



Detection of T4 DNA ligase using a solid-state electrochemiluminescence biosensing switch based on ferrocene-labeled molecular beacon

Xiaoying Wang^{a,b}, Ping Dong^a, Wen Yun^a, Ying Xu^a, Pingang He^{a,*}, Yuzhi Fang^{a,*}

^a Department of Chemistry, East China Normal University, Shanghai 200062, China

^b School of Public Health, Southeast University, Nanjing 210009, China

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ABSTRACT

A solid-state electrochemiluminescence (ECL) biosensing switch based on special ferrocene-labeled molecular beacon (Fc-MB) has been successfully developed for T4 DNA ligase detection. Such special switch system consisted of two main parts, an ECL substrate and an ECL intensity switch. The ECL substrate was made by modifying the complex of Au nanoparticle and Ruthenium (II) tris-(bipyridine) ($\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs) onto Au electrode. A molecular beacon labeled by ferrocene as the ECL intensity switch. The molecular beacon is designed with special base sequence, which could combine with its target biomolecule *via* the reaction of the repair and recombination of nucleic acids by DNA ligase. During the reaction, the molecular beacon opened its stem-loop, and the labeled Fc was consequently kept away from the ECL substrate. Such structural change resulted in an obvious increment in ECL intensity due to the decreased Fc quenching effect to the ECL substrate. The analysis results are sensitive and specific.

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1. Introduction

Nucleic acid ligation is one vital process in the repair, replication and recombination of nucleic acids and also an important tool for editing DNA both *in vivo* and *in vitro* [1–3]. Therein, DNA ligase plays an indispensable role in catalyzing the formation of phosphodiester bonds at single strand nick between adjacent 3'-hydroxyl and 5'-phosphate ends in double strand DNA [4–7]. Therefore, specific recognition and quantitative detection of DNA ligase are crucial to fundamental research as well as clinical practice. Currently, the ligation process is usually assayed by radial P labeling, denaturing gel electrophoresis, autoradiography and fluorescence [4]. But these methods are time consuming and not sensitive [8–11].

Molecular beacon (MB), a stem-loop structure oligonucleotide, was initially described in 1996 as a switch able to undergo spontaneous conformational change following hybridization with the complementary nucleic acid target [12,13]. It possesses high selectivity, fast speed, convenience and excellent sensitivity. MB has been used in the investigation of genetic disease, the studies of DNA-protein interactions, the monitoring of the nucleic acids ligation process and the detection of DNA/RNA hybridization in living cells [14–19]. Tang et al. have used MB to monitor the nucleic acid

ligation process in real-time, the sequence of which is designed to be complementary with the product of the ligation [9]. However, the MB, ligation substrate, nucleic acid hybridization complex and DNA ligase are all in solution. MB can only be used to detect DNA ligase in homogeneous solution.

In our recently studies, a solid-state electrochemiluminescence biosensing switch was developed successfully [20,21]. The biosensing system employed $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs modified Au electrode to emit ECL, and the ferrocene-labeled molecular beacon (Fc-MB) to control the ECL intensity with consideration of the high ECL quenching efficiency of Fc to $\text{Ru}(\text{bpy})_3^{2+}$. It has been found that the ECL intensity of the $\text{Ru}(\text{bpy})_3^{2+}$ -AuNPs modified Au electrode was correlated to the conformation of the Fc-MB. The Fc-MB was designed with special base sequence, which could be combined with its target biomolecule *via* the reaction of DNA hybridization or aptamer-protein combination. Therefore, the system can be applied to recognize the relevant biomolecules selectively.

Based on these studies, we attempt to combine the solid-state electrochemiluminescence biosensing switch with the template of nucleic acids ligation as well as the repair and recombination of nucleic acids by DNA ligase to detect DNA ligase. The Fc-MB is not only a probe for the detection of the ligation process, but also a template for the two oligos in the ligation process. Using this method, an efficient bioassay for monitoring the activity of T4 DNA ligase has been developed. In this protocol, the MB, ligation substrate, nucleic acid hybridization complex and DNA ligase were all captured to the surface of the electrode to avoid the interference by the target

* Corresponding authors. Tel.: +86 21 62233508/798; fax: +86 21 62233508.

