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Label free electrochemiluminescence protocol for sensitive DNA detection with a *tris*(2,2′-bipyridyl)ruthenium(II) modified electrode based on nucleic acid oxidation

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Abstract

Label free electrochemiluminescence (ECL) DNA detection based on catalytic guanine and adenine bases oxidation using *tris*(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] modified glassy carbon (GC) electrode was demonstrated in this work. The modified GC electrode was prepared by casting carbon nanotubes (CNT)/Nafion/$Ru(bpy)_3^{2+}$ composite film on the electrode surface. ECL signals of double-stranded DNA and their thermally denatured counterparts can be distinctly discriminated using cyclic voltammetry (CV) with a low concentration (3.04×10^{-8} mol/L for Salmon Testes-DNA). Most importantly, sensitive single-base mismatch detection of p53 gene sequence segment was realized with 3.93×10^{-10} mol/L employing CV stimulation (ECL signal of C/A mismatched DNA oligonucleotides was 1.5-fold higher than that of fully base-paired DNA oligonucleotides). Label free, high sensitivity and simplicity for single-base mismatch discrimination were the main advantages of the present ECL technique for DNA detection over the traditional DNA sensors.

Keywords: Electrochemiluminescence; DNA sensors; Label free; Single base mismatch

1. Introduction

DNA detection is of great importance to, for example, DNA sequencing, profiling, clinical test and mutated genes diagnosis associated with human diseases [1–3]. In order to meet all these objectives, a number of analysis methods have been developed over past decades, including classical fluorescence techniques [4], mass spectrum [5], electrochemical means [3,6,7] and recently emerged DNA sensors based on nanotechnology [8,9].

Among all these methods, electrochemical protocols can provide quite significant advantages such as simplicity, sensitivity, selectivity and low cost for the detection of DNA hybridization and damage. Since Paleček's pioneer work about half a century ago [10], a lot of efforts have been devoted to the research of electrochemical DNA biosensors [11,12]. One of the most interesting electrochemical approaches for DNA detection based on Thorp group's pioneer work [13] is the catalytic oxidation of guanine bases using *tris*(2,2′-bipyridyl)ruthenium (II) [$Ru(bpy)_3^{2+}$] and its analogues [14].

Recently, considerable advances have been made in DNA biosensors using $Ru(bpy)_3^{2+}$ electrochemiluminescence (ECL) [15] detection protocol, owing to its inherent sensitivity, selectivity, and wide linear range in the utility in different analytical areas such as clinical tests and biomolecules detection [16–20].

Rusling et al. developed direct ECL detection of DNA in poly(vinylpyridine)[PVP] ultra-thin film using cationic polymer $[Ru(bpy)_2(PVP)_{10}]^{2+}$ or $[Os(bpy)_2(PVP)_{10}]^{2+}$, and

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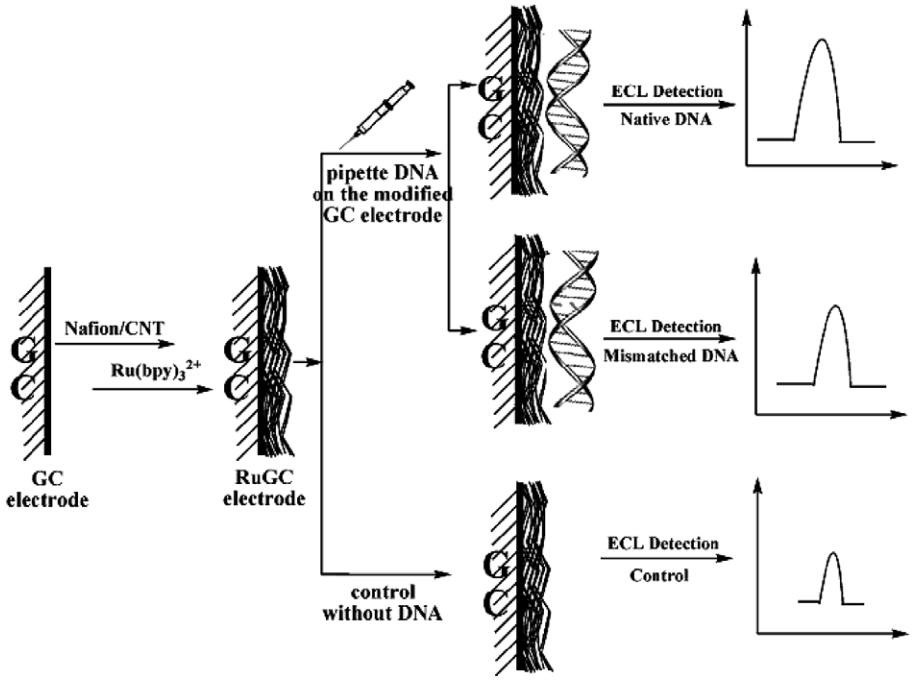

