

# Label-free supersandwich electrogenerated chemiluminescence biosensor for the determination of the HIV gene

Sanpeng Ruan · Zhejian Li · Honglan Qi · Qiang Gao · Chengxiao Zhang

Received: 11 January 2014 / Accepted: 1 April 2014 / Published online: 16 April 2014  
© Springer-Verlag Wien 2014

**Abstract** We describe a highly sensitive electrochemiluminescence (ECL) based method for the determination of the human immunodeficiency virus-1 (HIV-1) gene. A long-range self-assembled double strand DNA (ds-DNA) is used as a carrier, and the ruthenium complex  $\text{Ru}(\text{phen})_3^{2+}$  as an ECL indicator for signal amplification. The thiolated ss-DNA serving as a capture probe is firstly self-assembled on the surface of a gold electrode. After the target HIV-1 gene is completely hybridized with the capture probe, two previously hybridized auxiliary probes are hybridized with the target HIV-1 gene to form long-range supersandwich ds-DNA polymers on the surface of the electrode. Finally, the ECL indicator is intercalated into the supersandwich ds-DNA grooves. This results in a strongly increased ECL in tripropylamine solution because a large fraction of the intercalator is intercalated into supersandwich ds-DNA. The results showed that the increased ECL intensity is directly related to the logarithm of the concentration of the HIV-1 gene in the range from 0.1 pM to 0.1 nM, with a detection limit of 0.022 pM and using only 10  $\mu\text{L}$  of analyte samples. The method can effectively discriminate target HIV-1 gene (a perfectly matched ss-DNA) from a 2-base mismatched ss-DNA. This work demonstrates that the high sensitivity and selectivity of an ECL DNA biosensor can be largely improved by using supersandwich ds-DNA along with ECL indicators.

**Keywords** DNA hybridization · Electrogenerated chemiluminescence · Human immunodeficiency virus-1 gene · Supersandwich model

## Introduction

Highly sensitive method for the detection of DNA has attracted much attention due to its diverse applications including the identification of genetic diseases and disorders and the detection and characterization of genetic viruses, bacteria, and parasites [1]. Traditional methods for the detection of DNA on the basis of base-pair hybridization, such as gel electrophoresis or membrane blots, are slow and labor-intensive. DNA hybridization biosensors offer considerable promise for obtaining sequence-specific information in a simpler, faster and cheaper manner, compared to traditional hybridization assays [2]. Various DNA biosensors have been established including fluorescence imaging [3, 4], optical [5–7], electrochemical [8, 9], electrogenerated chemiluminescence (ECL) [10, 11], quartz-crystal microbalance [12], and surface plasmon resonance techniques [13]. In these DNA biosensors, ECL biosensors are very promising due to the combination of advantages of both electrochemical and chemiluminescent biosensors, such as high sensitivity and ease of control.

Much effort in the research of ECL DNA-based biosensors (DNA hybridization biosensors, aptasensor) has been made to improve the sensitivity and specificity. These include the employment of gold nanoparticles [14] and carbon nanotube [15] and dendrimers [16] as signal carriers for signal amplification, use of a hairpin DNA as a probe [17] for signal and recognition enhancement. In these ECL biosensors, the ss-DNA probe is generally labeled with an ECL reagent. The labeling

**Electronic supplementary material** The online version of this article (doi:10.1007/s00604-014-1252-3) contains supplementary material, which is available to authorized users.

S. Ruan · Z. Li · H. Qi · Q. Gao · C. Zhang (✉)  
Key Lab of Analytical Chemistry for Life Science of Shaanxi Province, School of Chemistry & Chemical Engineering, Shaanxi Normal University, Xi'an 710062, China  
e-mail: cxzhang@snnu.edu.cn

process of the DNA probe, however, is time-consuming and reduces the hybridization efficiency. Thus, increasingly attractive attention is to develop the label-free ECL DNA biosensors, mainly using ECL reagents such as  $\text{Ru}(\text{phen})_3^{2+}$  and  $\text{Ru}(\text{bpy})_3^{2+}$  as intercalator. Yin et al. [18] developed an ECL aptamer-based biosensor for the detection of thrombin based on a  $\text{Ru}(\text{phen})_3^{2+}$ -double-strand DNA composite film. Plaxco et al. [19] firstly reported a novel supersandwich approach for electrochemical detection of target DNA in complex matrices. They employed a modified signal probe containing a methylene blue (a redox moiety) label and a “sticky end.” When the ss-DNA target hybridizes this signal probe, the sticky end remains free to hybridize another target leading to the creation of a supersandwich structure containing multiple labels. This leads to large signal amplification upon monitoring by voltammetry. Xu et al. [20] reported an ECL method for the detection of  $\text{Hg}^{2+}$  by employing a label-free supersandwich model based on T– $\text{Hg}^{2+}$ –T coordination and the intercalation of  $\text{Ru}(\text{phen})_3^{2+}$ . Zhuo et al. [21] reported an ECL aptasensor for the detection of thrombin based on DNA supersandwich structure, in which hollow gold nanospheres were employed as tag-carriers for the aptamer, and  $\text{Ru}(\text{phen})_3^{2+}$  as an ECL signal reagent was intercalated into ds-DNA. Recently, Xiang et al. [22] reported an ECL method for the detection of DNA based on hybridization chain reaction for signal amplification and  $\text{Ru}(\text{phen})_3^{2+}$  as ECL intercalator. To the best of our knowledge, ECL DNA hybridization biosensors on the basis of supersandwich model using linear ss-DNA have not been reported.

