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ARTICLE TYPE

Electrochemical impedance sensor based on small molecule modified Au electrode for the recognition of a trinucleotide repeat

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A small molecule modified sensor was developed for the detection of XGG trinucleotide repeats (X=C, T) by electrochemical impedance spectroscopy. The sensor (NCD/MPA/Au) was fabricated by immobilizing the nucleic acid recognition molecule (NCD) on the surface of a gold electrode through a condensation reaction between the amino-terminal end of the NCD linker and carboxylic groups in 3-mercaptopropionic acid that were self-assembled on the electrode surface. After the sensor was incubated with trinucleotide repeats, electrochemical impedance spectroscopy were performed using $[Fe(CN)_6]^{3-/4-}$ as a redox marker ions. XGG repeats (X=C, T) could be selectively detected based on the differences in charge transfer resistance (ΔR_{ct}) even in the presence of other trinucleotide repeats. The relationship between ΔR_{ct} and lg[concentration of CGG repeats] for the sensor was linear from 1 nM to 1 μM, enabling the quantification of the number of repeats. The electrochemical impedance sensor provides the simple and rapid method to detect trinucleotide repeats without requiring labelling and immobilizations of DNA, making it promising for the early diagnosis of the neurodegenerative diseases; the sensor may be further extended to the detection of other special sequence of DNA.

1. Introduction

Specific sequences of nucleotide acids have attracted significant interests because the sequences have a great possibility to correlate with various diseases. Moreover, the recognition and rapid analysis of specific sequences of nucleotide acids is of great importance in the diagnosis, prevention, and treatment of numerous human diseases [1-5]. Trinucleotide repeats were known to be involved in a number of neurodegenerative diseases [5-11], including myotonic dystrophy (CTG), Fragile X syndrome (CGG) [12], Huntington disease (CAG), several spinocerebellar ataxias (CAG), and Friedreich ataxia (GAA) [7-8]. This discovery has prompted numerous studies regarding the structure, biology and sequence detection of trinucleotide repeats. Some of these studies have investigated the binding molecules, expansion and biological properties of trinucleotide acids [13-14]. The conventional methods of detection of trinucleotide repeats involve Southern blotting or gel electrophoresis [15-17]. The Nakatani group identified several organic molecules that were able to bind and recognize some trinucleotide repeats DNA [18-22].

Due to their low cost, low power consumption and portability, simple and rapid electrochemical methods for the detection of nucleic acids have gained increasing attention among chemical biologists, molecular biologists and some pharmacists involved in modern biomedicine. However, few studies have reported the electrochemical detections of trinucleotide repeats. Yang and Thorp demonstrated the electrochemical detection of CGG trinucleotide repeats using electrocatalysis by $Ru(bpy)_3^{2+}$ [23]. Palecek and coworkers proposed an electrochemical DNA hybridization assay for GAA trinucleotide repeats using multiple osmium-labelled reporter probes [24]. We also reported an electrochemical biosensor for the detection of CGG trinucleotide repeats based on changes in current [25-26]. These electrochemical methods mainly based on current detection such as square-wave voltammetry, differential pulse voltammetry.

Electrochemical impedance spectroscopy (EIS) is a powerful electrochemical technique for studying various surface processes and properties [27-29]. It has been widely used as a label-free detection method to study various molecular binding processes, such as antigen-antibody recognition [30], protein-DNA interactions [31-34], and DNA hybridization [35-39] as well as to monitor cellular processes. Currently, the majority of EIS biosensors use DNA or proteins self-assembled on the surface of an electrode to detect target molecules. However, to the best of our knowledge, no group has used organic small molecule modified electrodes to recognize specific sequences of nucleic acids by EIS.

