhancers [12,13]. We report herein the synthesis, characterization and analytical application of a novel electrocatalytic DNA threading intercalator in ultrasensitive DNA biosensors. The intercalator used in this study is a naphthalene diimide derivative grafted with two redox moieties at its termini. $Ru(bpy)_2Cl_2$ complex has been chosen as the electrocatalytic moiety because of its low redox potential and high electron-transfer rate. Experimental results suggested that the intercalator is highly selective to ds-DNA with a much enhanced stability constant. Moreover, the $Ru(bpy)_2Cl$ pendants effectively catalyze the oxidation of amine at potential as low as 0.63 V as compared to 0.90–1.2 V at a bare electrode [14]. The combination of the high affinity of the intercalator with ds-DNA and highly efficient electrocatalysis provides a generic platform for ultrasensitive detection of DNA.

2. Experimental

2.1. Chemicals

1(3-Aminopropyl)-imidazole (98%, AI) and 1,4,5,8-naphthalene tetracarboxylic dianhydride (>95%) were purchased from Sigma–Aldrich (St Louis, MO, USA). $Ru(bpy)_2Cl_2$ (99%) was from Avocado Research Chemicals Ltd. (Leysham, Lancester, UK). Trimethyloxysilane aldehyde (90%) was from United Chemical Technologies Inc (Bristol, PA, USA). Amine-terminated peptide nucleic acid (PNA) CP used in this work was custom-made by Eurogentec (Herstal, Belgium) and all other DNAs were from 1st Base Pte Ltd. (Singapore). Sequences of the PNA CP and DNAs are listed in Table 1. All other reagents of certified analytical grade were obtained from Sigma–Aldrich and used without further purification. ITO coated glass was from Delta Technologies Limited (Stillwater, MN, USA). A pH 8.5 10 mM Tris–HCl – 1.0 mM EDTA – 0.10 M NaCl (TE) buffer solution was used as the hybridization and detection buffer. A phosphate-buffer saline (PBS, pH 7.4), consisted of 0.15 M NaCl and 20 mM phosphate buffer, was used as the supporting electrolyte where amine is not wanted.

2.2. Apparatus

Electrochemical experiments were carried out using a CH Instruments model 660A electrochemical workstation coupled with a low current module (CH Instruments, Austin, TX, USA). A conventional three-electrode system, consisting of the ITO working electrode (diameter = 2.0 mm), a nonleak Ag/AgCl (3.0 M NaCl) reference electrode (Cypress Systems, Lawrence, KS, USA), and a platinum wire counter electrode, was used in all electrochemical measurements. Electrical contact was made to the ITO electrode using conducting epoxy and a copper wire. All potentials reported in this work were referred to the Ag/AgCl electrode. UV–vis spectra were recorded on an Agilent 8453 UV–vis spectrophotometer. Mass spectrometric experiments were performed with a Finnigan/MAT LCQ Mass Spectrometer (ThermoFinnigan, San Jose, CA, USA). All spectra were recorded at room temperature unless otherwise noted.

Table 1
Sequences of PNA capture probes and target DNAs.

PNA capture probe (N → C)	NH_2-AAC CAC ACA ACC TAC TAC CTC A
Complementary target DNA (5′ → 3′)	TGA GGT AGT AGG TTG TGT TGT GGT T
One-base-mismatched DNA (5′ → 3′)	TGA GGT AGT AGG TTG TGT TCT GGT T
Two-base-mismatched DNA (5′ → 3′)	TGA GGA AGT AGG TTG TGT TCT GGT T
Control DNA (5′ → 3′)	ATC TAG AAA TTC AGT GAG ATA CAT A