The aim of the present work is to develop a highly sensitive and label-free ECL method for the detection of HIV-1 gene based on a supersandwich DNA structure and  $\text{Ru}(\text{phen})_3^{2+}$  as intercalator. A HIV-1 gene (38-mer ss-DNA from HIV-1 U5 long terminal repeat gene sequence) [23] was chosen as target analyte (See Table 1). Schematic diagram of the label-free ECL hybridization biosensing based on supersandwich DNA model is shown in Fig. 1. The thiolated ss-DNA as a capture probe is

firstly self-assembled on the surface of a gold electrode. After the target HIV-1 gene is completely hybridized with the capture probe, previously hybridized two auxiliary probes are hybridized with the target HIV-1 gene to form long-range supersandwich ds-DNA polymers on the surface of the electrode. Finally,  $\text{Ru}(\text{phen})_3^{2+}$  is intercalated into the supersandwich ds-DNA grooves, resulted in greatly increasing ECL intensity in tripropylamine solution since a great amount of  $\text{Ru}(\text{phen})_3^{2+}$  are intercalated into supersandwich ds-DNA. In this paper, a label-free ECL supersandwich DNA hybridization biosensing for the detection of HIV-1 gene is fabricated and characterized, and the optimization of experimental conditions and analytical performance of this method are presented.

## Experimental

### Reagents

Tris(1,10-phenanthroline) ruthenium(II) dichloride ( $\text{Ru}(\text{phen})_3\text{Cl}_2\cdot\text{H}_2\text{O}$ ) and 6-mercapto-L-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)). Tripropylamine (TPA), potassium hexacyanoferrate (III) and other chemicals employed were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China, [www.sinoreagent.com](http://www.sinoreagent.com)). All oligonucleotides used as listed in Table 1 were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China, [www.sangon.com](http://www.sangon.com)). 0.10 M phosphate buffer saline (PBS, pH 7.4, 0.10 M  $\text{NaCl}$ +0.10 M  $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ ) was used as hybridization buffer and washing solution. All ss-DNA solutions were prepared using 0.10 M PBS (pH 7.4). All reagents were of analytical grade. Millipore Milli-Q water (18  $\text{M}\Omega\cdot\text{cm}$ ) was used throughout.

### Apparatus

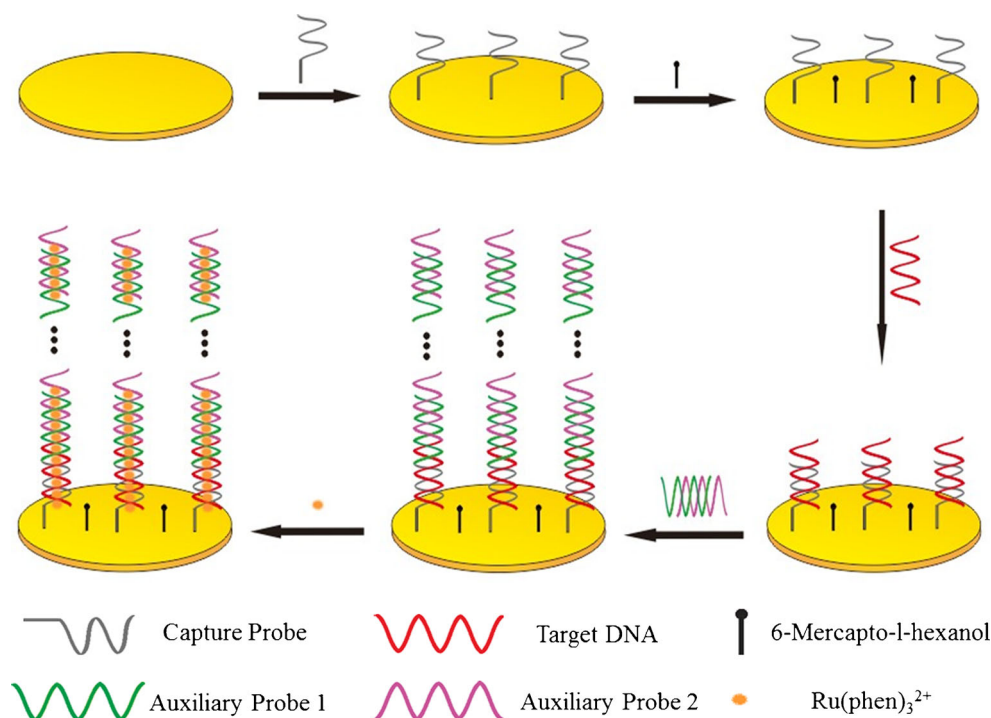
ECL measurements were performed with a MPI-E ECL detector (Xi'an Remax Electronics, Xi'an, China, [www.](http://www.)

