An ultrasensitive nucleic acid biosensor based on the catalytic oxidation of guanine by a novel redox threading intercalator†

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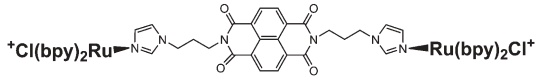
An ultrasensitive nucleic acid biosensor for the direct detection of attomoles nucleic acid in 1.0 μl droplets is described which can be used for detection of cancer marker genes in mRNA extracted from human breast tissues without a RT-PCR step.

Nucleic acid-based biosensors have potential applications that range from genotyping to molecular diagnostics.[1,2] The use of electrochemical techniques instead of fluorescence allows for simpler and smaller detectors.[3,4] The simplest of such systems would be direct electrochemistry of nucleic acid involving a solid electrode modified with an oligonucleotide probe that produces a measurable electrochemical signal upon hybridization of the immobilized probe to a specific target gene. However, it is generally believed that direct redox reaction of nucleic acid is irreversible and therefore requires a high overpotential, and often suffers from a pronounced fouling effect, resulting in rather poor selectivity and reproducibility.[5] Moreover, direct oxidation of water takes place at potentials close to that of nucleic acid oxidation and significantly lifts the background signal. The ability to directly detect nucleic acid selectively and sensitively has been a major goal of electrochemical research. A number of approaches have been proposed for direct electrochemical detection of nucleic acid.[3,6,7] In previous studies where transitional redox active metal complexes were used as homogenous catalysts, the analytical signal is superimposed onto an intrinsically large background current due to the oxidation of the catalyst and the catalytic oxidation of oligonucleotide capture probe (CP) by the catalyst.[6] Most of the catalytic oxidation current from CP can be eliminated by replacing guanine in CP with its analogues.[6] But little can be done to minimize the direct oxidation of the catalyst. For direct oxidation of nucleic acid, substantial improvements were achieved using the baseline-corrected adsorptive stripping square-wave voltammetry. As little as 15.4 fmol of nucleic acid was detected on a carbon paste electrode.[7] Our laboratory is interested in fabricating ultrasensitive nucleic acid biosensors using low redox potential biocatalysts/electrocatalysts as sensitivity enhancers.[8] The poor electron-transfer kinetics of nucleic acid can be addressed using nucleic acid oxidation catalysts and sensitive nucleic acid detection systems are expected. In addition, the low redox potential catalysts are beneficial in enhancing the detectability of nucleic acid owing to a minimized background current.[6] In this work, the feasibility of employing a novel low redox potential electrocatalytic 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) (Scheme 1) in an attempt to develop a sensitive electrochemical procedure for nucleic acid was studied. A remarkable improvement in the voltammetric response of nucleic acid and a noticeable enhancement of voltammetric sensitivity were observed. All of these were brought about by the combined catalytic function of the imidazole-complexed $[Ru(bpy)_2Cl]$ redox moieties and the high selectivity of PIND-Ru towards double-stranded DNA (ds-DNA). A cancer susceptibility gene, tumor protein p53 (TP53, 1182 bp) was selected as our target gene. Electrocatalytic signal was observed when as little as 7.5×10^{-18} moles (3.0 pg) of TP53 is present in the sample solution.

PIND was prepared following a general procedure for the synthesis of naphthalene diimide (ND).[9,10] 20-mer oligonucleotides were immobilized on the surface of each individual sensor and served as CPs. The sequences of the CPs are complementary to the sequence of the target gene in a region specific to that gene where no mutation is reported. TP53 in mRNA extracted from rat liver tissues was selected in this study. It was found that PIND-Ru intercalates very strongly to ds-DNA. To determine the mode of interaction of PIND-Ru with ds-DNA, UV-vis spectrophotometry of PIND-Ru in the presence of increasing amounts of salmon sperm DNA was investigated (Fig. 1A). In the UV-vis spectrophotometry, signatures of intercalative binding, where the fused planar aromatic ring system of a threading intercalator inserts itself between the base pairs of ds-DNA, are hypochromism and red shifts.[11] As shown in Fig. 1A, 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 ND absorbance band at 366 and 387 nm. Similar phenomena were previously observed with naphthalene diimide (ND) having aliphatic tertiary amine side chains.[11] The ND absorbance band hypochromism reached a plateau at the DNA base pair/PIND-Ru ratio >4.0, indicating that binding of PIND-Ru to ds-DNA takes place by preferential intercalation of the ND. To have a better estimation of the intercalating property, a competition experiment, similar to that proposed by Boger,[12] was designed using short hairpin oligonucleotides. A plot of the change in fluorescence *versus* equivalents of PIND-Ru provides a titration curve from which the stoichiometry of 1:1 was determined (Fig. 1B). The stability constant was found to be 3.0×10^7, corresponding to a ~75-fold enhancement over



