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RESEARCH PAPER

Label-free electrochemiluminescence biosensor for ultrasensitive detection of telomerase activity in HeLa cells based on extension reaction and intercalation of $Ru(phen)_3^{2+}$

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Abstract Telomerase is one of the most common markers of human malignant tumors, such as uterine, stomach, esophageal, breast, colorectal, laryngeal squamous cell, thyroid, bladder, and so on. It is necessary to develop some sensitive but convenient detection methods for telomerase activity determination. In this study, a label-free and ultrasensitive electrochemiluminescence (ECL) biosensor has been fabricated to detect the activity of telomerase extracted from HeLa cells. Thiolated telomerase substrate (TS) primer was immobilized on the gold electrode surface through gold-sulfur (Au-S) interaction and then elongated by telomerase specifically. Then, it was hybridized with complementary DNA to form double-stranded DNA (dsDNA) fragments on the electrode surface, and $Ru(phen)_3^{2+}$ has been intercalated into the dsDNA grooves to act as the ECL probe. The enhanced ECL intensity has a linear relationship with the number of HeLa cells in the range of 5~5000 and with a detection limit of 2 HeLa cells. The proposed ECL biosensor has high specificity to telomerase in the presence of common interferents. The relative standard deviations (RSDs) were <5 % at 100 HeLa cells. The proposed method provides a convenient approach for telomerase-related cancer screening or diagnosis.

Keywords Electrochemiluminescence · Telomerase · Label free · HeLa cell

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Introduction

Telomere has been found at the end of eukaryotic chromosome, which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes and plays a crucial role in maintaining the stability of chromosomes and activity of cells [1]. However, telomeres are progressively shortened with every cell division and then in turn limit cell division [2]. Telomerase, as a basic ribonucleoprotein reverse transcriptase, can elongate telomeres in cells by adding repetitive sequences ($(TTAGGG)_n$) to the 3′ ends of the chromosome [3, 4]. And for this exact reason, telomerase is essential for reversing telomere shortening and allowing immortal cells to divide repeatedly. About 85 % of human malignant tumors have been found with overexpression of telomerase [5, 6], making telomerase one of the most common cancer markers, and sensitive and rapid detection of telomerase activity is critical for early screening of cancers.

Various measuring methods have been established to evaluate the activity of telomerase. Among them, polymerase chain reaction (PCR)-based telomeric repeat amplication protocol (TRAP) has been developed as a conventional and typical approach to detect telomerase activity due to the employment of general amplification [7–10]. Nevertheless, it also suffers from the same drawbacks of all PCR-based assays, including being time consuming and temperature programmer

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dependent, imprecise quantitative information, polymerase inhibition by cell extracts, and so on [11–13]. To overcome these shortcomings, some alternative PCR-free methods have been developed for telomerase activity analysis, such as electrochemical detection [14–18], chemiluminescence assays [19, 20], colorimetric strategies [21–23], fluorescence methods [24–26], and so on.

Electrochemiluminescence (ECL) as an analytical technique is chemiluminescence triggered by electrochemical methods, which has a low noise background and simple equipment due to not using an external light source [27, 28]. Many ECL strategies have been proposed and implemented to detect telomerase activity [29–33]. For example, Zhou et al. proposed a novel ECL approach for telomerase activity detection which combined the modified TRAP with magnetic beads, as well as labeled gold nanoparticles [30]. Wu et al. constructed a label-free ECL biosensor for telomerase activity detection using porphyrin-functionalized graphene [31]. Zhang et al. demonstrated a sensitive ECL strategy to detect telomerase activity based on multifunctional gold nanoparticles modified with G-quadruplex deoxyribozyme and luminol [32]. Although these ECL methods are PCR free and sensitive to detect telomerase activity, most of them are still time consuming due to laborious labeling of ECL nanoprobes and complicated synthesis of nanoparticles or nanomaterials. There is still a critical demand to develop some simple, sensitive, and low-cost ECL strategies for telomerase activity detection.

Label-free ECL detection is more economical, time saving, and labor saving than label ECL detection [34]. In this study, a label-free and convenient ECL biosensor for telomerase activity detection had been proposed. A thiolated telomerase substrate primer was immobilized on the gold electrode surface and then incubated with telomerase for extension reaction. Once the telomeric repeats were formed on the electrode, the complementary DNA could hybridize with telomeric repeats to form double helixes. Then, $Ru(phen)_3^{2+}$ molecule intercalates into the grooves of double-stranded DNA and acts as an ECL probe [35]. The proposed ECL biosensor has been applied to detect telomerase activity extracted from human cervical carcinoma cells (HeLa) cells with high sensitivity. The proposed strategy provided a much more convenient approach for cancer screening and clinical diagnosis based on telomerase activity detection.

