Biosensors and Bioelectronics 26 (2011) 2215–2220

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Electrogenerated chemiluminescence detection of adenosine based on triplex DNA biosensor

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ARTICLE INFO

Article history:
Received 25 June 2010
Received in revised form
19 September 2010
Accepted 21 September 2010
Available online 29 September 2010

Keywords:
Tris(2,2′-bipyridine) ruthenium
Ferrocenemonocarboxylic acid
Biosensor
Aptamer

ABSTRACT

A novel electrogenerated chemiluminescence (ECL) biosensor based on the construction of triplex DNA for the detection of adenosine was designed. The ECL biosensor employs an aptamer as a molecular recognition element, and quenches ECL of tris(2,2′-bipyridine) ruthenium ($Ru(bpy)_3^{2+}$) by ferrocenemonocarboxylic acid (FcA). Through self-assembly technology, the ECL probe of thiolated hairpin adenosine aptamer tagged was self-assembled onto the surface of a gold electrode with an ECL signal producer $Ru(bpy)_3^{2+}$ derivative (Ru-DNA-1). The adenosine aptamer, including a section of triplex characteristic chain, formatted triplex DNA with two other DNAs (DNA-2, Fc-DNA-3) in the presence of triplex DNA binder coralyne chloride (CORA). Fc-DNA-3 was tagged with an ECL quencher ferrocenemonocarboxylic acid (FcA), a quenching probe. In the presence of adenosine, the aptamer sequence (Ru-DNA-1) prefers to form the aptamer–adenosine complex with hairpin configuration and the switch of the DNA-1 occurs in conjunction with the generation of a strong ECL signal owing to the dissociation of a quenching probe. Meanwhile, a control experiment was performed; the ECL-duplex biosensor was designed to detect adenosine. The detection limits were 2.7×10^{-10} mol L^{-1} and 2.3×10^{-9} mol L^{-1} for the ECL-triplex DNA biosensor and ECL-duplex DNA biosensor, respectively, which demonstrated that the ECL-triplex DNA biosensor improved the sensitivity and specificity greatly.

1. Introduction

DNA sensor has recently attracted much attention due to its diverse applications. Various DNA biosensors have been established including optical (Zhao et al., 2003; J. Zhang et al., 2008; S.S. Zhang et al., 2008; Ding et al., 2008), electrochemical (Park et al., 2002; Wang et al., 2003; Hu et al., 2008; Li et al., 2008; S.S. Zhang et al., 2009; X.R. Zhang et al., 2009), ECL (Xu et al., 1994), quartz-crystal microbalance (Su et al., 2004; Yamaguchi and Shimomura, 1993), and surface plasmon resonance techniques (Ito et al., 2007).

Among the many developed DNA sensors, the design of ECL biosensor has recently become one of the most active research areas because the sensors combine the advantages of both electrochemical chemiluminescent biosensors (Miao, 2008; Bertoncello and Forster, 2009; Forster et al., 2009), can provide high sensitivity with low background and ease of control. Therefore, this detection method has been utilized in a number of bioanalytical areas, including immunoassays and PCR product detection (Jie et al., 2010; Yu et al., 1995; Zhu et al., 2008; Zhou et al., 2008). Especially, aptamer-based ECL biosensors or biosensing method (Li et al., 2007) have garnered much attention over the last two decades due to the specific binding affinity of this kind of artificial oligonucleotide toward a variety of targets ranging from small molecules (Yao et al., 2009; Wang et al., 2005; Sankaran et al., 2006; Li et al., 2010), proteins (Zhou et al., 2009; Chen et al., 2010) and even to cells (Deng et al., 2010), substantially enable them to be used as recognition elements for biosensing applications (Nutiu and Li, 2003; Ren et al., 2010). All of the methods above mentioned have been involved with traditional duplex DNA structure, however, in recent years, triplex DNA has been more concerned for its special structure and properties.

