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Electrochemiluminescence biosensor for folate receptor based on terminal protection of small-molecule-linked DNA

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ABSTRACT

Owning to the characteristics such as high sensitivity and simplicity of apparatus, electrochemiluminescence (ECL) has become a powerful analytical technique and has been widely used. $Ru(phen)_3^{2+}$ can be intercalated into the grooves of dsDNA and act as an ECL probe efficiently, which has been applied to develop a sensitive ECL biosensor for folate receptor in this study. One ssDNA with a thiol group at its 3′ termini had been modified on the Au electrode first, and the other ssDNA with folic acid at its 3′ termini hybridized with the former one being modified on the electrode surface to form a dsDNA. In the absence of folate receptor, the 3′-terminus in the dsDNA region can be specificity hydrolyzed into mononucleotides by ExoIII and on dsDNA presents on the electrode surface, leading to the lower of ECL intensity detected. However, in the presence of the target (folate receptor), ExoIII failed to hydrolyze the dsDNA since the one 3′-terminus had been protected by the target and the other protected by the Au electrode, resulting in the enhancement of ECL intensity. The enhanced ECL intensity has a linear relationship with the logarithm of folate receptor concentration in the range of 0.66 nmol/L and 26.31 nmol/L with a detection limit of 0.1204 nmol/L. The proposed biosensor had been applied to detect HeLa cells concentration with satisfied results.

1. Introduction

Electrochemiluminescence (ECL), the smart combination of electrochemistry and chemiluminescence, has the predominant characteristics of high sensitivity, and easy control and manipulation, and has become an important and valuable detection method in analytical chemistry (McCord and Bard, 1991; Cao et al., 2002; Kulmala and Suomi, 2003; Richter, 2004; Zhu et al., 2010). $Ru(phen)_3^{2+}$ has a high ECL emission efficiency and can be intercalated into the grooves of dsDNA efficiently (Ling et al., 2001; Zhao et al., 2009; Ma et al., 2011). Many sensitive ECL biosensors had been developed based on this mechanism (Hu et al., 1998; Yin et al., 2009; Li et al., 2013; Qi et al., 2013). For example, Tang et al. (2010) designed a sensitive ECL biosensor for Hg^{2+} detection based on the formation of T–Hg^{2+}–T and the intercalation of $Ru(phen)_3^{2+}$ into the dsDNA. Chen et al. (2012) proposed an ultrasensitive ECL biosensor to detect specific DNA based on the fact that hybridization chain reaction can generate dsDNA polymers, which cause the intercalation of numerous ECL indicators ($Ru(phen)_3^{2+}$) into the dsDNA grooves and result in significant amplification of the ECL signal output.

Sensitive and specific determination of cancer-related biomarkers has drawn great attention in modern medicine and clinical diagnosis. Folate receptor (FR) is a glycosylphophatidylinositol-linked membrane glycoprotein; high expression levels of FR have occurred in many solid tumors, including ovarian cancer, endometrial cancer, lung cancer, breast cancers, and so on (Franzen, 2011). However, very little FR has been detected in normal tissues (notably choroid plexus, kidney, lung, and thyroid) (Holm et al., 1991; Lu et al., 2011). Aggressive or undifferentiated tumors at an advanced stage accompanied with an increased FR density indicate that FR can act as a clinical indicator (Toffoli et al., 1997). It had been reported that FR can bind with folic acid (FA) with high affinity (McHugh and Cheng, 1979), and this mechanism has been applied to develop many tumor-specific drug delivery systems and medical imaging of cancer cells or therapeutic agents (Konda et al., 2001; Leamon and Low, 2001; Kim et al., 2007; Sega and Low, 2008).