Experimental section

Materials

3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, bovine serum albumin (BSA), human serum albumin (HSA), tris(2-carboxyethyl) phosphine (TCEP), 6-mercapto-1-hexanol (MCH), tripropylamine (TPA), and dichlorotris(1,10-phenanthroline) ruthenium(II) hydrate ($Ru(phen)_3^{2+}$) were purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO) and used directly without further purification. Ethylene glycol-bis(aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), deoxynucleotide solution mixture (dNTPs), and all oligonucleotides used in this work were synthesized by Sangon Inc. (Shanghai, China), and their sequences were shown as follows:

Telomerase substrate (TS) primer: 5′-HS-$(CH_2)_6$-TTTTTTAATCCGTCGAGCAGAGTT-3′

Complementary DNA (cDNA): 5′-*CTAACCCTA*-3′ (cDNA contained the complementary sequence to the extended TS telomeric repeat strand)

Electrochemical impedance spectroscopy (EIS) characterization was performed in 0.5 M KCl solution containing 0.01 M $[Fe(CN)_6]^{3-/4-}$. ECL analysis was carried out in 0.2 M phosphate buffer solution (PBS) (pH = 7.4) containing 0.02 M TPA (acts as a co-react reagent). Other chemicals were all of analytical reagent grade. All solutions were prepared with deionized water, which had been purified with a Milli-Q purification system and was RNase free.

Instruments

ECL intensity versus potential was detected by a laboratory-made system, which contains a CHI660D electrochemical workstation (Chenhua Instruments, Shanghai, China) for electrochemical measurements and a BPCL ultra-weak luminescence analyzer (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China) for ECL emission detection. The photomultiplier tube (PMT) of the BPCL ultra-weak luminescence analyzer was operated at −800 V. The experiments were proceeded with a conventional three-electrode system: a 3-mm diameter gold electrode was employed as working electrode, an Ag/AgCl electrode saturated with KCl was used as reference electrode, and a platinum wire served as counter electrode.

Cell culture and telomerase extraction

Cell culture and telomerase extraction were prepared according to the conventional method described in refs. [31, 36]. HeLa were cultured in DMEM medium supplemented with 10 % fetal calf serum, and the cells were maintained at 37 °C in a humidified atmosphere (95 % air and 5 % CO_2). Cells were collected in the exponential phase of growth and then removed from the substrate by trypsinization, and 1×10^6 cells were dispensed in a 1.5-mL EP tube and washed twice with ice-cold PBS (pH 7.4). Subsequently, the cells were resuspended in ice-cold CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM

mercaptoethanol, 0.5 % CHAPS, 10 % glycerol), incubated for 30 min on ice, and centrifuged 20 min at 12,000 rpm, 4 °C. The supernatant was used immediately for telomerase assay or stored at −20 °C for further experiment.

Electrode pretreatment and telomerase substrate primer immobilization

The gold electrode (3 mm in diameter) was polished with 1.0, 0.3, and 0.05 μm alumina slurry in order to obtain a mirror surface and then washed by sonication in ethanol and deionized water for 3 min. Next, the gold electrode was subjected to cyclic potential scanning between −0.2 and 1.6 V in 0.5 M H_2SO_4 solution until a typical stable cyclic voltammogram was obtained. Further, the gold electrode was thoroughly rinsed with deionized water and dried with nitrogen gas. Next, the gold electrode was immersed in a thiolated TS primer (1 μM) solution at room temperature for 2 h in the dark to modify the TS primer on the gold electrode surface through gold-sulfur (Au-S) interaction, and subsequently, the unoccupied sites were blocked with 1 mM MCH for 1 h and then washed by deionized water again.

ECL biosensor fabrication and telomerase activity detection

Telomerase extracts were diluted in lysis buffer with respective number of cells and introduced into 50 μL of extension solution (20 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 1 mM EGTA, 63 mM KCl, 0.005 % Tween 20, 0.1 mg/mL BSA, 0.6 mM dNTPs). Subsequently, the TS primer-modified electrode was immersed into the extension solution and incubated at 37 °C for 2 h to proceed the extension reaction by telomerase.