A DNA triplex is formed upon binding of a pyrimidine or a purine single-stranded DNA to the major groove of a double helix, forming Hoogsteen or reverse-Hoogsteen hydrogen bonds with the purine strand of the duplex, which are already engaged in Watson–Crick hydrogen bonds (Casey and Glazer, 2001; Guntaka et al., 2003). This often provides an approach to the sequence-specific recognition of double-stranded DNA and subsequent gene regulation, including interfering with transcription, replication, repair and recombination (Guntaka et al., 2003; Faria and Giovannangeli, 2001; Rogers et al., 2005; Kalish and Glazer, 2005; Cheng et al., 2005). Chen reported the construction of a DNA nanomachine whose motion is triggered by changes in the solution pH value. Reversible formation and dissociation of a DNA triplex containing C+G-C triplets take place when the solution pH value changes between pH 5.0 and 8.0 (Chen et al., 2004). Jung designed the triplex-AuNP conjugates, it is possible that structures composed of triplex-forming AuNPs would generate

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doi:10.1016/j.bios.2010.09.037

swelling/shrinking of the whole structure in response to changes in pH value, and such variations might be applied to the design of a pH-dependent delivery system for small molecules, such as drugs and probe entities (Jung et al., 2006). Recently, triplex-binding strategy has been used as a means of labeling nanonetworks, for example, Tumpane et al. adopted the triplex recognition to label and functionalize nanostructures (Tumpane et al., 2007). This strategy is a simple model of a write-read-erase function of an information-rich DNA nanostructure driven by changes in pH. Also, triplex DNA molecular beacons, are as signaling probes based on a chemiluminescence resonance energy transfer (CRET) system for detection of small molecules (S.S. Zhang et al., 2009; X.R. Zhang et al., 2009).

However, to our best knowledge, ECL-triplex DNA biosensing method has not been reported. In the present work, a novel, simple and "signal on" biosensor based on ECL-triplex DNA is reported for the first time. The aim of the present work is to develop a high sensitivity ECL-triplex biosensing method for the determination of adenosine, using aptamer as a recognition element based on the triplex strand formation and the quenching ECL of $Ru(bpy)_3^{2+}$ by ferrocene. The characteristics of ECL-triplex biosensing method for the determination of adenosine and the analytical performance are performed. The sensor is used successfully for detecting adenosine.

2. Experimental

2.1. Reagents

All oligonucleotide sequences were customer-designed and synthesized by SBS Genetech Co. Ltd. (Beijing, China), and all stock solutions were prepared with triply distilled water and kept frozen. Dilute solutions of nucleotides with different concentration were prepared with the 0.01 mol L^{-1} phosphate buffer solution (PBS, pH 7.40, 0.10 mol L^{-1} NaCl + 0.01 mol L^{-1} KH_2PO_4/K_2HPO_4). Their sequences are given as follows:

- DNA-1 (adenosine aptamer labeled by tris(2,2′-b bipyridine)ruthenium derivative):
  - o 3′-HS-$(CH_2)_6$-ACC TGG GGG AGT ATT GCG GAG GAA GGT–NH_2-5′;
- DNA-2 (complementary to DNA1):
  - o 5′-TCA TAA CGC CTC CTT CCA-3′;
- DNA-2′ (complementary to DNA1):
  - o 5′-TCA TAA CGC CTC CTT CCA–NH_2-3′;
- DNA-3 (complementary to DNA-2, labeled by ferrocenemonocarboxylic acid and underline means the binding sequence of triplex characteristic chain):
  - o 5′-GCG GAG GAA GGT–NH_2-3′.

FcA was obtained from Strem (USA). Ruthenium(III) chloride hydrate and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (USA). Tripropylamine (TPA), 2,2′-bipyridine, 2,2′-bipyridyl-4,4′-dicarboxylic (dcbpy), 6-mercapto-1-hexanol (MCH) was obtained from Fluka (USA). Imidazole was obtained from Guoyao Chemical Company (China). Human serum, coralyne chloride (CORA) and other chemicals for buffers were purchased from Sigma. All DNA oligonucleotides were acquired from SBS Genetech. Co. Ltd. (China). All the reagents were analytical grade and used without further purification.

Dilute solutions of DNA and adenosine with different concentration were prepared with the 0.01 mol L^{-1} PBS. The triplex DNA hybridization buffer (pH 7.4) consisted of a PBS solution and 0.5 mol L^{-1} NaCl. After each step, the electrode was rinsed thoroughly with 0.01 mol L^{-1} PBS buffer solution. The ECL measurement was performed in 0.10 mol L^{-1} PBS (pH 7.40, 0.10 mol L^{-1} NaCl + 0.10 mol L^{-1} KH_2PO_4/K_2HPO_4).

2.2. Apparatus

The experimental setup for ECL measurements was Remex MPI-E client system (Xi'an Remex Analysis Instrument Co., China). A commercial cylindroid glass cell was used as an ECL cell. The conventional three-electrode system was composed of a gold electrode (diameter 1.5 mm), an Ag/AgCl reference electrode, and a platinum wire counter electrode. The electrochemical impedance spectroscopy (EIS) was performed on the CHI 660C electrochemical workstation (Shanghai CH Instrument, China). UV–vis absorption spectra were obtained with a Cary 50 probe UV-Vis spectrophotometer (Varian Co., Australia). A TDL-16B centrifuge (Shanghai Anting Science Instrument Co., China) was used for centrifugation.