E-mail addresses: pghe@chem.ecnu.edu.cn (P. He), yzfang@chem.ecnu.edu.cn (Y. Fang).

oligos and basal solution in detection. The method can be used to quantitate the T4 DNA ligase rapidly, sensitively and selectively.

2. Experimental

2.1. Reagents and apparatus

Oligonucleotides were purchased from Shenggong Bioengineering Ltd Company (Shanghai, China). The sequence of molecular beacon (MB): 5'-NH₂-(CH₂)₆-GGGCGTTGATGGTTCCACTTCTCGTGCGT TCAACG-(CH₂)₆-SH-3' (the underlined and italic sequences indicate the stem and loop of the MB), Fragment N1: 5'-CGCACCAGAAAGTGAACC-3' (18-nt oligo matching the loop of the MB), N2: 5'-CGCACCAGA-3' (9-nt oligo matching 3' half part of the loop of the MB), N3: 5'-AGTGAACC-3' (9-nt oligo matching 5' half part of the loop of the MB), N4: 5'-pAGTGAACC-3' (9-nt oligo matching 5' half part of MB's loop and was phosphorylated at its 5' end). T4 DNA ligase (5 U μL⁻¹) and diluted buffer (66 mM Tris-HCl containing 50% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM EDTA and 50 mM KCl, pH 7.5) were purchased from Dingguo Biological Technology Corporation of Shanghai. Ru(bpy)₃²⁺ (99.95%), HAuCl₄, 6-mercapto-1-hexanol (SH-(CH₂)₆-OH, >97.0%), cysteamine (SH-(CH₂)₂-NH₂) and 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC) were purchased from Sigma (USA). Ferrocenecarboxylic acid (FCA), dithiothreitol (DTT), horseradish peroxidase (HRP) and glucose oxidase (GOD) were purchased from Maoji Bioengineering Ltd Company of Shanghai. The following buffers were used: 66 mM Tris-HCl containing 0.8 M NaCl and 100 mM LiClO₄ (pH 7.5), 66 mM Tris-HCl containing 50 μM ATP, 20 mM MgCl₂, 10 mM DTT and 100 mM LiClO₄ (pH 7.5), 66 mM Tris-HCl containing 1.0 mM tri-*n*-propylamine (TPrA) and 5.0 mM LiClO₄ (pH 8.7) was used as the detecting solution. Other reagents were of analytical reagent grade. All of the solutions were prepared with ultrapure water from a Millipore Milli-Q system.

ECL was recorded with MPI-E electrogenerated chemiluminescence analyzer (Xi'an Remax Electronic Science Tech., Co. Ltd), a CHI 660A electrochemical analyzer (CHI instruments Inc., USA) was used to carry out impedance measurements.

2.2. Preparation of Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode

The Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode was prepared according to previously published protocols [20]. Briefly, a self-assembled monolayer of cysteamine is prepared onto Au electrode firstly (cysteamine-derivated Au electrode), then, the Ru(bpy)₃²⁺-AuNPs composite is assembled onto the cysteamine-derivated Au electrode to form the luminescent substrate. The as-prepared electrode was Ru(bpy)₃²⁺-AuNPs electrode. The Fc-MB is attached onto the above electrode via Au-S interaction, and the electrode was then treated with SH-(CH₂)₆-OH solution to occupy the unassembled surface of Ru(bpy)₃²⁺-AuNPs as well as adjust the Fc-MB distribution on the electrode surface. Thus, the Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode was obtained which has a solid-state electrochemiluminescence biosensing switch of Fc-MB-Ru(bpy)₃²⁺-AuNPs.