Fig. 1. Schematic diagram of GC electrode modification and DNA ECL detection procedures.

also chemically induced damage by styrene oxide of DNA [21,22]. Single-base mismatch detection, however, has not been reported through catalytic oxidation of DNA with an ECL detection on a modified electrode [23,24]. Landers' group recently reported an interesting quenching ECL method for quantitive and sequence-specific DNA detection [25]. By employing luminophore-active species as labels on biomolecules such as DNA and proteins, ECL opens a rapid, selective, and sensitive avenue for bioassay and detection. Bard's group employed $Ru(bpy)_3^{2+}$ as ECL label combined with tri-*n*-propylamine [TPrA] as a coreactant to determine DNA [19].

Here we presented a label free ECL protocol for DNA detection using carbon nanotubes (CNT)/Nafion/$Ru(bpy)_3^{2+}$ composite film modified glassy carbon electrode (abbreviated as RuGC) (Fig. 1). ECL signals can distinctly discriminate double-stranded Salmon Testes-DNA and their thermally denatured counterparts using cyclic voltammetry (CV) with a low concentration (3.04×10^{-8} mol/L). Moreover, sensitive single-base mismatch detection of p53 gene sequence segment was realized with 3.93×10^{-10} mol/L employing CV stimulation (ECL signal of C/A mismatched DNA oligonucleotides was 1.5-fold higher than that of fully base-paired DNA oligonucleotides).

2. Experimental

2.1. Chemicals and materials

Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate and Nafion (perfluorinated ion-exchange powder, 5 wt% solution in a mixture of lower aliphatic alcohols and water) were obtained from Aldrich (Milwaukee, WI, USA). The multi-wall carbon nanotubes (CNT) were purchased from Shenzhen Nanotech. Port. Co. Ltd. (Shenzhen, China) and were purified according to a literature method [26]. Other reagents and chemicals were at least analytical reagent grade. All aqueous solutions were prepared with water purified by a Milli-Q system (Millipore, Bedford, MA, USA) and stored at 4 °C in a refrigerator.

Salmon Testes (ST) double-stranded (ds) DNA were purchased from Sigma. Poly(guanadylic acid) (5′) poly[G], poly(adenylic acid) (5′) poly[A] and oligonucleotides containing specific sequences (see Table 1) were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China).

The concentrations of oligonucleotides were determined using the 260 nm UV absorbance and the corresponding extinction coefficient. The extinction coefficient of Salmon Testes DNA was taken as 6600 M^{-1} cm^{-1}. The extinction coefficients of single strands were calculated by the sum of the extinction coefficients of the individual bases: $\varepsilon(dA) =$ 15,400 M^{-1} cm^{-1}, $\varepsilon(dG) = 11{,}500$ M^{-1} cm^{-1}, $\varepsilon(dC) =$ 7400 M^{-1} cm^{-1}, $\varepsilon(dT) = 8700$) M^{-1} cm^{-1}.