2.3. Synthesis of $Ru(bpy)_2$(dcbpy)NHS-labeled DNA probe

$Ru(bpy)_2$(dcbpy)NHS was synthesized according to the literature (Terpetschnig et al., 1995; Shimdzu et al., 1985). The ECL probe, $Ru(bpy)_2$(dcbpy)NHS-DNA-1 (abbreviated as Ru-DNA-1) was synthesized according to the literature procedures (J. Zhang et al., 2008; S.S. Zhang et al., 2008) with a little difference. Briefly, 250 μL of 1.0×10^{-6} mol L^{-1} DNA-1 was added to the mixture of 200 μL of 1.0×10^{-4} mol L^{-1} $Ru(bpy)_2$(dcbpy)NHS and 10 μL of 0.1 mol L^{-1} tetraborate buffer (pH 8.5). The acquired solution was incubated at room temperature for 12 h with gentle shaking. Followed by addition of 100 μL of 3 mol L^{-1} NaAc and 2 mL ethanol, the mixture was frozen in refrigerator for at least 12 h. Finally the ECL DNA probe tagged with $Ru(bpy)_2$(dcbpy)NHS were collected by centrifugation at 12,000 rpm for 30 min. The precipitate was washed with cold 70% ethanol twice and dried in air. The dried precipitate was re-dissolved in 250 μL of 0.01 mol L^{-1} PBS. The solution of $Ru(bpy)_2$(dcbpy)NHS modified DNA-1 (abbreviated as Ru-DNA-1) was stored at 4 °C for later use.

2.4. Synthesis of quenching probe

The ECL quenching probe, Fc-DNA-3 probe was prepared by chemically bonding FcA to the terminal 5′-amino of the DNA-3 in the presence of a water-soluble EDC (Yang et al., 2005). Briefly, 20 mg of imidazole was first added into 200 μL of 1 mmol L^{-1} FcA, and the solution was adjusted to pH 7.0 by 1 mol L^{-1} HCl. 20 mmol L^{-1} EDC was then added into this prepared solution to activate those carboxylic groups on FcA. After 30 min, 280 μL of 10 μmol L^{-1} DNA-3 was added into the resulting FcA-EDC solution with stirring. After 12 h, the reaction was stopped by adding 100 μL of 3 mol L^{-1} sodium acetate and 1 mL ethanol. The above solution was refrigerated for at least 12 h and centrifuged at 12,000 rpm for 30 min, and the sediment was obtained as Fc-DNA-3. The final product was washed with 70% cold ethanol, centrifuged, resuspended in 0.01 mol L^{-1} PBS and stored at 4 °C for later use.

2.5. Fabrication of ECL biosensors

The cleaned electrode was immersed in 200 μL of the aptamer probe solution with a desired concentration and incubated for an appropriate time at 37 °C to immobilize the ECL probe on the surface of the electrode. Then the aptamer probe immobilized electrode was immersed in 0.01 mol L^{-1} PBS containing 0.01 mol L^{-1} MCH for 1 h to block the uncovered surface of the electrode. The blocked electrode was immersed in the mixture of DNA-2 and the Fc-DNA-3 probe in 0.01 mol L^{-1} PBS (pH 7.4, 0.5 mol L^{-1} NaCl) with triplex DNA binder CORA for 1 h. The triplex DNA structure was formed. After each step, the electrode was thoroughly rinsed with 0.01 mol L^{-1} PBS. The resulting electrode was used as a triplex DNA ECL biosensing electrode.

The ECL-duplex DNA biosensor was constructed as follows: Ru-DNA-1 was immobilized on the surface of Au electrode the same as mentioned above. Then the electrode was immersed in the complementary DNA sequence tagged with ferrocene (Fc-DNA-2′) solution in 0.01 mol L^{-1} PBS at 37 °C for 1 h to hybridize to form the duplex DNA biosensor.

2.6. Detection of adenosine

The ECL biosensing electrode was immersed in 200 μL of 0.01 mol L^{-1} PBS containing different concentrations of adenosine for 1 h, followed by thoroughly washing with 0.01 mol L^{-1} PBS to remove unbound adenosine.