2.3. Synthesis of PIND-Ru

The synthesis of PIND-Ru is outlined in Fig. 1. PIND was prepared following a general procedure for the synthesis of naphthalene diimide (ND) [15] (1H NMR (300 MHz $CDCl_3$) δ 8.76 (4H), 7.54 (2H), 7.26 (2H), 4.27 (4H), 4.12(4H), 2.31 (4H) and 1.83 9(2H)). Mass spectrometric tests on PIND using electron-spray ionization mass spectrometry (ESI-MS) showed predominant peaks at m/z 483.3 and 242.3, corresponding to PIND+H^+, (and (PIND+2H^+)/2, which are in good agreement with the molecular weights of the desired compounds. PIND-Ru was synthesized in a single-step ligand-exchange reaction. To a solution of $Ru(bpy)_2Cl_2$ (0.52 mmol) in 8.0 mL fresh-distilled ethylene glycol was added PIND (0.25 mmol) in small portions over 10 min and the result mixture was refluxed for 30–40 min. The completion of the ligand-exchange reaction was monitored by cyclic voltammetry. The purple reaction mixture was then poured slowly into 100 mL of rapid stirred ethanol saturated with KCl. The precipitate was collected by suction filtration through a fine fritted funnel. The crude product was washed with PBS, dissolved in 3.0–5.0 mL of ethanol and precipitated again from KCl saturated ethanol. The precipitate was further purified by crystallization from ethanol giving the pure product in 70% yield. The product showed a single pair of reversible redox waves at a gold electrode with an $E_{1/2}$ of 0.63 V in PBS. To ensure a complete double ligand-exchange at the two imidazole termini of PIND, slight excess of $Ru(bpy)_2$ (10–15%) is required.

2.4. Immobilization of CP on ITO electrode

The pretreatment and silanization of the electrode were performed according to the method of Zhang [16]. Arrays of electrodes (10–50) can be fabricated by patterning the ITO substrate with a hydrophobic membrane after silanization. The unreacted aldehyde moieties were blocked by butylamine in a 1.0 mM butylamine solution in the acetate buffer. The reduction of the imines was carried out by a 5-min incubation of the electrode in a 2.5 mg mL^{-1} sodium borohydride solution made of PBS/ethanol (3/1). The electrode was then soaked in vigorously stirred hot water (90 – 95 °C) for 2 min, copiously rinsed with water, and blown dry with a stream of nitrogen. The reason of using PNA instead of DNA CP is twofold: To minimize electrostatic interaction and to increase the specificity of hybridization. The neutral character of the PNA backbone alleviates electrostatic interaction between surface-immobilized CP and cationic PIND-Ru, producing a high signal/noise ratio. The mismatch discrimination of PNA is in many cases much better than that of DNA [17], offering a much higher specificity.

2.5. Hybridization and detection

The hybridization of the target DNA and its electrochemical detection were carried out in three steps. First, a 2.0 μL aliquot of hybridization solution containing the target DNA was uniformly spread onto the biosensor and the biosensor was placed in a moisture saturated environmental chamber maintained at 60 °C for 30 min. It was then washed thoroughly with a stirred blank hybridization solution at 60 °C for 5 min and incubated at

