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Facile electrochemical biosensor based on a new bifunctional probe for label-free detection of CGG trinucleotide repeat

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ABSTRACT

We have developed a simple and label-free electrochemical assay to detect CGG trinucleotide repeat. For this purpose, a new bifunctional probe (FecNCD2) was developed, in which a recognition part (naphthyridine carbamate dimmer, NCD) was connected with an electro-active part (ferrocenyl group) using a chain of –CO–NH–CH_2–CH_2–. The results of circular dichroismic measurements indicated that FecNCD2 exhibited a superior performance for selective binding to CGG trinucleotide repeats compared to a previous bifunctional electrochemical probe connected with shorter linker –CH_2– (FecNCD1). Then, the electrochemical properties of FecNCD2 were evaluated and were found to show a good redox response due to the ferrocene moiety. Owing to the high performances of FecNCD2, the label-free electrochemical biosensor for CGG repeats was constructed by immobilizing them onto gold disk electrode and by using FecNCD2 as an electrochemical probe in solution. Further CGG repeats in solution were confirmed to be detectable using the CGG modified biosensor in competitive experiments, i.e., by treating it in test solutions containing FecNCD2 and d$(CGG)_{10}$ or others. No interference of ct-DNA on the CGG detection was also confirmed with this approach. The strategy should have significant potential for the development of versatile and low-cost biosensor for early diagnosis and treatment of neurodegenerative diseases associated with trinucleotide repeats.

1. Introduction

Considerable interest has been attracted to the development of electrochemical methods for detecting nucleic acid due to portable and affordable device (Drummond et al., 2003; Gooding, 2002; Palecek and Fojta, 2005). The development of electrochemical sensor for DNA hybridization (Wang, 2000, 2002; Gooding, 2002) represents up-to-date hot topics not only for electrochemists, but also for chemical biologists, molecular biologists and some medicine involved in modern biomedicine. In particular, the detection of specific sequences of nucleic acids is necessary in diverse fields including molecular diagnosis of certain bacterial and viral infections as well as human genetic diseases.

In the electrochemical detection, ferrocene-labeled DNA has been applied for hybridized target DNA induced a large change in electrochemical signal. As an example, it was been used for detection of special sequences of nucleic acids such as mismatch DNA and aptamers (Khor et al., 2011). Recently metal nanoparticles or quantum dots labeled DNA (Ye et al., 2011) have been widely applied to detect DNA hybridization electrochemically. However, there are still some technological limitations in the detection, which is mainly due to time-consuming, expensive and complicated synthesis labeling of nucleic acid.

Thus, DNA biosensors have been developed with alternative methods, such as introducing nanomaterial and external redox active molecules as electron transfer mediator. Indirect electrochemical DNA sequences detection is mainly based on electrochemical active molecules such as organic dyes and metal complexes, (Kara et al., 2002; Tani et al., 2001; Ju et al., 2003; Millan and Mikkelsen, 1993; Levicky et al., 1998; Yu et al., 2003; Cusumano et al., 2006; Erdem et al., 1999; Zhu et al., 2004; Wang et al., 1996; Li et al., 2006), but with low selectivity. A report dealt

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with the detection of abasic site DNA using small molecules with electroactivity (Morita et al., 2006). However, for the purpose of facile electrochemical detection, the design of probes should be desirable. The advanced design would lead to highly selective and sensitive detection for nucleic acid special sequence, especially those responsible for certain types of disease such as neurodegenerative diseases.

A number of neurodegenerative diseases were caused by the expansion of trinucleotide repeats (Pearson and Sinden, 1998; Campuzano et al., 1996; Paulson and Fischbeck, 1996; Wells, 1996; Sutherland and Richards, 1995), which has prompted a considerable amount of researches regarding the structure, biology and sequence detection of trinucleotide repeats. To date, more than 12 human genetic diseases including Myotonic dystrophy, Fragile X syndrome, Huntington disease, several spinocerebellar ataxias, and Friedreich ataxia, have been found to be associated with the expansion of trinucleotide repetitive sequences such as CTG, CGG, or GAA repeats (Pearson and Sinden, 1998a; Campuzano et al., 1996). The $d(CGG)_n$ trinucleotide expansion in the coding sequence of the FMR1 (Fragile X mental retardation 1) gene causes Fragile X syndrome disease, which leads to the gradual damage of specific areas of the brain (Fu et al., 1991). Some researchers had studied binding molecules and expansion of CGG trinucleotide acid and biological property. However, the studies on electrochemical detections of trinucleotide repeats are very limited. Yang and Thorp (2001) demonstrated the electrochemical detection of CGG trinucleotide repeats using electrocatalysis by $Ru(bpy)_3^{2+}$. Palecek and coworkers proposed electrochemical DNA hybridization assay for GAA trinucleotide repeats by multiply osmium-labeled reporter probes (Fojta et al., 2004a, 2004b).

We have previously reported that the synthetic naphthyridine carbamate dimer (NCD) stabilizes the DNA containing CGG/CGG triad including $d(CGG)_n$ trinucleotide repeats (Peng and Nakatani, 2005; Hagihara et al., 2012). These studies have indicated that the selective binding of naphthyridine dimmer to guanine-rich special DNA sequences was due to full complementary hydrogen bonding between the naphthyridine group and guanine base. In these studies, all interactions were investigated mainly by gel electrophoresis and optical methods such as fluorescence, UV–vis, CD and surface plasmon resonance. Furthermore, since there is a strong demand for a simple and efficient approach to realize early gene and prenatal diagnoses and to detect hereditary neurological diseases, we have explored feasibility of detecting GG mismatch DNA by the electrochemical method with FecNCD1 (Scheme 1) endowing NCD electrochemical activity active and on the basis of different diffusion coefficients of free and bound FecNCD1 (He et al., 2013). Although successful results were obtained with FecNCD1, for early diagnosis and treatment of Fragile X syndrome disease, it would be very important to develop a probe which has ability to selectively detect CGG trinucleotide acid and can be used for a facile electrochemical detection.