2.7. ECL measurement

The electrode was transferred into 10 mL homemade quartz ECL cell. A three-electrode system was used at room temperature with modified Au electrode (1.5 mm in diameter) as the working electrode, an Ag/AgCl (sat.) as the reference electrode, and a platinum wire as the counter electrode. The ECL measurement was performed at a constant potential of +1.00 V in 2.0 mL of 0.10 mol L^{-1} PBS containing 0.10 mol L^{-1} TPA. A high voltage of −900 V was supplied to the photomultiplier to determine the luminescence intensity.

2.8. Adenosine detection in human serum

Human serum was diluted 5-fold by buffer (0.10 mol L^{-1} PBS) to produce a 20% serum sample. The extraction recoveries of adenosine from serum samples were measured based on the calibration graph using pure adenosine in PBS at comparable levels. The concentration of adenosine detected in the diluted serum can be converted to the concentration in the original serum by multiplying the results by five.

3. Results and discussion

3.1. Fabrication of the ECL-triplex biosensor and detection process

Scheme 1A and B illustrates the fabrication and detection process of adenosine of the ECL-triplex biosensors. The ECL-triplex biosensor contains three nucleic acid strands. Through self-assembly technology, Ru-DNA-1 was self-assembled onto the surface of a gold electrode with an ECL signal producer. The adenosine aptamer, including a section of triplex characteristic chain, formatted triplex DNA with the other two DNA chains. One of its complementary DNA sequence, was DNA-2. The other of its complementary DNA sequence, was DNA-3 tagged with an ECL quencher FcA (Fc-DNA-3). As an effective and cheap triplex DNA binder (Lytton-Jean et al., 2007), CORA raises the stability of triplex biosensor. The three DNA chains were combined in the presence of triplex DNA binder CORA. The ECL-triplex DNA biosensor was constructed. In the absence of adenosine, the ECL probe immobilized on the surface of the electrode maintained a rigid, linear triple-helix conformation, which led to a weak ECL signal, probably due to the quenching ECL of $Ru(bpy)_3^{2+}$ by ferrocene and the ECL element moving away from the electrode surface.

In the presence of adenosine, the aptamer sequence (Ru-DNA-1) prefers to form the aptamer–adenosine complex with hairpin configuration and the structure switching (Zayats et al., 2006) of the Ru-DNA-1 occurs in conjunction with the generation of a strong ECL signal owing to the dissociation of Fc-DNA-3. The ECL vs potential and the inset the CV is shown in Fig. S1. The concentration of adenosine was quantified by an increase of ECL peak height ΔI ($\Delta I = I - I_0$), where I_0 is the ECL peak height before the detection of adenosine, and I is the ECL peak height after the detection of adenosine.