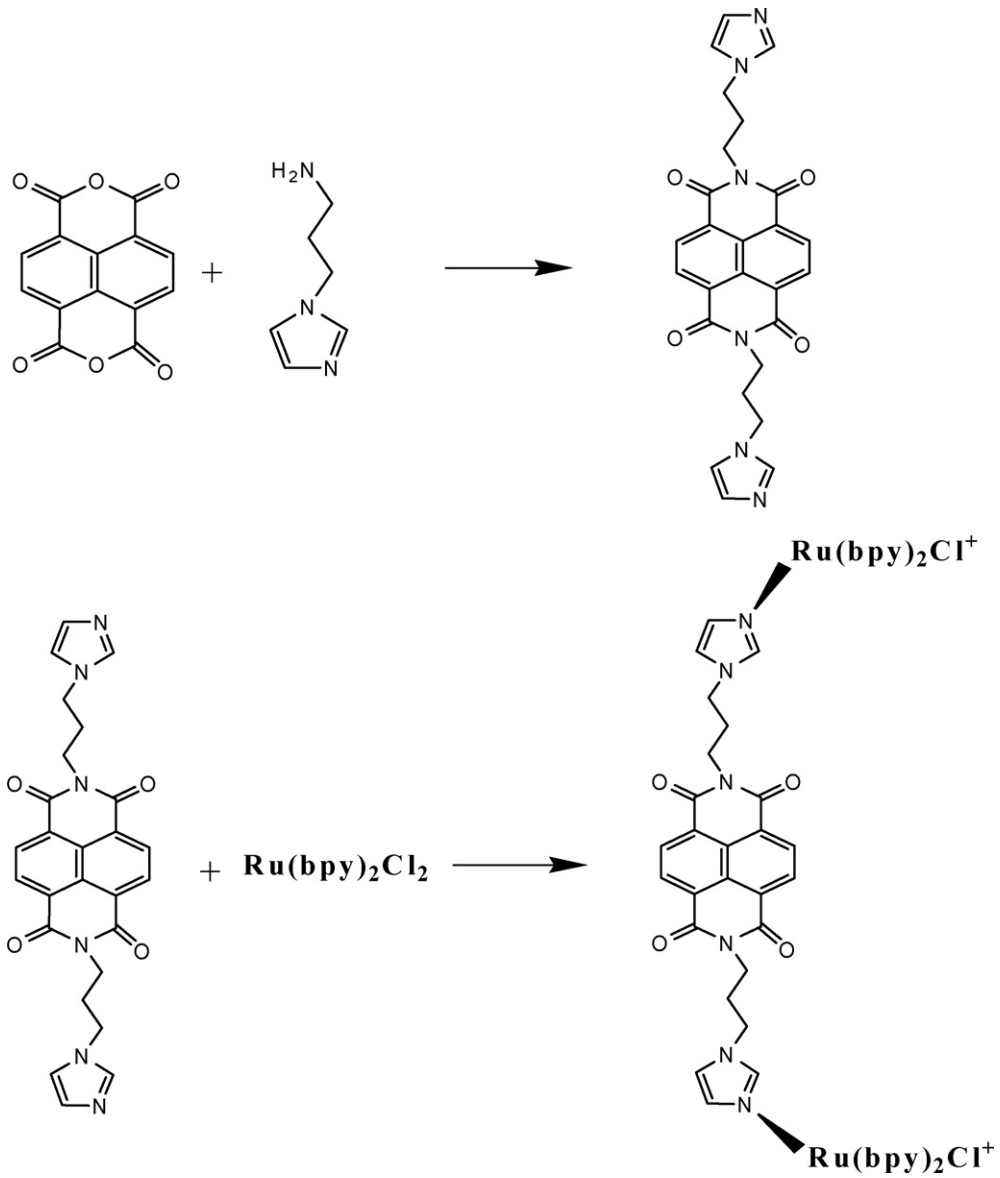

Fig. 1. Synthetic route to PIND-Ru threading intercalator.

35 °C for 10 min with a 5.0 μL aliquot of 50 μg mL^{-1} of PIND-Ru in the hybridization buffer. Thereafter the biosensor was thoroughly washed with a stirred NaCl-saturated phosphate buffer (pH 7.4) containing 10% ethanol at room temperature for 5 min. The steady-state electrocatalytic oxidation current was measured amperometrically at 0.65 V.

3. Results and discussion

3.1. Formation of PIND-Ru

The formation of PIND-Ru can be conveniently monitored by cyclic voltammetry. During reflux in ethylene glycol, cyclic voltammetric tests were conducted every 5 min. Fig. 2 shows two typical voltammograms obtained in the first 30 min. Before adding PIND to $Ru(bpy)_2Cl_2$, one pair of reversible voltammetric peaks centered at 0.40 V were obtained, corresponding to the well-known redox processes of $Ru(bpy)_2Cl_2$. Upon adding PIND and refluxing, a new pair of voltammetric peaks appeared at 0.63 V, indicating the formation of PIND-Ru (Fig. 3, trace 2). Both electron transfer processes