Recently, we developed bi-functional probes that were both electrochemically active and able to bind nucleic acids by combining of naphthyridine carbamate dimmers (NCD) and an electro-active (ferrocenyl group) molecule, and applied them for the electrochemical detection of DNA based on current changes [25-26]. In this work, the recognition molecule (naphthyridine carbamate dimmer, NCD) of nucleic acids was immobilized on the surface of a gold electrode by a classical coupling reaction between carboxyl and amino groups (as shown in Figure 1). The binding of DNA to the NCD molecule on the surface of the gold

electrode would result in increased negative charges at the electrode surface, hindering the charge transfer of negatively charged redox molecules in solution. This produced a change in the charge transfer resistance (R_{ct}) that could be detected using electrochemical impedance spectroscopy (EIS). CGG trinucleotide repeats were readily detected by monitoring differences in the charge-transfer resistance (ΔR_{ct}). Our approach is simple, free-labelling, and has potential applications in the early diagnosis and prevention of neurodegenerative diseases such as Fragile X syndrome.

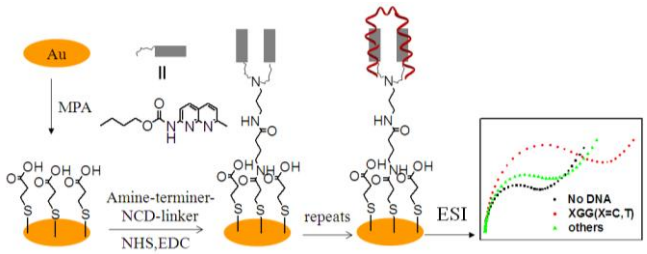

Figure 1. Schematic of the small molecule modified Au electrode and the recognition for the trinucleotide repeat DNAs in EIS measurements.

2. Experimental

2.1. Materials and apparatus

3-Mercaptopropionic acid (MPA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98%), N-hydroxysuccinimide (NHS, 98%) and 2-(N-morpholino)-ethanesulfonic acid (MES) were purchased from Aladdin Reagent Database Inc. (Shanghai, China). All the chemicals were of analytical grades and solutions were prepared with PBS and ultrapure water. All oligonucleotides were synthesized and purchased from the Invitrogen Corporation (China). The DNA sequences are shown in the supporting information.

All the electrochemical measurements were performed on a CHI660A electrochemical workstation (CHI Instrumental, Chenhua Corp., Shanghai, China). A standard three-electrode system, with a small molecule modified gold electrode as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a platinum foil as the auxiliary electrode, was used in the measurements. The supporting electrolyte solution for the electrochemical impedance spectra experiments was 0.5 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ (1:1) solution.

2.2. Synthesis of the amino-terminal NCD linker

Naphthyridine carbamate dimmer (NCD) was synthesized as described in previous literature (Fig.S1, [25-26, 40]). The NCD linker was prepared by the condensation of the amine group in NCD with the aldehyde group in the linker (4-[(tert-butoxy)carbonylamino]-N-(3-oxopropyl)butanamide). The reaction was performed in methanol under sodium cyanoborohydride at room temperature overnight. The pure NCD-linker solid was obtained by purification from a silicon gel ($CHCl_3$/CH_3OH 10/1, R_f = 0.4 for 6/1 v/v $CHCl_3$/CH_3OH) with a yield of 45.00% (details can be found in the supporting information). The NCD-linker (20 mg) was deprotected in 2.0 mL of 4 M HCl/EA to get the amino-terminal NCD linker that would be used in the next preparation of the NCD modified electrode.

2.3. Preparation of the recognition molecule NCD modified electrode

Prior to each experiment, Au disk electrodes (diameter, 2mm, 99%Au) were polished successively by aluminium slurries (0.1-1 μM) with ultrapure water on polishing cloths. After rinsing with ultrapure water, the electrodes were ultrasonically rinsed in ethanol and ultrapure water, respectively. Afterwards, the cleaned gold electrode was subsequently voltammetrically cycled between -0.5-1.6 V at 0.1 V S^{-1} in 0.5 M sulphuric acid for 20 cycles.

