Electrochemical DNA Biosensor Improved by Imidazo[4,5-f]1,10-phenanthroline Iron(III) as an Indicator based on Pt-nanoparticles and Carbon Nanotubes Modified Electrode

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A novel and sensitive electrochemical DNA biosensor has been developed for the detection of DNA hybridization. The biosensor was proposed by using imidazo[4,5-*f*]1,10-phenanthroline iron(III) (Fe(phen)$_2$IP·3ClO$_4$·2H$_2$O, abbreviated as [Fe(phen)$_2$IP]$^{3+}$), as an electroactive indicator based on multi-walled carbon nanotubes/platinum nanoparticles (MWCNTs/Ptnano) modified glassy carbon electrode (GCE). The interaction between [Fe(phen)$_2$IP]$^{3+}$ and double-stranded DNA (dsDNA) was investigated by cyclic voltammetry (CV). The experiment results showed that [Fe(phen)$_2$IP]$^{3+}$ presented an excellent electrochemical activity on GCE and could intercalate into the double helix of double-stranded DNA. The target ssDNA of the human hepatitis B virus (HBV) was quantified in a linear range from 1.13×10^{-11}-1.13×10^{-9} M (r = 0.9892) with a detection limit of 5.56×10^{-12} M (3σ, n = 11).

Key Words: Fe(phen)$_2$IP·3ClO$_4$·2H$_2$O, Carbon nanotubes, Pt-nanoparticles, DNA Hybridization, Electrochemical DNA biosensor.

INTRODUCTION

In recent years, DNA biosensor has become more and more important, particularly with the advances in genetics, criminology, pathology, pharmacogenetics, food safety and many other fields. Due to its intrinsic specificity, the hybridization between complementary single-stranded DNA (ssDNA) chains has been used as a bio-recognition event in the design of a great number of DNA biosensors. This recognition event has been applied in various ways, such as optical, mechanical or electrochemical[1,2]. Compared to other methods, electrochemical DNA biosensors are attractive devices for their abilities of converting DNA hybridization event into an analytical electronic signal to obtain sequence-specific information and are applied in clinical, environmental or forensic investigations[3]. While immobilization of DNA probes onto the electrode surface has always been the focus of the preparation of electrochemical DNA biosensors. DNA can be immobilized on sensor surfaces with methods similar to those used for enzyme-based biosensors: adsorption, covalent immobilization and avidin (or streptavidin) biotin interaction[4]. Many workers have studied the immobilization of DNA on electrodes surfaces by a wide variety of methods[5]. Recently, the modification of electrochemical sensors with carbon nanotubes (CNTs) has attracted considerable attention in the field of DNA sensing technology, because such modification can drastically improve the effective surface area of the electrodes and carbon nanotubes are rather chemically stable and well conductive, so that carbon nanotubes-modified electrodes always show high performance in electrochemical analysis, especially high sensitivity. Many different schemes for electrochemical DNA sensing based on carbon nanotubes have been reported[6-10].

Many attempts have been conducted to deposit various metal particles, such as platinum, palladium, copper, nickel or ruthenium, on the surface of carbon nanotubes[11-13]. The hybrid materials could potentially display the combined properties of nanocrystals and carbon nanotubes and also additional novel properties[14-16] to increase the sensitivity.

On the other hand, the binding mechanisms between DNA and various redox substances, namely metal complexes[17,18], anticancer or antivirus drugs[19] and organic dyes[20,21], have been extensively investigated. In our laboratory, many efforts were also conducted to explore the interaction between metal complexes and DNA[22-23]. It has been found that phenanthroline based metal complex has a wide range of biochemical and pharmacological effects including antioxidation, antiinflammation, antiplatelet, antithrombotic action and antiallergic effects, which is widely used as the intercalators for its planar structure[24-26].