Therefore, in the present work, we developed a simple, label-free electrochemical biosensor to detect CGG trinucleotide repeat by synthesizing a new bifunctional electrochemical probe (FecNCD2), which contained a recognition part of NCD and an electrochemically active ferrocenyl group (Scheme 1). The selective binding of this probe to CGG trinucleotide repeats would lead to significant and selective changes in electrochemical signals (Fig. 1). The new approach represents an important strategy for the development of electrochemical sensors for DNA detection, which should provide further insight into the early diagnostics and therapies of special sequence responsible diseases.

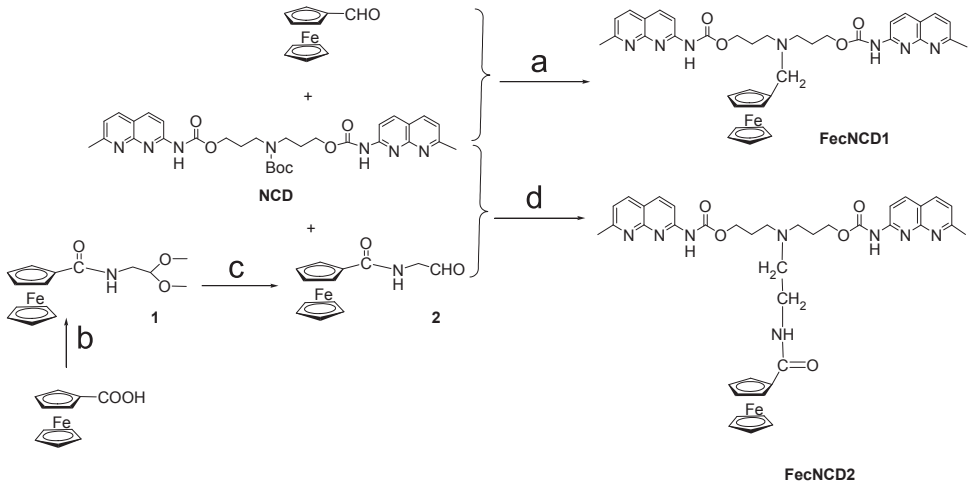
2. Experimental

2.1. Synthesis of N-(2, 2-dimethoxyethylamine) ferrocenyl formamide (1)

Ferrocenecarboxylic acid (115.02 mg, 0.50 mmol), HBTU (379.30 mg, 1.00 mmol) and DIPEA (520 mL, 3.00 mmol) were dissolved in 10 mL CH_2Cl_2, and stirred for 1 h in ice bath. Then 2, 2-dimethoxyethylamine (54.50 mL, 0.50 mmol) was added to the mixture, and the mixture was stirred for 4 h at ambient temperature. The mixture was washed sequentially with saturated $NaHCO_3$ and H_2O before drying over Na_2SO_4 and filtering off Na_2SO_4. The filtrate was evaporated to dryness in vacuo in a rotary evaporator. The residue was purified by silica gel column chromatography on a $1 \times 20\ cm^2$ column using 300 mL of 100:2 (v/v) $CHCl_3/CH_3OH$ (R_f=0.5, 30:1 v/v $CHCl_3/CH_3OH$) to get pale-yellow solid. Yield, 140.9 mg, 88.85%. ^1H NMR (600 MHz, $CDCl_3$, δ): 4.67 (*s*, 2H), 4.46 (*t*, 1H, *J*=6 Hz), 4.34 (*s*, 2H), 4.20 (*s*, 5H), 3.51 (*t*, 2H, *J*=6 Hz), and 2.79 (*s*, 6H). ESI-MS: $[M^+]$ calcd for $C_{15}H_{19}FeNO_3$, 317.16; found 317.9.

2.2. Synthesis of N-(2-oxoethyl) ferrocenyl formamide (2)

Compound 1 (158.58 mg, 0.50 mmol) and PPTS (502.00 mg, 2.00 mmol) were dissolved in 20 mL acetone/H_2O (v/v, 7/3) and stirred for 5 h while refluxing. The mixture was washed



Scheme 1. Synthesis of bifunctional electrochemical probes: FecNCD1 and FecNCD2. (a) (1) 4 M HCl/ethyl acetate, $CHCl_3$ and (2) $NaBH(OAc)_3$, CH_2Cl_2, refluxing, 6 h. (b) HBTU, DIPEA, CH_2Cl_2, 2, 2-dimethoxy-ethylamine, ice-bath, then RT. (c) PPTS, acetone/H_2O, 7/3, refluxing. (d) The reaction conditions were the same as those of (a).