3.2. Fabrication of the ECL-duplex biosensor and detection process

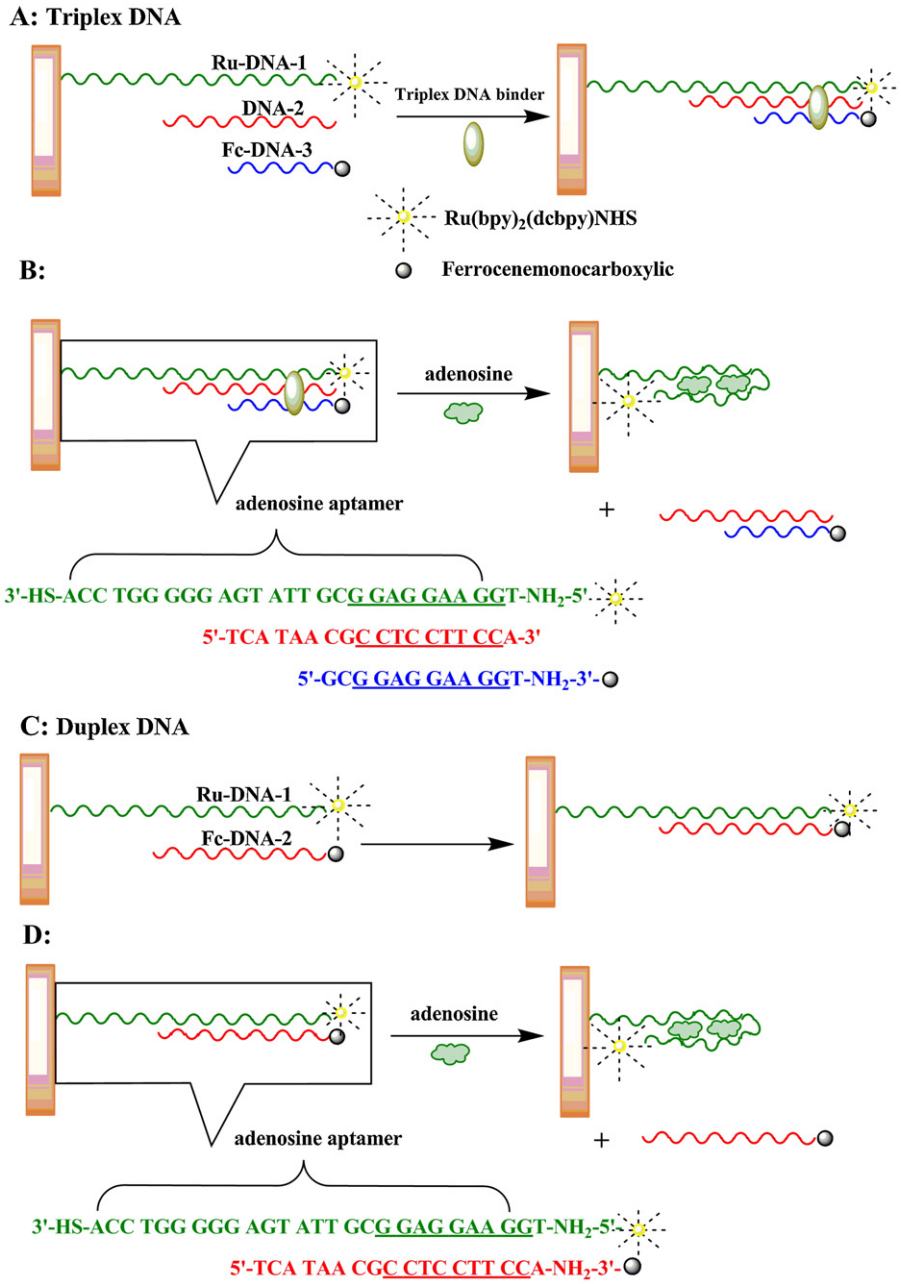
The design of the ECL-duplex biosensor sensor is shown in Scheme 1C and D. The ECL-duplex biosensor sensor contains two nucleic acid strands. Through self-assembly technology, Ru-DNA-1 was self-assembled onto the surface of a gold electrode. Fc-DNA-2′ was its complementary DNA sequence tagged with an ECL quencher ferrocene. The ECL-duplex DNA biosensor was constructed. The detection process was the same as one of the triplex biosensor.

3.3. Characterization of ECL-triplex DNA biosensor

The fabrication process of ECL biosensing electrode and the interaction between fabricated biosensing electrode and adenosine was characterized by electrochemical impedance spectroscopy (EIS) as shown in Fig. 1. EIS was a powerful tool to monitor the whole procedure in preparing modified electrodes, which could provide valuable information for exploring the changes of the surface of gold electrode in different modification stages. The results showed that EIS of the bare Au electrode exhibited an almost straight line, which was characteristic of a mass diffusion limiting electron-transfer process. When Ru-DNA-1 was self-assembled onto the gold electrode, it wasn't a line, the interfacial electron-transfer resistance (Ret) occurred. This is attributed to the fact that self-assembled Ru-DNA-1 having a single strand nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force to $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. A short-chain alkyl molecule MCH was employed to occupy the left bare sites on gold electrodes for eliminating the unspecific adsorption, led to the Ret increased. When DNA-2 was hybridized with Ru-DNA-1 to form the duplex DNA biosensor, the Ret was further increased. While DNA-2 and Fc-DNA-3 were hybridized with Ru-DNA-1 to form the triplex DNA structure, the Ret increased greatly, which was higher than duplex DNA structure. This is probably attributed to the fact that an insulating layer on the surface of the duplex DNA biosensing electrode is generated and the transfer of redox couple $[Fe(CN)_6]^{3-/4-}$ to the surface of gold electrode is prohibited (Cai et al., 2006). This indicates that the triplex DNA biosensor was constructed.

The corresponding ECL intensity–time curves of the various electrodes in 0.1 mol L^{-1} PBS containing 0.1 mol L^{-1} TPA were presented in Fig. S2. A weak ECL signal appeared when the triplex DNA was immobilized on Au electrode, while a sharp increase of the ECL signal is observed when the modified Au electrode was reacted with a desired concentration of adenosine solution. The results verify that the ECL of the Ru-DNA-1 can be efficiently quenched by the Fc-DNA-3 and through the action between adenosine and its aptamer chain, the aptamer sequence (Ru-DNA-1) prefers to form the aptamer–adenosine complex with hairpin configuration and the switch of the Ru-DNA-1 occurs. The mechanism for quenching ECL has been explained as bimolecular energy or electron transfer between $Ru(bpy)_3^{2+*}$ and ferrocerium (Fc^+), the oxidized species of Fc, along with suppression of radical reactions (Cao et al., 2006).