In this piece of work, carboxyl-functionalized multiwall carbon nano-tubes (MWCNTs-COOH) modified with Pt nanoparticles and Fe(phen)$_2$IP·3ClO$_4$·2H$_2$O were utilized in

the DNA electrochemical biosensor. Pt nanoparticles was combined to the MWCNTs-COOH with the help of nafion by electrostatic force. Oligonucleotide probes with an amino group at the 5'-phosphate end can form covalent bonds with the Pt nanoparticles on the MWCNT-COOH/Pt_{nano}/GCE modified GCE. A phenanthroline base complex, imidazo [f]1,10-phenanthroline ferrum(III) ([Fe(phen)$_2$IP]·3ClO$_4$·2H$_2$O) has been synthesized and characterized used as an indicator for the detection of hybridization between the probe DNA and the target sequence. This novel sensor and the related procedure would be of lower cost and more convenience compared to many DNA sensors developed in the past decade while still possess relatively high selectivity and sensitivity.

EXPERIMENTAL

Multi-walled carbon nanotubes (with a diameter of about 40-60 nm and length of around 1-10 µm) with carboxylic groups were obtained from Shenzhen Nanotech Co. Ltd. (Shenzhen, China). Nafion-perfluorinated ion-exchange resin (5 wt. %), sodium dodecyl sulfate (SDS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide solution (EDC) and *N*-hydroxysuccinimide solution (NHS) were purchased from Sigma. Salmon sperm DNA was purchased from Shanghai Huashun Biological Engineering Company ($A_{260}/A_{280} > 1.8$), the concentration was determined by the ultraviolet absorption at 260 nm ($\varepsilon = 13200$ M^{-1} cm^{-1}).

24-Base synthetic ssDNA were purchased from SBS Genetech Company (Beijing China): 24-base probe sequence(S1): 5'-NH2-GAG CGG CGC AAC ATT TCA GGT CGA-3'. Target sequence(S2): 5'-TCG ACC TGA AAT GTT GCG CCG CTC-3' a non-complementary sequence (S3): 5'-GAG CGG CGC AAC ATT TCA GGT CGA-3'.

All ssDNA were dissolved in TE buffer (10 mM *tris*-HCl, 1 mM EDTA, pH8.0) and kept frozen. Other chemicals employed were all of analytical reagent grade and deionized water was used throughout the experimental work.

The cyclic voltammetric and differential pulse voltammetric measurements were performed with a CHI 832 electrochemical analyzer (Shanghai Chenhua Instrument Company, China). The three-electrode system consisted of a glassy carbon working electrode, an Ag/AgCl reference electrode (saturated KCl) and a counter electrode made of platinum. All electrochemical measurements were conducted in a 10 mL cell at room temperature. A pHS-3D digital acidometer (Rex Electric Chemical Products Department, Shanghai Precision Scientific Instrument Co. Ltd.) were used.

Synthesized of Fe(phen)$_2$IP·3ClO$_4$·2H$_2$O: Imidazo[4,5-*f*] [1,10] phenanthroline (IP) was synthesized according to literature[27]. A 0.058 g IP was added to a 12 mL hot solution dissolved 0.068 g FeCl$_3$·6H$_2$O and 0.100 g 1,10-phenanthroline (phen). The mixture was refluxed for 5 h with continuous stirring, which was then allowed to cool at room temperature. After 1 mL sodium perchlorate solution (2.5 M) was added, the colour of the solution turned to crimson immediately and solid precipitate appeared. The precipitate was recrystallized from hot water and the crimson crystal of [Fe(phen)$_2$IP]·3ClO$_4$·2H$_2$O was obtained finally. Elementary analysis: calc. (found) for [Fe(phen)$_2$IP]·3ClO$_4$·2H$_2$O: C: 44.13 (44.25); H: 3.2 (3.07);