Table 1
Oligonucleotides synthesized in this experiment

Sequence 1 (**Seq 1**)	5′ GCA GGG GC***C*** GCC GGT 3′ (primary sequence)
Sequence 2 (**Seq 2**)	5′ ACC GGC ***G***GC CCC TGC 3′ (complete complementary sequence)
Sequence 3 (**Seq 3**)	5′ ACC GGC ***A***GC CCC TGC 3′ (single-base mismatch complementary sequence)

2.2. Instrumentation

The electrochemical measurements were performed with a CHI 832 workstation (CH Instruments, Austin, TX, USA) using a three-electrode system. The working electrode was glassy carbon (Φ, ~1 mm) coated with the CNT/Nafion/$Ru(bpy)_3^{2+}$ composite film. A KCl-saturated Ag/AgCl electrode and a platinum wire electrode were used as the reference and the auxiliary electrode respectively. The ECL emission was detected with a Model MCFL-A Chemiluminescence Analyzer Systems (Xi'An Remax Science & Technology Co. Ltd., Xi'An, China). The voltage of photo multiplier tube used in Model MCFL-A Chemiluminescence Analyzer was set at 800 V in the process of detection. The ECL peak intensities were used for quantitative analysis in our study. For ST-DNA, the ECL peak intensities at 1.1 V were used for quantitative analysis (Fig. 2b). For oligonucleotides, the ECL peak intensities at 1.25 V were used for quantitative analysis (Fig. 4b).

Absorption spectra were recorded on a Cary 500 Scan UV–Vis-NIR Spectrophotometer (Varian, Harbor City, CA, USA) at room temperature.

2.3. Electrochemical and ECL detection of DNA

Details of the preparation and characterization of CNT/Nafion/$Ru(bpy)_3^{2+}$ composite film modified glassy carbon electrode (shortened as RuGC) has been published [27]. The prepared electrode was washed thoroughly with water and stored in buffer atmosphere before use.

In our experiment, DNA detection was realized as following procedure (Fig. 1): 5 μL of DNA sample solution was pipetted onto the RuGC electrode, the electrode was then allowed to dry in air atmosphere. The DNA pre-treated GC electrode was then placed in a conventional electrochemical cell containing 2 mL 10 mM acetate buffer solution (pH 5.50, 50 mM NaCl) to perform electrochemical and ECL measurement. Along with cyclic voltammograms (CVs) stimulation was performed, scanning from 0 to 1.35 V (vs. Ag/AgCl) at scan rate of 50 mV/s, the ECL signals were recorded with a photo multiplier tube.

To discriminate the ECL signals of native ST-DNA and thermally denatured ST-DNA, native double-stranded (ds) ST-DNA was pretreated by heating a native double-strand ST-DNA in a boiling water bath for about five minutes and then cooling immediately in an ice bath.

For single-base mismatch detection, p53 gene sequence segments (**Seq 1** and its complementary sequence (**Seq 2** or **Seq 3**)) were hybridized by heating the solution containing two single stranded oligonucleotides (equivalent molar) to 90 °C for 5 min and then cooled slowly to room temperature (1 ~ 2 h).

3. Results and discussion

3.1. Effect of pH on ECL intensity

It is known that catalytic DNA oxidation using $Ru(bpy)_3^{2+}$ undergoes a proton-coupled reaction pathway [28,29]. When ECL from $Ru(bpy)_3^{2+*}$ was used as a detection signal, the response of ECL is highly dependent on reaction buffer pH. In our present experiment, pH influence was investigated and optimized ECL response was gotten at pH 5.50 buffer solution (data are not shown here). So, 10 mM acetate buffer solution, pH 5.50, containing 50 mM NaCl was used in this study.

3.2. ECL and CV measurement of DNA

Previously reported electrochemical studies on DNA absorbed onto indium tin oxide electrodes used CV to measure catalytic guanine bases oxidation [30]. Here we employed CV and ECL to explore catalytic DNA oxidation using a modified RuGC electrode. Fig. 2 shows the electrochemical and corresponding ECL signals of the RuGC electrode with 3.04×10^{-8} mol/L ST-DNA (ds) in 10 mM acetate buffer containing 50 mM NaCl (pH = 5.50).