Small-molecule-linked DNA has become a versatile tool to investigate the interaction between small organic molecules and their protein receptors. It had been reported (Zhao et al., 2013) that the presence of FR on the terminal of DNA can prevent

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exonuclease I (ExoI) from approaching and cleaving the phosphodiester bond adjacent to the 3′ terminus of the ssDNA. Many sensitive FR determination approaches had been developed based on this mechanism. For example, Wu et al. (2009) introduced a novel electrochemical strategy for FR determination based on the terminal protection with a detection limit of 3 pmol/L. Cao et al. (2012) proposed a novel electrochemical method for FR detection which coupled the terminal protection and nicking endonuclease-assisted amplification strategy. Wang et al. (2013) also developed a sensitive electrochemical biosensor which combined the protecting effect of FR toward FA modified DNA and the signal amplification of supersandwich DNA structure. Based on the specific FA–FR interaction that can prevent enzymolysis by exonuclease III (ExoIII) from the 3′ terminus of the dsDNA, a convenient fluorescence biosensor for FR has been proposed by Wei et al. (2012). But to the best of our knowledge, no sensor which combines the merits of high sensitivity of ECL and high selectivity of FR–FA interaction has been reported yet.

This study is based on the specific FA–FR interaction that can prevent the dsDNA from enzymolysis by ExoIII and the fact that Ru $(phen)_3^{2+}$ can be intercalated into the grooves of dsDNA and act as ECL probe. A sensitive ECL biosensor for FR determination has been developed and been applied to detect HeLa cells concentration with satisfied results.

2. Experimental section

2.1. Apparatus

The detection system contains a CHI660D electrochemical system (Chenhua Instruments, Shanghai, China), a BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Science, Beijing, China) and a computer to record the data. All experiments were carried out with a conventional three-electrode system, which was composed of a gold electrode as the working electrode, a platinum wire as the counter electrode and Ag/AgCl (saturated with KCl) as the reference electrode. The voltage of the PMT was set at 850 V in the detection process.

2.2. Chemicals

Dichlorotris (1,10-phenanthroline) ruthenium(II) hydrate ($Ru(phen)_3^{2+}$), bovine hemoglobin (BHb), chronic viral hepatitis (CVH), lysozyme (LZM), horseradish peroxidase (HRP), bovine serum albumin (BSA), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), folate receptor 2 human (FR), folic acid (FA), 1-ethyl-3-(3-dimethylaminopropyl) carbodi-imide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), 6-mercaptohexanol (MCH) and tripropylamine (TPA) were purchased from Sigma-Aldrich Chemical Co., Ltd.. Sulfuric acid, potassium chloride and absolute ethyl alcohol were purchased from Alfa Aesar China (Tianjin) Co., Ltd. and used as received. Exonuclease III was purchased from Thermo Scientific China (Shanghai) Co., Ltd.

Labeled DNA oligonucleotides were purchased from Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Sequences of oligonucleotide probes used in this work are listed as follows:

ssDNA1: 5′-GCTA GAGA TTTT CCAC ACTG ACT-SH-3′

ssDNA2: 5′-AGT CAGT GTGG AAAA TCTC TAGC-NH_2-3′

The functional ssDNA2–FA was prepared according to the previous literature (Fahlman and Sen, 2002). ssDNA2 was briefly incubated with folic acid solution (50 μmol/L) in the presence of the carbo-diimide cross-linking agent for 2 h, which was mixed by 10 μL EDC (1 mmol/L) and 10 μL NHS (5 mmol/L); by this means, NH_2 at the 3′ end of the ssDNA2 combined with FA molecule via a conjugate link to form ssDNA2–FA.

All solutions were prepared with Milli-Q water (Milli-Q, Millipore, 18.2-MΩ resistivity) and stored at 4 °C. DNA hybridization buffers contain 100 mmol/L NaCl, 10 mmol/L $MgCl_2$ and 10 mmol/L Tris–HCl buffer solution (pH 7.4). Buffer for ECL detection was 0.2 mol/L phosphate buffer (pH 7.5), prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4, containing 20 mmol/L TPA.