**Table 1** Sequence of oligonucleotides used in this work

Oligonucleotides	Sequence (from 5' to 3')
HIV-1 gene	GCT AGA GAT TTT CCA CAC TGA CTA AAA GGG TCT GAG GG
Capture probe	CAG TGT GGA AAA TCT CTA GC-( $\text{CH}_2$ ) <sub>6</sub> -SH
Auxiliary probe 1	<b>TAC TCC CCC AGG TGC CCC TCA GAC CCT TTT AGT</b>
Auxiliary probe 2	<b>GCA CCT GGG GGA GTA ACT AAA AGG GTC TGA GGG</b>
Completely mismatched ss-DNA compared with HIV-1 gene	CCT TTT AGT CAG TGT GGA AAA TCT CTA GCA GTG GC
Two-base mismatched ss-DNA compared with HIV-1 gene	GCT AGA GAT <u>TGG</u> CCA CAC TGA CTA AAA GGG TCT GAG GG

The italic fragment at the 3' end of auxiliary probe 1 is complementary with the italic fragment of auxiliary probe 2. The bold fragment at the 5' end of auxiliary probe 2 can hybridize with the bold fragment of auxiliary probe 1. The underlined bases are the mismatched bases of two-base mismatched ss-DNA compared with HIV-1 gene

**Fig. 1** Schematic diagram of the label-free ECL DNA hybridization biosensing based on supersandwich ds-DNA structure



chinaremex.com). A commercial cylindroid glass cell was used as an ECL cell (diameter, 25 mm). A three-electrode system composed of a biosensor or a disk gold electrode (2.0 mm diameter with an effective electrode area of 3.42 mm<sup>2</sup>) [24] as working electrode, a platinum plate as counter electrode, and an Ag/AgCl (saturated KCl) as reference electrode, respectively. For detecting ECL, the cell was placed directly in front of a photomultiplier (PMT, operated at -900 V) and the PMT window was only opened toward the working electrode to eliminate the blank CL and the ECL from the counter electrode.

#### Immobilization of capture probe

A disk gold electrode was mechanically polished with 0.3 and 0.05 μm alumina slurry, ultrasonicated for 3 min in ultrapure water, ethanol, and ultrapure water respectively to remove any remaining polishing agent, and then electrochemically cleaned by a linearly scanning potential between -0.2 and +1.5 V in 0.10 M H<sub>2</sub>SO<sub>4</sub> until a stable cyclic voltammogram was obtained [25], and finally washed with water, dried with nitrogen.

Ten microliters of 1 μM capture probe was drop-coated on the surface of the cleaned gold electrode, allowed to stay for 4 h to thiol-self-assembly the capture probe on the surface of the electrode. The resulted electrode was thoroughly washed with 0.10 M PBS to remove the unbound capture probe, and then immersed in 100 μL of 2.0 mM MCH prepared using 0.10 M PBS for 1 h to block the uncovered surface of the gold electrode, and washed and dried under a stream of nitrogen gas. The ECL biosensor obtained was stored in 0.10 M PBS.

#### ECL measurements

First, 10 μL of a fixed concentration of target HIV-1 gene was dropped onto the surface of the ECL biosensor fabricated above, and incubated for 90 min in a sealed humidity chamber at 37°C. Second, 10 μL of a freshly prepared auxiliary probe solution containing 1 μM auxiliary probe 1 and 1 μM auxiliary probe 2 (Table 1) was dropped onto the surface of the electrode and then incubated for 90 min in a sealed humidity chamber at 37°C to form long-range supersandwich ds-DNA polymers on the surface of the electrode. Finally, the resulted electrode was immersed in 100 μL of 0.1 mM Ru(phen)<sub>3</sub><sup>2+</sup> for 90 min at room temperature. After each binding step, the resulted electrode was washed with 0.10 M PBS.

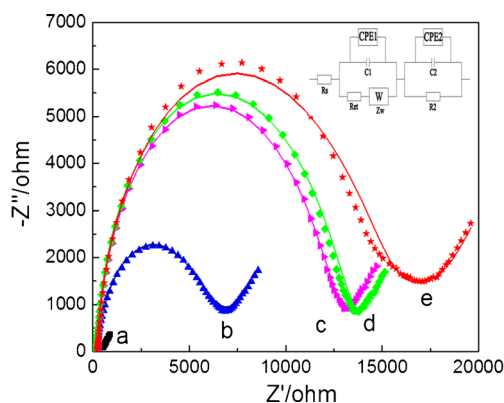
ECL measurements were performed at the resulted electrode prepared-above in 1 mL of 0.10 M TPA (pH 7.4) in the potential range from 0 to +1.3 V (versus Ag/AgCl) with a scan rate of 50 mV/s. The concentration of target HIV-1 gene was quantified by the increased ECL intensity ( $\Delta I = I - I_0$ ), where  $I_0$  is the ECL peak height in the absence of the target HIV-1 gene and  $I$  is the ECL peak height in the presence of the target HIV-1 gene.

## Results and discussion

#### Characteristics of the electrode assembling process

Electrochemical impedance spectroscopy (EIS) technique has proven to be one of the most powerful tools for the