2.3. The specific recognition of T4 DNA ligase onto Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode

The Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode was incubated in 500 μL of 66 mM Tris-HCl (pH 7.5) containing 0.8 M NaCl, 100 mM LiClO₄ and the two short complementary DNA at room temperature for 2 h to form a DNA complex with a nick. By this way, the dsDNA with a nick electrode was formed.

The resultant electrode was incubated with T4 DNA ligase in 66 mM Tris-HCl (pH 7.5) containing 50 μM ATP, 20 mM T4 DNA ligase MgCl₂, 10 mM DTT and 100 mM LiClO₄ for 15 min. Then the

electrode was washed with the same buffer to decrease the non-specific binding at most, and the dsDNA electrode was obtained.

2.4. Electrogenerated chemiluminescence detection

A three-electrode system was used with the modified Au electrode (2 mm in diameter) as the working electrode, an Ag/AgCl (sat.) as the reference electrode and a platinum wire as the counter electrode. Cyclic voltammetry mode with continuous potential scanning from 0.0 to 1.2 V and scanning rate of 0.1 V s⁻¹ was applied to achieve ECL signal in 66 mM Tris-HCl containing 1.0 mM TPrA and 5.0 mM LiClO₄ (pH 8.7) at room temperature. A high voltage of -800 V was supplied to the photomultiplier for luminescence intensity determination.

3. Results and discussion

3.1. Recognition of T4 DNA ligase using the solid-state electrochemiluminescence biosensing switch

The ligation system is composed of a DNA ligase, two oligos to be ligated and a ferrocene-labeled molecular beacon (Fc-MB), in which the combined sequences of the two oligos are complementary to the loop sequence of the Fc-MB. Fig. 1 presents an illustration of the ligation process. In the beginning, each oligo is hybridized to one-half of the loop of the Fc-MB to form a DNA complex with a nick as shown in step a in Fig. 1. It cannot open the Fc-MB stem completely, but can slightly destabilize the stem. Once ligase is added, the ligation reaction closes the nick to form a longer DNA strand which is complementary to the Fc-MB, resulting in the Fc-MB opening completely and the Fc being pulled away from the electrode (step b).

The corresponding ECL intensity-potential curves of the electrodes are presented in Fig. 2. As shown in the figure, compared to the ECL signal of Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode without hybridization with any DNA (curve a), the ECL signal should be a slight increase for the slight torsion of the stem of Fc-MB and part of Fc being shifted from original position after the process of hybridization with the two short complementary DNA (curve b). The ligation reaction closes the nick to form a longer DNA strand which is complementary to the MB, resulting in the stem of Fc-MB being separated and Fc being pulled away from the electrode. So a remarkable increase of ECL signal was presented simultaneity (curve c). Thus, the difference of ECL intensity before and after the ligation (ΔI_{ECL}) can be used to quantify the T4 DNA ligase.

3.2. The characterization of solid-state electrochemiluminescence biosensing electrode

The fabrication processes of solid-state electrochemiluminescence biosensing electrode and the interaction between fabricated biosensing electrode and T4 DNA ligase were characterized by electrochemical impedance spectroscopy (EIS). For EIS measurements, [Fe(CN)₆]^{3-/4-} was employed as the redox probe and Nyquist plots were used to calculate the R_{et} for modified electrodes. As shown in Fig. 3, the R_{et} for bare Au electrode was 246.4 Ω, and increased to 469.7 Ω for the cysteamine-derivated Au electrode, then declined to 327.9 Ω for the Ru(bpy)₃²⁺-AuNPs electrode. It is attributed to the electronic conductive capacity of Au nanoparticles. The results showed that 10 μM Fc-MB was self-assembled onto the Ru(bpy)₃²⁺-AuNPs electrode, the R_{et} increased to 737.6 Ω. This is attributed to fact that self-assembled biosensing switch of the Fc-MB having negative charges on its phosphate backbone makes an electrostatic repulsive force to [Fe(CN)₆]^{3-/4-}. The R_{et} of dsDNA with a nick electrode and dsDNA electrode was 1164.3 Ω and 2069.2 Ω, respectively. The successive increase in