2.3. Cell culture

Human cervical carcinoma cells (HeLa) were obtained from our in-house frozen cell stock cryopreserved in ampoules in a large repository. These cell lines were maintained in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (FBS, 10%), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 at 37 °C. The medium was replaced by fresh medium three times per week until the cells grew 70–80% confluence. The cell lines were then undetached from the flasks after a 3-min incubation with the Hank's balance salt solution containing 0.05% trypsin and 0.5 mmol/L of EDTA–4Na. RPMI 1640 medium containing 10% FBS (to deactivate trypsin) was added to the cell lines. After washing cells with RPMI 1640 medium several times, cell lines at final density 10^5 cells/mL were cultured in 96-well plates (0.1 mL per well) for the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasodium bromide) assay. After being re-suspended in PBS (0.1 M, pH 7.2) with desired concentration, the cell suspensions were prepared for further use.

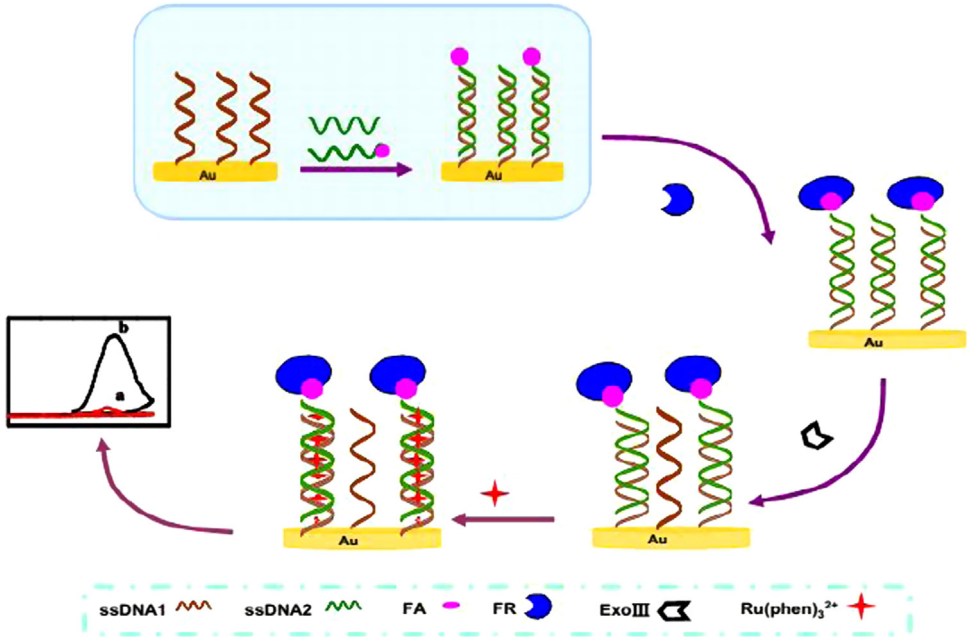
2.4. Preparation of the modified electrodes

A gold electrode was polished with aqueous slurries of 1.0 μm, 0.3 μm and 0.05 μm α-Al_2O_3 powders on a polishing microcloth and sonicated with deioned water, ethanol for 3 min. Finally, the gold electrode was rinsed with deioned water, and then electrochemically activated by consecutive potential scanning between −0.4 and 1.6 V in 0.5 mol/L sulfuric acid until stable cyclic voltammogram (CV) curves were obtained. The activated gold electrode was made to interact with 1 μmol/L ssDNA1 solution for 2 h, and then the surface of the gold electrode was passivated with 1 mmol/L MCH. SsDNA1 can be immobilized on the gold electrode through thiol–Au interaction. Then ssDNA1-modified electrode was immersed in the 5 μmol/L ssDNA2–FA solution for 5 h in 37 °C water bath. Since ssDNA1 is the complementary strand with FA–ssDNA2, the ssDNA1 was then subjected to hybridization with the ssDNA2–FA chains and formed the dsDNA. Thus dsDNA–FA-modified electrode was obtained for the following experiment. (Note: after each step, the electrode should be rinsed with Tris–HCl buffer solution to eliminate the physical adsorption).