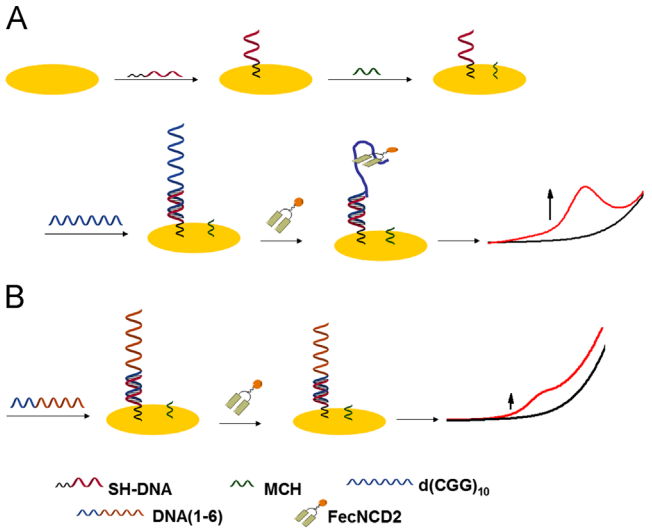


Fig. 1. Schematic diagram of trinucleotide repeat DNA detection by FecNCD2 with trinucleotide repeat DNA-fabricated Au electrode. (A) CGG trinucleotide repeats (blue wave line) modified biosensor would present an obvious electrochemical signal after incubation with FecNCD2 because of the binding between FecNCD2 with CGG trinucleotide repeats. (B) The biosensors modified by other trinucleotide repeats (orange and blue wave lines) would show no or less apparent electrochemical signal due to poor binding of FecNCD2 with other repeats.

sequentially with saturated $NaHCO_3$ and H_2O before drying over Na_2SO_4 and filtering off Na_2SO_4. The filtrate was evaporated to dryness in vacuo on a rotary evaporator. The residue was purified by silica gel column chromatography on a $1 \times 20\ cm^2$ column using 300 mL of 100:2 (v/v) $CHCl_3/CH_3OH$ (R_f=0.6, 20:1 v/v $CHCl_3/CH_3OH$) to get pale-yellow solid for the next step directly. Yield, 141 mg, 52.01%.

2.3. Synthesis of FecNCD2

1.5 mL 4 M HCl/EA was added into NCD (0.30 mmol, 180 mg) and 8 mL $CHCl_3$ solution in iced water, and the mixture was stirred for 1 h at room temperature. The mixture was evaporated in vacuo in a rotary evaporator to get deprotected product. The pH of the solution of aldehyde 2 (60 mg, 0.22 mmol) and the deprotected product (0.71 g, 0.28 mmol) in 10 mL dehydrated CH_2Cl_2 was adjusted to approximately 6 by adding triethylamine or acetic acid. Sodium triacetoxyborohydride $NaBH(OAc)_3$ was added to the solution, and the mixture was stirred for overnight while refluxing. The solvent was evaporated, and the product was extracted with chloroform and aqueous $NaHCO_3$. The combined organic layer was washed with water and dried over Na_2SO_4. The solvent was evaporated and the crude product was purified by column chromatography on silica gel chloroform/methanol 10/1. R_f is 0.5 (5:1 v/v $CHCl_3/CH_3OH$). Yield of pale yellow solid, 53.92%. ^1H NMR (600 MHz, $CDCl_3$, δ): 8.26 (*d*, 2H, J=4.2 Hz), 8.11 (*d*, 2H, J=9 Hz), 7.97 (*d*, 2H, J=7.8 Hz), 7.26 (*d*, 2H, J=8.4 Hz), 6.48 (*s*, 1H), 4.75 (*s*, 2H), 4.35 (*t*, 4H, J=6.6 Hz), 4.31 (*s*, 4H, Fc), 4.20 (*s*, 5H, Fc), 3.46 (*s*, 2H), 2.75 (*s*, 6H), 2.64 (br, 6H), and 1.92 (*t*, 4H). $^{13}C\{H\}$NMR (150 MHz, CH_3OD, δ): 170.22, 162.93, 154.13, 153.17, 153.09, 138.87, 136.23, 122.13, 117.76, 112.50, 77.20, 76.99, 76.78, 76.12, 70.17, 69.50, 67.93, 63.47, 53.07, 49.71, 36.98, 29.50, 26.37, and 25.40. ESI-MS: [$(M+H)^+$] calculated for $C_{39}H_{42}FeN_8O_5$, 759.2; found, 758.65. HR-FAB-MS: [$(M+H)^+$] calculated for $C_{39}H_{42}FeN_8O_5$, 759.2706, found, 759.2715.

2.4. Circular dichroismic spectral studies

Circular dichroismic (CD) experiments were performed at a scanning speed of 500 nm/min on a Jasco-810 spectropolarimeter (Jasco, Easton, MD, USA) at room time temperature. The CD spectral of trinucleotide repeats (2.5 μM) were measured from 200–400 nm wavelength with different concentrations of probes in 10 mM PBS (pH 7.0, 100 mM NaCl). A buffer blank correction was performed for all spectra.

2.5. Measurements of melting temperature

Melting curves were performed on a Chirascan circular dichroism spectrophotometer (Applied Photophysics), and the software Prism 4 was used to calculate the T_m values. The absorbance of trinucleotide repeats sequence (2.50 μM) in 10 mM phosphate buffer solution (with 100 mM NaCl, pH 7.0) was monitored at 260 nm from 20 °C to 94 °C with a heating rate of 1 °C/min in the absence and presence of ligand (50 μM).

2.6. Electrochemical measurement

All electrochemical measurements including cyclic voltammogram (CV), square wave voltammetry (SWV) and electrochemical impedance spectroscopy (EIS) were performed with a conventional three-electrode system. Au electrode (2.0-mm in diameter, CH Instruments) was used as the working electrode, a saturated calomel electrode (SCE) and a platinum wire were used as the reference electrode and the auxiliary electrode respectively. Cyclic voltammogram (CV) was recorded in 10 mM PBS (pH 7.0) at a scan rate of 0.1 V s^{-1} in a potential range of −0.1–0.7 V. Square wave voltammograms (SWV) were recorded in 10 mM PBS in a potential range of −0.1–0.65 V with a modulation amplitude of 4 mV, a step potential of 25 mV, and a frequency of 15 Hz. All impedance spectra were performed using AC modulation of the frequency range from 0.1 Hz to 100,000 Hz in a 1 M KNO_3 solution containing 0.5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1).

