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A reagentless DNA biosensor based on cathodic electrochemiluminescence at a C/C_xO_{1-x} electrode

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ABSTRACT

A reagentless signal-on electrochemiluminescence (ECL) biosensor for DNA hybridization detection was developed based on the quenching effect of ferrocene (Fc) on intrinsic cathodic ECL at thin oxide covered glassy carbon (C/C_xO_{1-x}) electrodes. To construct the DNA biosensor, molecular beacon (MB) modified with ferrocene (3′-Fc) was attached to a C/C_xO_{1-x} electrode via the covalent bound between labeled amino (5′-NH_2) and surface functional groups. It was found that the immobilization of the probe on the electrode surface mainly depended on the fraction of surface carbonyl moiety. When a complementary target DNA (cDNA) was present, the stem-loop of MB on the electrode was converted into a linear double-helix configuration due to hybridization, resulting in the moving away of Fc from the electrode surface, and the restoring of the cathodic ECL signal. The restoration of the ECL intensity was linearly changed with the logarithm of cDNA concentration in the range of 1.0×10^{-11} to 7.0×10^{-8} M, and the detection limit was ca. 5.0 pM (S/N = 3). Additionally, single-base mismatched DNA can be effectively discriminated from the cDNA. The great advantage of the biosensor lies in its simplicity and cost-effective with ECL generated from the electrode itself, and no adscititious luminophore is required.

1. Introduction

In recent years, DNA biosensors have received significant attention due to their potential applications in clinical diagnostics [1], food analysis [2], forensic study [3] and environmental monitoring [4]. All of these applications require a low-cost and fast DNA hybridization detection tool. To the best of our knowledge, fluorescent-based DNA chips is one of the most used conventional large-scale gene analysis tools [5]. However, the equipment is expensive, and labeling or modifying step is usually required, which costs time, money, and furthermore can increase the potential for errors in the analysis due to additional steps. Therefore, studies on label-free method for DNA hybridization detection are vital important, owing to its simplicity, rapidity and cheapness. According to the previous reports, most of the label-free DNA biosensing technologies rely on electrochemistry [6–8], surface plasmon resonance method [9], colorimetry [10], chemiluminescence [11,12] and electrochemiluminescence (ECL) [13]. Though tedious labeling procedures can be avoided by these methods, adscititious signal indicators such as methylene blue, gold nanoparticles, $Ru(bpy)_3^{2+}$ etc. are still required. Recently, with the rapid development of the studies on the optical properties of quantum dots, DNA biosensors based on the ECL of quantum dots have also been reported [14,15], however, the complicated labeling procedure is still required in these works.

Hot electron induced cathodic ECL is similar to common ECL except that light emission is initiated by hot energetic electrons from a thin film-covered electrode during cathodic pulse polarization [16]. The significant advantage of cathodic ECL lies in that a low detection limit and a wide linear range can be achieved at the same time [17,18]. This method has been previously used for biorecognition detection in DNA hybridization [19], however, the complicated labeling process of probe with luminophore is required. Furthermore, the oligonucleotides are physically adsorbed on the surfaces by both electrostatic and hydrophobic interactions, which would result in a poor stability and reproducibility.

The electrodes used in cathodic ECL are different from those used in common ECL. In order to generate energetic electrons by direct tunneling during cathodic pulse polarization, a thin oxide film with a thickness no more than 5 nm is required. In previous reports, Al/Al_2O_3 and Si/SiO_2 electrodes are commonly used [17–19]. A C/C_xO_{1-x} electrode was fabricated by electrochemical oxidizing glassy carbon substrate in 0.1 M NaOH in our previous work. The properties of the C_xO_{1-x} film were carefully characterized by XPS and ellipsometry. A thin oxide film with a thickness of 2.82 nm was formed on the electrode surface, which allowed the generation of energetic electron during cathodic pulse polarization. Cathodic ECL at C/C_xO_{1-x} electrode was firstly found, based on

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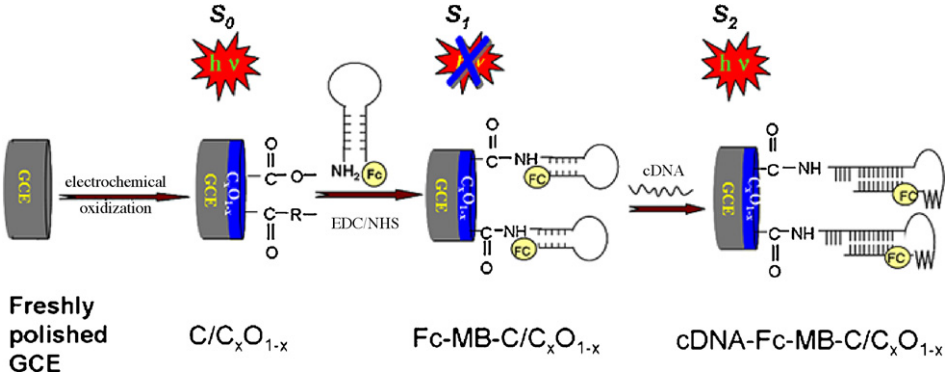

Scheme 1. Schematic diagram of the reagentless Fc-MB DNA biosensor.

which a label-free immunosensor was fabricated due to the surface carbonyl group that can covalently interact with antibodies.