2.5. FR detection procedure

The dsDNA–FA-modified electrode had been immersed into the solution containing various concentrations of target FR for 2 h in a 37 °C water bath to allow complete interaction between FA and FR. Finally, through acceding to 2 U/μL ExoIII, the modified electrodes were hydrolyzed completely in about 1 h in which dsDNA–FA–FR modified electrode was fabricated. Then the dsDNA–FA–FR modified electrode was immersed in 1 mmol/L $Ru(phen)_3^{2+}$ solution for 5 h to make $Ru(phen)_3^{2+}$ molecules intercalate into dsDNA structures.

Then the modified electrode was immersed in a detection cell containing 2 mL of PBS (pH 7.4) and 20 mmol/L of TPA (working as an ECL coreactant). Cyclic voltammetry (CV) in the range of 0.5–1.25 V (vs. Ag/AgCl) with a scan rate of 100 mV/s was



Scheme 1. The mechanism of the ECL sensor for folate receptor based on the terminal protection of small-molecule-linked DNA.

performed and the ECL signal was recorded simultaneously. The intensity of peak ECL signal was selected for quantitative detection. Electrochemical impedance spectroscopy (EIS) of the gold electrode before and after modification is examined in the mixed solution of 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ containing 0.1 mol/L KCl. The frequency range is 1.0 Hz–100 kHz and potential is 0.214 V.

3. Results and discussion

3.1. Principle of the proposed biosensor

Scheme 1 shows the protocol of the biosensor for FR determination. The ssDNA1 with a thiol group at its 3′ termini is self-assembled on a gold electrode via Au–S bonding. SsDNA2 with a small molecule recognition element (FA) at its 3′ termini hybridized with the ssDNA1 modified on the electrode surface to form dsDNA–FA. Since ExoIII catalyzes the stepwise removal of mononucleotides from 3′ termini of dsDNA, 3′ termini of ssDNA1 had been linked on the gold electrode and prevented from being hydrolyzed; so in the presence of ExoIII, ssDNA2–FA will be completely hydrolyzed from the 3′ end and no dsDNA will be present on the electrode surface. However, as FR is added into the test solution, which can bind with FA specifically to form the dsDNA–FA–FR and protect the dsDNA from digestion by the ExoIII, dsDNA is still present on the electrode surface. Ru(phen)$_3^{2+}$ can be stably intercalated into the dsDNA section and acts as ECL probe (Yuan et al., 2011); so a strong ECL signal can be detected at this situation. The enhanced ECL intensity of the system has a relationship with the FR concentration, based on which a sensitive ECL sensor for FR detection can be developed.

A simple experiment had been performed to verify our assumption. Fig. 1(A) shows ECL of dsDNA–FA-modified electrode in 0.2 mol/L PBS containing 20 mmol/L TPA (pH 7.5) before (a) and after (b) reacting with 26.31 nmol/L FR (the inset figure is the corresponding CV curves). If the solution contained no FR, very weak electrochemical and ECL signal had been detected. If FR had been added into the solution, a large increase in the anodic current of Ru(phen)$_3^{2+}$ oxidation at 1.10 V was observed. Meanwhile, a strong ECL signal was detected also. This indicates that FR can prevent dsDNA–FA from hydrolysis and the Ru(phen)$_3^{2+}$ can intercalate into the dsDNA groove to act as the ECL probe. These results demonstrate the feasibility of our proposal.