2.7. Immobilization of DNA and preparation of the biosensor

Prior to immobilization, Au electrodes (2 mM in diameter, CH Instruments) were polished successively with alumina slurries (0.1–1 μM) in Milli-Q water on polishing cloths. After polishing, the electrodes were rinsed with Milli-Q water several times before sonicating once in ethanol and then twice in Milli-Q water for 5 min. Then, it was electrochemically cleaned in 0.5 M sulfuric acid by cycling the electrode potential 10 times between −0.1 and +1.5 V vs. SCE, continuously rinsed in Milli-Q water for 5 min two times. The cleaned gold surface was incubated for 12 h in a solution containing 50 μL of 2.0 μM thiolated DNA (5′-HS–$(CH_2)_6$–GGC CAC GAG TTG ACA-3′; HS-DNA). A monolayer of 6-mercapto-1-hexanol (MCH) was formed by immersing the electrode into a 1 mM MCH aqueous solution for 90 min in order to minimize the nonspecific adsorption of target DNA or probe molecules. The SH-DNA and MCH modified Au electrode was incubated in 4.0 μM trinucleotide repeat DNA solutions at 65 °C in water bath for 5 min. Then the solution, in which the electrode was soaked, was taken out from water bath and cooled down slowly in room temperature for 1 h to allow the hybridization. Finally, the modified electrode was incubated continually in the trinucleotide repeat DNA solutions at 3–5 °C for another 7 h for better hybridization. In order to minimize the physical adsorption of trinucleotide repeat DNA, the modified electrode was rinsed with a flow of PBS buffer (10 mM, pH 7.0) for a few seconds and then soaked in PBS buffer for 5 min. Finally, the DNA-modified electrode was incubated with a 20 μM FecNCD2 in PBS for 30 min at room temperature. The electrode was rinsed with a PBS prior to electrochemical detection in order to remove non-specifically bound of FecNCD2 with DNA. The fabrication of the modified Au electrode is schematically shown in Fig. 1.

3. Results and discussion

3.1. Design and synthesis of bifunctional probe, FecNCD2

Due to the advantages of electrochemical analysis, a bifunctional probe containing recognition part for CGG trinucleotide repeats and an electrochemically active part were designed by conjugating ferrocenyl group with a recognition molecule (NCD). In our previous work, FecNCD1 with a short –CH_2– link between NCD and ferrocene was synthesized (Scheme 1) for the selective detection of GG mismatch in the solution (He et al., 2013). However, the introduction of the ferrocenyl group would change the steric configuration, which probably affects the selective binding. As the binding ability and selectivity of the previous FecNCD1 with the short linker toward CGG trinucleotide repeats in DNA were not sure to have better performances or not, in the present work. FecNCD2 that possesses a longer –CO–NH–CH_2–CH_2– linker was designed and synthesized. As shown in Scheme 1(b)–(d), N-(2-oxoethyl) ferrocenyl formamide was condensed with deprotected hydrochloride salt NCD, and then sodium triacetoxyborohydride reduction to give FecNCD2, which was fully confirmed by mass spectra, ^{1}H NMR, ^{13}C{H} NMR, and HR-FAB-MS.

3.2. Optical detection of the interaction

Previous studies have revealed that NCD binds to a CGG/CGG triad selectively through the complementary hydrogen bonding between naphthyridine and guanine bases (Peng and Nakatani, 2005; Hagihara et al., 2012). After ferrocene combined with NCD, the molecular structure FecNCD should have some change compared to free NCD, which would cause some steric hindrance for the binding. Empirically, a longer link should have a smaller steric effect for the recognition domain, resulting in a better binding performance. In order to explore the selectivity, the biophysical and biochemical properties of some trinucleotide repeats in the presence of FecNCD1 and FecNCD2 were investigated by thermal melting profiles and circular dichroism (CD).

Thermal melting profiles did not exhibit typical sigmoidal shape (as shown in Fig. S1), whereas the melting profile of the free d$(CGG)_{10}$ showed an inflection point about 82 °C, which exhibited an intrinsic hairpin structure. The stability of d$(CGG)_{10}$ was enhanced in the presence of FecNCD1 and FecNCD2 due to no obvious inflection point. Because of the uncertain thermal melting profiles, the selective binding was difficult to discuss here.

On the other hand, CD of some trinucleotide repeats in the absence and presence of the probes provided strong evidences for the selective bindings. The CD spectra of free d$(CGG)_{10}$ (Fig. 2A) showed a slightly positive broad signal at around 280 nm, and a negative signal at 255 nm, which indicated an unclear intrinsic hairpin structure. However, distinct changes in CD signals were observed in the presence of FecNCD2 with a significantly increasing positive peak at 249 and 260 nm, an increasing negative peak at 230 nm and another positively induced band around 342 nm. The results indicated that the secondary structure of d$(CGG)_{10}$ was significantly altered and was more stable due to binding of FecNCD2. By contrast, with increasing concentration of FecNCD1, slight signal changes were found without any induced CD band (Fig. 2B). Consequently, the results indicated well that FecNCD2 having the longer linker, possessed the better binding for d$(CGG)_{10}$ trinucleotide repeats.