N: 11.1 (11.18). The formula of the [Fe(phen)$_2$IP]$^{3+}$ is shown in **Scheme-I**.
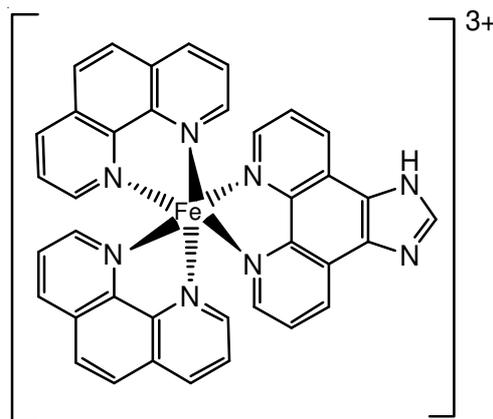
Scheme-I: Structure of [Fe(phen)$_2$IP]$^{3+}$

Preparation of platinum nanoparticles and modification electrode: Platinum nanoparticles (Pt_{nano}) were prepared according to the literature[28]. In brief, H$_2$PtCl$_6$·6H$_2$O (4 mL 5 % aqueous solution) was added to deionized water (65 mL) and heated to 80 ºC with stirring in a 100 mL flask. After adding 12 mL of sodium citrate (1 % aqueous solution), the resulting solution was maintained at 80 ± 0.5 ºC for about 4 h. The course of the reduction was followed by absorption spectroscopy and the end of the reaction was marked by the disappearance of the absorption bands of PtCl$_6^{2-}$ (Fig. 1).
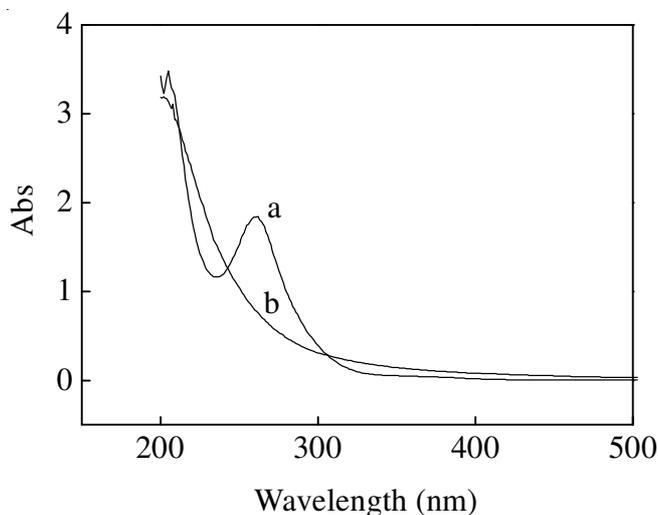
Fig. 1. Absorption bands of PtCl$_6^{2-}$ before (curve a) and after (curve b) reaction

Glassy carbon electrode was carefully polished with polishing paper and subsequently with alumina until a mirror finish was obtained. After 5 min of sonication to remove the alumina residues, the electrode was immersed in concentrated H$_2$SO$_4$ for 3 min followed by thorough rinsing with water and ethanol. The electrode was then transferred to the electrochemical cell for cleaning by cyclic voltammetry between -0.5 and +1.2 V (versus Ag/AgCl) in 50 mM phosphate buffer, pH 7.2, until a stable cyclic voltammetric profile was obtained. The prepared electrode was dried and use immediately for modification. An

amount of 2 mg of MWCNTs were dissolved in a mixture of 100 µL of naflon and 900 µL of Pt_{nano} solution[29], as the stock solution. After about 40 min of sonication, uniformly dispersed MWCNTs and Pt_{nano} were formed. GCE was then modified by a 5 µL drop of MWCNTs-COOH/Pt_{nano} and dried in air. After the electrode was thoroughly rinsed with water, the MWCNTs/Pt_{nano}-modified electrodes were prepared.

Immobilization of ssDNA on MWCNTs-COOH/Pt_{nano}-modified GCE: The immobilization of ssDNA probe on the MWCNTs-COOH/Pt_{nano} modified GCE was carried out as follows: The MWCNTs-COOH/Pt_{nano} modified GCE was immersed in a 30 mM acetate buffer (pH 5.2) containing 10 mM EDC for 1 h. The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)-attached electrode was washed with acetate buffer and subsequently incubated in a 2.25×10^{-5} M oligonucleotide solution containing 0.1 M EDC and 10 mM acetate buffer (pH 5.2) for 12 h with stirring at room temperature. Then the electrode was washed with a 0.2 % sodium dodecyl sulfate phosphate buffer (pH 7.3) for 5 min to remove the unbound DNA probes and prevent the nonspecific binding. Thus the oligonucleotide probe was immobilized through the formation of amide bonds between the -COOH on the MWCNTs and -NH$_2$ of the ssDNA at 5' end.