The pretreated, clean Au electrodes were dried with nitrogen and then immersed in a 2 mM MPA ethanol solution (100μL) overnight to obtain a carboxyl-terminated self-assembled monolayer on the Au electrode. The MPA modified electrode (MPA/Au) was washed with ethanol and ultrapure water to remove excess MPA molecules, and then immersed into the activation solution containing1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC, 50 mM) and N-hydroxysulfosuccinimide (NHS, 50 mM) for 2 h. After thoroughly rinsing with 0.1 M MES buffer (pH6.0) and ultrapure water, the activated gold electrode was immersed into 0.01 mM PBS (pH 7.4) containing 500 μM amino-terminal NCD-linker overnight in order to form a peptide bond. The NCD-linker modified electrode (NCD/MPA/Au) (Figure 1) was rinsed with PBS and ultrapure water before use.

2.4. Measurements

The NCD/MPA/Au electrodes were incubated with a test DNA solution in 0.01 M PBS (pH 7.4) for 2 hours, and subsequently immersed in a traditional three-electrode system for electrochemical measurements using a saturated calomel electrode (SCE) as the reference electrode and a platinum foil as the auxiliary electrode. The CV were scanned from -0.2 to 0.6 V at a scan rate of 100 mV/s in a 0.5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ and 0.5 mol L^{-1} KNO_3 solution. Square wave voltammograms (SWV) were recorded in 10 mM PBS in a potential range of -0.1 to 0.65 V with modulation amplitude of 4 mV, step potential of 25 mV, and frequency of 15 Hz. The EIS was also collected in the same electrolyte solution within a frequency range from 0.5 Hz to 100 kHz. The commercial software Zview2 was used to fit the data from the Nyquist plot, providing the real (Z') and imaginary component (-Z'') of resistance of the immobilized layers, as well as the equivalent circuit for understanding the surface structure. From the values of R_{ct}, the quantitative signal could be determined as a function of DNA concentration from the increasing R_{ct} after the NCD/MPA/Au sensor was incubated with varying DNA concentrations [41].

3. Results and discussion

3.1. Successful preparation of the NCD/MPA/Au sensor

EIS can provide detailed information about the surface changes occurring during the modification process. The fabrication of MPA and NCD onto the surface of the electrode was characterized by impedance. As shown in Figure 2A, we

observed an increase in R_{ct} from the successive blocking of the electrode surface with the sequential attachment of MPA, activation of MPA, and condensation of NCD on the electrode surface. The EIS curve of bare gold electrode was approximately a straight line in $[Fe(CN)_6]^{3-/4-}$, indicating that the redox reactions of $[Fe(CN)_6]^{3-/4-}$ occurred smoothly on the electrode surface. Here, the equivalent circuit (Figure 2A) was used to fit the impedance data for each step with commercial software Zview. As illustrated in the inset of Fig. 2A, the circuit includes the electrolyte resistance between the working and reference electrodes (R_s, the solution resistance), Warburg impedance (Z_W), electron transfer resistance (R_{ct}) caused by charge transfer reaction (Faraday process) and the diffusion of ions from the electrolyte to the interface, and the interfacial double layer capacitance was treated as a constant phase element (CPE).