The resulting telomerase extension product-modified electrode was followed by a thorough rinse with Tris-HCl buffer. Then, the resulting electrode was immersed in the reaction buffer (2 μM cDNA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM KCl, 0.1 mM $Ru(phen)_3^{2+}$) for 3 h at 37 °C, allowing the telomerase extension products to hybridize with cDNA, and in the meantime, $Ru(phen)_3^{2+}$ molecules were intercalating into double-stranded DNA (dsDNA) grooves. After this, the electrode was washed with Tris-HCl buffer to reduce the nonspecific binding.

The ECL signal from the modified electrode was measured in the potential range of 0.6~1.6 V versus Ag/AgCl in 0.2 M PBS (pH 7.4) containing 0.02 M TPA, and the scan rate was settled at 100 mV/s. Each sample was replicated three times and the averaged value was adopted as the reference result. The error bars show the standard deviation of three replicate determinations.

Results and discussion

Principle of the proposed ECL biosensor for telomerase activity

The principle of the label-free ECL biosensor for telomerase activity analysis is illustrated in Fig. 1. The thiolated TS primer was firstly immobilized on the gold electrode surface via Au-S covalent bonds. Since the TS primer could be discriminated specifically by telomerase extracted from cancer cells, once the telomerase was introduced into the system, the TS primer could be elongated by telomerase in the presence of dNTPs. So, a much longer single-stranded DNA (ssDNA) was formed on the gold electrode surface by the incessant addition of TTAGGG repeat units onto the 3′ end of the TS primer. Subsequently, cDNA, which contained the complementary sequence to telomeric repeat unit, was added into the solution to hybridize with the extended TS telomeric repeat strand to form a dsDNA. Since $Ru(phen)_3^{2+}$ can intercalate into the grooves of dsDNA and acts as an ECL probe, the addition of $Ru(phen)_3^{2+}$ would result in the enhancement of the ECL signal detected. In the absence of telomerase, the TS primer could not be extended; hence, no hybridization occurred after cDNA was introduced into the system, and no dsDNA fragment was gathered on the electrode surface as well; so, only a weak ECL signal had been detected. Since the enhanced ECL signal has a relationship with the activity of telomerase, a sensitive and convenient biosensor for telomerase activity analysis can be developed.

Characterization of the modified electrode

EIS has been applied to monitor the electrode modification procedures. The equivalent circuit model of the working electrode has been adapted to fit EIS data (shown in the upper right corner of Fig. 2A), and the R_s, R_{ct}, C_{dl}, and Z_w represent the electrolyte solution resistance, surface charge transfer resistance, double-layer capacitance, and Warburg impedance, respectively. Specially, in the impedance spectra, the semicircle diameter of impedance equals the electron transfer resistance (R_{ct}), and R_{ct} increases with the constant blocking by the transfer of redox probe. $[Fe(CN)_6]^{3-/4-}$ was employed as the redox probe. As shown in Fig. 2A, the bare Au electrode (curve a) showed a very small semicircular domain, corresponding to the direct transfer of electrons on the electrode surface. After the modification of thiolated TS primer on the electrode surface (curve b), the R_{ct} increased markedly due to the electrostatic repulsion between the negatively charged phosphate backbone of the TS primer and $[Fe(CN)_6]^{3-/4-}$. After blocking of the electrode with MCH, the R_{ct} increased further (curve c), and the reason lies in that MCH molecules formed a more compact layer that hindered the interfacial electron transfer. After the extension reaction with telomerase

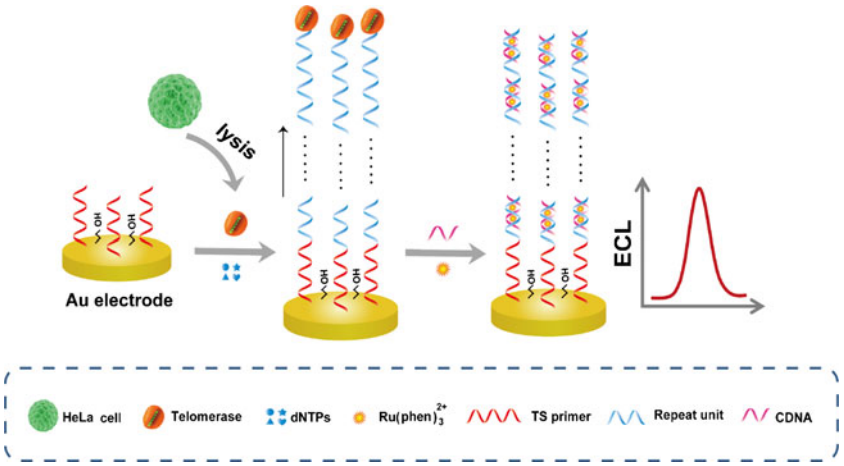

Fig. 1 The protocol of the ECL biosensor for telomerase activity analysis based on extension reaction and intercalation of $Ru(phen)_3^{2+}$