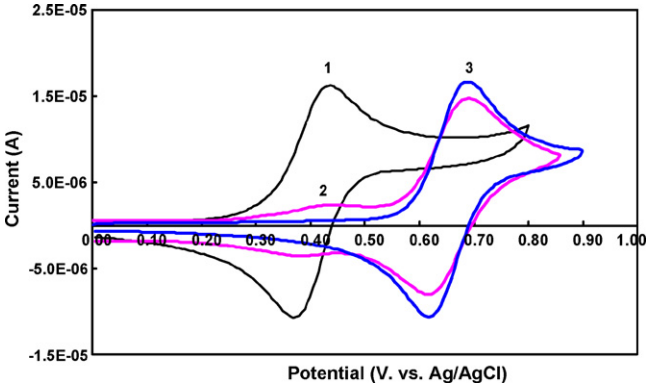

Fig. 2. Cyclic voltammograms of (1) $Ru(bpy)_2Cl_2$, (2) $Ru(bpy)_2Cl_2$ after 30 min of refluxing with PIND in ethylene glycol and (3) purified PIND-Ru. Supporting electrolyte for (3) PBS, potential scan rate 100 mV s^{-1}.

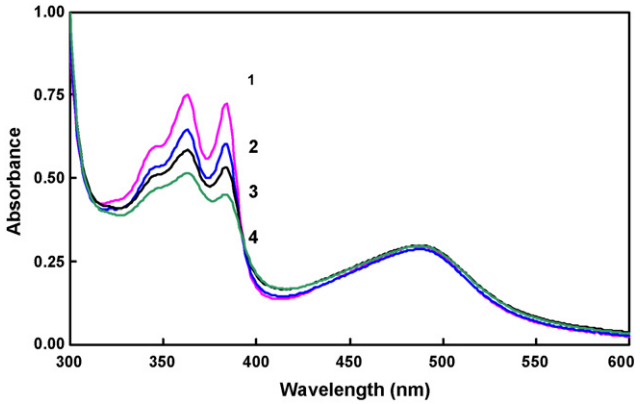

Fig. 3. UV–vis spectra of 25 μM PIND-Ru as a function of increasing concentration of salmon sperm DNA (in base pair) of (1) 0, (2) 25, (3) 50 and (4) 100 μM.

are clearly resolved have all the characteristics of reversible processes. The intensity of the voltammetric peak at 0.63 V increased gradually with reaction time with a simultaneous decrease in peak currents of those at 0.40 V. Both of the redox pairs reached a steady-state after 30 min of refluxing. The minute voltammetric peaks at 0.40 V are indicative of the excess of $Ru(bpy)_2Cl_2$. Voltammetric tests of the purified PIND-Ru showed only one pair of voltammetric peaks implying that the purification process is very effective (Fig. 2, trace 3).

3.2. Electrochemical properties of PIND-Ru

As illustrated in trace 3 in Fig. 2, PIND-Ru exhibited exactly as expected for a highly reversible redox couple in solution. Little change was observed after numerous repetitive potential cycling between −0.20 and +0.80 V, good stability of PIND-Ru in solution. A typical diffusion-controlled voltammogram, exhibiting an ideal Nernstian behavior of a one-electron exchange system: the peak current is proportional to the square root of the potential scan rate, i_p (μA) = $48v^{1/2}$ (i_p: peak current and v: scan rate), the peak-to-peak potential separation is very close to the theoretical value of 59 mV and potential scan rate independent [18], was observed at potential scan rates <500 mV s^{-1}. Such results ascertain the ruthenium redox centers are allowed to reach the electrode surface and proceed to reversible heterogeneous electron transfer.