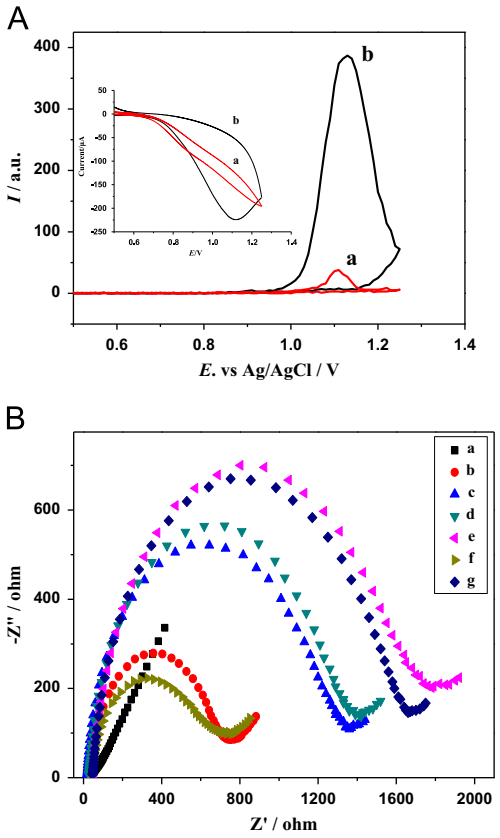


Fig. 1. (A) The ECL response without (a) and with (b) 26.31 nmol/L folate receptor in 0.2 mol/L PBS (pH 7.5) containing 20 mmol/L TPA. (B) Nyquist plots at different modification stages: (a) bare gold electrode; (b) ssDNA1, (c) dsDNA, (d) dsDNA–FA and (e) dsDNA–FA–FR modified electrodes; (f) dsDNA–FA and (g) dsDNA–FA–FR modified electrode hydrolyzed by ExoIII. The data were recorded in the presence of 5.0 mmol/L $[Fe(CN)_6]^{3-/4-}$ containing 0.1 mol/L KCl and upon application of a biasing potential of 0.214 V, applying 5 mV alternating voltage in the frequency range of 1–100K Hz.

3.2. Characterization of the modified electrodes

Faradic electrochemical impedance spectroscopy (EIS) has been applied to characterize the difference stages of the electrode surface modification (Lisdat and Schafer, 2008). The impedance spectra consist of a semicircular portion at high frequencies and a linear part at low frequencies. The semicircle relates to an electron transfer-limited process, while the linear part corresponds to a diffusion-limited process. The change in the diameter of the semicircle reflects the change in the interfacial charge-transfer resistance (R_{ct}) (Cao et al., 2010). Fig. 1(B) shows the nyquist plots of EIS for the electrode modification at different stages. Firstly, the impedance spectrum of the bare gold electrode (curve a) shows nearly no semicircle domain, indicating the small interfacial charge transfer resistance. After the immobilization of the thiolated ssDNA1, a semicircle can be observed (curve b). The electrostatic repulsion between ssDNA1 with negatively charged phosphate backbone can prevent redox probe $[Fe(CN)_6]^{3-/4-}$ from transferring on the electrode surface, the increment of the electron-transfer distance also causes the EIS enhancement. Furthermore, the hybridization of ssDNA2 with ssDNA1 on the electrode surface results in the further increment of the diameter of the semicircle (curve c). This can be explained by the double negative charged phosphate backbones immobilizing on the electrode and the longer electron-transfer distance which further prevent $[Fe(CN)_6]^{3-/4-}$ charge transfer on the electrode interface. If ssDNA2 had been modified with FA first (curve d), the impedance is almost the same as that of curve c. The reason lies in the fact that FA is a small molecule and dsDNA–FA-modified electrode has similar electrostatic repulsion between DNA and $[Fe(CN)_6]^{3-/4-}$ with the dsDNA modified electrode. Moreover, after the electrode is treated with target protein (FR) (curve e), the interfacial electron transfer resistance increased greatly. It can be explained by the fact that FR has a big molecular mass, which causes significant steric hindrance for $[Fe(CN)_6]^{3-/4-}$ to reach the electrode surface, which also indicates the successful preparation of ssDNA2–FA. Nevertheless, after the dsDNA–FA complex modified gold electrode is treated by ExoIII, the diameter of the semicircle becomes small, closely corresponding to the thiolated ssDNA1 and MCH modified electrode (curve f). The reason lies in the fact that dsDNA has been digested after treatment with ExoIII, ssDNA2 had been hydrolyzed from the 3′-terminus of the DNA and the digestion product will leave the electrode. While, the dsDNA–FA–FR complex modified gold electrode is treated by ExoIII (curve g), the diameter of the semicircle has decreased a little. The reason lies in the fact that macromolecular FR binding to FA in dsDNA–FA chimeras could protect the conjugated dsDNA from degradation by the 3′ end double-strand specific ExoIII, leaving the dsDNA–FA–FR intact. And some dsDNA without bonding FA will hydrolyzed by ExoIII, resulting in the little decrease of the charge transfer resistance.