The UV–vis spectra of the $Ru(bpy)_2$(dcbpy)NHS, unmodified DNA probe, Ru-DNA-1 were recorded by the spectrophotometer as shown in Fig. 2. The absorption spectrum of the $Ru(bpy)_2$(dcbpy)NHS (curve a) exhibits a characteristic peak at 450 nm, assigned to metal-to-ligand charge-transfer band. Curve b exhibited the characteristic absorbance of DNA at about 260 nm which had no characteristic absorbance appeared between 260 nm and 500 nm. The appearance of characteristic absorbance of DNA at about 260 nm displayed in curve c indicated that the



Scheme 1. Schematics of the ECL-triplex DNA biosensor (A) and the application in detection of adenosine (B), dublex DNA biosensor (C) and the application in detection of adenosine (D).

$Ru(bpy)_2(dcbpy)NHS$ had been successfully labeled on the 5′-terminus of DNA-1 probe.

3.4. Optimization of the self-assembly time

To improve the sensitivity of ECL quantification of adenosine, the self-assembly time of the aptamer probe was optimized when the concentration of the adenosine was 10^{-8} mol L^{-1}. Fig. S3a shows the effect of the self-assembly time on the increased ECL intensity (ΔI). It was obvious that ΔI increased upon raising the concentration from 1 to 4 h, and then it started to level off, attributed to the steric and electrostatic hinderance arising from the more tightly packed probe, which were necessary for highly hybridization efficiency. Thus the self-assembly time of 4 h was selected for the subsequent assays after confirming the workable range of buffer conditions for adenosine.

3.5. Optimization of the triplex DNA hybridization

The concentration of salt was an important influence condition for the stabilization of the triplex DNA structure. The concentration of NaCl was changed as shown in Fig. S3b, when the concentration of the adenosine was 10^{-8} mol L^{-1}. At first, ΔI increased with the increase of the concentration of NaCl from 0.1 to 0.5 mol L^{-1}. Thus, the concentration of NaCl was 0.5 mol L^{-1} in the PBS buffer solution for the triplex DNA hybridization.

The triplex DNA hybridization time was another influence condition for the assays. The influence of the hybridization time was investigated as shown in Fig. S3c, when the concentration of the adenosine was 10^{-8} mol L^{-1}. The results show that ΔI reaches the maximum at 70 min. So the hybridization time of 60 min was routinely used for these assays.

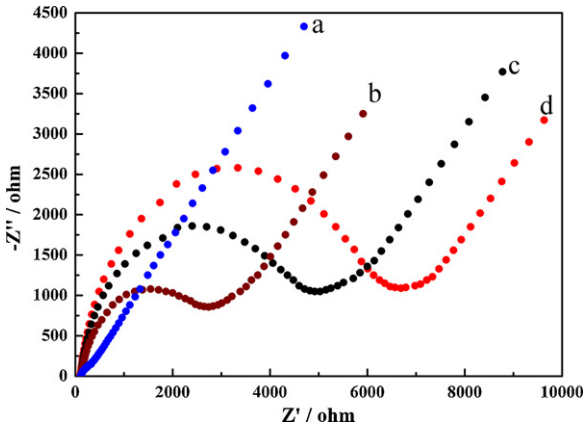


Fig. 1. Electrochemical impedance spectra (Nyquist plots) corresponding to Au electrode (a), after immobilization of Ru-DNA-1 (b), hybridization with DNA-2 (c), and triplex DNA formed (d). The data were recorded in the presence of 2.5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$, applying 5 mV alternative voltage in the frequency range of 0.05 Hz to 100 kHz. Data were recorded in a PBS solution (10 mmol L^{-1}, pH 7.4).