The catalytic enhancement in current and ECL intensity due to catalytic DNA oxidation is apparent in Fig. 2. Current response of native ds-DNA was about 1.17-fold larger when compared the current responses of the control buffer

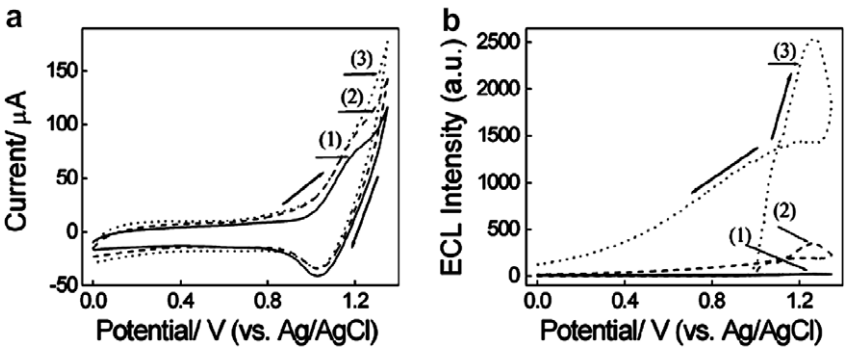

Fig. 2. CVs (a) and corresponding ECL intensity-potential curves (b) of RuGC electrode in the absence (line 1) and presence of 3.04×10^{-8} mol/L ST-DNA (ds) (line 2) and 3.04×10^{-8} mol/L thermally denatured ST-DNA (ds) (line 3) in 10 mM acetate buffer containing 50 mM NaCl (pH = 5.50). Scan rate = 50 mV/s.

solution (Fig. 2a, lines 1 and 2). The RuGC electrode with DNA gave electrochemical responses that were larger in the forward direction and smaller in the reverse direction while comparing with that of the electrode without DNA (Fig. 2a, lines 1 and 2), which is a typical characteristic of electrocatalytic process. It can be seen clearly that in the presence of DNA, ECL signals were much higher comparing with responses of the RuGC electrode in buffer solution only (Fig. 2b, lines 1 and 2). Further, continuous scanning of the RuGC electrode containing DNA produced much smaller currents and ECL signals on the second scan, and by the fifth scan, the signals had decreased to the values of, within experimental error, the RuGC electrode in buffer solution without DNA. This result indicates that the electrochemical catalytic oxidation of nucleic acid is irreversible.

3.3. ECL and CV measurement of thermally denatured DNA

Previous work has demonstrated that single-stranded (ss) DNA is much more easily oxidized than double-stranded (ds) DNA using $Ru(bpy)_3^{2+}$as catalytic reagents [21,22,31]. Here we further investigated the difference of electrochemical and ECL signals between native ds-ST-DNA and thermally denatured ds-ST-DNA. As shown in Fig. 2, both electrochemical and ECL signals of native ds-ST-DNA (line 2) were lower than that of thermally denatured ds-ST-DNA (line 3). The reason of these different responses of native ds-ST-DNA and thermally denatured ds-ST-DNA can be explained as follows: the double-helix structure of native ds-ST-DNA can shield purine bases from close contact with catalyst $Ru(bpy)_3^{2+}$. Meanwhile, thermal treatment of native ds-ST-DNA can cause unwinding of the double-helix structure, thus leading to the efficient contact of purine bases with catalyst $Ru(bpy)_3^{2+}$.

Further analysis indicated that ECL signals gave a more efficient discrimination than electrochemical signals did. ECL intensity of thermally denatured ST-DNA (ds) was about 5-fold larger than their native analogues (Fig. 2b, lines 2 and 3). Discrimination of electrochemical responses, however, was not so apparent comparing that of ECL. As shown in Fig. 2a, CV response of thermally denatured ST-DNA (ds) is slight higher than that of their native counterparts. These results suggest that ECL yield is sensitive to the hybridization state of oligonucleotides, which is a key feature of base mismatches detection [3,6,8].