An important issue is whether FecNCD2 had selective binding for different kinds of trinucleotide repeats. Therefore, the CD spectra of various types of trinucleotide repeats corresponding diseases were investigated in the presence of FecNCD2, as shown in Figs. S2–S7. The CD spectra of d$(CCG)_{10}$ indicated an apparent intrinsic secondary structure, whereas the signal intensity slightly decreased with increasing concentration of FecNCD2 (Fig. S2), which indicated that the presence of FecNCD2 made the secondary structure of d$(CCG)_{10}$ slightly unstable. For d$(GAA)_{10}$ (Fig. S3), the binding of FecNCD2 stabilized the secondary structure, but the degree was much weaker compared to d$(CGG)_{10}$. The spectra of d$(TGG)_{10}$ (Fig. S4) indicated that it did not form an intrinsic hairpin structure, and its secondary structure does not have major changes even in the presence of FecNCD2. For d$(CTG)_{10}$, d$(CAG)_{10}$, and d$(ATT)_{10}$, almost no changes were observed in the CD spectra in the presence of FecNCD2. These results confirmed that FecNCD2 had the ability to selectively recognize trinucleotide repeat d$(CGG)_{10}$.

3.3. Electrochemical characterization of FecNCD2

Since it was found that the present FecNCD2 has better binding and selectivity for CGG trinucleotide repeat, FecNCD2 was applied to the electrochemical detection of CGG trinucleotide repeats. At first, in order to evaluate the electrochemical responses of FecNCD2, cyclic voltammograms (CV) of FecNCD2 in PBS was recorded for different concentrations (Fig. S8). While the oxidation and reduction peak currents increased with the increasing concentration up to 30 μM, the increase has become very small over 30 μM. This would be due to poor water-solubility of FecNCD2 in PBS.

As shown in Fig. 3A(c), the CV of 30 μM FecNCD2 exhibited clear oxidation and reduction peaks at 0.464 V and 0.409 V respectively, which correspond to the ferrocenium/ferrocene couple (ΔE_p=0.055 V, I_{pa}/I_{pc}=1.37) (Campos-fernandez et al., 2000), indicating quasireversible redox process. The CV of NCD without ferrocene group showed no redox peaks as in Fig. 3A(b). The plots of the peak current of FecNCD2, I_{pa} and I_{pc}, vs. the root of sweep rate (Fig. 3B and Fig. S9) were linear, which indicated that the redox reaction of FecNCD2 was a diffusion-controlled process. The solid curves represent the results for best-fit parameters (a: I_{pa}=−0.19201+6.4474 $v^{1/2}$, R^2=0.99; b: I_{pc}=0.45063–4.3297 $v^{1/2}$, R^2=0.99). From the slope of Line a in Fig. 3B, the diffusion coefficient of free FecNCD2, D_f, was determined to be 2.33×10^{-6} cm^2/s (for details, please see Supporting information). The results confirmed the good redox property of the present FecNCD2.

3.4. Immobilization of DNA and preparation of the biosensor

Hybridization of DNA results in an increased negative charge at the electrode surface, which would hinder charge transfer of negatively charged redox molecules in solution. These would cause a change in charge transfer resistance (R_{ct}) that can be detected using electrochemical impedance spectroscopy (EIS) (Bardea et al., 1999). So EIS is one of the most effective methods to monitor time-dependent adsorption and assembly of nucleic acids on the transducer surface, and to characterize DNA hybridization events (Katz and Willner, 2003; Revenga-Parra et al., 2011). Here the immobilization of DNA on the surface of gold electrode was characterized by ESI. As shown in Fig. 4A, the R_{ct} of a bare gold electrode is very small, which showed that the redox reactions of $[Fe(CN)_6]^{3-/4-}$ should smoothly occur on the electrode surface. The properties of the electrode interface would be changed when HS-ssDNA, with the negatively charged phosphate backbone, was self-assembled on the gold electrode surface by S–Au bonding. The R_{ct} of HS-ssDNA modified electrode was raised to 3 kΩ, which indicated a slight deceleration of the redox reactions of $[Fe(CN)_6]^{3-/4-}$. After the electrode was closed by MCH, the R_{ct} of HS-ssDNA–MCH modified electrode dramatically increased to 31 kΩ. Furthermore, the hybridization with trinucleotide repeats DNA should hinder the charge transfer, judging from the R_{ct} value of 36 kΩ. These impedance data indicated successful immobility of SH-DNA and hybridization of trinucleotide repeat DNA with SH-DNA.

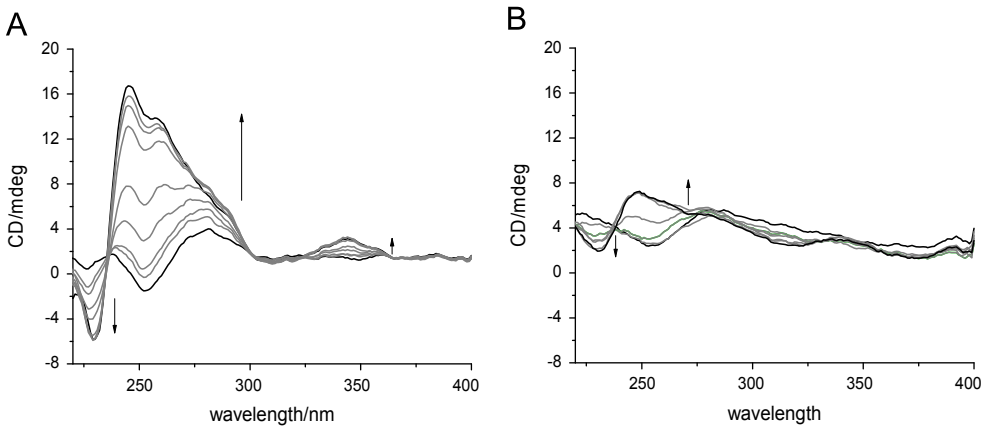


Fig. 2. (A) CD spectral of $d(CGG)_{10}$ (2.5 μM) in the absence and presence of FecNCD2 with increasing concentrations of FecNCD2 (as the direction of the arrow): 0, 2.5, 5.0, 7.5,12.5, 17.5, 25.0, 30.0, 35.0, 40.0, 45.0, and 50.0 μM. (B) CD spectral of $d(CGG)_{10}$ (2.5 μM) in the absence and presence of FecNCD1 with increasing concentration: 2.5, 7.5, 15.0, 22.5, 30.0, 37.5, and 50 μM.