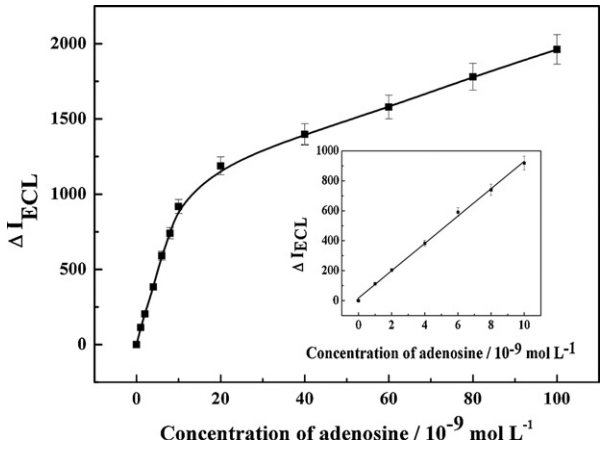


Fig. 3. ECL-triplex DNA biosensor for detection of adenosine of different concentration (varying the concentration of adenosine from 1.0×10^{-9} to 1.0×10^{-7} mol L^{-1}). Inset: standard working curve of relative adenosine. ECL experiments were carried out in 0.10 M PBS (pH 7.40, 0.10 mol L^{-1} NaCl + 0.1 mol L^{-1} KH_2PO_4/K_2HPO_4) containing 0.1 mol L^{-1} TPA at a scan rate of 100 mV/s, the PMT voltage was −900 V.

The effect of pH of the PBS buffer solution for triplex DNA hybridization was investigated in the pH range from 6.8 to 8.0 as shown in Fig. S3d. ΔI reached the maximum at pH 7.4. Thus, the pH 7.4 was used as an optimal condition.

Then we explored the action time between adenosine and the ECL-triplex biosensor as shown in Fig. S4 that is important for the exact detection of adenosine. The results showed that 90 min is enough for adenosine to sufficiently react.

3.6. Sensitivity of the ECL-triplex DNA biosensor and ECL-duplex DNA biosensor

The sensitivity of the aptamer-based ECL-triplex DNA biosensing assay was investigated by using different concentrations of adenosine. Experiments showed that the ECL intensity increased with the increase of the concentration of the adenosine ranging from 1.0×10^{-9} to 1.0×10^{-7} mol L^{-1} as shown in Fig. 3. The linear range for the concentration of adenosine was from 1.0×10^{-9} to 1.0×10^{-8} mol L^{-1}. Regression equation was $Y = A + BX = 17.167 + 91.253X$ (X was the concentration of adenosine, 10^{-9} mol L^{-1}; Y was the increased ECL intensity, ΔI_{ECL}) with a coefficient $R = 0.999$. A detection limit of 2.7×10^{-10} mol L^{-1} of adenosine can be estimated using 3σ (where σ is the standard deviation of a blank solution, $n = 11$). The detection limit obtained using the present sensing system is 10,000-fold lower than the literature value achieved using label-free and reagentless aptamer-based sensor (Zayats et al., 2006), more than 6 orders of magnitude lower than the detection limit reported using a colorimetric method based on AuNP aggregation (Liu and Lu, 2004), 100 times lower than that of the ferrocene-labeled aptamer probe (Wu et al., 2007), and 1 order of magnitude lower than that of SPR sensor (Wang et al., 2010).

A control experiment was performed with the ECL-duplex DNA biosensor. Compared with the triplex biosensor, the average blank value was higher and the sensitivity of the control experiment was lower. The linear range for the concentration of adenosine was from 1.0×10^{-8} to 1.0×10^{-7} mol L^{-1}, the sensitivity of the proposed method was found to be increased about 1 order of magnitude than that of ECL-duplex DNA biosensor (Fig. 4). In the process of our experiment, we found that the average blank value of triplex biosensor was lower than that of duplex biosensor. According to the ECL quenching mechanism (Cao et al., 2006), FcA quenches ECL of $Ru(bpy)_3^{2+}$ through an electron-transfer mechanism, the electron transfer is believed to occur between the $Ru(bpy)_3^{2+}$ and

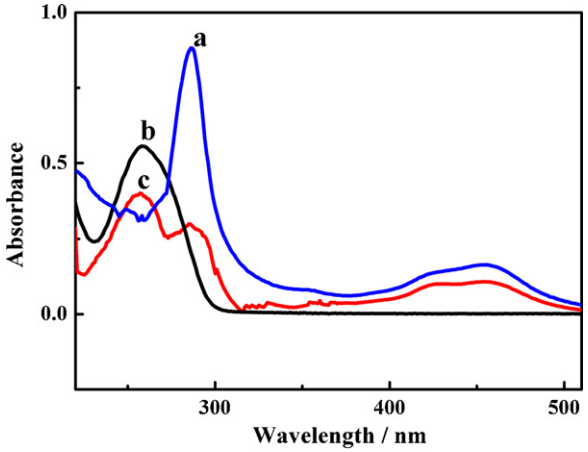


Fig. 2. The UV–vis spectra: (a) 3.9×10^{-5} mol L^{-1} $Ru(bpy)_2(dcbpy)NHS$, (b) 2.8×10^{-6} mol L^{-1} unmodified DNA probe and (c) 1.0×10^{-5} mol L^{-1} DNA probe labeled with $Ru(bpy)_2(dcbpy)NHS$ (Ru-DNA-1).