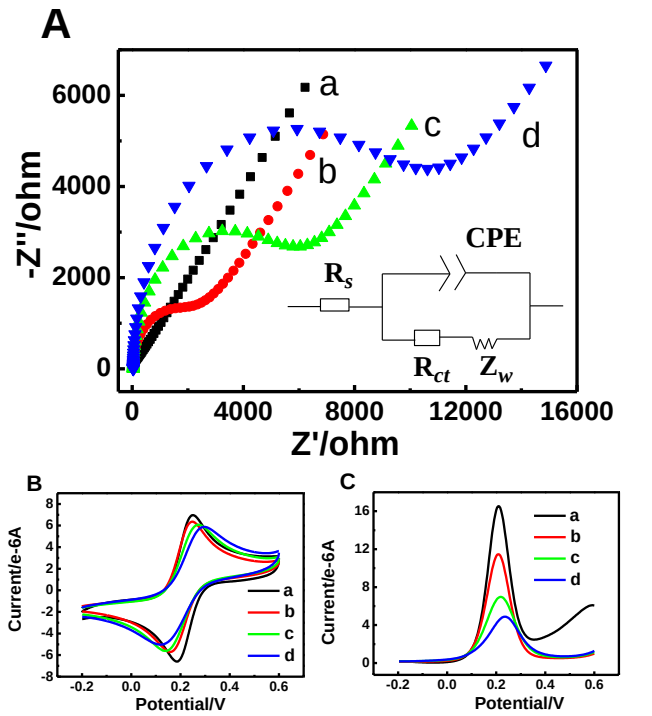

Figure 2. (A) The Nyquist plots of impedance spectra (a frequency range from 0.5 Hz to 100 kHz), (B) CV curves and (C) SWV curves obtained from (a) the bare gold electrode, (b) the MPA/Au, (c) the NCD/MPA/Au, and (d) the NCD/MPA/Au following incubation with CGG_{10}. The spectra were carried out in 0.5 mM $Fe(CN)_6^{3-/4-}$. Scan rate, 100 mV/s; SWV, modulation amplitude of 4 mV, step potential of 25 mV, and frequency of 15 Hz.

The impedance spectra were analyzed with the equivalent circuit (the inset in Fig 2A.), and the fitting results were showed in Table S1 (in the supporting information). After the electrode was incubated with the MPA solution, a layer of MPA was formed. Curve b in Fig. 2A revealed an increasing impedance (R_{ct} = 2288 Ω), indicating that MPA was successfully self-assembled on the surface of the electrode. The MPA/Au surface was activated with an activation solution containing EDC and NHS to improve the reaction activity of carboxylic acid. After coupling the carboxyl on the gold electrode surface to the amine group of the NCD linker, the diameter of semicircle increased to thousands of Ohms (R_{ct} = 6089 Ω) due to an electrostatic repulsive force of small molecule to $Fe(CN)_6^{3-/4-}$ [42]. These data demonstrated that the NCD linker was successfully bonded to carboxyl group on the surface of the gold electrode.

Based on previous study [19, 25-26], NCD is able to strongly bind to CGG trinucleotide repeats selectively. Firstly, the NCD/MPA/Au sensor was incubated with CGG trinucleotide repeats DNA in PBS. After interaction, the diameter of semicircle significantly increased, which indicated successful capture of the CGG trinucleotide repeats by the NCD/MPA/Au. The increase in R_{ct} value (11035 Ω) was attributed primarily to DNA on the surface of the gold electrode, which blocked the interfacial electron transfer reaction of $Fe(CN)_6^{3-/4-}$. Compared to the NCD/MPA/Au sensor, only a small increase in R_{ct} was observed for MPA/Au and bare Au (as observed in Fig. S2 and S3). The small increases may be due to the nonspecific adsorption of DNA. These data demonstrate that the NCD modified electrode was successfully prepared and that the sensor exhibited a clear electrochemical impedance response upon the interaction of the NCD with DNA.

To exclude the possible false positive impedimetric responses in our assay, a series of control CV and SWV measurements were conducted (Fig. 2B and C). During the stepwise construction of the electrode and the binding of DNA, the CV peak current decreased in response to an increase in hindrance (Fig. 2B). The E_{pa} value shifted to more positive potentials, and E_{pc} shifted to negative potentials (the data can be seen in Table S2). Square wave voltammetry (SWV) revealed an obvious decrease in the current and a positive shift in the potential with the stepwise construction of the sensor and the binding of DNA (Fig. 2B, the data are summarized in Table S3). The results of both the CV and SWV were consistent with the increase in R_{ct} observed in the EIS measurements. Importantly, it was apparent that the change in R_{ct} in the EIS measurements was much more sensitive than the currents in CV and SWV measurements.

3.2 Optimization of the incubation time

The value of R_{ct} was dependent on the incubation time of the biosensor with the trinucleotide repeats because that the interaction between NCD and DNA bases follows dynamic equilibrium thermodynamics. It was evident that the semicircular domains of the impedance spectra increased with increasing incubation times from 0 to 120 minutes (Figure S4). After 2 hours, the diameters of the semicircles almost became constant, which meant that the binding had reached saturation. Thus, we used 120 minutes as the incubation time in all the experiments.