Herein, the quenching effect of ferrocene (Fc) on the intrinsic cathodic ECL at C/C_xO_{1-x} electrode was firstly investigated. A new strategy for developing reagentless DNA biosensor was proposed based on the efficient quenching effect of Fc on the ECL intensity. The schematic representation is outlined in Scheme 1. By applying a cathodic pulse polarization, ECL with intensity of S_0 is produced at C/C_xO_{1-x} electrode. The ECL signal is quenched (S_1) when amino modified Fc-MB probe is covalently bounded to the electrode surface (Fc-MB-C/C_xO_{1-x}). When the probe encounters the complementary target DNA (cDNA), the stem is forced to be apart due to the hybridization. Fc is moved away from the electrode surface (cDNA-Fc-MB-C/C_xO_{1-x}), and thus, leads to the restoration of ECL signal (S_2).

2. Experimental

2.1. Chemicals and materials

The labeled oligonucleotides were synthesized by TaKaRa biotechnology (Dalian) Co., Ltd. (China), purified via C18 HPLC, and confirmed by mass spectroscopy. Their concentrations were quantified by OD_{260} based on their individual absorption. The probe DNA sequence was selected according to Ref. [20], which was derived from the oligonucleotide sequences that unique to Francisella organisms. It was designed with an 18 base loop and a 4 bp stem, with $-NH_2$ attached to the 5′ end and ferrocene (Fc) attached to the 3′ end. The base sequences are as follows: ferrocene labeled molecular beacon capture probe (Fc-MB): 5′-NH_2-GCG CGT AAC GTC CTC CCC CAA GGC GC-Fc-3′, complementally target DNA (cDNA): 5′-GCC TTG GGG GAG GAC GTT AC-3′, one-base mismatched: 5′-GCC TTG GGG AAG GAC GTT AC-3′. 0.2 M boric acid borate buffer solution (pH 8.0) containing 0.1 M Na_2SO_4 and 1 mM $K_2S_2O_4$ was used for ECL and cyclic voltammetry (CV) measurement. DNA immobilization buffer was 10 mM TE buffer solution (pH 8.0). Washing buffer was 50 mM phosphate buffer (PBS) (pH 7.4 with 0.9% NaCl). All solutions were prepared with MilliQ water (18 MΩ cm resistivity) from a Millipore system.

Glassy carbon electrodes (GCE, Ø 4 mm) (Lanli Technology Co. Ltd., Tianjin, China) were used as the working electrodes. Before electrochemical oxidization, the GCE was firstly polished with emery paper of mesh 1500# and 2000# for 2 min, and washed with deionized water. The C/C_xO_{1-x} electrode was prepared by electrochemically oxidizing the freshly polished GCE in 0.1 M NaOH at 1.8 V for 20 min. A re-C/C_xO_{1-x} electrode was obtained by reducing C/C_xO_{1-x} electrode in 0.1 M H_2SO_4 at −1.0 V for 5 min. The counter electrode was a platinum foil (area ca. 200 mm^2), the reference electrode was an Ag/AgCl (saturated KCl) electrode.

2.2. Apparatus

An electrochemical working station (VMP3, Princeton Applied Research Co., Ltd., USA) was used as pulse generator. The pulse amplitude was −16 V (vs. Ag/AgCl) with a pulse width of 0.4 ms, and the duration time of 9.6 ms. The whole time period for applying pulse polarization equaled to the time for collecting ECL signals was 20 s. ECL signals were recorded by an Ultra-weak Chemiluminescence Analyzer (BPCL-K, Institute of Biophysics, Academia Silica, Beijing, China) controlled by a personal computer with 0.1 s sample interval. While collecting ECL signals, the cell was placed directly in front of the photomultiplier (PMT operated at −800 V) and the PMT window was opened towards the working electrode only. X-ray photoelectron spectroscopy (XPS) was carried out at VG ESCALAB 250 (Thermo Nicole, USA) X-ray photoelectronic spectrometer with an Al $K_{\alpha 1,2}$ X-ray source (1486.60 eV). All the spectra were obtained with a spot of 500 μm at a vacuum less than 5.0×10^{-10} mbar. The deconvolution of C 1s XPS spectra was performed by using Gauss–Lorentz distribution function.

2.3. Construction and characterization of DNA biosensor

The DNA biosensor was fabricated according to the procedure reported before [21]. Briefly, before immobilization, the C/C_xO_{1-x} electrode was activated by dipping into the mixed solution containing 8 mM EDC and 5 mM NHS for 1 h at room temperature, and then washed thoroughly with 50 mM PBS (pH 7.4). After being dried with nitrogen, the activated C/C_xO_{1-x} electrodes were incubated in the immobilization buffer which contained Fc-MB probe at appropriate concentrations for 2 h at 37 °C. The electrode surface was then washed with PBS to remove the unbound oligonucleotides. Then the fabricated sensor was incubated in 100 μL of different concentrations of cDNA at 45 °C for 0.5 h. After binding, electrodes were extensively rinsed with washing buffer and dried under a stream of nitrogen prior to characterization.

Electrochemical impedance spectra (EIS) experiments were performed in 5 mM $Fe(CN)_6^{4-/3-}$ dissolved in 50 mM PBS (pH 7.4) with a frequency between 0.01 Hz and 200 kHz and an ac amplitude of 10 mV.