3.3. Intercalation with DNA

The mode of interaction of PIND-Ru with ds-DNA was determined by UV–vis spectrophotometry of PIND-Ru in the presence of increasing amounts of salmon sperm DNA (average size ≤2000 base pairs) was investigated (Fig. 3). In the UV–vis spectrophotometry, signatures of intercalative binding are hypochromism and red shifts [19]. As shown in Fig. 3, addition of DNA to PIND-Ru at a DNA base pair/PIND-Ru ratio of 4.0 resulted in a 40% decrease and a 2-nm-red-shift of the naphthalene diimide (ND) absorbance band at 366 and 387 nm, as a result of the intercalating ND stacked in between DNA base pairs [20] Similar phenomena were previously observed with ND having aliphatic tertiary amine side chains [19]. The ND absorbance band hypochromism reached a plateau at the DNA base pair/PIND-Ru ratio >4.0, and a constant hypochromism was observed, indicating that binding of PIND-Ru to ds-DNA takes place by preferential intercalation of the ND. Furthermore, the insertion of intercalating unit increases the length of DNA strand and increases of the viscosity of a DNA solution [21]. The viscometric study showed that an 8% increase in viscosity was observed upon adding PIND-Ru to the DNA solution, further confirming its intercalative binding.

A fluorescent intercalator displacement (FID) assay using a 5-base pair hairpin oligonucleotide was then conducted to determine the binding constant to ds-DNA using the method proposed by Boger [22]. In the FID assay, a fluorescent intercalator first saturates the ds-DNA. A non-fluorescent intercalator, in this case PIND-Ru, was then introduced with gradual increase in concentration. The fluorescent and non-fluorescent intercalators bind to similar sites in the ds-DNA, creating a competition for a fixed number of binding sites. Changes in fluorescent intensity are thus expected as the ds-DNA-bound fluorescent intercalator is displaced by PIND-Ru. Ethidium bromide (EB), one of the most widely-used DNA threading intercalators, [20] fulfill these criteria and thus was used in this study [23]. The binding constant K_a, was found to be 4.0×10^7 M, corresponding to 10^2 enhancement over ND [24]. This considerable enhancement may be brought about by the much stronger electrostatic interaction. After intercalation of the ND group with ds-DNA, the two cationic $Ru(bpy)_2Cl^+$ groups form ion-pairs with phosphates on each side of the ds-DNA, reinforcing the stability of the intercalation. Similar stability enhancement has previously been observed [12]. The excellent binding properties prompted subsequent application of this intercalator in DNA detection.

3.4. Analytical applications of PIND-Ru in ultrasensitive DNA biosensors

PIND-Ru was evaluated as the electroactive indicator for possible applications in nucleic acid biosensors. In the first experiments, 2.0 μL aliquots of 200 nM of target DNA in TE were hybridized with their corresponding complementary PNA CP coated biosensors,