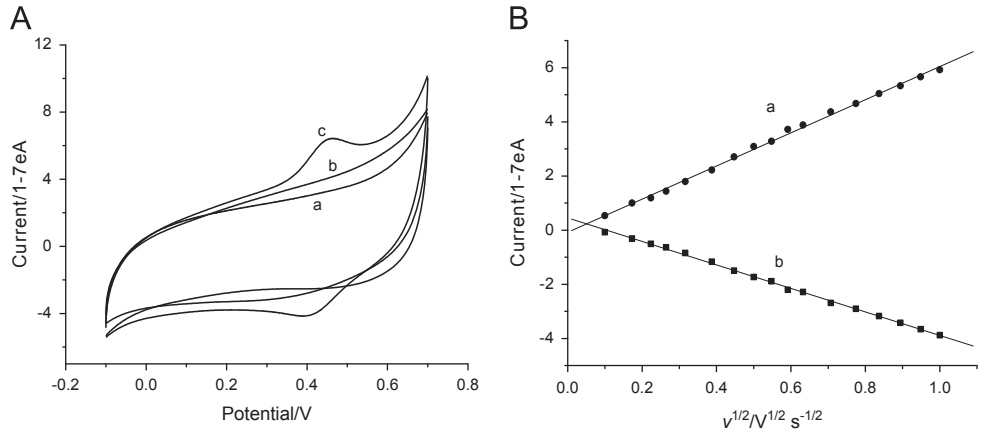


Fig. 3. (A) Cyclic voltammograms of blank (a), 50 μM NCD (b), 50 μM FecNCD2 and (c) in PBS (pH 7.0) with the sweep rate of 0.1 mV/s. (B) The relationship of the peak currents (a: I_{pa} and b: I_{pc}) of FecNCD2 (50 μM) and the square root of sweep rates (as the direction of the arrow): from 0.01 to 1 V s^{-1}.The solid curves represent the results for best-fit parameters ($R2=0.99$ for a, $R2=0.99$ for b).

Whether the biosensor gives a successful electrochemical response or not after incubation with the bifunctional probe is our most concerned issue. Obviously the CGG modified biosensor did not show any electrochemical signal before incubation with FecNCD2 PBS solution (Fig. 4B(d)). In contrast, after incubation with FecNCD2 PBS, the clear SWV current, a typical oxidative peak of FecNCD2, was observed around 0.47 V (Fig. 4B(e)). Also, electrochemical impedance ($R_{ct}=26$ kΩ) has decreased after the incubation with FecNCD2 as shown in Fig. 4A(e). In order to obtain the optimal electrochemical signal, the SWV spectra of the CGG modified biosensor were performed after incubation with FecNCD2 for different incubation time. The data showed that the current reached a platform after incubation for 30 min, which indicated that saturated interaction was already achieved (Figs. S10 and S11).

Ferrocene is known to give good electrochemical response (Fig. S12), so the SWV signal of biosensor should reasonably come from the ferrocenyl groups of FecNCD2 bound with CGG repeats. In order to eliminate the doubts, electrochemical signal of CGG biosensor was observed after incubating in 20 μM ferrocene and 20 μM NCD . As a result, no electrochemical signal was found (Fig. S13), indicating that ferrocene alone has no interaction with CGG repeats. So the combination of NCD and the electrochemical activity of ferrocene in FecNCD2 would be both indispensable. After all, as the results of electrochemical measurements, FecNCD2 was found to have an excellent ability to bind CGG repeats with significant changes in electrochemical signal.

3.5. *Selectivity of FecNCD2 for trinucleotide repeats*

Since the biosensor modified with $d(CGG)_{10}$ has exhibited sufficient electrochemical signal after incubation with FecNCD2, it was clarified that the present bifunctional probe can be used in principle to detect trinucleotide repeats. However, as another, important key factor would be the selectivity in electrochemical detections. For example, the selectivity should be a key issue in the early diagnosis of the Fragile X syndrome disease.

According to the above CD results, the probe FecNCD2 has found to have the ability to bind $d(CGG)_{10}$ trinucleotide repeat selectively. So the biosensors immobilized with different trinucleotide repeats were fabricated according to the same procedures. In the incubation step, all the biosensors were immersed into a 20 μM FecNCD2 for 30 min at room temperature. Finally, the biosensors were rinsed in 2 mL PBS before SWV analysis in order to minimize nonspecific interaction of FecNCD2 and DNA. As shown in Fig. 4C and D, the CGG modified biosensor presented a highest current signal of 1.09×10^{-6} Å. The biosensors modified by other trinucleotide repeats showed no apparent current signal due