3. Results and discussion

3.1. Quenching effect of Fc on the cathodic ECL at C/C_xO_{1-x} electrode in the solution

As one of the most used redox-active moiety in electrochemistry, Fc has been attached to oligonucleotides and used for

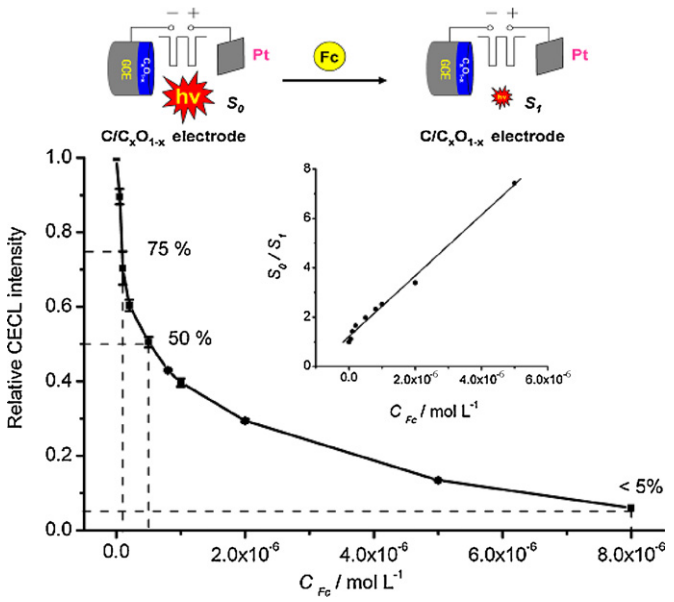

Fig. 1. Quenching effect of Fc on cathodic ECL intensity at C/C_xO_{1-x} electrode. Pulse amplitude: −16 V (vs. Ag/AgCl), pulse width: 0.4 ms, duration time: 9.6 ms and total time: 20 s. Insert: intensity Stern–Volmer quenching plot for cathodic ECL at C/C_xO_{1-x} electrode by Fc. Solution: 0.2 M BBS (pH 8.0) containing 0.1 M Na_2SO_4 and 1 mM $K_2S_2O_8$.

fabricating electrochemical DNA biosensors [22]. The quenching effect of Fc on the $Ru(bpy)_3^{2+}$ ECL system was investigated by Cao et al. in aqueous solution [23]. On this basis, a few DNA hybridization assays were successfully fabricated [24–26].

Cathodic ECL at C/C_xO_{1-x} electrode during pulse polarization was found to be closely related to the surface properties as well as the thickness of the C_xO_{1-x} oxide film. Herein, the quenching effect of Fc on the cathodic ECL at C/C_xO_{1-x} electrode was investigated, as shown in Fig. 1. The ECL intensity was dramatically decreased to 75% and 50% in the presence of 0.1 and 0.5 μM of Fc, respectively, suggesting that the quenching effect was efficient. When 8.0 μM of Fc was added to the solution, the ECL intensity decreased to 5% of that in the absence of Fc. Further increasing of Fc concentration did not change the intensity significantly. The quenching effect can be treated with Stern–Volmer equation [23]:

$$\frac{S_0}{S} = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q] \quad (1)$$

where S and S_0 are the ECL intensity with/without quencher, [Q] is the concentration of quencher, K_{sv} is the quenching constant, τ_0 is the lifetime for the exciting state and K_q is the quenching rate constant. The quenching effect of Fc on the ECL at C/C_xO_{1-x} electrode is presented as Stern–Volmer plot in the insert (Fig. 1). Here K_{sv} is estimated to be 1.23×10^6 L mol^{-1} S^{-1} (r = 0.9961). Supposing that K_q is at its maximum value of 2.0×10^{10} L mol^{-1} S^{-1} [27], τ_0 is calculated to be 62 μs. Such a long lifetime might be due to the electron and holes trapped in the surface state of the oxide film layer [28]. Analogous to the F-center luminescence at oxide covered aluminum electrode [29], we assumed that a similar luminescent center was present in the C_xO_{1-x} film. The possible mechanisms are proposed as follows:

$$e_{aq}^- + S_2O_8^{2-} \rightarrow SO_4^{2-} + SO_4^{\bullet-} \quad (2)$$

$$L + SO_4^{\bullet-} \rightarrow L^* + SO_4^{2-} \quad (3)$$

$$L^* \rightarrow L + h\nu \quad (4)$$

Hot electrons were injected into the aqueous electrolyte solution from the C/C_xO_{1-x} electrode during cathodic pulse polarization, which resulted in the generation of (e_{aq}^-) as reducing mediators, and subsequently the strong oxidizing radicals (e.g. $SO_4^{\bullet-}$) from the added coreactant (e.g. $S_2O_8^{2-}$) (Eq. (2)). Electrons trapped in the surface state of the luminescent center within the C_xO_{1-x} oxide film would be captured by $SO_4^{\bullet-}$ [30], leading to the formation of the excited-state of luminescent species (L*), and finally the cathodic ECL (Eqs. (3) and (4)).