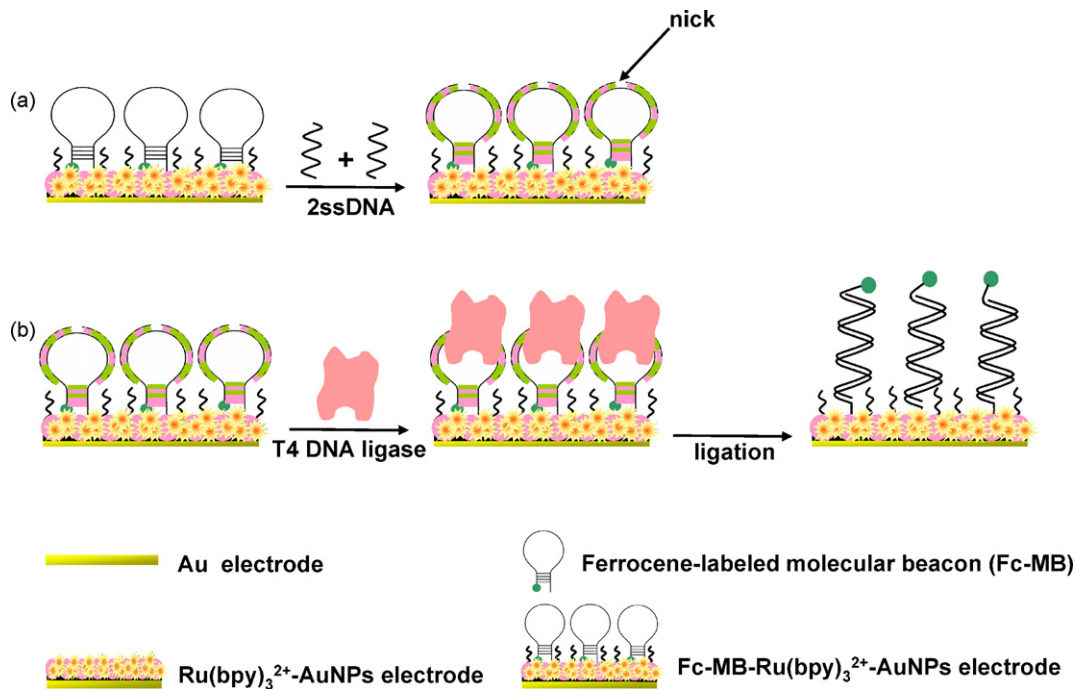


Fig. 1. The specific recognition of T4 DNA ligase by using the Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode. (a) The two half-matching oligos hybridize with the Fc-MB to form a nick. (b) The T4 DNA ligase binds to the nick and catalyzes the ligation of two short oligos to form a longer oligo.

the R_{et} illustrates the successful modification of the biomolecules onto Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode. It also indicates that the interactions between immobilized Fc-MB and the two short complementary DNA, dsDNA with a nick and T4 DNA ligase arise successively.

3.3. The selectivity of solid-state electrochemiluminescence biosensing switch

Two control experiments were performed for evaluating the selectivity of solid-state electrochemiluminescence biosensing switch. In order to confirm the repair and recombination specificity of T4 DNA ligase to target nucleic acids, ECL experiments were performed for different samples, such as Fc-MB with N1 (sample a), Fc-MB with N2 and N4 (sample b), Fc-MB with N2 and N3 (sample c) and Fc-MB only (sample d). The difference between N3 and N4 is that whether the 5' end of DNA fragment was modified with a

phosphate group or not, and it is a necessary condition for repair and recombination nucleic acids in DNA ligation reaction [5–7]. As presented in Fig. 4, there was no ECL change for samples a, c and d. It implied that there was no significant change in Fc-MB stem-loop structure after the addition of T4 DNA ligase into these sample solutions. However, the ECL of sample b increased rapidly and reached a plateau after 15 min, which is contributed to the ligation reaction of T4 DNA ligase to link N2 and N4 into one long DNA strand that is complementary to Fc-MB (displayed in Fig. 1). As a result, the Fc-MB opened completely, and ferrocene molecule was away from the Ru(bpy)₃²⁺-AuNPs electrode, which finally led to the increase of the ECL intensity. An almost complete ligation reached after 15 min.