The different discriminatory ability of CV and ECL can be explained as follows: For CV signals, the background current originates from $Ru(bpy)_3^{2+}$, and the catalytic current originates from co-reactant (DNA in our study) and $Ru(bpy)_3^{2+}$. Since $Ru(bpy)_3^{2+}$is an electrochemical active molecule, the background current is relative large compared with the catalytic current of DNA and $Ru(bpy)_3^{2+}$. Thus the current signal difference between the $Ru(bpy)_3^{2+}$alone and the DNA and $Ru(bpy)_3^{2+}$is small. For ECL signal, the background ECL signal originates from the reaction of $Ru(bpy)_3^{2+}$and H_2O, and the catalytic ECL signal originates from co-reactant (DNA in our study) and $Ru(bpy)_3^{2+}$. In this case, H_2O is not an ECL active molecule while DNA is an ECL active molecule, and the ECL signal is relative small compared with the catalytic ECL signals of DNA and $Ru(bpy)_3^{2+}$. Thus the ECL signal difference between the $Ru(bpy)_3^{2+}$ alone and the DNA and $Ru(bpy)_3^{2+}$ is larger than the current signal difference between the $Ru(bpy)_3^{2+}$ alone and the DNA and $Ru(bpy)_3^{2+}$.

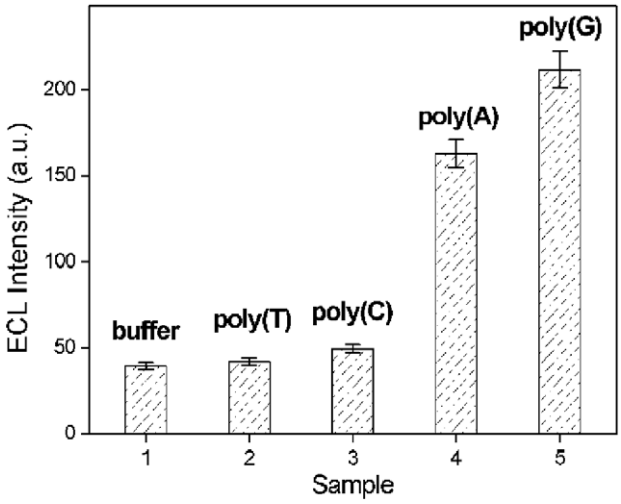

Fig. 3. ECL responses of RuGC electrode in the absence (sample 1) and presence of 8.34×10^{-10} mol/L poly[T] (sample 2), 8.34×10^{-10} mol/L poly[C] (sample 3), 8.34×10^{-10} mol/L poly[A] (sample 4) and 8.34×10^{-10} mol/L poly[G] (sample 5) in 10 mM acetate buffer containing 50 mM NaCl (pH = 5.50).

3.4. ECL responses of DNA bases

As Rusling et al. reported previously, catalytic DNA oxidation experiment using ECL detection in ultra-thin film containing $[Ru(bpy)_2(PVP)_{10}]^{2+}$ or $[Os(bpy)_2(PVP)_{10}]^{2+}$ appears to involve mainly the guanine bases [21,22]. To further confirm that, here we investigated the ECL response of poly[G], poly[A], poly[C] and poly[T] with the RuGC electrode in 10 mM acetate buffer solution. As shown in Fig. 3, both poly[G] and poly[A] gave higher ECL responses while poly[C] and poly[T] gave nearly the same ECL responses when comparing that in the controlled pure buffer solution. Quantitative analysis results of the ECL intensity indicates that poly[A] gives ca. 4-fold larger ECL signal than that in the controlled buffer solution while poly[G] gives ca. 5.5-fold larger ECL signal. This result suggests that the two purine bases both guanine and adenine in DNA can generate ECL signals, although the majority of the catalytic oxidation reactions involve the guanine bases [13,14,21,22,32].

3.5. Single-base mismatch detection with ECL

Many of human diseases are connected with a single-base mutation, so a lot of study has been devoted to the detection of such a small change in DNA duplex. Discrim-