characteristics of interfacial properties in the fabrication process of the biosensors and the hybridization process [26, 27]. Figure 2 shows the Nyquist plots of the gold electrode subjected to the step-by-step modification process, using  $[\text{Fe}(\text{CN})_6]^{4-/3-}$  as the redox indicator. The inset shows the equivalent circuit applied to fit the impedance spectra, where  $R_s$  is the solution resistance,  $R_{ct}$  is the interfacial electron transfer resistance,  $Z_w$  is a Warburg impedance, C1 and C2 are the double layer capacitances, CPE1 and CPE2 are the constant phase elements, and R2 is the resistance that develops between the MCH layer and the electrode surface. It can be seen that the simulating curves (solid line) are quite well fitted with the experimental data (dot). After the capture probe was self-assembled on the surface of gold electrode, the interfacial electron transfer resistance ( $R_{ct}$ ) value increases from  $260\ \Omega$  to  $5537\ \Omega$  (Fig. 2 curve a and curve b). This is attributed to the fact that the self-assembled capture probe having a single strand nucleic acid with negative charges on its phosphate backbone makes an electrostatic repulsive force to  $[\text{Fe}(\text{CN})_6]^{4-/3-}$  [28, 29]. This indicates that the capture probe is successfully assembled onto the surface of gold electrode. After blocking with 2 mM MCH, the  $R_{ct}$  value increases from  $5,537\ \Omega$  to  $12,700\ \Omega$  (Fig. 2 curve c), indicating that the capture probe-uncovered surface of the gold electrode is blocked. After the resulted electrode is hybridized with the target HIV-1 gene, the  $R_{ct}$  value slightly increases from  $12,700\ \Omega$  to  $13,490\ \Omega$  (Fig. 2 curve d), indicating that the HIV-1 gene is hybridized with the capture probe on the surface of the gold electrode. A relatively large increase of the  $R_{ct}$  ( $16,310\ \Omega$ , Fig. 2 curve e) is observed after two hybridization auxiliary probes prepared are introduced into the HIV-1 gene



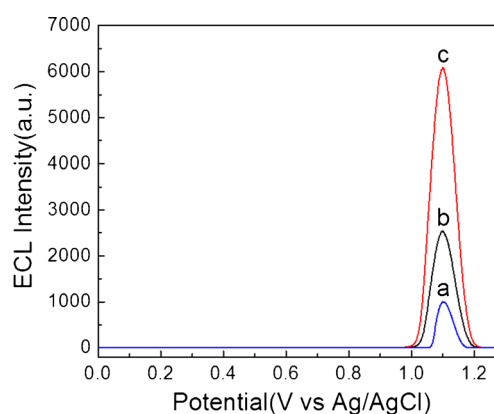
**Fig. 2** Nyquist plots of electrochemical impedance spectra obtained in 0.10 M PBS (pH 7.4)–5.0 mM  $\text{K}_4[\text{Fe}(\text{CN})_6]$ –5.0 mM  $\text{K}_3[\text{Fe}(\text{CN})_6]$ –0.10 M NaCl. Experimental data (dot). Fitted data (solid line). Electrodes used from a to e were: (a) bare gold electrode, (b) capture probe modified gold electrode, (c) capture probe modified gold electrode after blocking with 2 mM MCH, (d) capture probe/MCH modified gold electrode after interaction with  $1.0 \times 10^{-9}$  M target ss-DNA, (e) capture probe/MCH modified gold electrode after interaction with 1  $\mu\text{M}$  auxiliary probe 1 and auxiliary probe 2. Experimental conditions: The biased potential of 0.225 V, the frequency from 100 kHz to 0.1 Hz, and the amplitude of 5.0 mV

hybridized electrode. This indicates that long-range supersandwich ds-DNA polymers are formed on the surface of the electrode. This coincides with the characterization obtained by atomic force microscopy [23]. The EIS results indicate that the desired immobilization and hybridization are successfully accomplished on the electrode surface.

### Feasibility of supersandwich ECL approach

Figure 3 shows ECL intensity-potential profiles at different electrodes. From Fig. 3 (line a), it can be seen that a small ECL peak (1,000 a. u.) appears. This is attributed to the fact that the small amount of  $\text{Ru}(\text{phen})_3^{2+}$  is electrostatically bound to the negative charged capture probe immobilized on the gold electrode [30]. Compared Fig. 3 line b with line a, the ECL peak height increases from 1,000 a. u. to 2,500 a. u. after target HIV-1 gene is hybridized with the capture probe. This is attributed to the fact that the ECL reagent  $\text{Ru}(\text{phen})_3^{2+}$  is intercalated to the double strands formed by the capture probe and the target HIV-1 gene. It was found that the ECL peak height greatly increases from 2,500 a. u. to 6,100 a. u. after two auxiliary probes were further hybridized with the target HIV-1 gene on the surface of the electrode (Fig. 3, line c). This is attributed to the fact that the supersandwich ds-DNA is formed and a large number of  $\text{Ru}(\text{phen})_3^{2+}$  is intercalated to the long-range supersandwich ds-DNA. This indicates that a remarkable amplification is achieved. A supersandwich ECL DNA hybridization biosensing method is, therefore, feasible.

In order to understand the hybridizations among the capture probe and the target HIV-1 gene as well as auxiliary probes, the changes of Gibbs free energy ( $\Delta G$ ) were predicted with the computer program RNA Structure version 4.5 at



**Fig. 3** ECL intensity-potential profiles at different electrodes. (a) capture probe-assembled gold electrode; (b) capture probe/MCH modified gold electrode after interaction with  $5.0 \times 10^{-11}$  M HIV-1 gene; (c) capture probe/MCH modified gold electrode after interaction with 1  $\mu\text{M}$  auxiliary probe 1 and auxiliary probe 2 after interaction with  $5.0 \times 10^{-11}$  M HIV-1 gene. The intercalation time of  $\text{Ru}(\text{phen})_3^{2+}$  was 90 min. The ECL measurements were performed with a scan rate of 50 mV/s in 0.10 M PBS (pH 7.4) containing 0.10 M TPA

25 °C [31]. The absolute values of the predicted  $\Delta G$  from the capture probe/HIV-1 gene (-24.9 kcal/mol), HIV-1 gene/auxiliary probe 1 (-23.1 kcal/mol), and auxiliary probe 1/auxiliary probe 2 (-23.0 kcal/mol) are much higher than those of capture probe/auxiliary probe 1 (-4.1 kcal/mol) and HIV-1 gene/auxiliary probe 2 (-4.8 kcal/mol). From the changes of Gibbs free energy, it is anticipated that the capture probe can be hybridized with HIV-1 gene and HIV-1 gene with auxiliary probe 1, and auxiliary probe 1 with auxiliary probe 2. However, the capture probe could be not hybridized with auxiliary probe 1, and HIV-1 gene could be not hybridized with auxiliary probe 2. The predicted results with the computer program RNA Structure can support our design and the preliminary ECL results described above.