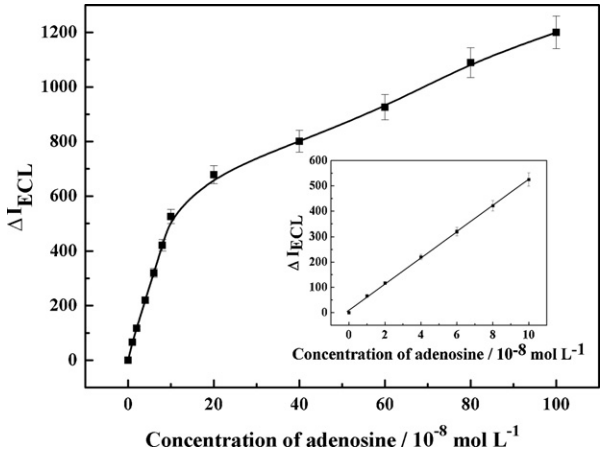


Fig. 4. ECL-duplex DNA biosensor for detection of adenosine of different concentration (varying the concentration of adenosine from 1.0×10^{-8} to 1.0×10^{-6} mol L^{-1}). Inset: standard working curve of relative adenosine. The experimental conditions were as in Fig. 3.

FcA species because the complementary DNA sequences bring the ECL reporter and the quencher molecule into close proximity. ECL quenching occurs through both direct electron transfer from $Ru(bpy)_3^{2+}$ to the quencher molecule and charge transfer through the base pairs of the DNA to the quencher molecule. Compared with the common double helix DNA sequences, the tripled-strands DNA increases the compactness upon triple-helix formation (Brucale et al., 2005), so it is then expected to improve the charge transfer to the quencher molecule. So the triplex biosensor enhances the ECL quenching efficiency by FcA, in accordance with the lower ECL blank value of triplex biosensor than that of control duplex biosensor.

In addition, we also found that the ECL value of triplex biosensor was higher than that of control duplex biosensor in the presence of adenosine with the same concentration. The triplex structure is relatively unstable at room temperature and will only form in the presence of triplex DNA binding molecules (Lytton-Jean et al., 2007). Compared to the duplex, the stability of the triplex is much more sensitive to the change of the condition. So the triplex biosensor is expected to be more sensitive to the presence of adenosine in this experiment.

3.7. Selectivity of the ECL-triplex DNA biosensor

The evaluation of the selectivity of ECL-triplex DNA biosensing method was performed, different ECL signals of the proposed ECL-triplex DNA biosensing electrode after immersing the electrode in 10^{-8} mol L^{-1} adenosine, cytidine or uridine. On the basis of structure-switching signaling aptamers for the detection of small molecules, cytidine and uridine, did not induce any significant changes in the ECL signal as compared to that of adenosine. This indicates that the ECL-triplex biosensing method has a good selectivity.

3.8. Measurement of adenosine in the serum

To explore their potential application in real sample analysis, the triplex DNA biosensor designed here was used to quantify adenosine in 20% human serum. Because the concentration of other electroactive species and non-electroactive species such as protein, fat and sugar in serum sample were also diluted, the effect of other electroactive species on the detection was slight, the foul block on the electrode surface was reduced markedly. The statistical data for the quantization of adenosine were shown in Table S1, which indicated recoveries ranging from 90 to 100% and coefficients of variation less than 10%. The results suggest that the biosensor was successful in detecting the respective target in the real-world samples.

4. Conclusions

In the present work, a novel ECL-triplex DNA biosensor for the detection of adenosine was constructed. Herein, we use an aptamer as a molecular recognition element and quenching ECL of $Ru(bpy)_3^{2+}$ by FcA. The biosensor presents relative sensitive adenosine detection with a low detection limit of 2.7×10^{-10} mol L^{-1} which is more sensitive than the duplex DNA biosensor. It shows great promise to use ECL-triplex biosensing method for detecting adenosine. Currently, the application of this method in the sequential determination of other proteins was still in process in our laboratory.

Acknowledgement

This work was supported by the Education Administration Foundation of Shandong Province (No. J09LB09).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.09.037.

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