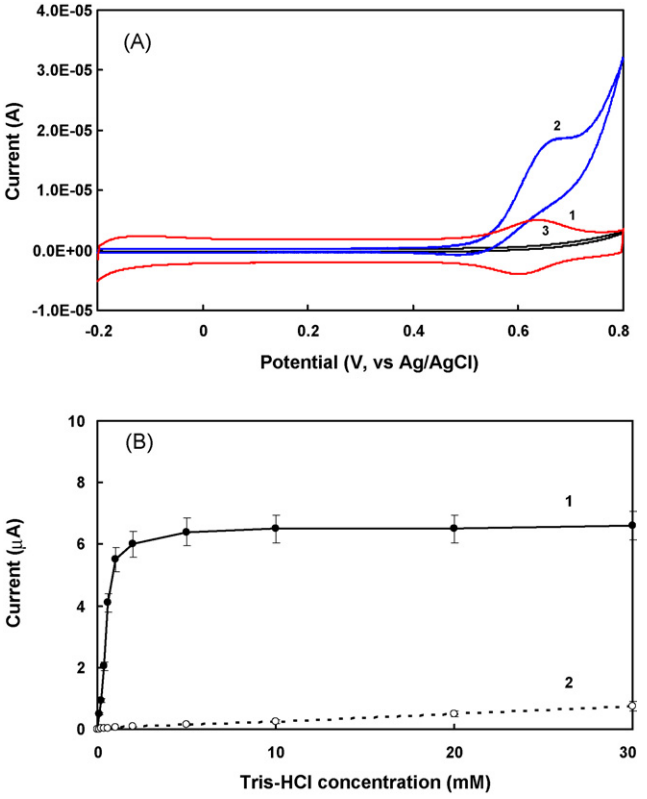

Fig. 4. (A) Cyclic voltammograms of a biosensor hybridized to 1.0 nM of target DNA in (1) PBS and (3) TE buffer; and (2) a control biosensor in TE buffer. Potential scan rate 100 mV s^{-1} for clarity, (1) was scaled up 10 times. (B) Effect of Tris–HCl concentration on the oxidation current in amperometry of (1) a biosensor and (2) the control. 1.0 nM target DNA, applied potential 0.65 V.

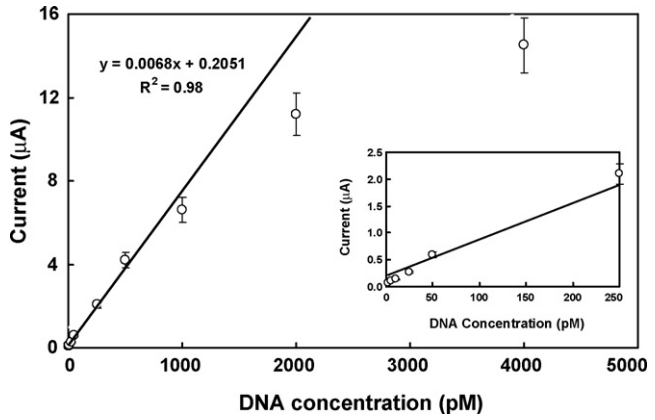

Fig. 5. Amperometric responses of different target DNA concentrations at 0.65 V in TE buffer. Insert: the amperometric responses at low concentration end.

respectively. For comparison, the same biosensors were treated with 2.0 μL aliquots of 200 nM of non-complementary DNA (control). Upon hybridization, the target DNA was selectively bound to its complementary CP and became fixed on the biosensor surface. A subsequent incubation with a 5.0 μL aliquot of 50 μg mL^{-1} of PIND-Ru in the hybridization solution brought PIND-Ru to the biosensor surface. It was found that extensive washing with NaCl saturated 10 mM phosphate buffer containing 10% ethanol removed most of the non-specific adsorption of PIND-Ru. A steady-state cyclic voltammogram of the biosensor hybridized to the complementary target DNA is shown in trace 1 in Fig. 4. A pair of current peaks at $E_{1/2}$ = 0.63 V, corresponding to the reversible redox reaction of Ru^{2+}/Ru^{3+}, were observed. These current peaks were more than 20 times higher than that of a non-complementary target DNA (not shown) and are proportional to the concentration of the target DNA, suggesting that the intercalating unit of the reporter molecules selectively interacts with the ds-DNA that has been formed upon hybridization of CP and the target DNA, giving a greatly enhanced analytical signal compared to non-hybridized CP. Cyclic voltammetric studies revealed a detection limit of 0.10 nM and a dynamic range up to 50 nM.