#### Optimization of experimental conditions

Important experimental parameters including intercalation time of  $\text{Ru}(\text{phen})_3^{2+}$ , the concentration of auxiliary probes and the hybridization time were optimized according to the experiment protocol described in ECL Measurements.

As expected, both the concentration of  $\text{Ru}(\text{phen})_3^{2+}$  and intercalation time of  $\text{Ru}(\text{phen})_3^{2+}$  influence the sensitivity due to their affecting the intercalation quantity of  $\text{Ru}(\text{phen})_3^{2+}$  into the grooves of the supersandwich ds-DNA. Fig. S1 (Electronic Supplementary Material, ESM) shows dependence of the intercalation time on the increased ECL intensity ( $\Delta I$ ) for the detection of  $5.0 \times 10^{-11} \text{ M}$  target HIV-1 gene. From Fig. S1 can be seen that the  $\Delta I$  linearly increases about 333 % with increasing intercalation time from 15 min to 60 min, and then slowly increases about 105 % with increasing intercalation time from 60 min to 90 min. Therefore, 90 min was chosen as the intercalation time of 0.1 mM  $\text{Ru}(\text{phen})_3^{2+}$  in following experiments. This is quite shorter than that reported in ECL aptasensors using  $\text{Ru}(\text{phen})_3^{2+}$  intercalated into ds-DNA as ECL indicator [18, 21].

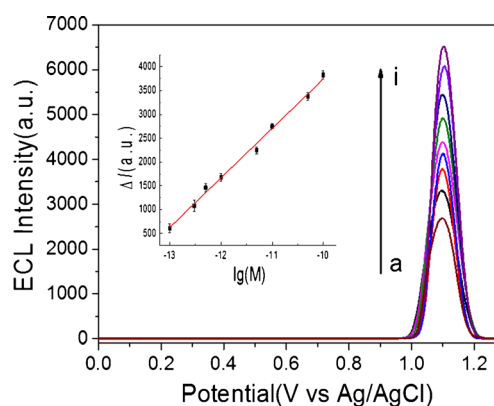
Fig. S2 (ESM) shows effect of the concentrations of two auxiliary probes on the ECL intensity for the detection of  $1.0 \times 10^{-11} \text{ M}$  target HIV-1 gene. It should be noted that the same concentrations of the two auxiliary probes were chosen since the hybridization ratio of the two auxiliary probes is one to one and the concentration of the target HIV-1 gene is much lower than that of the auxiliary probe. From Fig. S2, it can be seen that the ECL intensity sharply increases from 2,318 to 5,433 about 234 % as the concentrations of auxiliary probes increases from 0 to 1.0  $\mu\text{M}$ . This is attributed to the fact that amount of the long-range hybridized ds-DNA polymers increases and thus amount of  $\text{Ru}(\text{phen})_3^{2+}$  intercalated into ds-DNA increases. This indicates that the long-range supersandwich ds-DNA is formed. And the ECL intensity slightly increases from 5,433 to 5,739 about 106 % as the

increase of the concentrations of auxiliary probes from 1.0 to 2.0  $\mu\text{M}$ . This is attributed to long range electron transfer and the steric hindrance of the long-range hybridized ds-DNA polymers [32]. On consideration of the sensitivity and saving the reagents, therefore, the concentrations of auxiliary probes were chosen as 1.0  $\mu\text{M}$  in the subsequent experiments. These results indicate that in the case of tested experiment, the ECL intensity is 2.34 fold of that in the absence of the auxiliary probes. The amplification of supersandwich model is, therefore, evident. The signal amplification of supersandwich model coincides with those reported previously in the literatures [22, 23, 33].

Fig. S3 (ESM) shows effect of super-hybridization time of auxiliary probes on the increased ECL intensity for the detection of  $1.0 \times 10^{-11} \text{ M}$  target HIV-1 gene. It can be seen that the increased ECL intensity nearly linearly increases from 540 to 2,908 with increasing the hybridization time from 10 min to 60 min, and slowly increases from 2,908 to 3,306 with increasing the hybridization time from 60 min to 150 min. This change tendency coincides with the report by electrochemical method using  $[\text{Ru}(\text{NH}_3)_6]^{3+}$  as intercalator [23]. This suggests that the positive charge of the intercalator may be dominated rather than the size of intercalator. In subsequent experiments, 90 min was employed as the super-hybridization time to obtain high sensitivity.