Hybridization and electrochemical detection: Hybridization reaction was carried out by immersing the ssDNA probe captured MWCNTs-COOH/Pt_{nano}/GCE into a stirred hybridization solution (0.3 M PBS buffer) containing different concentration of DNA target for 40 min at 37 ℃. After that, the electrode was rinsed three times with a 0.2 % sodium dodecyl sulfate phosphate buffer (pH 7.3) to remove the non-hybridized target DNA.

Intercalation of hybridization indicator: The accumulation of [Fe(phen)$_2$IP]$^{3+}$ on the hybridized electrode surface was performed by immersing the electrode into 0.20 M B-R buffer solution (pH 5.0) containing 2.0×10^{-4} M [Fe(phen)$_2$IP]$^{3+}$ for 20 min at room temperature. Then, the electrode was washed with ultrapure water to remove the remnant [Fe(phen)$_2$IP]$^{3+}$ on the electrode surface.

Electrochemical detection: The electrochemical detection of hybridization was performed in a 10 mL of electrochemical cell with hybridized working electrode, an Ag/AgCl electrode and a platinum wire counter electrode. The differential pulse voltammetric measurements were conducted from 0.70-1.2 V (*versus* Ag/AgCl) in a 0.1 M phosphate buffer (pH 7.3). The peak current related to the [Fe(phen)$_2$IP]$^{3+}$ at about + 0.94 V was taken as the electrochemical measurement signal.

The procedure of the operation of the sensor, including the modification of MWCNT/ Pt_{nano} immobilization, hybridization and detection of DNA, was illustrated in **Scheme-II**.

RESULTS AND DISCUSSION

Cyclic voltammetry study of the interaction between [Fe(phen)$_2$IP]$^{3+}$ and dsDNA: Appropriate amount of dsDNA and [Fe(phen)$_2$IP]$^{3+}$ was mixed and incubated in 0.20 M B-R buffer (pH 5.0) for 20 min at 25 ℃. The interaction characterization of [Fe(phen)$_2$IP]$^{3+}$ with dsDNA was performed by cyclic voltammetrics. As to cyclic voltammetric scanning, the potential scanning range was from 1.2 to 0.4 V; the scanning