Similar to the mechanism for quenching effect of Fc on the $Ru(bpy)_3^{2+}$ ECL system [22], energy and/or electron transfer between Fc and the excited L* are supposed to be the main reasons for the quenching effect in the present system. Namely, the quenching effect of Fc on the cathodic ECL at C/C_xO_{1-x} electrode can be partly attributed to the energy transfer between L* and Fc (Eq. (5)). Alternatively, Fc is oxidized to form ferrocenium (Fc^+) by the strong oxidant $SO_4^{\bullet-}$ (Eq. (6)). The resultant Fc^+, acting as an efficient e_{aq}^- scavenger [31], can further react with the generated e_{aq}^- (Eq. (7)), and then quenches the ECL signals (Eq. (8)). The possible mechanisms are proposed in the following two pathways:

$$Fc + L^* \rightarrow L + Fc^+ \quad (5)$$

Or:

$$Fc + SO_4^{\bullet-} \rightarrow SO_4^{2-} + Fc^+ \quad (6)$$

$$Fc^+ + e_{aq}^- \rightarrow Fc \quad (7)$$

$$Fc + L^* \rightarrow L + Fc^+ \quad (8)$$

3.2. The characterization of the DNA biosensor

In order to explore the possibility of utilizing the quenching ability of Fc on the cathodic ECL at C/C_xO_{1-x} electrode in DNA hybridization detection, a 26 base pair amino modified Fc-MB was selected as the probe sequence. Schematic diagram of the C/C_xO_{1-x} electrode at three different modified steps (i.e. C/C_xO_{1-x}, Fc-MB-C/C_xO_{1-x} and cDNA-Fc-MB-C/C_xO_{1-x}) are shown in Fig. 2A. The corresponding CV behaviors of the above three electrodes obtained in borate buffer solution (0.2 M, pH 8.0) are shown in Fig. 2B. It is found that the activated C/C_xO_{1-x} electrode shows no voltametric signal (curve a), while at the probe modified electrode a quasi-reversible redox wave of Fc is observed (curve b). The results demonstrate that Fc-MB probe was successfully immobilized onto the electrode surface. The corresponding voltametric signal decreases (curve c) when the probe encounters the cDNA, showing that Fc is moved away from the electrode surface due to the hybridization.

Electrochemical impedance spectroscopy (EIS) is an effective technique to monitor the surface feature and understand the chemical transformation [32]. The semicircle diameter at higher frequencies corresponds to the electron-transfer resistance (R_{ct}) and the linear part at lower frequencies corresponds to the diffusion process. The larger of the semicircle diameter, the larger the value of R_{ct} is. The above procedures can be confirmed by the corresponding Nyquist plots of EIS shown in Fig. 2C. An increase in R_{ct} in curve (b), compared with that of C/C_xO_{1-x} electrode (curve a), indicates the immobilization of the probe DNA at the electrode and the electrostatic repulsive effects between negative charges of the DNA backbone and the electroactive redox probe [$Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$]. The further increase in R_{ct} in curve (c) after the probe incubated with cDNA might be resulted from successful hybridization of cDNA with the immobilized probe on electrode surface.

Fig. 2D shows the corresponding cathodic ECL intensity at different modified steps. As expected, ECL signal at C/C_xO_{1-x} electrode (curve a) is nearly completely quenched after the immobilization of Fc-MB probe (curve b), and is restored after hybridization (curve c) with cDNA.