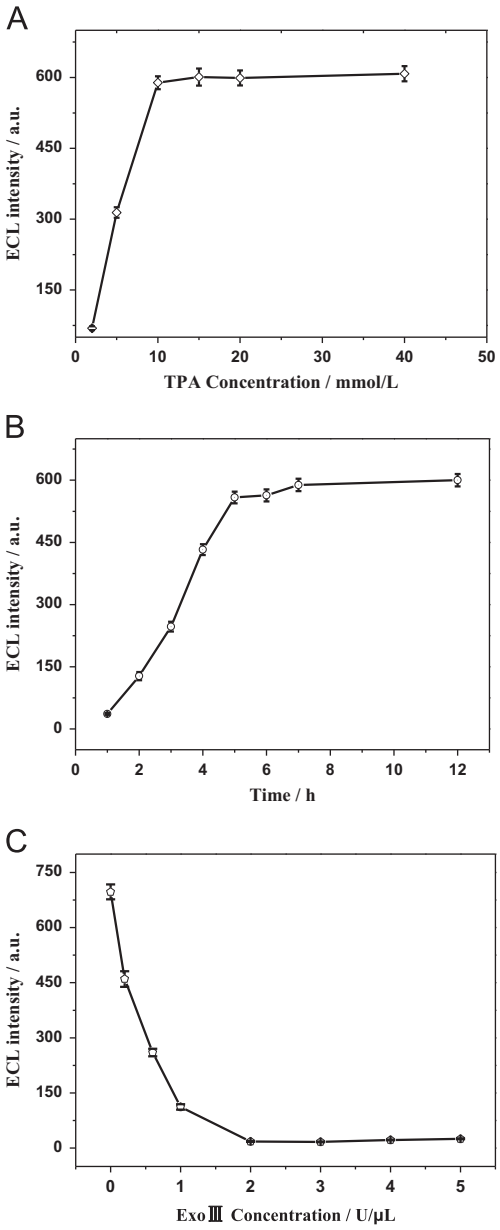


Fig. 2. (A) ECL intensity at different TPA concentrations. The RSD are 5.8%, 3.6%, 2.3%, 3.0%, 2.7% and 2.6%. (B) ECL intensity at different incubation times in Ru $(phen)_3^{2+}$ solution. The RSD are 5.8%, 7.7%, 4.8%, 3.1%, 2.5%, 2.6%, 2.5%, and 2.5%, respectively. (C) ECL intensity at different ExoIII concentrations. The RSD are 2.9%, 4.6%, 3.9%, 6.4%, 6.7%, 5.9%, 4.9%, and 4.8%, respectively. The concentration of Ru $(phen)_3^{2+}$ is 1 mmol/L, FR is 1 μmol/L, and TPA in (B) and (C) is 20 mmol/L. The error bars show the standard deviation of three replicate determinations.

3.3. Optimization of the reaction conditions

To ensure the thorough reaction, excessive amount of FR (1 μmol/L) and $Ru(phen)_3^{2+}$ (1 mmol/L) had been used during the preparation of the biosensor. The concentration of TPA will cause great effect on the ECL intensity of the system. As shown in Fig. 2 (A), the ECL intensity boosts up gradually, while the ECL intensity trends to a stabilized platform at 10 mmol/L TPA. In order to make sure that TPA concentration is high enough, 20 mmol/L is chosen in the following studies.

The effect of the concentration of ExoIII on the ECL intensity has also been studied. As shown in Fig. 2(B), the ECL intensity decreases with the increasing of ExoIII concentration from 0 to 2 U/μL, and then reaches a constant. Hence, 2 U/μL of ExoIII has been chosen in the following study.