(extracted from 5000 HeLa cells), the extension of the TS primers resulted in a further increase of the R_{ct} (curve d), because the numbers of elongated ssDNA were formed on the electrode surface. The hybridization of elongated TS primers with cDNA led to a further increased R_{ct} value (curve e), which can be explained by the fact that the double negative-charged phosphate backbones hindered $[Fe(CN)_6]^{3-/4-}$ charge transfer, and this also indicated that dsDNA had been formed on the electrode surface. In contrast, in the absence of telomerase, after the extension reaction and hybridization under the same conditions, the R_{ct} value was almost the same with that of curve c (curve f). The reason lay in that TS primers could not be elongated without telomerase, and then the following hybridization between elongated TS primers and cDNA could not occur. Therefore, the increase of the R_{ct} value stepwise confirmed the successful modification of the electrode. To further characterize the modified electrode, the ECL and electrochemical signals had been recorded in the presence and absence of telomerase (extracted from 5000 HeLa cells). As shown in Fig. 2B (the inset figure is the corresponding cyclic voltammetry (CV) curves), in the presence of telomerase, obvious ECL and CV signals were recorded (curve g), and these indicate that a large amount of dsDNA fragments was induced by the elongation and hybridization on the electrode surface and a large amount of $Ru(phen)_3^{2+}$ had been accumulated on the electrode surface. In the absence of telomerase, both ECL and CV signals were weak (curve h), and the reason lies in that no elongation and hybridization occurred without telomerase. These results confirmed the feasibility of the proposed ECL biosensor.

Optimization of the reaction conditions

In order to reach the best performance of the proposed biosensor, some experimental parameters were optimized. The incubation time of extension reaction directly affected the final length of elongated ssDNA, which had been optimized firstly. As shown in Fig. 3A, the ECL signal increased significantly with the enhancing of incubation time from 0.5 to 2 h and then reached a plateau after 2 h. This means the ssDNA was hardly

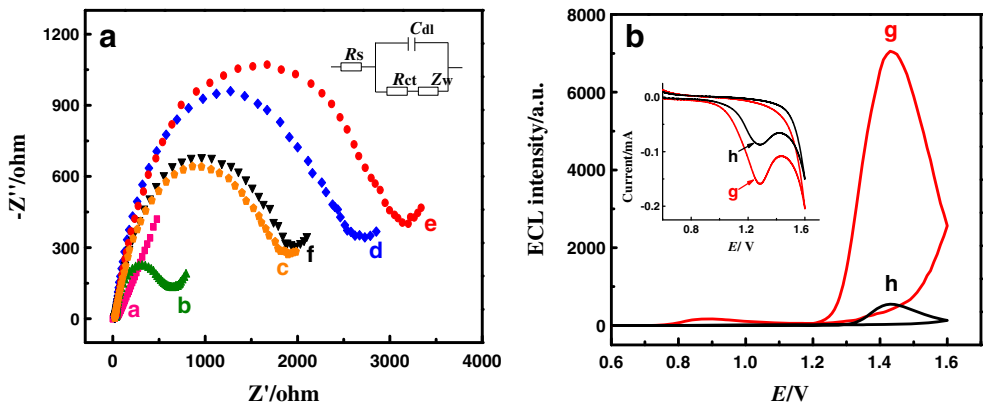

Fig. 2 (**A**) Nyquist diagrams of electrochemical impedance spectra of bare Au electrode (**a**), TS-modified electrode (**b**), MCH-blocked electrode (**c**), TS primer elongated by telomerase extracts from 5000 HeLa cells (**d**), telomerase extension products hybridized with cDNA (**e**), and execution of the elongation and hybridization in the absence of telomerase extracts (**f**). (**B**) ECL signals of the developed sensing system in the presence (**g**) and absence (**h**) of telomerase extracts from 5000 HeLa cells (the *inset figure* is the corresponding CV curves)