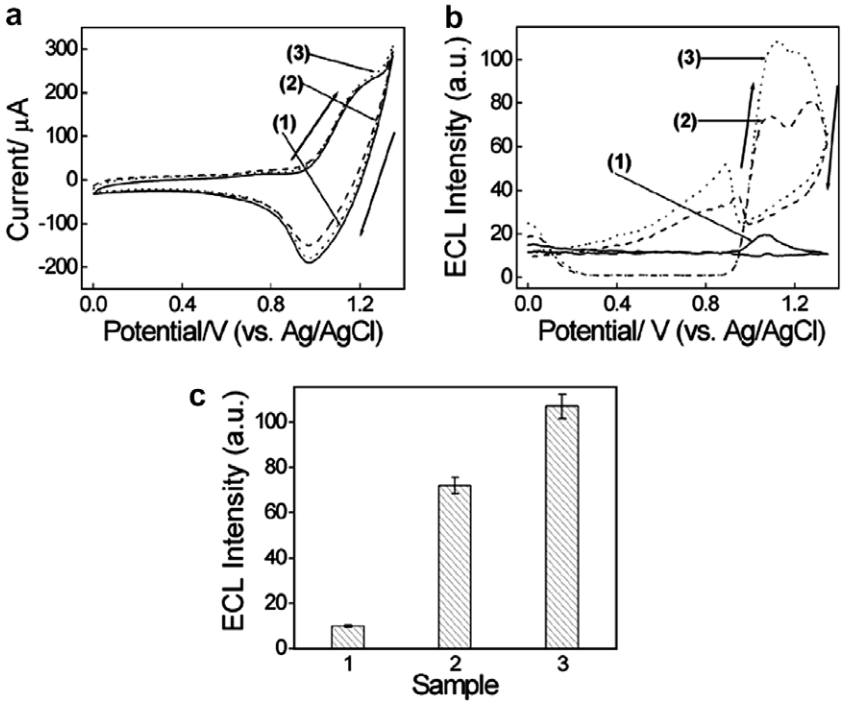

Fig. 4. CVs (a) and corresponding ECL intensity-potential curves (b) of RuGC electrode in the absence (line 1) and presence of 3.93×10^{-10} mol/L oligonucleotides containing 5′ GCA GGG GCC GCC GGT 3′ (**Seq 1**) hybridized to its fully base-paired complement (**Seq 2**) (line 2) and complement introduced with single-base mismatches (**Seq 3**) (line 3) in 10 mM acetate buffer containing 50 mM NaCl (pH = 5.50). Scan rate = 50 mV/s. (c) Quantitative data of ECL intensities obtained from panel b (sample 1: buffer solution; sample 2: **Seq 1** hybridized to its fully base-paired complement **Seq 2**; sample 3: **Seq 1** complement introduced with single-base mismatches **Seq 3**).

ination of single-base mismatch has been studied extensively using electrochemical methods [3,12]. Thorp et al. had effectively distinguished single-base mismatches via DNA and $Ru(bpy)_3^{2+}$ interaction [13]. Based on the above-mentioned ECL detection of difference between native ds-ST-DNA and thermally denatured ds-ST-DNA, we felt that this strategy could be used for analysis of base-mismatches, which also disrupt the native DNA double-helix structure. In this study, we realized single-base mismatches detection of p53 gene sequence segment with ECL method through hybridization protocol. Mutations of p53 gene, which is a tumor suppressor gene, have been found at high frequency in many human cancers [33]. Hybridization was completed by treating oligonucleotides containing p53 gene sequence segment (**Seq 1**) and its complementary counterparts (**Seq 2** and **Seq 3**, see Table 1).

Fig. 4 shows the combined CV and ECL results of the single-base mismatch detection. CV here can hardly distinguish single-base mismatches clearly. ECL in this study, however, is capable of discriminate the single-base mismatch at a low concentration (3.93×10^{-10} mol/L). Shown in Fig. 4c is the quantitative ECL data. *C*/*A* mismatch comparing with *C*/*G* Watson-Crick base pairing gave a 1.5-fold larger ECL intensity. The calibration curve of oligonucleotides containing p53 gene sequence segment (**Seq 1**) hybridized to its fully base-paired complement (**Seq 2**) was linear from 3.93×10^{-9} mol/L to 1.965×10^{-7} mol/L with $R^2 = 0.9990$ (Fig. 5).