#### Analytical performance

Figure 4 shows the ECL profiles of the ECL biosensor fabricated after interaction with different concentrations of target HIV-1 gene under the optimized conditions. From Fig. 4, it can be seen that the ECL intensity increases with an increase of the concentration of HIV-1 gene. The inset in Fig. 4 shows the



**Fig. 4** The ECL profiles of the ECL biosensor after interaction with different concentrations of HIV-1 gene under the optimized conditions. Inset: the linear relationship between increased ECL intensity ( $\Delta I$ ) and the logarithm of HIV-1 gene sequence concentration. The concentrations of HIV-1 gene: (a) 0 M; (b)  $1.0 \times 10^{-13} \text{ M}$ ; (c)  $3.0 \times 10^{-13} \text{ M}$ ; (d)  $5.0 \times 10^{-13} \text{ M}$ ; (e)  $1.0 \times 10^{-12} \text{ M}$ ; (f)  $5.0 \times 10^{-12} \text{ M}$ ; (g)  $1.0 \times 10^{-11} \text{ M}$ ; (h)  $5.0 \times 10^{-11} \text{ M}$ ; (i)  $1.0 \times 10^{-10} \text{ M}$ . ECL measurement was as the same as Fig. 3. The illustrated error bars represent the standard deviation of three repetitive measurements

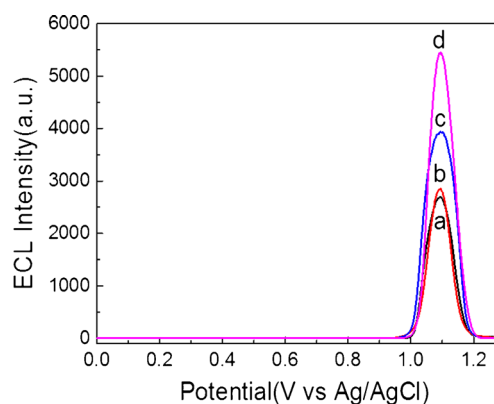
**Table 2** Comparison of the proposed method with some reported genosensors

Detection method	Detection limit	Signal amplification strategy	Refs
Differential pulse voltammetry	$1 \times 10^{-14}$ M	Probe labeled with carbon nanotubes loaded with silver nanoparticles	[36]
Square wave voltammetry	$1 \times 10^{-10}$ M	Label free methylene blue as indicator.	[34]
Square wave voltammetry	$1 \times 10^{-13}$ M	Labeled with methylene blue Supersandwich	[19]
Differential pulse voltammetry	$2 \times 10^{-18}$ M	Label free, supersandwich degassed with nitrogen for 15 min.	[23]
Electrochemical impedance spectroscopy	$1.7 \times 10^{-9}$ M	Label free Supersandwich	[33]
Quartz crystal microbalance	$1 \times 10^{-9}$ M	Label free	[12]
Chemiluminescence	$7.1 \times 10^{-17}$ M	HRP-labeled rolling circle amplification DNAzyme amplification	[37]
Fluorescence	$8 \times 10^{-16}$ M	Bioconjugated nanoparticles	[6]
Fluorescence image	$4 \times 10^{-11}$ M	FAM-labeled	[3]
Surface plasmon resonance	–	Label free DNA microarrays, gold nanoparticles	[13]
ECL	$5.0 \times 10^{-12}$ M	Labeled gold nanoparticle-modified electrodes	[14]
ECL	$9.0 \times 10^{-15}$ M	Carbon-nanotubes loaded ECL Labeled sandwich conjugate	[15]
ECL	$9 \times 10^{-11}$ M	Labeled Hairpin DNA probe	[17]
ECL	$6.7 \times 10^{-12}$ M	Labeled gold nanoparticle-modified electrode	[35]
ECL	$1.5 \times 10^{-14}$ M	Label free Chain reaction amplification supersandwich	[22]
ECL	$2.2 \times 10^{-14}$ M	Label free supersandwich	This work

quantitative relationship between the increased ECL intensity and the logarithm of the concentration of HIV-1 gene. The increased ECL intensity was directly related to the logarithm of the concentrations of target HIV-1 gene in the range from  $1.0 \times 10^{-13}$  to  $1.0 \times 10^{-10}$  M. The linear regression equation was  $\Delta I = 14126.5 + 1038.2 \lg C$  (unit of C is M) with a correlation coefficient of 0.9921. The detection limit was calculated to be  $2.2 \times 10^{-14}$  M HIV-1 gene ( $S/N=3$ ). Comparison of this method with some reported genosensors with the detection limit and the signal amplification strategy is listed in Table 2. The detection limit obtained is considerably lower than that of the genosensors reported in label free model [12, 33, 34] and labeled model [3, 14, 17, 19, 35]. It is higher than that of the genosensors reported with nanomaterials amplification [6, 15, 36] or chain reaction amplification [22] or rolling circle amplification DNAzyme amplification [37]. Although the detection limit obtained by differential pulse voltammetry with a label free supersandwich model is quite lower than that obtained in our work, it needs degassing tested solution with nitrogen for 15 min [23]. This is time-consuming and there may present unstable problems from  $\text{Ru}(\text{NH}_3)_2^{2+}$ . Therefore, an excellent detection limit obtained is considerably satisfactory.