3.3 Selectivity of the NCD/MPA/Au for XGG10 (X=C,T)

The naphthyridine carbamate dimer (NCD) has been stabilized the repeat DNA with GG rich such as $(CGG)_n$ and $(TGG)_n$. In the presence of NCD, the repeat can form a hairpin secondary structure reasonably resulted from complementary hydrogen bonding between naphthyridine group and guanine and from the conformational freedom of two heterocycles by a long linker in NCD, as described in previous studies [19-20]. However, it was necessary to determine whether the small molecule modified gold electrode (the NCD/MPA/Au sensor) would exhibit sufficient selectivity in EIS. The sensor had been immersed in varies trinucleotide repeat solutions for 2 hours at room temperature to investigate its selectivity by EIS. Specially recognized DNA would be much captured on the electrode

surface through the hydrogen bonding interactions, subsequently increasing R_{ct}. Thus, a lack of change in the R_{ct} value indicates that the sensor cannot recognize the DNA. As shown in Fig. 3, the sensor exhibited the largest semicircle after incubation with CGG repeats. Furthermore, it also showed higher electron transfer resistance for TGG repeats. However the impedance values for other repeats such as CCG, GAA, CAG, CTG, and ATT, were much weaker in comparison. Small increases in R_{ct} (about 1000 Ω) result from the nonspecific adsorption of DNA on the sensor surface. Fig. 3B clearly demonstrates the very good selectivity for CGG trinucleotide repeats as well as the recognization for TGG trinucleotide repeats. The impedance spectra were analyzed with the equivalent circuit (the inset in Fig 2A.), and the fitting results were showed in Table S4.

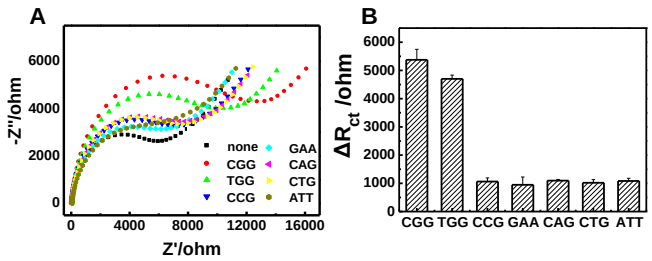

Figure 3. (A) Nyquist plots of the impedance spectra from the NCD/MPA/Au sensor after incubation with 5 μM of various trinucleotide repeats for 2 hours at room temperature. (B) The R_{ct} change (ΔR_{ct}) of the NCD/MPA/Au biosensor after incubation with CGG_{10}, TGG_{10}, CCG_{10}, GAA_{10}, CTG_{10}, CAG_{10} and ATT_{10} (PBS: pH 7.4). These experiments were repeated at least three times, which showed better relative standard deviation (RSD) about 2.82-11.1 %. (The definition of ΔR_{ct} can be seen in the supporting information)

3.4 Sensitive performance of the biosensor

The sensitivity of the NCD/MPA/Au sensor was investigated by studying the relationship between the changes in R_{ct} and the concentration of the CGG trinucleotide repeats (Fig. 4). Fig. 4A shows the gradual increase in ΔR_{ct} with the increasing concentration of CGG repeats. The ΔR_{ct} increased gradually from 0 to 1 μM, then plateaued more than 1 μM (Fig. 4B). The inset in Fig. 4B displayed the linear relationship between ΔR_{ct} and concentration in the concentration range from 1 nM to 1 μM, with a linear fit of Y (ΔR_{ct}) = 1370.61748 X (lg[C_{DNA}])+ 563.5137 and a correlation coefficient R^2=0.99731.