Scheme 1 Structure of PIND-Ru.

† Electronic supplementary information (ESI) available: experimental details, synthetic procedures and characterization of PIND-Ru. See http://www.rsc.org/suppdata/cc/b4/b411803k/

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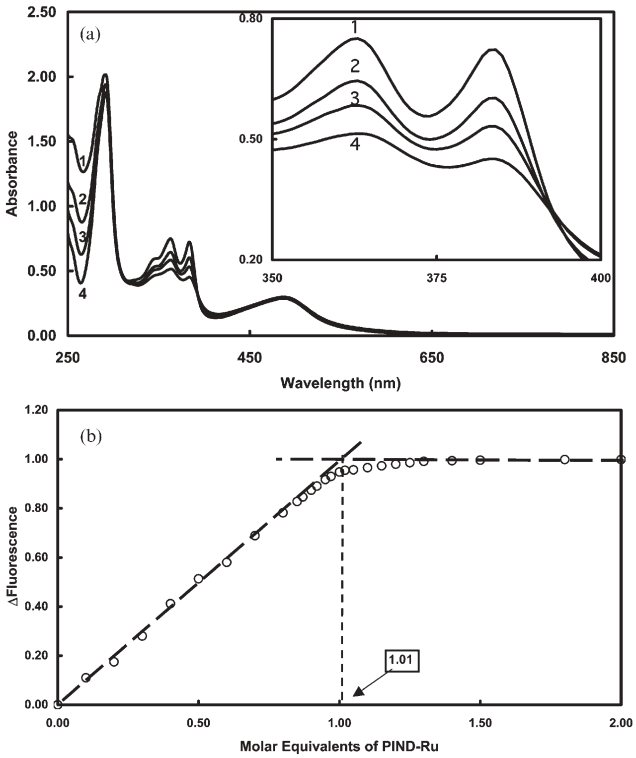

Fig. 1 **(A)** UV-Vis spectra of 25 μM PIND-Ru (resolution 0.10 nm) as a function of increasing concentration of salmon sperm DNA (in base pair) of (1) 0, (2) 25, (3) 50 and (4) 100 μM. **(B)** Fluorescent intercalator displacement titration curve of PIND-Ru against a hairpin oligonucleotide containing the sequence 5′-AATTT-CCCCC-AAATT. Insert: Enlarged UV-Vis adsorption spectra of the intercalative binding region.

ND. A plausible explanation for the stability enhancement would be that after the ND group has intercalated with ds-DNA, the two cationic $Ru(bpy)_2Cl$ groups in PIND-Ru form ion-pairs with phosphates on each side of the ds-DNA, making ND more tightly fixed in between the base pairs of ds-DNA.