To assay the specificity of this method, the ECL response of the DNA biosensor fabricated was examined under the optimized conditions for  $1.0 \times 10^{-11}$  M target HIV-1 gene,  $1.0 \times 10^{-9}$  M two-base mismatched target HIV-1 gene, and  $1.0 \times 10^{-9}$  M completely mismatched HIV-1 gene,

respectively. As shown in Fig. 5, the increased ECL intensity from completely mismatched HIV-1 gene is only 5.6 % of that from the perfect-matched HIV-1 gene while the increased ECL intensity from two-base mismatched HIV-1 gene is only 44.9 % of that from the perfect-matched HIV-1 gene although the concentrations of completely mismatched HIV-1 gene and two-base mismatched HIV-1 gene are higher than two orders of the perfect-matched target HIV-1 gene. These results indicate that the label-free supersandwich ECL method has a highly selectivity to effectively discriminate the perfect-



**Fig. 5** The ECL response for different gene sequences (a) blank (without target HIV-1 gene on the electrode); (b) completely mismatched HIV-1 gene ( $1.0 \times 10^{-9}$  M); (c) two-base mismatched HIV-1 gene ( $1.0 \times 10^{-9}$  M); (d) perfect-matched HIV-1 gene ( $1.0 \times 10^{-11}$  M). ECL measurement was as the same as Fig. 3

matched HIV-1 gene from the two-base mismatched HIV-1 gene and completely mismatched HIV-1 gene.

## Conclusions

A label-free ECL hybridization biosensing for the detection of target HIV-1 gene with high sensitivity and selectivity has been designed on the basis of supersandwich ds-DNA structure and ruthenium complex as an intercalated signal-producing compound. The detection limit of  $2.2 \times 10^{-14}$  M was achieved. The perfect-matched HIV-1 gene can be effectively discriminated from the two-base mismatched HIV-1 gene. Two ss-DNA auxiliary probes used can form a long-range self-assembled ds-DNA on the basis of themselves hybridization and target HIV-1 gene hybridization, and achieve 2.34 fold signal amplification. This work demonstrates that the high sensitivity and selectivity of an ECL DNA biosensing method could be greatly improved using supersandwich ds-DNA and ECL indicators. The strategy presented could be easily extended to various analytical platforms including ECL and electrochemical biosensors for the detection of many kinds of analytes or their interactions such as DNA/RNA, DNasezyme/target, and aptamer/target.

**Acknowledgments** Financial supports from the National Science Foundations of China (No. 21027007, 21275095) are gratefully acknowledged.

## References

- Hvastkovs EG, Buttry DA (2007) Electrochemical detection of DNA hybridization via bis-intercalation of a naphthylimide-functionalized viologen dimer. *Anal Chem* 79:6922–6926
- Wang J (2002) Electrochemical nucleic acid biosensor. *Anal Chim Acta* 469:63–71
- Benoit V, Steel A, Torres M, Yu YY, Yang HJ, Cooper J (2001) Evaluation of three-dimensional microchannel glass biochips for multiplexed nucleic acid fluorescence hybridization assays. *Anal Chem* 73:2412–2420
- Dharmadi Y, Gonzales R (2004) DNA microarrays: experimental issues, data analysis, and application to bacterial systems. *Biotechnol Prog* 20:1309–1324
- Taton TA, Lu G, Mirkin CA (2001) Two-color labeling of oligonucleotide arrays via size-selective scattering of nanoparticle probes. *J Am Chem Soc* 123:5164–5165
- Zhao XJ, Tapeç-Dytioco R, Tan WH (2003) Ultrasensitive DNA detection using highly fluorescent bioconjugated nanoparticles. *J Am Chem Soc* 125:11474–11475
- Ahn S, Walt DR (2005) Detection of Salmonella spp. using microsphere-based, fiber-optic DNA microarrays. *Anal Chem* 77:5041–5047
- Wang J, Musameh M, Lin YH (2003) Solubilization of carbon nanotubes by nafion toward the preparation of amperometric biosensors. *J Am Chem Soc* 125:2408–2409
- Gasparac R, Taft BJ, Lapierre-Delvin MA, Lazarek AD, Xu JM, Kelley SO (2004) Ultrasensitive electrocatalytic DNA detection at two- and three-dimensional nanoelectrodes. *J Am Chem Soc* 126:12270–12271
- Xu XH, Yang HC, Mallouk TE, Bard AJ (1994) Immobilization of DNA on an aluminum(III) alkanebisphosphonate thin film with electrogenerated chemiluminescent detection. *J Am Chem Soc* 116:8386–8387
- Miao WJ, Bard AJ (2003) Electrogenerated chemiluminescence. 72. determination of immobilized DNA and C-reactive protein on Au(111) electrodes using tris(2,2'-bipyridyl)ruthenium(II) labels. *Anal Chem* 75:5825–5834
- Su XD, Robelek R, Wu YJ, Wang GY, Knoll W (2004) Detection of point mutation and insertion mutations in DNA using a quartz crystal microbalance and MutS, a mismatch binding protein. *Anal Chem* 76:489–494
- Ito M, Nakamura F, Baba A, Tamada K, Ushijima H, Lau KHA, Manna A, Knoll W (2007) Enhancement of surface plasmon resonance signals by gold nanoparticles on high-density DNA microarrays. *J Phys Chem C* 111:11653–11662
- Wang H, Zhang CX, Li Y, Qi HL (2006) Electrogenerated chemiluminescence detection for deoxyribonucleic acid hybridization based on gold nanoparticles carrying multiple probes. *Anal Chim Acta* 575:205–211
- Li Y, Qi HL, Fang F, Zhang CX (2007) Ultrasensitive electrogenerated chemiluminescence detection of DNA hybridization using carbon-nanotubes loaded with tris(2,2'-bipyridyl)ruthenium derivative tags. *Talanta* 72:1704–1709
- Ma F, Zhang Y, Qi HL, Gao Q, Zhang CX, Miao WJ (2012) Ultrasensitive electrogenerated chemiluminescence biosensor for the determination of mercury ion incorporating G4 PAMAM dendrimer and Hg(II)-specific oligonucleotide. *Biosens Bioelectron* 32:37–42
- Zhang J, Qi HL, Li Y, Yang J, Gao Q, Zhang CX (2008) Electrogenerated chemiluminescence DNA biosensor based on hairpin DNA probe labeled with ruthenium complex. *Anal Chem* 80:2888–2894
- Yin XB, Xin YY, Zhao Y (2009) Label-free electrochemiluminescent aptasensor with attomolar mass detection limits based on a Ru(phen)<sub>3</sub><sup>2+</sup>-double-strand DNA composite film electrode. *Anal Chem* 81:9299–9305
- Xia F, White RJ, Zuo XL, Patterson A, Xiao Y, Kang D, Gong X, Plaxco KW, Heeger AJ (2010) An electrochemical supersandwich assay for sensitive and selective DNA detection in complex matrices. *J Am Chem Soc* 132:14346–14348
- Yuan T, Liu ZY, Hu LZ, Zhang L, Xu GB (2011) Label-free supersandwich electrochemiluminescence assay for detection of sub-nanomolar Hg<sup>2+</sup>. *Chem Commun* 47:11951–11953
- Gui GF, Zhuo Y, Chai YQ, Liao N, Zhao M, Han J, Zhu Q, Yuan R, Xiang Y (2013) Supersandwich-type electrochemiluminescent aptasensor based on Ru(phen)<sub>3</sub><sup>2+</sup> functionalized hollow gold nanoparticles as signal-amplifying tags. *Biosens Bioelectron* 47:524–529
- Chen Y, Xu J, Su J, Xiang Y, Yuan R, Chai YQ (2012) In situ hybridization chain reaction amplification for universal and highly sensitive electrochemiluminescent detection of DNA. *Anal Chem* 84:7750–7755
- Chen X, Hong CY, Lin YH, Chen JH, Chen GN, Yang HH (2012) Enzyme-free and label-free ultrasensitive electrochemical detection of human immunodeficiency virus DNA in biological samples based on long-range self-assembled DNA nanostructures. *Anal Chem* 84:8277–8283
- Qi HL, Zhang Y, Peng YG, Zhang CX (2008) Homogenous electrogenerated chemiluminescence immunoassay for human immunoglobulin G using N-(aminobutyl)-N-ethylisoluminol as luminescence label at gold nanoparticles modified paraffin-impregnated graphite electrode. *Talanta* 75:684–690
- Yin BC, Wu D, Ye BC (2010) Sensitive DNA-based electrochemical strategy for trace Bleomycin detection. *Anal Chem* 82:8272–8277