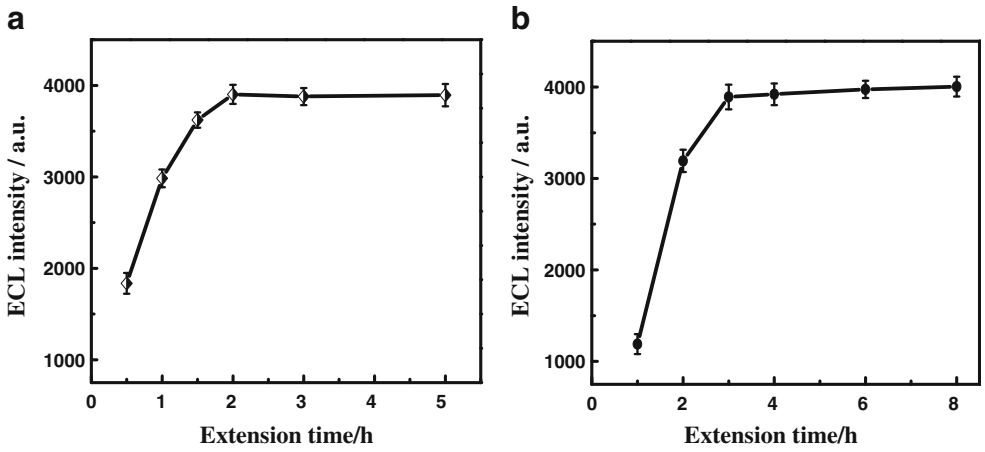

Fig. 3 Optimization of the experimental conditions with telomerase extracts from 100 HeLa cells: (**A**) effect of extension time at 37 °C on ECL intensity and (**B**) effect of the reaction time for hybridization of elongated TS primers with cDNA and intercalating $Ru(phen)_3^{2+}$ into dsDNA grooves on ECL intensity

elongated by telomerase and the extension reaction was completed after 2 h; so, 2 h was adopted as the optimal incubation time for extension reaction.

The reaction time for hybridization of elongated TS primers with cDNA and the intercalating of $Ru(phen)_3^{2+}$ into dsDNA grooves also cause a great effect on the sensitivity of the ECL biosensor. As shown in Fig. 3B, the ECL intensity of the system increased with the increase of the reaction time firstly and then reached a plateau after 3 h. This means 3 h was adequate for hybridization of elongated TS primers with cDNA and intercalation of $Ru(phen)_3^{2+}$ into dsDNA grooves on the electrode surface. So, 3 h had been chosen as the best condition for the following study.

Calibration curve for telomerase activity analysis

The proposed ECL biosensor has been applied to detect telomerase extracts from different numbers of HeLa cells under optimized conditions. As shown in Fig. 4A, the ECL peak intensity increased gradually with the enhanced HeLa cell number in the range of 5~50,000. This indicates that more telomerase could elongate more TS primers to produce more hybridized sites for cDNA, and eventually lead to a high ECL signal detected. Figure 4B showed the relationship between the enhanced ECL intensity (ΔECL, the difference of the ECL intensity at the presence and absence of telomerase) and the number of HeLa cells. ΔECL has a linear relationship with the logarithm of the number of HeLa cells from 5 to 5000. The regression equation was $\Delta ECL/counts = -643.96 + 1954.8 \lg N$ ($R^2 = 0.9906$), where N is the number of HeLa cells. The limit of detection is calculated to be as low as 2 HeLa cells ($S/N = 3$). Compared with the early reported ECL approaches, such as porphyrin-functionalized graphene-based ECL sensor [33] and bifunctionalized luminol-gold nanoparticle-based ECL sensor [35], the proposed ECL biosensor in this study was much more simple and sensitive for telomerase activity analysis.