rate was 0.20 V s^{-1}; the sample interval was 0.001 V and the quiet time was 2 s (Fig. 2). Electrochemical study on [Fe(phen)$_2$IP]$^{3+}$ and its interaction with dsDNA were performed. There were a couple of quasi-reversible redox peaks for [Fe(phen)$_2$IP]$^{3+}$ (curve a). The anodic peak potential $E_{pa} = 0.949$ V, cathodic peak potential $E_{pc} = 0.870$ V; Curve b was the voltammogram obtained when $C_{DNA} : C_{[Fe(phen)2IP]^{3+}} = 1 : 1$, $E_{pa} = 0.952$ V, $E_{pc} = 0.876$ V; curve c was the voltammogram obtained when $C_{DNA} : C_{[Fe(phen)2IP]^{3+}} = 5 : 1$, $E_{pa} = 0.956$ V, $E_{pc} = 0.881$ V. The peak potential separation (ΔE_p) was 9 mV and its formal potential (E°´) was 0.919 V. Pang and Abruna[30] had reported the discrimination of binding modes between small molecules and DNA. If E°´ shifted to more negative value, the interaction mode was electrostatic binding. On the contrary, if E°´ shifted to more positive value, the interaction mode was intercalative binding. Therefore, we attributed the interaction between [Fe(phen)$_2$IP]$^{3+}$ and dsDNA to be intercalation.
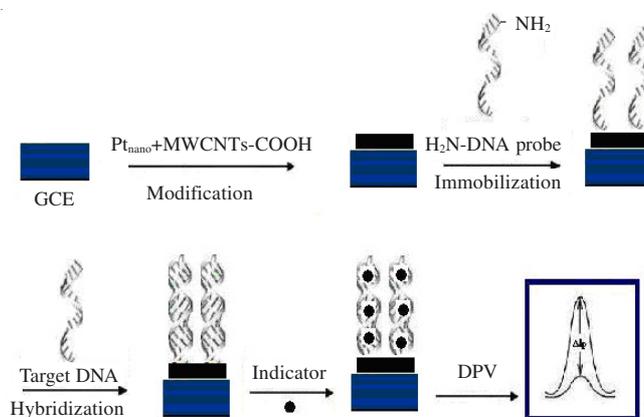
Scheme-II: Schematic representation of the electrochemical detection of DNA hybridization based on platinum nanoparticles combined with MWCNTs
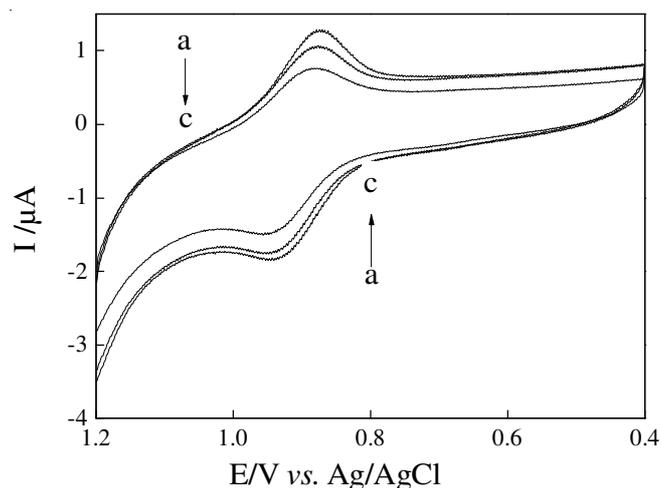
Fig. 2.   CVs of [Fe(phen)$_2$IP]$^{3+}$; C[Fe(phen)$_2$IP]$^{3+}$: 0.20 mM; CDNA: (a) 0, (b) 0.20 mM, (c) 1.00 mM

Optimization of experimental parameters: The influence of experimental conditions, including the pH of 0.20 M B-R buffer and the reaction time, were explored for optimum analytical performance. pH 5 was adopted because the maximum peak current change (before and after addition of dsDNA) was obtained. After incubation of 20 min the peak

current reached a constant value, therefore, 20 min was chosen as the reaction time. The hybridization time has also been studied as shown in Fig. 3, which reveals the optimum hybridization time is 40 min.
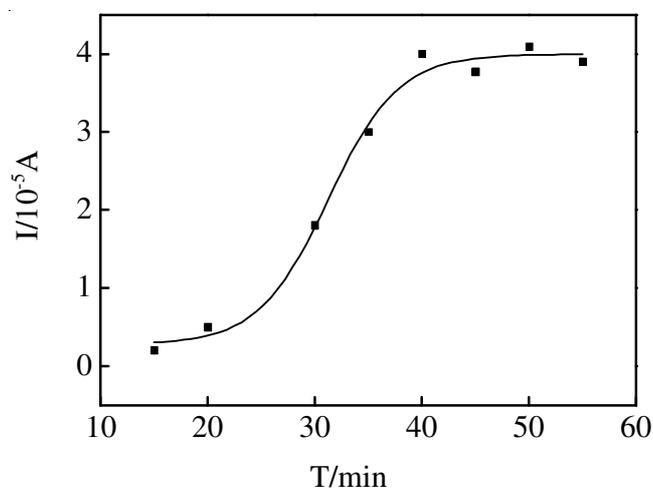
Fig. 3 Effect of the hybridization time on peak current