To further enhance the sensitivity of the biosensor, the hybridized biosensor was tested in PBS containing various electrochemically oxidazable substrates. It was found that primary and secondary amines can be electrocatalytically oxidized by PIND-Ru at 0.63 V, whereas electrochemical oxidation of amine at bare electrodes occurs at potentials between 0.90 and 1.2 V [14]. Since the hybridization buffer already contains a secondary amine, Tris–HCl, no additional substrate will be needed if Tris–HCl can be electrocatalytically oxidized by PIND-Ru, which simplifies the detection procedure. Indeed, Tris–HCl in the hybridization buffer can be efficiently oxidized at 0.63 V at the biosensor treated with PIND-Ru, as compare to >0.80 V at the same biosensor without PIND-Ru treatment (Fig. 4, traces 2 and 3). The current was enhanced by a factor of $\sim 10^3$ as compared to that of the control electrode at the same potential. And the cathodic current of PIND-Ru was largely suppressed (Fig. 4, trace 2). These results suggest that there is a very strong catalytic effect by PIND-Ru. The catalytic current was found to be pH dependent and the maximum catalytic current was obtained in the pH range of 8.2–9.5. Therefore, subsequent experiments were performed at pH 8.5. As expected, the electrocatalytic oxidation current is strongly dependent on the concentration of Tris–HCl. The oxidation current increased with increasing Tris–HCl concentration and plateaued beyond 5.0 mM (Fig. 4B, trace 1). It was observed that a high background current was observed at the control electrode when high Tris–HCl concentration (>20 mM) was employed, which significantly lower the signal-to-noise ratio (Fig. 4B, trace 2). Subsequent experiments were therefore conducted in the standard pH 8.5 TE hybridization buffer (10 mM Tris–HCl).

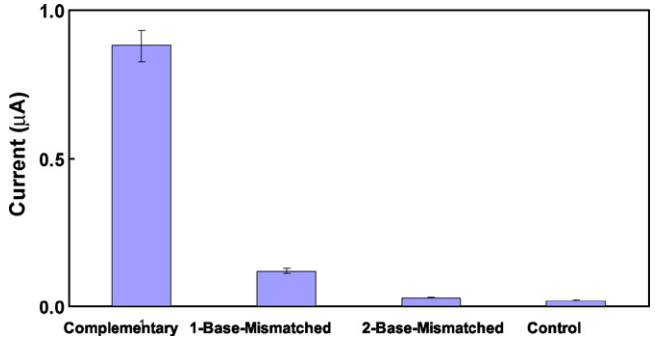

Fig. 6. Amperometric responses of biosensors hybridized with complementary and mismatched target DNAs at 100 pM. Applied potential 0.65 V, supporting electrolyte TE buffer.

Taking advantage of the successful electrocatalysis, amperometry was applied for the detection of targets DNA at lower concentrations. It was found that the catalytic current, observed in amperometry at 0.65 V, is proportional to the target DNA concentration in the range of 2.0 pM to1.0 nM (Fig. 5). A detection limit of 1.5 pM was found, 60-fold higher than that of the direct detection discussed in the previous section. Values of relative standard deviation were found to be 18% at 2.0 pM and 10% at 1.0 nM. Taking the sample volume into consideration, as little as 3.0 attomoles of DNA was successfully detected using the proposed method. Compared to the previous results of direct DNA oxidation assays, the sensitivity of the DNA assay was greatly improved by adopting the catalytic threading intercalator scheme.

To evaluate the capability of the proposed method in discriminating mismatches, three target DNAs, namely, complementary, one-base-mismatched, and two-base-mismatched, were tested at 100 pM. As shown in Fig. 6, the current increments of 0.12 and 0.030 μA were detected for one- and two-base mismatched target DNAs, respectively, whereas a 0.88 μA current increment was obtained for the fully complementary target. These results demonstrated the successful usage of a PIND-Ru in electrochemical DNA biosensor with improved sensitivity and selectivity.

4. Conclusion

In conclusion, the present work showed that PIND-Ru selectively interacts with ds-DNA with a much improved stability. The redox moieties of the interacted PIND-Ru showed excellent catalytic activity towards oxidation of amines. The dramatically improved signal-to-noise characteristics in amperometry and the high sensitivity are attributed to the effective catalytic oxidation of amine at the much reduced overpotential and high affinity of PIND-Ru towards ds-DNA. These findings are of particular significance for the design of ultrasensitive DNA biosensors and biosensor arrays. Such electrocatalytic amplification strategies could enable the development of a simple, low-cost and portable electrochemical detection system providing fast, cheap and simple solutions for molecular diagnosis, particularly for early cancer diagnosis, point-of-care and field uses. Efforts to fabricate electrochemical protein arrays for multiplexing and incorporate the array into a handheld electrochemical detector are currently underway.

Acknowledgment

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