Another control experiment was carried out by using 5 U mL⁻¹ T4 DNA ligase, horseradish peroxidase (HRP), glucose oxidase (GOD) or mixed sample to react with N2 and N4. It could be found

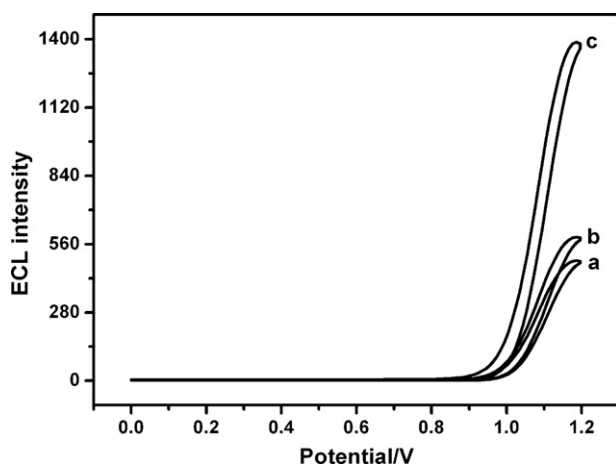


Fig. 2. ECL intensity-potential curves for various electrodes: (a) Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode, (b) dsDNA with a nick electrode and (c) dsDNA electrode.

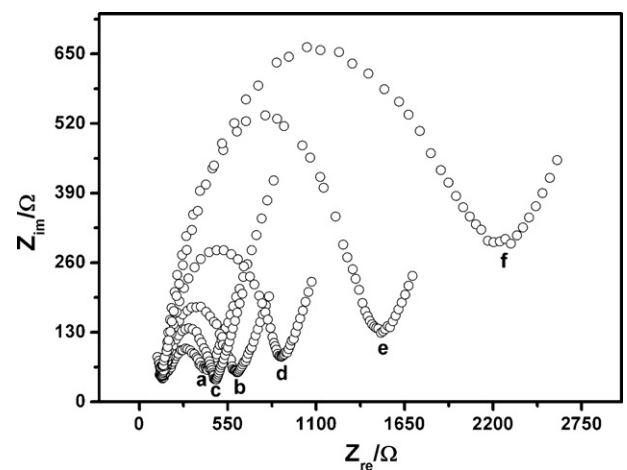


Fig. 3. Nyquist plots for the impedance measurement in 10 mM [Fe(CN)₆]^{3-/4-} solution for the bare Au electrode (a), cysteamine-derivated Au electrode (b), Ru(bpy)₃²⁺-AuNPs electrode (c), Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode (d), dsDNA with a nick electrode (e) and dsDNA electrode (f).