PIND-Ru was evaluated as an electroactive indicator for possible applications in nucleic acid detection. Under optimized conditions, the oligonucleotides were selectively bound to their complementary CPs and became fixed on the biosensor surface upon hybridization. Thorough rinsing with the hybridization buffer washed off most of the non-hybridization related oligonucleotides. PIND-Ru was brought to the biosensor surface during a subsequent incubation with a 5.0 μl aliquot of 100 μg ml^{-1} of PIND-Ru in the hybridization solution. It was found that extensive washing with NaCl saturated 10 mM phosphate buffer removed most of the non-DNA related PIND-Ru uptake. Cyclic voltammograms for the biosensors after hybridization are shown in Fig. 2A. For the non-complementary $poly(T)_{40}$, one pair of minute voltammetric peaks were observed at the redox potential of PIND-Ru (0.62 V) after hybridization (Fig. 2A trace 1), largely due to pure electrostatic interaction between PIND-Ru and CP on the biosensor surface. For the complementary $poly(AT)_{20}$ $poly(AG)_{20}$ and $poly(G)_{40}$, slight positive shifts (8.0 ± 2.0 mV) in the redox potential were observed and the peak currents increased substantially (Fig. 2A traces 2, 3 and 4). The observed current, 0.30 μA after hybridization to 200 nM of $poly(AT)_{20}$, results therefore from 1.3 pmol of active and intercalated PIND-Ru. This

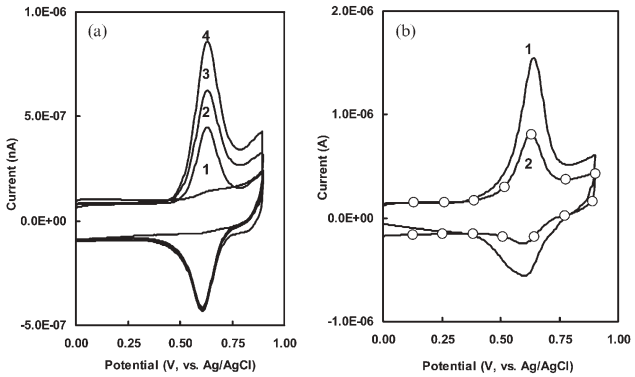

Fig. 2 **(A)** Cyclic voltammograms of 200 nM of (1) $poly(T)_{40}$ hybridized to a non-complementary CP coated electrode, and 200 nM of (2) $poly(AT)_{20}$, (3) $poly(AG)_{20}$ and (4) $poly(G)_{40}$ hybridized to their complementary CP coated electrode, respectively. Hybridization was performed in TE at 53 °C for 30 min under open-circuit condition. Intercalation was carried out with 5.0 μl aliquots of 100 μg ml^{-1} of PIND-Ru in TE. Voltammograms were recorded in PBS at a scan rate of 100 mV s^{-1}. **(B)** cyclic voltammograms of TP 53 hybridized to (1) perfectly-matched and (2) one-base-mismatched biosensors. Hybridization was carried out in 1.0 μg mRNA in TE. Potential scan rate 100 mV s^{-1}.

number represents <0.50% of PIND-Ru contained in the assayed droplet. Taking 1.2×10^{-11} mol cm^{-2} (midrange of the estimated values) as the surface CP coverage and assuming that a maximum PIND-Ru/base ratio of 1/4,[11] 0.13 pmol of the target DNA is hybridized. Thus, 13% of the target DNA and 15% of the surface-bound CP were actually hybridized, comparable to the values found in the literature.[13,14] Interestingly, when $poly(AG)_{20}$ and $poly(G)_{40}$ were hybridized with their corresponding complementary CP coated biosensors, noticeable increments in anodic current and slight decreases in cathodic current were observed (Fig. 2A traces 3 and 4). The increment increased almost linearly with increasing guanine content, indicating that guanine bases in the oligonucleotides are catalytically oxidized at 0.62 V by the intercalated PIND-Ru.[15] These results clearly demonstrated that PIND-Ru selectively interacts with ds-DNA and the PIND-Ru–ds-DNA adduct has a very slow dissociation rate, which paves the way for developing an ultrasensitive DNA biosensors. Better sensitivity is expected when working with genomic nucleic acid samples. Consequently, utilizing the intercalated PIND-Ru as the electroactive indicator for the direct detection of cancer susceptibility genes in mRNA was evaluated, taking the full length TP53 gene in mRNA as our target gene. Upon hybridization at 53 °C for 30 min, TP53 mRNA from the mixture was selectively bound to the biosensor surface. Thorough rinsing with the hybridization buffer washed off all of the non-hybridization-related mRNA. A typical cyclic voltammogram of the biosensor after applying PIND-Ru is shown in Fig. 2B. As seen in trace 1 in Fig. 2B, a considerably higher peak current was observed for the anodic process, indicating that a larger amount of electrons is involved in the oxidation process, most probably due to the captured long TP53 mRNA molecules that bring many more guanine bases to the biosensor surface. Integration of oxidation or reduction current peak at a low scan rate $\leqslant 10$ mV s^{-1} yields a surface coverage of 3.8 pmol in terms of electroactive Ru^{2+}/Ru^{3+} sites. The total amount of PIND-Ru, 1.9×10^{-11} mole cm^{-2}, is equivalent to