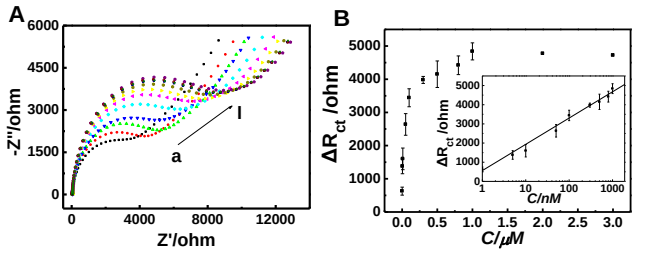

Figure 4. (A) Nyquist plots of the NCD/MPA/Au sensor after incubation with different concentrations of CGG repeats (from a to l were 0.001, 0.005, 0.01, 0.05, 0.1, 0.3, 0.5, 0.8, 1.0, 2.0, and 3.0 μM, respectively) in 0.01 M PBS (pH 7.4) containing 0.5 mM $Fe(CN)_6^{3-/4-}$ and 0.5 M KNO_3. (B) The relationship between the R_{ct} changes and the concentration of the CGG trinucleotide repeat from 1 nM to 1 μM. (R^2=0.99731, the diameter of the electrode: 2 mm).

3.4 Interference

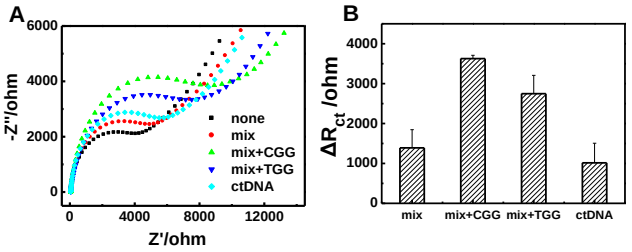

Figure 5. (A) Nyquist plots of the NCD/MPA/Au sensor after incubated with mixed solutions. (B) The R_{ct} changes of the NCD/MPA/Au sensor in the mixed test solutions. Here, mix indicates a mixture of $(CCG)_{10}$, $(GAA)_{10}$, $(CTG)_{10}$, $(CAG)_{10}$ and $(ATT)_{10}$ trinucleotide repeats and excludes $(CGG)_{10}$ and $(TGG)_{10}$ repeats. The concentration of each repeats was 4 μM.

Generally, a variety of DNA trinucleotide repeats would coexist in test sample, especially in the diagnosis of diseases from clinical samples. To improve the practical utility of our method, the NCD/MCA/Au sensor would be incubated in a mixture of trinucleotide repeats. The resulting impedance plots can be observed in Figure 5A. For the mixture of CCG, GAA, CTG, CAG and ATT trinucleotide repeats that excluded CGG and TGG repeats, the ΔR_{ct} was measured to be about 1391 Ω. This low value demonstrates that the NCD/MPA/Au sensor did not detect CGG or TGG repeats in the test solution containing a mixture of DNA sequences. When CGG and TGG repeats were added separately to the mixture, obvious R_{ct} increases were observed, with ΔR_{ct} values of 3627 Ω and 2748 Ω respectively. In addition, the NCD/MP/Au sensor was also incubated with a full matched double strand ct-DNA. Only a small ΔR_{ct} (1015 Ω) was observed even in the 100 μM ct-DNA, demonstrating that the sensor did not interact specifically with ct-DNA. Thus, our sensor has the potential to be used for the detection of trinucleotide repeats DNA.

Conclusions

In conclusion, we developed an electrochemical impedance sensor based on an organic molecule for the detection of trinucleotide repeats by EIS. NCD immobilized gold electrodes were able to capture the XGG (X=C,T) trinucleotide repeats selectively, as indicated by an increase in the charge transfer resistance. Based on these changes in charge transfer resistance (ΔR_{ct}), XGG repeats (X=C,T) could be selectively detected even in the presence of other trinucleotide repeats. In addition, the amount of CGG repeats could be determined according to the linear relationship between ΔR_{ct} and lg[concentration] from 1 nM to 1 μM. Compared with current electrochemically methods, the electrochemical impedance sensor allows the very simple and rapid detection of trinucleotide repeats without the labelling and immobilizations of DNA and exhibits excellent selectivity and sensitivity. The sensing assay provides a feasible method for the simple and highly reliable detection of specific DNA sequence

and is a very promising option for the early diagnosis and treatment of neurodegenerative diseases.

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Notes and references

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‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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