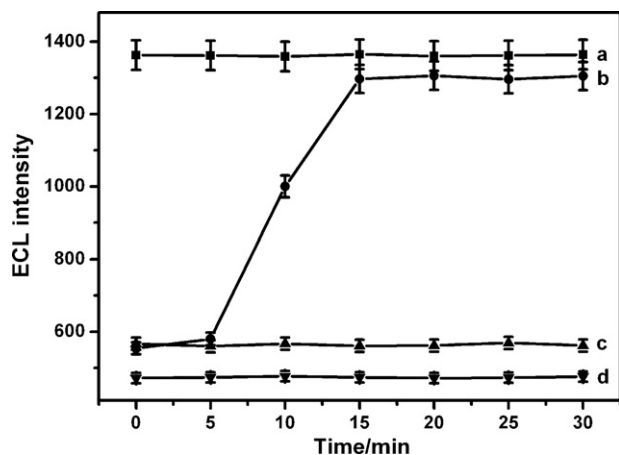


Fig. 4. ECL measurements for the repair and recombination specificity of T4 DNA ligase to target nucleic acids. The Fc-MB-Ru(bpy)₃²⁺-AuNPs electrode incubated with N1 and T4 DNA ligase (a), N2, N4 and T4 DNA ligase (b), N2, N3 and T4 DNA ligase (c), and T4 DNA ligase (d).

that only the samples containing T4 DNA ligase showed an obvious increase of ECL intensity as shown in Fig. 5. However, the change of the ECL intensity for the dsDNA with a nick electrode reacting with HRP or GOD can be neglected. It is therefore can be concluded that the ECL intensity increase is caused by the T4 DNA ligase combining with the nick as showed in Fig. 1, which suggested the unique repair and recombination specificity of the T4 DNA ligase to the nick between Fc-MB and the two short complementary DNA, N2 and N4. Above results also indicate that the solid-state electrochemiluminescence biosensing switch has a good selectivity for discriminating the two short DNA to other complementary DNA and T4 DNA ligase from other enzyme.

3.4. Optimization of experimental conditions

The ligation process can be affected by many molecular species including biomolecules and metal ions. The effect of four molecular species, including ATP, Mg²⁺, K⁺ and Na⁺ was studied. The experimental results show that the ligation did not take place unless ATP or Mg²⁺ was added to the ligation mixtures. The ligation reached its maximum rate at 50 μM of ATP or at 20 mM of Mg²⁺, respectively. The high concentration of K⁺ and Na⁺ obviously hindered the ligation process. The initial ligation velocity decreased proportionally to the concentrations of K⁺ or Na⁺, and the velocity was close to 0 when the concentrations were ~300 mM. In addition, the activ-

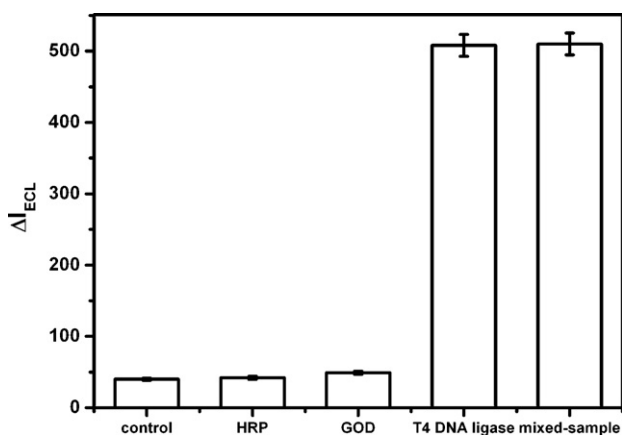


Fig. 5. Comparison of the ΔI_{ECL} of the dsDNA with a nick electrode when incubated with T4 DNA ligase, HRP, GOD and mixed sample (T4 DNA ligase/HRP/GOD).