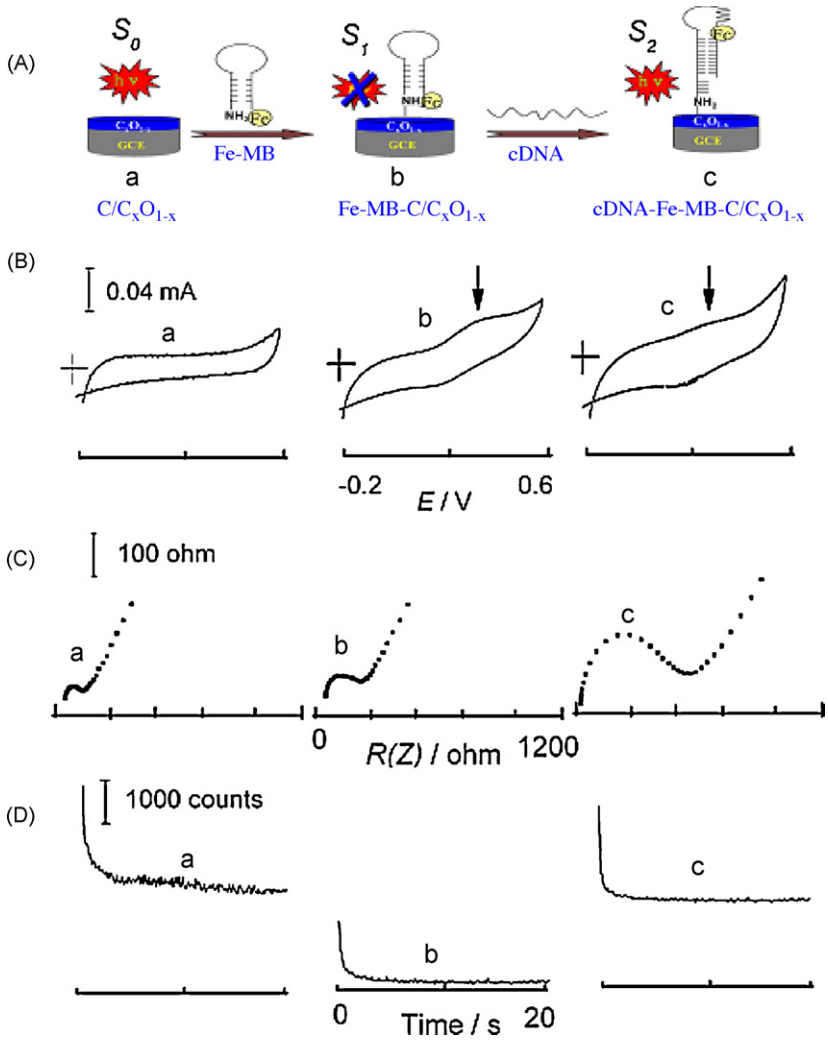

Fig. 2. Schematic diagram (A), CV (B), Nyquist plots (C) and ECL response (D) of the modified C/C_xO_{1-x} electrode at three stages: (a) activated with EDC/NHS (C/C_xO_{1-x}), (b) immobilized with 1.0 μM Fc-MB probe (Fc-MB-C/C_xO_{1-x}) and (c) after exposure of Fc-MB-C/C_xO_{1-x} electrode to 2.0 μM complementary DNA (cDNA-Fc-MB-C/C_xO_{1-x}). EIS experiments were performed in 5 mM $Fe(CN)_6^{4-/3-}$ dissolved in 50 mM PBS (pH 7.4) with a frequency between 0.01 Hz and 200 kHz (ac amplitude: 10 mV). Scan rate for CV measurement was 100 mV/s. Other conditions are as in Fig. 1.

It was previously shown that cathodic ECL at thin oxide film covered electrode was sensitive to the film thickness. A thickness of 5 nm was a threshold value, that is, ECL intensity increased slightly up to a thickness of 5 nm, and began to decrease after this value [33]. Thus, it is probable that the decreasing of the ECL intensity at Fc-MB-C/C_xO_{1-x} electrode can be attributed to the quenching effect of the immobilized Fc-MB probe, as well as the change of the film thickness covered on the electrode surface. In order to make sure what is dominant in the quenching effect, a control experiment is carried out by employing an Fc free MB sequence as the probe.

Although the length of the probe with 26 bases is estimated to be 4.81 nm (0.37 nm per base) according to Ref. [34], the thickness of the modified DNA sequence depends on the configuration of the sequence. Therefore, the exact thickness of the modified DNA probe is hard to know. Here, it was found that ECL intensity before and after the immobilization of Fc free probe, and that after hybridization were nearly the same (signal lost < 8%), indicating that the DNA layer covered on the C/C_xO_{1-x} electrode surface did not affect the ECL signals. It can be concluded that the quenching effect is mainly attributed to the Fc-MB probe rather than the change in the film thickness.

3.3. Effect of surface functional groups on the immobilization of Fc-MB probe

Carboxyl and/or phenolic are reported to be the main moieties for covalently interaction with NH_2 modified DNA probe [35,36]. In order to give insight into the nature of this assumption, the effects of the surface functional groups at pretreated electrodes on the immobilization of the probe were examined. Herein, a freshly polished GCE, a C/C_xO_{1-x} electrode and a re-C/C_xO_{1-x} electrode were selected. The electrode surfaces before probe modification were characterized by XPS technique, and the probe modified electrode surfaces were monitored by EIS.

As shown in Fig. 3, XPS C 1s spectra recorded were deconvoluted using a five peaks model [37]. Four peaks were indentified as graphitic carbon and carbon bounded to different functional groups such as phenolic, carbonyl and carboxyl. Detailed results about the fractions of different functional groups on the electrode surface were easily read out as shown in the pie chart. On the freshly polished GCE, the fractions of oxidized carbon (phenolic, carbonyl and carboxyl) are quite small (ca. 14%). After the electrode was oxidized (C/C_xO_{1-x} electrode), a significant increase of these fractions was observed, especially for those of carbonyl (from 3.11% to 16.3%)