Selectivity, reproducibility, and stability of the ECL biosensor

To validate the selectivity of the proposed ECL biosensor for telomerase activity detection, the telomerase extracts from 100 HeLa cells with and without heat treatment, HSA (0.1 mg/

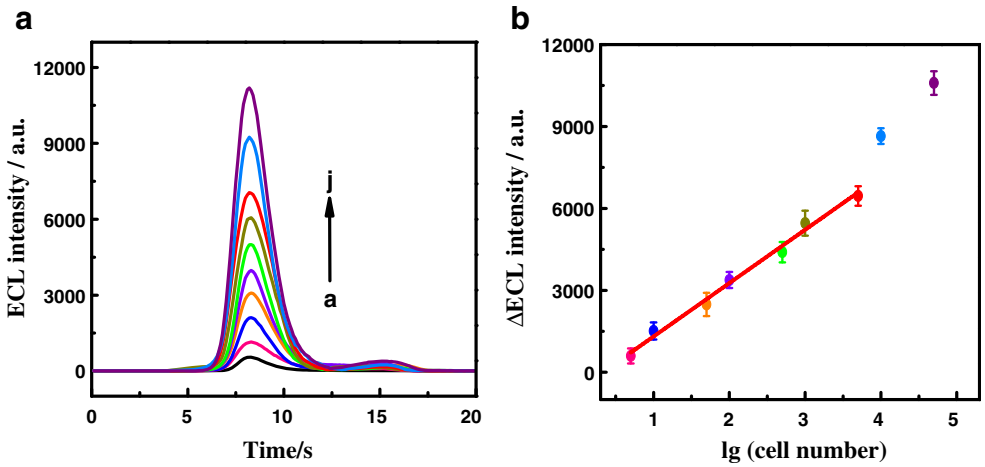

Fig. 4 (**A**) ECL response corresponding to the analysis of telomerase extracts from different numbers of HeLa cells: (**a**) 0, (**b**) 5, (**c**) 10, (**d**) 50, (**e**) 100, (**f**) 500, (**g**) 1000, (**h**) 5000, (**i**) 10,000, and (**j**) 50,000. (**B**) The calibration curve between the logarithm of the number of HeLa cells and ΔECL

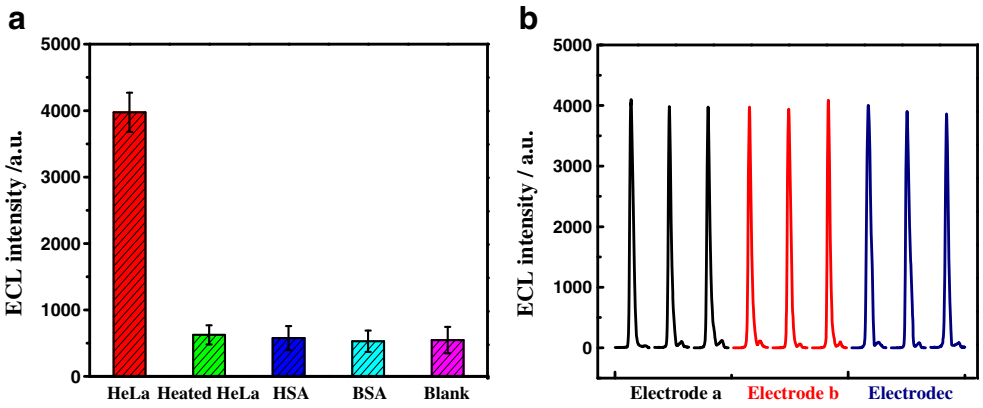

Fig. 5 (**A**) Selectivity of the proposed ECL biosensor for telomerase activity analysis. (**B**) ECL curves of the different electrodes with three duplicate assays in the presence of telomerase extracts from 100 HeLa cells

mL), BSA (0.1 mg/mL), and the blank sample were investigated. As shown in Fig. 5A, ECL signals of the heat-inactivated extracts, HSA, and BSA were all negligible, while a greatly enhanced ECL signal was detected in the presence of HeLa cell extracts, demonstrating that the proposed ECL biosensor had high specificity for telomerase activity analysis.

From a practical point, reproducibility and stability were also essential for telomerase activity detection. The ECL signals from different ECL biosensors with telomerase extracts from 100 HeLa cells had been compared. As shown in Fig. 5B, the relative standard deviations (RSDs) of intra-assays and inter-assays ($n = 3$) were <5 %, revealing that the proposed strategy had good precision and reproducibility. The stability of the proposed biosensor was tested by storing modified electrodes at 4 °C for 2 weeks, and it was found that there were no obvious decreases compared with the freshly prepared electrodes, indicating the good stability of the proposed ECL biosensor.

Conclusion

In summary, we have fabricated a label-free and convenient ECL biosensor for the detection of telomerase activity. This work exhibited high sensitivity for detecting the activity of telomerase extracted from HeLa cells with a detection limit of 2 HeLa cells. The proposed ECL strategy provides a much more convenient approach for telomerase-related cancer screening or diagnosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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