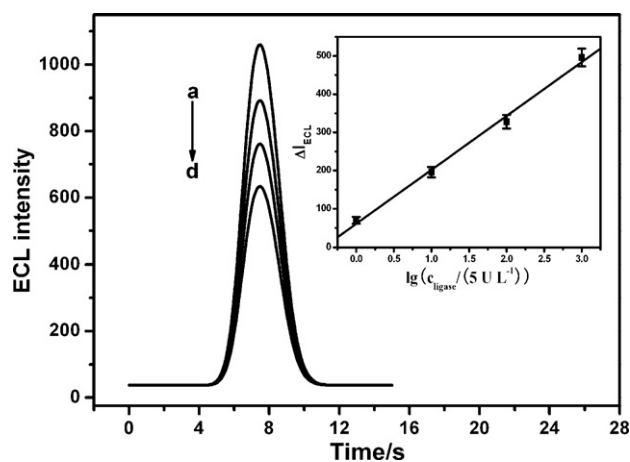


Fig. 6. ECL intensity–time curves for various dsDNA electrodes. The concentrations of T4 DNA ligase were $5 \times 10^{-3} \text{ U } \mu\text{L}^{-1}$ (a), $5 \times 10^{-4} \text{ U } \mu\text{L}^{-1}$ (b), $5 \times 10^{-5} \text{ U } \mu\text{L}^{-1}$ (c) and $5 \times 10^{-6} \text{ U } \mu\text{L}^{-1}$ (d), respectively. Inset: the calibration curve of T4 DNA ligase detection. ECL curves were measured in 66 mmol/L Tris–HCl containing 1.0 mM TPrA and 5.0 mM LiClO₄ (pH 8.7). Scan rate: 0.1 V s^{-1} , scan range: 0.0–1.2 V.

ity of T4 DNA ligase has been maintained by dithiothreitol (DTT). Therefore, the 66 mM Tris–HCl buffer solution (pH 7.5) containing 50 μM ATP, 20 mM MgCl₂ and 10 mM DTT was selected as the ligation buffer solution.

The efficiency of the ligation, which effects on the ECL intensity of the second ECL determination, is in correlation with ligation time and ligation temperature. The ECL intensity increased with the increment the ligation time, and reached its plateau regions at the ligation time of 15 min. Thus, the optimal ligation time was chosen at 15 min. The influence of the hybridization temperature on the response is investigated, the ECL signal increased rapidly from 5 to 30 °C, then changed slowly from 35 to 40 °C, and reduced notably after 50 °C. So, 37 °C was chosen as the hybridization temperature at the following experiment.

3.5. The calibration curve of T4 DNA ligase detection

According to the Michaelis–Menten equation, the initial reaction rate catalyzed by enzymes is directly proportional to the concentration of the enzyme within a certain concentration range [22]. When the initial reaction rate of the T4 DNA ligation reaction was expressed as the increased rate of the ECL intensity, it was namely the increased value of the ECL intensity (ΔI_{ECL}) in a certain period of time. The sensitivity of the solid-state electrochemiluminescence biosensing switch was investigated. Fig. 6 shows the ECL profiles of the different concentration of T4 DNA ligase after binding with dsDNA electrode. The ECL intensity was enhanced when the T4 DNA ligase concentration increased, and the change of the ECL intensity, ΔI_{ECL} , was found to be linear with the logarithm of T4 DNA ligase concentration in the range from $5 \times 10^{-6} \text{ U } \mu\text{L}^{-1}$ to $5 \times 10^{-3} \text{ U } \mu\text{L}^{-1}$ (as shown in the inset). The equation for the resulting calibration plot was $y = 141 \lg x + 14$ (x : the concentration of T4 DNA ligase divide $5 \text{ U } \mu\text{L}^{-1}$, y : ΔI_{ECL}) with correlation coefficient of 0.9975 and detection limit of $2.5 \times 10^{-6} \text{ U } \mu\text{L}^{-1}$.

4. Conclusions

An efficient bioassay for monitoring the T4 DNA ligase has been developed using a solid-state electrochemiluminescence biosensing switch based on Fc-MB. The Fc-MB is not only a probe for the detection of the ligation process, but also a template for the two oligos in the ligation process. The conformation change information of the Fc-MB before or after the ligation was a reflection of the ECL

signal changes of the whole modified electrode. Thus, the ECL signal changes of the modified electrode can be detected once the nucleic acids were ligated. By this way, the ligation process can be monitored simultaneously and accurately. This method provides a new and effective platform for studying a wide variety of nucleic acids ligation processes and interactions between protein (enzyme) and nucleic acid. Furthermore, it is meaningful to the deeply study the dynamics of them.

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