Electrochemical characteristics of MWCNT-COOH/ Pt_{nano}/GCE: Cyclic voltammetry was employed to MWCNT-COOH/P_{tnano}/GCE from 0.6 to 1.1 V. The electrochemical responses of bare GCE (curve a), MWCNTs-COOH/GCE (curve b) and MWCNT-COOH/Pt_{nano}/GCE (curve c) in 2.00×10^{-4} M [Fe(phen)$_2$IP]$^{3+}$ were shown in Fig. 4. Compared with the bare GCE and the MWCNT-COOH/Pt_{nano}/GCE, the background current of the MWCNTs-COOH/Pt_{nano}/GCE was apparently large, showing that modification of the electrode with MWCNT-COOH/Pt_{nano}/GCE has the best electrical properties while the enlarged electrode surface area could significantly enlarge effective binding for ssDNA.
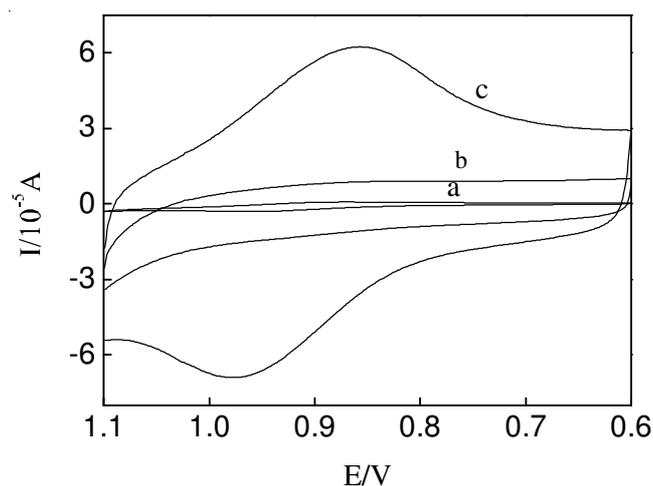
Fig. 4. Cyclic voltammetric curves of [Fe(phen)$_2$IP]$^{3+}$ on different electrodes; (a) GCE; (b) MWCNTs-COOH/GCE; (c) MWCNTs-COOH/Pt_{nano}/GCE

Selectivity of the prepared electrochemical DNA biosensor: [Fe(phen)$_2$IP]$^{3+}$ was used as DNA hybridization indicator for the detection of a 24-*mer* oligonucleotide which is specific for hepatitis B virus and the selectivity of this assay was investigated. The differential pulse voltammetric curves

obtained in 0.20 M B-R buffer (pH 5.0) were shown in Fig. 5, on MWCNTs-COOH /Pt_{nano}/GCE with probe ssDNA (S$_1$) immobilized (curve a), after the hybridization of complementary ssDNA (S$_2$) (curve c), after the applying of non-complementary ssDNA (S$_3$) (curve b) respectively. Each measurement was performed after [Fe(phen)$_2$IP]$^{3+}$ was sufficiently accumulated and was repeated on at least three GCEs and the mean value of the three closest readouts was adopted as the measurement result. It was found that there was only a small differential pulse voltammetric peak response of [Fe(phen)$_2$IP]$^{3+}$ for S$_1$ modified MWCNTs-COOH/Pt_{nano}/GCE, indicating that only negligible [Fe(phen)$_2$IP]$^{3+}$ was bound to ssDNA (S$_1$) on modified MWCNTs-COOH/Pt_{nano}/GCE. A significant increase in the differential pulse voltammetric signal was observed after the ssDNA modified MWCNTs-COOH /Pt_{nano}/GCE was hybridized with the complementary ssDNA (S$_2$) (curve c). In addition, as expected, there was no significant current difference observed before and after the non-complementary ssDNA (S$_3$) was applied on ssDNA probe (S$_1$) modified MWCNTs-COOH/Pt_{nano}/GCE (curve b), indicating that the non-specific absorption was negligible. A good selectivity was thus obtained for the novel electrochemical DNA biosensor.
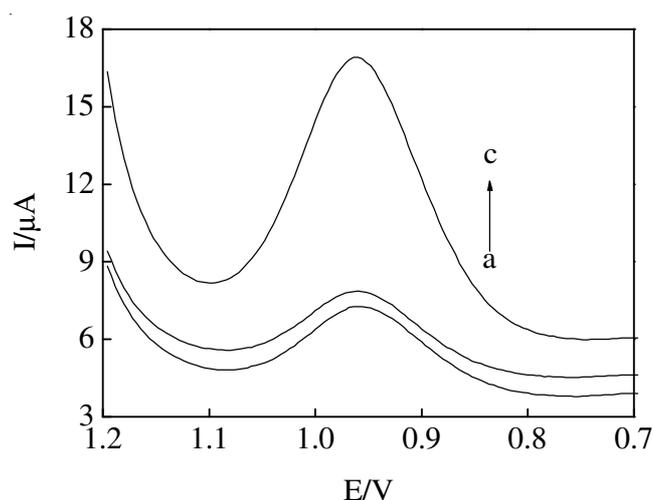
Fig. 5. DPV response of the [Fe(phen)$_2$IP]$^{3+}$ recorded on MWNTs/Pt_{nano}/GCE; (a) probe (S$_1$); (b) hybridized with 1.60×10^{-8} M S$_3$; (c) hybridized with 1.60×10^{-8} M S$_2$; Amplitude: 50 mV; pulse period: 0.2 s; pulse width: 60 ms