The incubation time of dsDNA modified electrode in the Ru $(phen)_3^{2+}$ solution has a significant effect on the signal intensity of the present ECL biosensor. The effect of incubation time for intercalation of $Ru(phen)_3^{2+}$ on the hybrid of 23-mer ssDNA1 and ssDNA2 has also been studied (shown in Fig. 2(C)). The ECL intensity increases with the increasing of intercalation time, and which reaches its maximum beyond 5 h. So 5 h has been selected.

3.4. Linear relationship and detection limits

Fig. 3(A) shows ECL response at different FR concentrations in PBS solution (pH 7.5) containing 20 mmol/L TPA. The ECL intensity

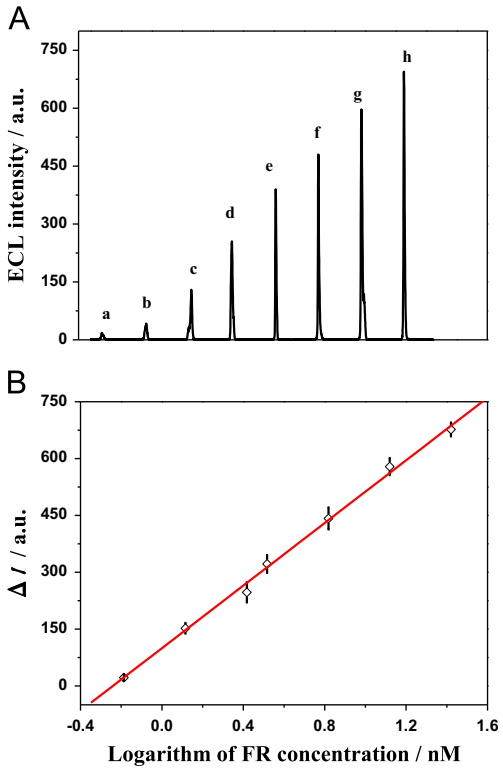


Fig. 3. (A) ECL intensity at different FR concentrations. (a–h) 0, 0.6530, 1.306, 2.613, 3.290, 6.579, 13.16, and 26.31 nmol/L. (B) Plot of ECL intensities vs. logarithm of the FR concentrations. The error bars show the standard deviation of three replicate determinations. The RSDs are 8.2%, 9.2%, 10.8%, 7.3%, 6.5%, 3.9%, and 2.7%,. The concentration of $Ru(phen)_3^{2+}$ is 1 mmol/L and TPA is 20 mmol/L.

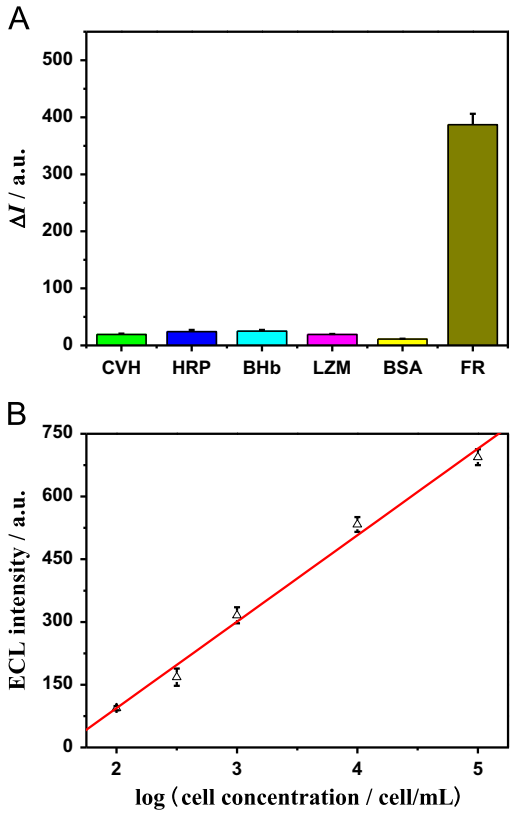


Fig. 4. (A) Specificity of the sensing system for FR over the other proteins: the concentration of FR was 5 nmol/L and the other interference proteins were 2, 1, 0.5, 0.5, and 0.5 μmol/L. The RSDs are 8.9%, 4.7%, 9.2%, 5.2%, 8.0%, and 3.9%. (B) The calibration cure between the value of ECL intensity and logarithm of HeLa cell concentrations. The RSDs are 5.3%, 12.4%, 6.0%, 3.3%, and 2.7%. The error bars show the standard deviation of three replicate determinations. The concentration of Ru $(phen)_3^{2+}$ is 1 mmol/L and TPA is 20 mmol/L.