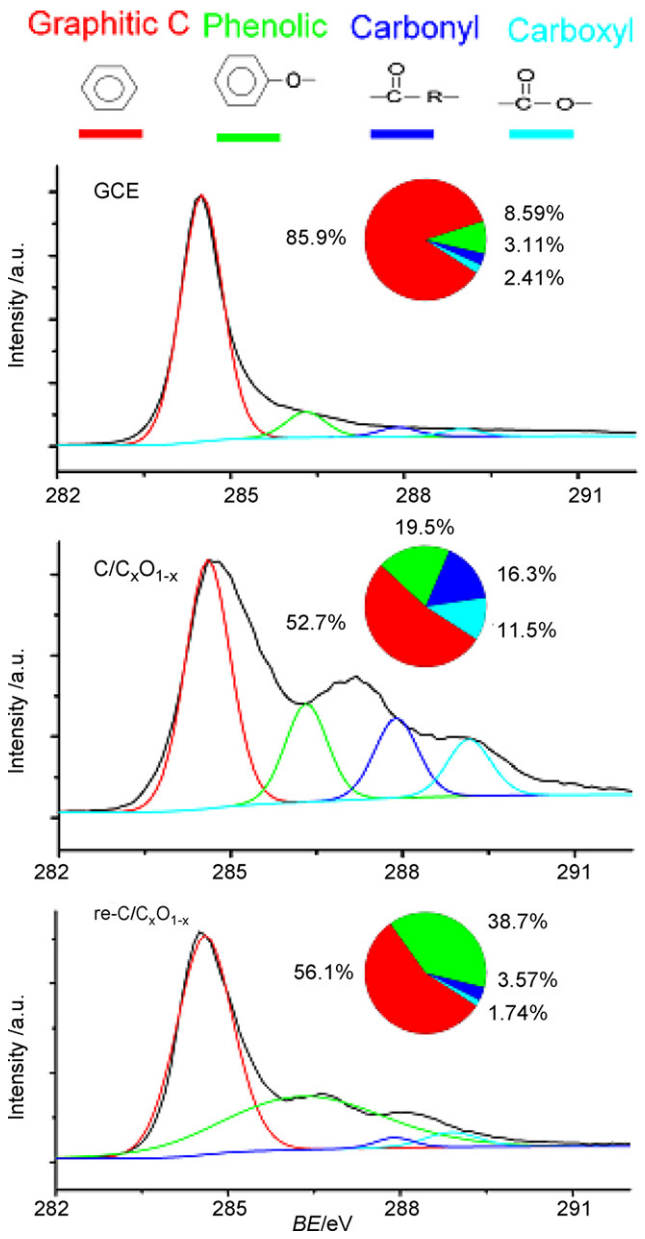

Fig. 3. Deconvoluted XPS C 1s spectra for the surface of freshly polished GCE, C/C_xO_{1-x} electrode and re-C/C_xO_{1-x} electrode.

and carboxyl (from 2.41% to 11.5%). When the C/C_xO_{1-x} electrode was further reduced to a re-C/C_xO_{1-x} electrode, the total fractions of these oxidized carbons did not change, though, the fraction of phenolic increased to ca. two times, and that of carbonyl and carboxyl decreased almost to that of freshly polished GCE. The results suggest that carbonyl and carboxyl were converted to phenolic during the process of reduction, which was in accordance with our previous study [38].

Fig. 4 shows the corresponding EIS at the probe modified electrodes. The amount of probe immobilized on the electrode surface can be estimated by R_{ct}. The more the probe is immobilized, the larger R_{ct} is. It was found that the amount of probe immobilized on C/C_xO_{1-x} electrode is much more than that at freshly polished GCE or re-C/C_xO_{1-x} electrode (Fig. 4). A further understanding on the effect of surface function groups on the probe immobilization process can be obtained, if the fractions of these groups (Fig. 3) were taken into consideration. For example, at C/C_xO_{1-x} electrode, the amount of immobilized probe is the most among the three selected

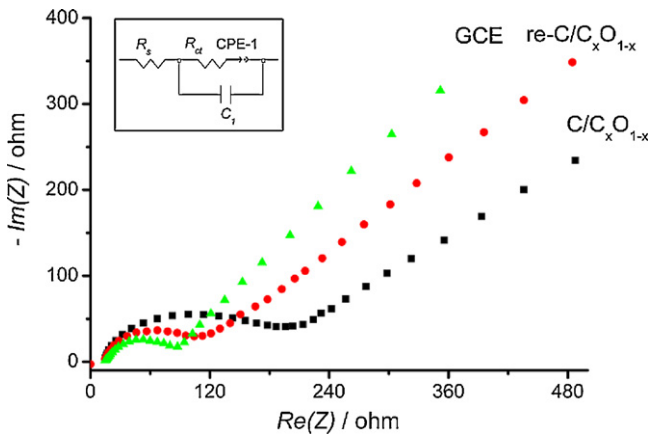

Fig. 4. Nyquist plots for freshly polished GCE, C/C_xO_{1-x} electrode and re-C/C_xO_{1-x} electrode after the immobilization of Fc-MB probe. The conditions for EIS detection are as in Fig. 2.

electrodes, and the fractions of carbonyl and carboxyl are the most as well. Otherwise, when the electrode was further reduced to a re-C/C_xO_{1-x} electrode, the fractions of these two moieties decreased, which in turn resulted in the decreasing of the amount of immo-