26. Li GJ, Liu LH, Qi XW, Guo YQ, Sun W, Li XL (2012) Development of a sensitive electrochemical DNA sensor by 4-aminothiophenol self-assembled on electrodeposited nanogold electrode coupled with Au nanoparticles labeled reporter ssDNA. *Electrochim Acta* 63:312–317
27. Huo ZH, Zhou YL, Liu Q, He XL, Liang Y, Xu MT (2011) Sensitive simultaneous determination of catechol and hydroquinone using a gold electrode modified with carbon nanofibers and gold nanoparticles. *Microchim Acta* 173:119–125
28. Fu YZ, Yuan R, Xu L, Chai YQ, Zhong X, Tang DP (2005) Indicator free DNA hybridization detection via EIS based on self-assembled gold nanoparticles and bilayer two-dimensional 3-mercaptopropyltrimethoxysilane onto a gold substrate. *Biochem Eng J* 23:37–44
29. Katz E, Willner I (2003) Probing biomolecular interactions at conductive and semiconductive surfaces by impedance spectroscopy: routes to impedimetric immunosensors, DNA-sensors, and enzyme biosensors. *Electroanalysis* 15:913–947
30. Li Y, Qi HL, Gao Q, Zhang CX (2011) Label-free and sensitive electrogenerated chemiluminescence aptasensor for the determination of lysozyme. *Biosens Bioelectron* 26:2733–2736
31. Mathews DH, Sabina J, Zuker M, Turner DH (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol* 288:911–940
32. Herne TM, Tarlov MJ (1997) Characterization of DNA probes immobilized on gold surfaces. *J Am Chem Soc* 119:8916–8920
33. Zhou LY, Zhang XY, Wang GL, Jiao XX, Luo HQ, Li NB (2012) A simple and label-free electrochemical biosensor for DNA detection based on the super-sandwich assay. *Analyst* 137:5071–5075
34. Zhang DD, Peng YG, Qi HL, Gao Q, Zhang CX (2010) Label-free electrochemical DNA biosensor array for simultaneous detection of the HIV-1 and HIV-2 oligonucleotides incorporating different hairpin-DNA probes and redox indicator. *Biosens Bioelectron* 25: 1088–1094
35. Li Y, Qi HL, Yang J, Zhang CX (2009) Detection of DNA immobilized on bare gold electrodes and gold nanoparticle-modified electrodes via electrogenerated chemiluminescence using a ruthenium complex as a tag. *Microchim Acta* 164:69–76
36. Gao M, Qi HL, Gao Q, Zhang CX (2008) Electrochemical detection of DNA hybridization based on the probe labeled with carbon-nanotubes loaded with silver nanoparticles. *Electroanalysis* 20:123–130
37. Bi S, Li L, Zhang SS (2010) Triggered polycatenated DNA scaffolds for DNA sensors and aptasensors by a combination of rolling circle amplification and DNAzyme amplification. *Anal Chem* 82:9447–9454