increased with increasing FR concentration. Fig. 3(B) shows the relationship between concentration of FR and ECL intensity. The ECL intensity increases linearly with the logarithm concentration of FR in the range of 0.66–26.31 nmol/L. The regression equation is

$$\Delta I/\text{a.u.} = 99.5 + 413.3 \log C_{FR}, \quad R = 0.9995$$

where ΔI is the difference in ECL intensity in the presence and absence of FR, C_{FR} is the FR concentration (nmol/L) and R is the regression coefficient. The detection limit for FR is calculated to be 0.1204 nmol/L (defined as $S/N=3$), which is better than the early reported fluorescence biosensor (Wei et al., 2012) and the electrochemical detection method (Wang et al., 2013). Furthermore, the early reported fluorescent(Wei et al., 2012) or electrochemical methods (Wu et al., 2009; Wang et al., 2013) need time consuming dialysis process, so the whole detection procedure needs several days. In this study, dsDNA–FA was modified on the electrode surface directly and can be separated from the complex solution easily, this avoids the time consuming dialysis process; the total time needed is about 15 h.

To test the repeatability of the proposed method, the enzymolysis at the same FR concentration (at 3.290 nmol/L) were examined for five times, the relative standard deviation (RSD) of the ECL response ($n=5$) was found to be 5.3%, this indicates the proposed method owns high reproducibility. If the prepared biosensor had been stored at 4 °C for three weeks, the ECL response has no apparent difference with the freshly prepared one, which shows that this modified electrode has good stability.

3.5. Specificity and application of the biosensor

The specificity of the proposed biosensor had been tested. The concentration of FR was set at 5 nmol/L. The concentrations of the interfering substance were 2 μmol/L of CVH, 1 μmol/L of HRP, and 0.5 μmol/L of BHb, LZM and BSA. As shown in Fig. 4(A), the enhancement in ECL intensities in the presence of interfering substance was little, but increased greatly in the presence of FR, suggesting that the proposed biosensor also owns good specificity.

After this very encouraging result for the biosensor, we next asked whether the proposed biosensor could be applied to detect the cell concentration which contained FR. In this case, HeLa cells with over-express FR were detected in our experiments. As shown in Fig. 4(B), the ECL intensities had a relationship with the logarithm of cell concentration in the range of 10^2–10^5 cell/mL. The detection limit of the proposed method (lower than 100 cell/mL) had been compared with the early reported methods (shown in Table 1); the results indicated that the proposed method owns the character of lower detection limit. So the proposed sensing system was selective towards FR rich-HeLa cells and could be used as a diagnostic tool for the detection of cancer cells.

4. Conclusions

In this work, a novel ECL sensor for FR assay based on the specific FA–FR interaction and which can prevent enzymolysis of dsDNA by ExoIII system has been demonstrated. The greatest advantages of the proposed sensor are the high sensitivity and good selectivity, even in the presence of high concentration of other common proteins. Furthermore, the proposed biosensor is also time saving when compared with the early reported methods. Using this small molecule–protein interaction strategy,

Table 1
The comparing of the performance of the different reported methods.

Detection technology	Linearity range (cells/mL)	Detection limit (cells/mL)	Refs.
Electrochemical assay	Up to 5000	250	Castillo et al. (2013)
Electrochemical impedance assay	500–5,000,000	500	Zheng et al. (2012)
Electrochemical impedance assay	1000–100,000	1000	Yang et al. (2011)
ECL	100–100,000	100	Present study

we anticipate that a lot of ECL biosensors can be developed by our methodology.

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