3.6. Interpretation for the ECL signal at 0.8 V

As shown in Figs. 2b and 4b, ECL signals were observed also at about 0.8 V. The ECL signal in this region could be assigned to $Ru(bpy)_3^{3+}$ long time stability and relative insufficient DNA in the composite film: When CV stimulation was applied to the system, $Ru(bpy)_3^{2+}$ was oxidized to $Ru(bpy)_3^{3+}$ on the electrode at ca 1.0 V, then $Ru(bpy)_3^{3+}$

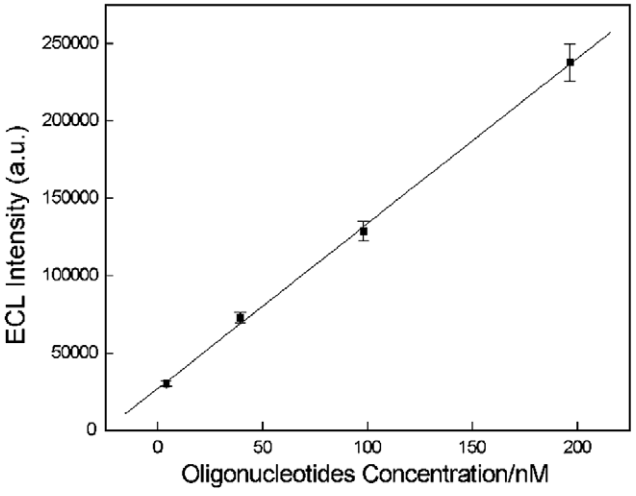

Fig. 5. Calibration curve of oligonucleotides containing 5′ GCA GGG GCC GCC GGT 3′ (**Seq 1**) hybridized to its fully base-paired complement (**Seq 2**) in 10 mM acetate buffer containing 50 mM NaCl (pH = 5.50).

could further be reduced with co-reactant DNA to produce excited state $Ru(bpy)_3^{2+*}$. ECL signal was obtained when $Ru(bpy)_3^{2+*}$ decays to the ground state $Ru(bpy)_3^{2+}$ (see the Scheme below).

$$Ru(II)_{CNT/Nafion} \rightarrow Ru(III)_{CNT/Nafion} + e^- \quad (1)$$

$$Ru(III)_{CNT/Nafion} \rightarrow Ru(II)_{CNT/Nafion} + G^{\bullet} \quad (2)$$

$$G^{\bullet} + Ru(III)_{CNT/Nafion} \rightarrow G_{ox} + Ru(II)^{*}_{CNT/Nafion} \quad (3)$$

$$Ru(II)^{*}_{CNT/Nafion} \rightarrow Ru(II)_{CNT/Nafion} + h\nu\ (615\ nm) \quad (4)$$

$Ru(bpy)_3^{2+}$ was immobilized on the electrode surface through ion exchange, therein $Ru(bpy)_3^{2+}$ with relative high concentration in composite film was obtained. When CV scanned forward, the onset of luminescence occurred at ca. 1.0 V, at which $Ru(bpy)_3^{2+}$ was oxidized to $Ru(bpy)_3^{3+}$. The ECL signal arose deeply when $Ru(bpy)_3^{3+}$ reacted with DNA and a ECL peak at 1.2 V was observed. As for the backward scanning, since the co-reactant DNA had been consumed during the forward scanning and was insufficient in the composite film due to diffusion limit [27,34], the un-reacted $Ru(bpy)_3^{3+}$ could not be changed into $Ru(bpy)_3^{2+*}$ efficiently in our system since $Ru(bpy)_3^{3+}$ was stable for 6 hours in aqueous system [35]. Thus a broad ECL peak originated the reaction of $Ru(bpy)_3^{3+}$ with H_2O could be obtained at about 0.8 V during the backward scanning.

4. Conclusions

In summary, an interesting label free protocol for sensitive DNA detection using RuGC electrode combining ECL technique and guanine and adenine bases catalytic oxidation was demonstrated. ECL signals of double-stranded DNA and their thermally denatured counterparts can be easily distinguished with a low concentration (3.04×10^{-8} mol/L for ST-DNA). Most importantly, sensitive single-base mismatch detection of p53 gene sequence segment was realized with 3.93×10^{-10} mol/L employing CV stimulation (ECL signal of C/A mismatched DNA oligonucleotides was 1.5-fold higher than that of fully base-paired DNA oligonucleotides). Label free, high sensitivity and simplicity for single-base mismatch discrimination were the main advantages of the present ECL technique for DNA detection over the traditional DNA sensors. In principle, such label free detection protocol demonstrated in this work would meet the criteria for sensitive, selective, and reagentless DNA assay.

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