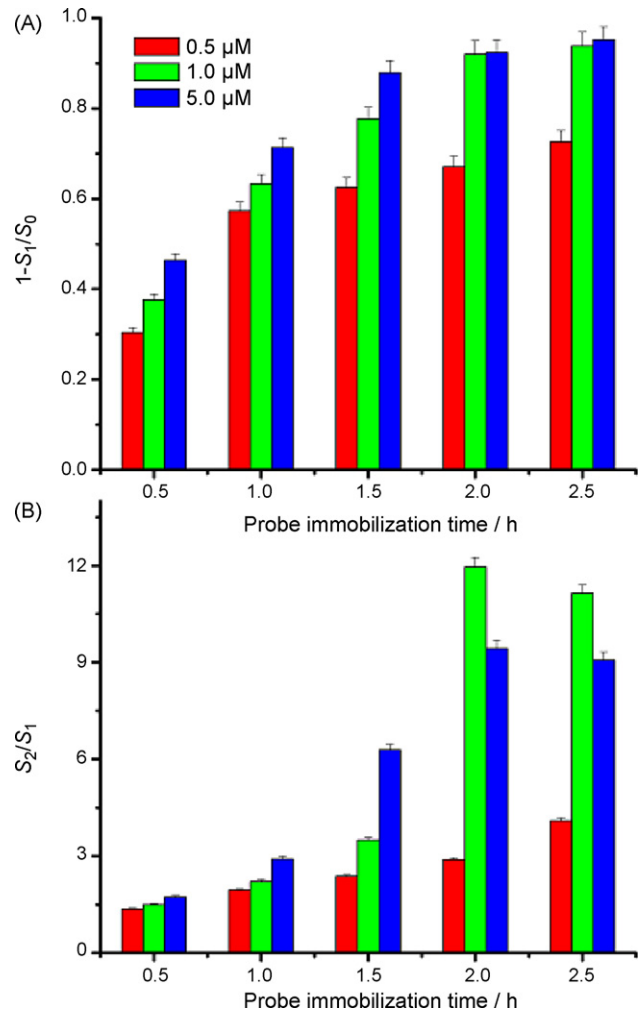

Fig. 5. Effect of probe immobilized time on the quenching efficiency $(1 - S_1/S_0)$ (A) and the hybridization efficiency (S_2/S_1) (B). The incubation Fc-MB probe concentrations were of 0.5, 1.0 and 5.0 μM. The concentration of cDNA is 10 μM. ECL measurement conditions are as in Fig. 1.

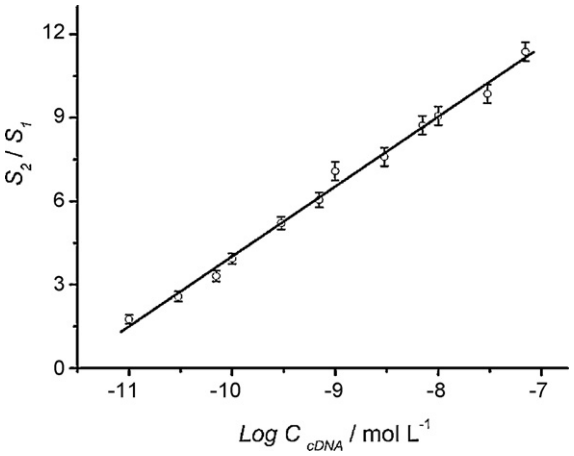

Fig. 6. Calibration curve for the detection of cDNA. The signals are average values of three parallel measurements. The concentration of immobilized Fc-MB is 1.0 μM, and the immobilized time is 2 h. ECL measurement conditions are as in Fig. 1.

bilized probe. It seems that the immobilization of the probe on the electrode surface is mainly dependent on the fractions of carbonyl and carboxyl, especially on that of carbonyl. Also, it is almost irrelevant to the fraction of phenolic. The result is unexpected, and it provides a new knowledge for understanding the covalent bounding of amino modified biomolecule to the surface of a carbon substrate.

3.4. The optimum conditions for sensor construction

The responses of the DNA biosensor would be affected by the coverage of Fc-MB probe on the electrode surface. The influence can be viewed from two aspects. Firstly, the quenching efficiency (denoted as $1 - S_1/S_0$) of Fc on the cathodic ECL intensity is strongly dependent on the probe coverage. Secondly, the surface coverage of immobilized Fc-MB probe also has a profound effect on the hybridization efficiency (denoted as S_2/S_1) with the target DNA [39]. A good response of the sensor to cDNA would be obtained with a high S_2/S_1 ratio.

Generally, the coverage of Fc-MB probe on the electrode surface relies on the incubation probe concentration and the immobilization time. Here, three different incubation probe concentrations (0.5, 1.0 and 5.0 μM) were selected to examine the immobilization time period on the value of $1 - S_1/S_0$ and the S_2/S_1. As shown in Fig. 5A, a higher incubation probe concentration would result in a higher probe coverage, and thus, a higher value of $(1 - S_1/S_0)$. When the incubation probe concentration is fixed, the quenching efficiency increases with the immobilization time period in the first 1.5 h. A stable value of $(1 - S_1/S_0)$ is observed (ca. 95%) as the immobilization time is longer than ca. 2 h, indicating that a saturated surface coverage is obtained.

On the other hand, experiments were conducted to examine the effect of probe coverage on the response of the biosensor to cDNA. As shown in Fig. 5B, the value of S_2/S_1 increases with the probe immobilization time or the probe coverage, in the first 2 h for all the three cases. Further increase of the immobilization time tends to decrease the value of this ratio, which is attributed to the electrostatic hindrance arising from the more tightly packed probe [40]. A highest S_2/S_1 ratio is obtained when 1.0 μM of the Fc-MB probe is used and incubated for 2 h. Therefore, in the following experiment the biosensor is fabricated under this optimum condition (Fig. 5).

3.5. Performance of DNA biosensor

The fabricated DNA biosensor has a good response to cDNA over a wide range of concentrations as shown in Fig. 6. A linear relationship between the logarithm of the concentration of cDNA and the ratio of S_2/S_1 was found to be in the range of 1.0×10^{-11} and 7.0×10^{-8} M, the regression equation is