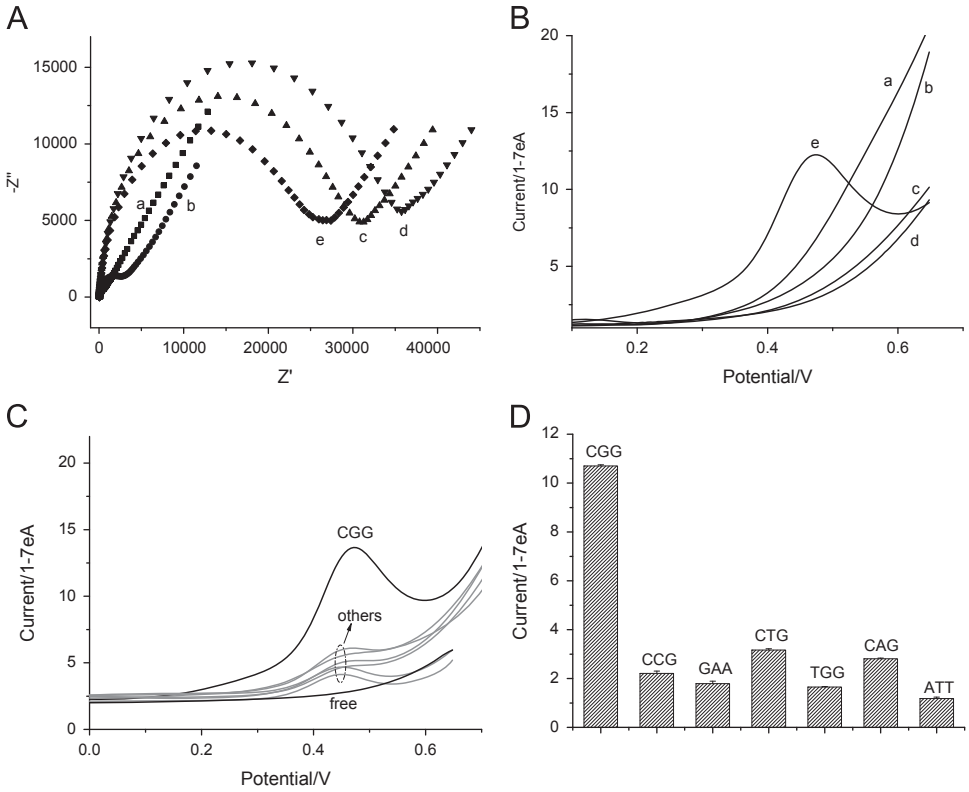


Fig. 4. (A) Electrochemical impedance spectroscopy (EIS) of bare Au electrode (a), HS-ssDNA modified electrode (b), MCH-treated modified electrode (c), trinucleotide repeat DNA hybridized electrode (d), and biosensors after incubation for 30 min with 20 μM FecNCD2 in PBS (pH 7.0) (e). The real and imaginary components of the Nyquist diagram were labeled *Z*′ and *Z*″, respectively. (B) Square wave voltammograms (SWV) of the biosensor in PBS (pH 7.0). Here the indications (a, b, c, d, and e) were the same as those of (A). (C) SWVs of the biosensors with different trinucleotide repeats after incubation with 20 μM FecNCD2 for 30 mins at room temperature. (D) Peak current dependence of the biosensors with $d(CGG)_{10}$, $d(CCG)_{10}$, $d(GAA)_{10}$, $d(CTG)_{10}$, $d(TGG)_{10}$, $d(CAG)_{10}$ and $d(ATT)_{10}$. These experiments were repeated for a minimum of three times, which showed better relative standard deviation (RSD) about 1.4–4.9%.

to less binding of FecNCD2 with repeats, which was consistent with the CD results. These results verified that the biosensor possessed an excellent selectivity for $d(CGG)_{10}$ trinucleotide repeat owing to the excellent performances of FecNCD2.

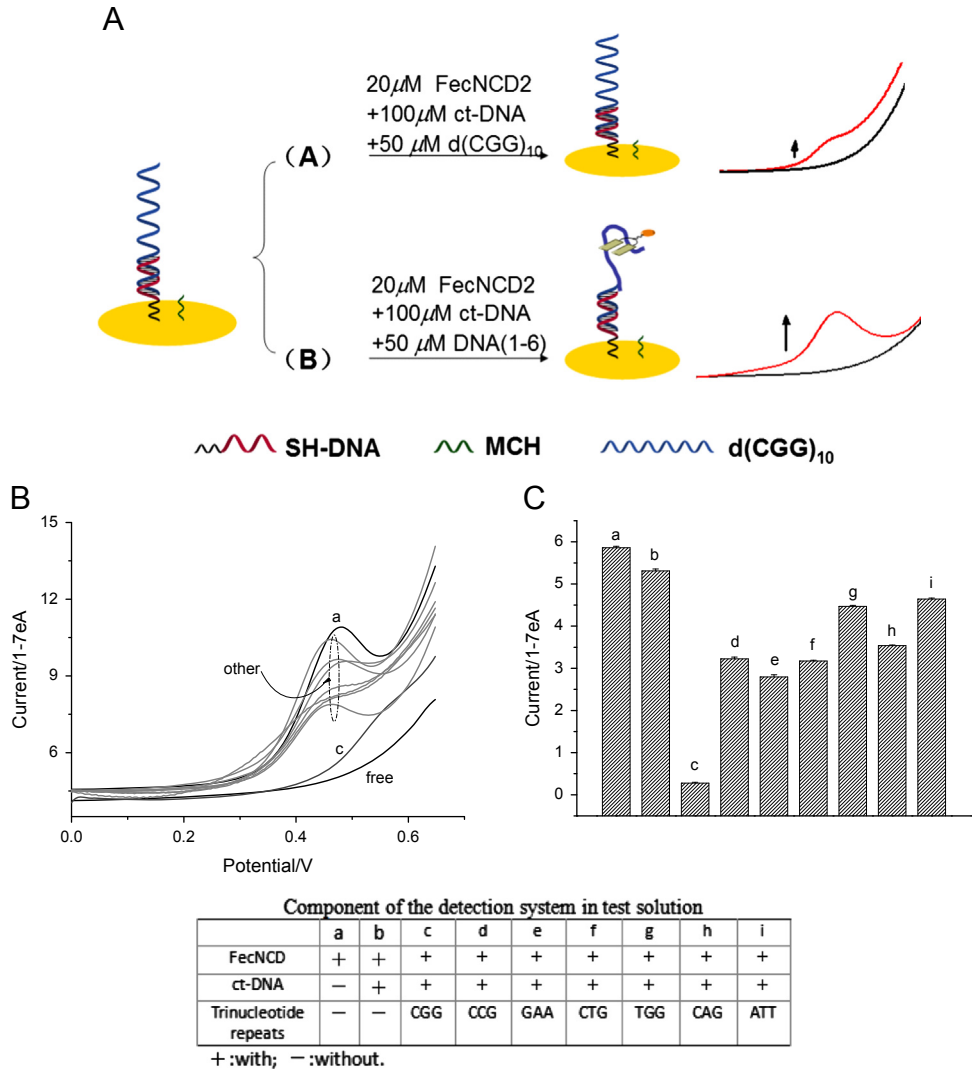
3.6. Detection of CGG trinucleotide repeats in presence of interference ct-DNA