Sensitivities for the detection of target ssDNA: The sensitivity of the prepared DNA electrochemical sensor was investigated using differential pulse voltammetric method. The differential peak currents were measured against various concentrations of target ssDNA (Fig. 6). The cathodic peak currents difference was in linear relation with the concentration of target ssDNA in a range from 1.13×10^{-11}- 1.13×10^{-9} M. The regression equation was found to be $\Delta I_{pc} = 2.1804$ μA $+ 0.6390$ (CS$_2$/10^{-9} M) μA with a correlation coefficient of 0.9892. A detection limit of 5.56×10^{-12} M for the target ssDNA was estimated using 3σ (where σ is the standard deviation of the blank solution, n = 11).

Conclusion

Interaction between [Fe(phen)$_2$IP]$^{3+}$ and double-stranded salmon sperm dsDNA was studied using cyclic voltammetry.
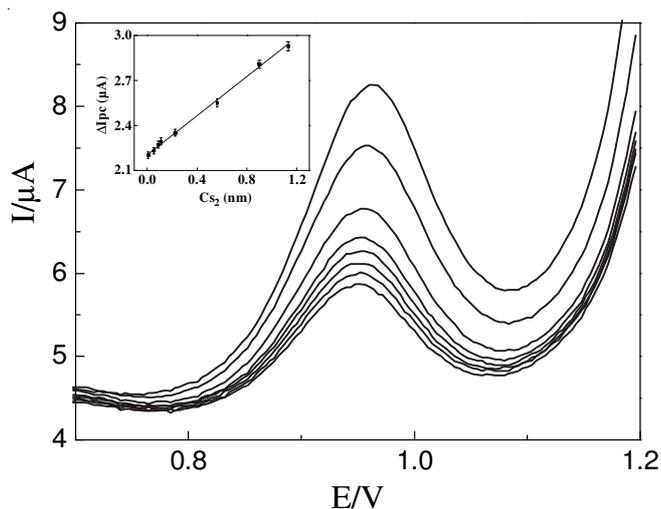
Fig. 6. Relationship curves obtained with different amounts of complementary target DNA

It was shown that $[Fe(phen)_2IP]^{3+}$ could be intercalated into double helices of the dsDNA. MWCNTs-COOH/Pt_{nano} were used to prepared a novel covalent electrochemical DNA biosensor for the enhancement of sensitivity of sequence-specific ssDNA detection with a detection limit of 5.56×10^{-12} M and a linear range from 1.13×10^{-11} M-1.13×10^{-9} M.

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