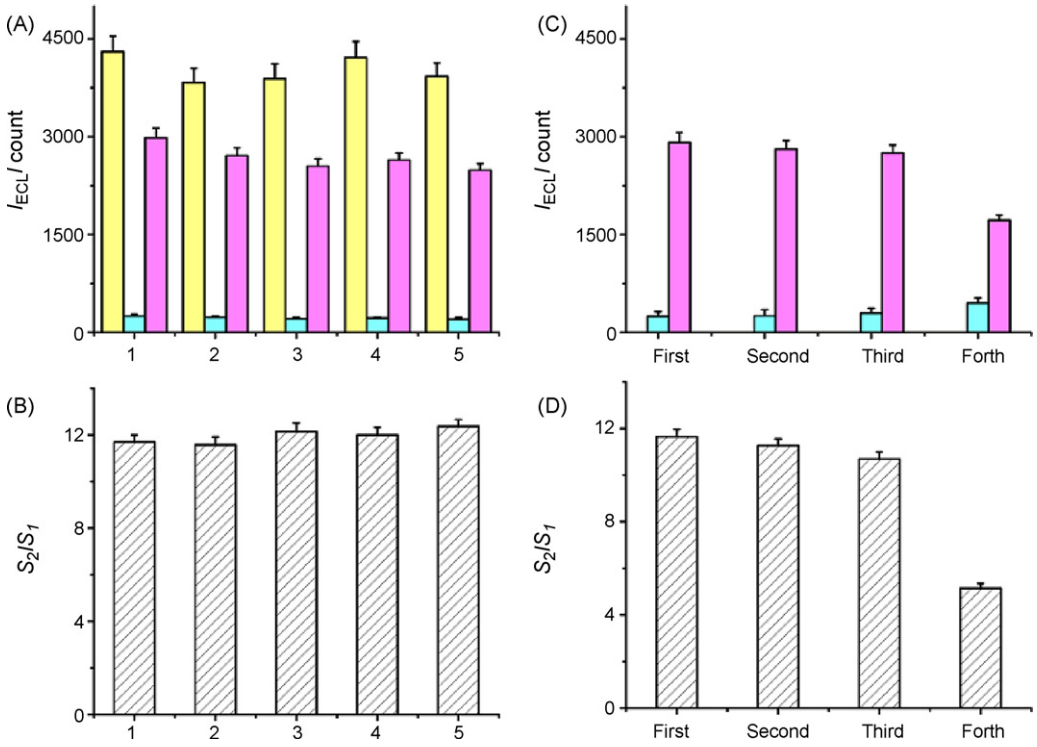

Fig. 7. (A) ECL signals and (B) the signal ratio of S_2/S_1 obtained at five parallel sensors. (C) ECL signals and (D) the signal ratio of S_2/S_1 at one sensor before and after regeneration. Yellow bars: S_0; cyan bars: S_1; magenta bars: S_2. The concentration of immobilized Fc-MB is 1.0 μM, and the immobilized time is 2 h, the concentration of cDNA is 1.0 μM. ECL measurement conditions are as in Fig. 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

$S_2/S_1 = 2.45 \log C_{cDNA} + 28.51$ ($r = 0.9966$). Our results indicate the fabricated biosensor can be used to detect target DNA in the picomolar range of ca. 5.0 pM (S/N = 3), which was about two orders of magnitude lower than that of the electrochemistry based reagentless sensor (ca. 1.0 nM) [41] and equivalent to that of the cathodic ECL based one (ca. 10.0 pM) [19].

The specificity of the present ECL hybridization assay was examined by detecting the ECL response of perfectly complementary targets and one-base mismatched strands at three concentrations of 0.1, 1.0 and 10.0 nM. The ratios of the ECL intensities for the two sequences were 100:72.4, 100:68.9 and 100:70.4, respectively. The results indicate that the single-base mismatched DNA strands can be differentiated from the cDNA with the proposed ECL hybridization assay.

The reproducibility of the reagentless ECL biosensor was evaluated from the ECL response to cDNA by using five parallel sensors that made at different electrodes. As shown in Fig. 7A, ECL signals of S_0, S_1 and S_2 are more or less the same for the five sensors. The relative standard deviation for S_2/S_1 as shown in Fig. 7B was 3.46%, indicating that the present method was stable and repeatable. The long term stability was also examined by comparing the ECL signals (S_1) at the fabricated sensor right after it was prepared and that after kept in refrigerator at 4 °C. No significant lost of the signal was observed after the sensor was stored for more than one week, suggesting that the sensor was stable for at least one week. In addition, a preliminary investigation on the regeneration of the sensor shows that the fabricated DNA biosensor can be regenerated by rinsing with hot water (90 °C) for ca. 5 s for three times. As shown in Fig. 7C, the ECL signal of the test beacon after the thirdly hybridization was approximately 90% of the first hybridization. However, a marked decline of S_2/S_1 (ca. 50%) (Fig. 7D) was observed after three cycles, which might be attributed to the denaturing of the probe and the physically removing of the probe from the electrode surface.

4. Conclusion

The quenching effect of Fc on the intrinsic cathodic ECL at C/C_xO_{1-x} electrodes was investigated, based on which a reagentless signal-on DNA biosensor by using Fc-MB as probe was developed. Surface carbonyl and carboxyl are found to be the main moieties for covalently bounding with NH_2 modified Fc-MB probe. The conditions for fabricating the biosensor are optimized according to the dependence of the sensor sensitivity on the probe coverage. Additionally, this DNA sensor also exhibits high selectivity to cDNA and single-base mismatched one. The main advantage of the present sensor lies in that ECL is generated form the electrode itself, no luminophore or luminophore labeled DNA probe is need. Although the mass production screen-printed electrodes (SPCEs) are attractive for fabricating disposable biosensors, they are not suitable for fabricating regeantless biosensors. We firstly make use of the intrinsic cathodic ECL at oxide covered electrodes to fabricate DNA biosensor, which opens a new window for constructing low-cost reagentless biosensors.

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