In order to simplify the experiment procedure and apply the real detection trinucleotide repeats conveniently, the detection approach was improved to perform the competition experiment. Here, "competitive" means the interactions of FecNCD2 with trinucleotide repeats modified on the electrode surface and in the test solution. That is, with the CGG modified biosensor, if a test solution includes both FecNCD2 and $d(CGG)_{10}$, it is expected that the binding of FecNCD2 with $d(CGG)_{10}$ on the electrode should be significantly suppressed by the interaction between FecNCD2 and CGG repeats in the test solution (in Fig. 5A). Conversely, the CGG biosensor was expected to produce a clear electrochemical signal when a test solution contains FecNCD2 and other trinucleotide repeats such as $d(CCG)_{10}$, $d(TGG)_{10}$, $d(GAA)_{10}$, $d(CTG)_{10}$, $d(CAG)_{10}$ and $d(ATT)_{10}$, because of the poor binding of FecNCD2 toward them.

The SWV current signal of CGG modified biosensor decreased with increasing concentration of CGG repeat in test solution in presence of 20 μM FecNCD2 probe (Figs. S14 and S15). The current signal reached a plateau, decreased to almost zero when the concentration of CGG repeat was more than 5 μM. The result indicated that the saturation binding point was at about 4:1 mol ratio of FecNCD2 and $d(CGG)_{10}$, which was consistent to previous report for formation of hairpin secondary structure (Nakatani et al., 2005; Peng and Nakatani, 2005; He et al., 2009).

Generally, some gene sequence responsible diseases coexist with full matched DNA strands especially in real diagnosis of diseases. In order to improve detection practicality, the interference ct-DNA was added in the test solution containing trinucleotide repeats. The selectivity of the CGG modified biosensor was evaluated by SWV spectra after incubation with 200 μL test solution containing 50 μM trinucleotide repeats and interference 100 μM ct-DNA together with 20 μM FecNCD2.

After incubation in the test solution of ct-DNA and FecNCD2, the CGG modified biosensor showed large current intensity similar with the solution containing only FecNCD2 (Fig. 5B and C), which indicated that the interference ct-DNA did not affect the recognition of FecNCD2 for CGG repeat. This means that FecNCD2 has no interaction with ct-DNA. On the other hand, no obvious SWV current (93.1% decreasing in current) was observed after incubation with test solution containing CGG repeat, ct-DNA and FecNCD2. The SWV currents were decreased to some extents after incubation with test solutions containing other repeats, 38%, 45%, 35%, 21%, 30%, and 16% for $d(CCG)_{10}$, $d(GAA)_{10}$, $d(CTG)_{10}$, $d(TGG)_{10}$, $d(CAG)_{10}$ and $d(ATT)_{10}$ respectively. However, a remarkable difference from the results for $d(CGG)_{10}$ could be easily recognized as shown in Fig. 5B and C. These results demonstrated that CGG modified biosensor can be directly used to detect CGG trinucleotide repeats with good selectivity even in the presence of interference ct-DNA. The biosensor would permit us simple and effective detections of pathogenic gene sequence CGG of Fragile X syndrome disease.



Component of the detection system in test solution

	a	b	c	d	e	f	g	h	i
FecNCD	+	+	+	+	+	+	+	+	+
ct-DNA	−	+	+	+	+	+	+	+	+
Trinucleotide repeats	−	−	CGG	CCG	GAA	CTG	TGG	CAG	ATT

+:with; −:without.

Fig. 5. (A) Schematic diagram of competitive experiments for observing interference of ct-DNA; (B) SWVs of the biosensor with $d(CGG)_{10}$ after incubation for 30 min with the test solutions, whose contents are listed in the table as a, b, c, d, e, f, g, h and i, and (C) peak current change dependence of the biosensors. These experiments were repeated for a minimum of three times, which showed better relative standard deviation (RSD) about 1.2–4.2%.

4. Conclusions

The bifunctional probe, FecNCD2, synthesized in the present work, was found to bind with CGG trinucleotide repeat selectively. Owing to the ferrocenyl group, FecNCD2 exhibited good performances for the electrochemical detection of CGG trinucleotide repeat DNA. After modifying trinucleotide repeats on the electrode, FecNCD2 worked as an electrochemical probe to show remarkable electrochemical signal of the biosensor. Furthermore, CGG-modified biosensor was directly used to detect different trinucleotide repeats in test solution through the competitive experiments. The results demonstrated that CGG modified biosensor had an excellent ability to detect CGG repeat selectively. In comparison with other methods including electrochemical assay, the present protocol has excellent selectivity and should provide a very simple and rapid detection of DNA without labeling and immobilizations.

As revealed in the present work, the design and application of bifunctional probe have significant potential in the development of novel biosensor. The strategy would permit new types of electroanalysis for trinucleotide repeats with low-cost assay, simple and rapid operations, and would be very useful for early diagnosis and treatment of the neurodegenerative diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.05.022.

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