Table 1 QCM Data of CP coated quartz crystal resonators after hybridization to TP53 in 1.0 μg mRNA, and after PIND-Ru intercalation

CP Coated Resonator	TP53 Hybridization			PIND-Ru Intercalation			
	Δf (Hz)	Δm (ng)	Hybridized TP53 ($\times 10^{15}$ mole)	Δf (Hz)	Δm (ng)	PIND-Ru Loading ($\times 10^{12}$ mole cm^{-2})	PIND-Ru/Base Ratio
1	17	15	38	5.4	4.7	15	1/14
2	18	16	41	7.4	6.5	22	1/11
3	16	14	36	5.5	5.0	17	1/12
4	19	17	43	6.9	6.1	21	1/12

32% of the CP being hybridized and fully intercalated. To have a better understanding of the hybridization efficiency and PIND-Ru loading level, a series of QCM measurements were carried out on TP53 after hybridization, and after PIND-Ru intercalation. The results are summarized in Table 1. As shown in Table 1, ~40 fmoles of TP53 was hybridized. This number represents ~1.6% of the surface-bound CP was actually hybridized a much lower value than that of short oligonucleotides (20–50-mers) reported in the literature.[13,14] It is not surprising that the hybridization efficiency decreases drastically with increasing the size of the analyzed gene. In addition, the QCM experiments showed that one PIND-Ru molecule intercalated per 11–14 bases of TP53, suggesting that some of the PIND-Ru molecules intercalated into the secondary structure of TP53,[16,17] further enhancing the sensitivity of the method. PIND-Ru loading density was found to be in the range of 1.5–2.2 $\times$ 10^{-11} mole cm^{-2}, which is in good agreement with that obtained in voltammetric tests.

The dynamic range for the quantification of TP53 gene was established using purified cDNA transcribed from the mRNA of TP53 and diluted to different concentrations with TE buffer before use. For control experiments, non-complementary CPs were used in the biosensor preparation. It was found that the current increased linearly with cDNA concentration from 2.5 to 350 pM with a detection limit of 1.5 pM, corresponding to 0.60 ng ml^{-1}. Taking the sample volume into consideration, as little as 7.5 attomoles of TP53 cDNA was successfully detected using the proposed method. The selectivity of the biosensor was evaluated in 1.0 μg mRNA by analyzing one-base mismatched DNA under hybridization conditions set for the perfectly matched sequence. The current increment for the one-base mismatched was only ~40% of that for the perfectedly matched sequence (Fig. 2B trace 2), readily allowing discrimination between the perfectly matched and mismatched oligonucleotides. Compared to the previous results of direct nucleic acid oxidation assays, the sensitivity of genomic nucleic acid assay was greatly improved by adopting the catalytic threading intercalator scheme. The appeal of the non-labeling electrochemical method for the direct detection of nucleic acid is that genes from real-world sample which contains many of the redox active units (guanine and intercalated PIND-Ru) exhibit high sensitivity. The advantage of the PIND-Ru catalyzed system described here is that both guanine and PIND-Ru are oxidized at 0.62 V where little background current exists. Moreover, genes with more guanines give more sensitive signals. The combination of the two enables picomolar detection limit and a dynamic range up to 350 pM.

In conclusion, the findings in the present communication are of particular significance for the design of ultrasensitive non-labeling nucleic acid biosensors and biosensor arrays. Such ultrasensitive electrocatalysis strategies may 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.

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