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Hairpin probes based click polymerization for label-free electrochemical detection of human T-lymphotropic virus types II

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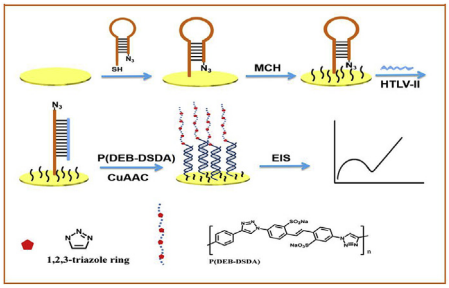
HIGHLIGHTS

- In absence of enzymes and nanomaterials, a novel and label-free amplification electrochemical impedance technique for the detection of HTLV-II was developed.
- The DNA detection based on click-chemistry EIS amplification has considerable simplicity, label-free, cheap "green" process, highly selective and flexibility with respect to reporter design.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel, simple, and label-free amplification electrochemical impedance method for quantitative detection of Human T-lymphotropic virus types II(HTLV-II) via click chemistry-mediated of hairpin DNA probes (hairpins) with polymers was developed. The hairpins were firstly attached to the gold electrode surface by an S-Au bond, the azido terminals of hairpins were close to the electrode surface, which make it difficult to be approached. After hybridizing with HTLV-II, the hairpins were unfolded and experienced a big configuration change, which made the azido terminals of the hairpins available to conjugate with alkynyl-containing polymer, called P(DEB-DSDA), formed by 1,4-diacetylenebenzene (DEB) and 4,4′-Diazido-2,2′-stilbenedisulfonic acid disodium salt tetrahydrate (DSDA) via Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). With amount of P(DEB-DSDA) conjugated with the hairpin probes via click polymerization, its electrochemical signal can have a great amplification. Under optimized experimental conditions, this new probe showed a low detection limit of 0.171 pM with a good liner in the range of 1 pM-1 nM. Meanwhile, the biosensor also exhibited good selectivity and reliability in detection of real serum samples, indicating that it has great application potential in clinical DNA diagnosis and detection.

1. Introduction

Human T-lymphotropic virus (HTLV) is human retroviruses including types I (HTLV-I) and II (HTLV-II), with strong association with adult T cell lymphoma/leukemia, progressive neurodegenerative disease and rare lymphocytic neoplasms [1–3]. As a close

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relative of HTLV-I, HTLV-II is closely related to neurological disease, inflammatory responses, respiratory responses and increased risk of mortality [4,5]. However, the reliable and sensitive detection of the retroviral DNA is very difficult, which mainly because its small size, low abundance and high sequence homology among the HTLV family members. Taking into account its vital roles in biomedical research, an effective method for detecting of HTLV-II DNA sensitively and selectively is very necessary.

Currently, a series of powerful amplification technique, such as polymerase chain reaction [6–8], loop-mediated isothermal amplification [9,10], rolling circle amplification [11,12], hybridization chain reaction [13], as well as ligase chain reaction [14], have been developed for detection of target nucleotides. However, the drawbacks of them, such as, complicated procedures, amplicon cross contamination, high cost, poor specificity and low sensitivity, may not conducive to their wide applications. Therefore, the development of new strategies with feasible, low-cost, and high efficiency is urgently needed.

In recent years, label-free electrochemical biosensors, especially the label-free biosensors based on electrochemical impedance spectroscopy (EIS) have received widespread attention because of simple to operate, small dimension, high sensitivity and inexpensiveness. EIS has been proved to be a effective strategy for efficient, fast and label-free detection of proteins [15,16], DNAs [17–23], and low molecular weight molecules in the biological field [24,25]. Since its firstly reported by Sharpless in 2001 [26], click chemistry has undoubtedly become one of the versatile and beneficial chemistry tools in the field of chemistry and materials science [27–32]. It has many significant advantages, such as high efficiency, excellent selectively and mild reaction conditions [33]. Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is one of the most prominent click reactions, under mild conditions with Cu(I) as catalyst, the 1,2,3-triazole can be rapidly formed by the reaction of azide and terminal alkyne. This reaction has been widely used in numerous areas, such as drug discovery, surface modification, dendrimer design, and series of other biological reactions [34,35]. At present, it has been tried to integrate into the biosensing of DNA [36,37]. For example, Hu developed an electrochemical biosensing method to detect and identify sequence-specific DNA via click chemistry, and in this method CuAAC was used as a straightforward and effective strategy to the immobilize of ethynylferrocene [36]. Sun and Peng reported a highly specific and capable approach using CuAAC for the discrimination of SBM under mild conditions [37]. However, as far as we know, the electrochemical biosensing strategy based on click chemistry has not yet been used to detect the HTLV virus yet.

In this report, a novel and label-free amplification electrochemical impedance technique for the simple and rapid detection of HTLV-II based on exploiting click-chemistry was reported for the first time. Herein, the hairpin DNA probes (hairpins) were first immobilized onto the surface of gold electrode by self-assembly. This immobilized capture probes were used to specific recognize the target DNA (tDNA) in subsequent steps. The six complementary bases at each terminal of hairpin sequence prone to form a thermostable double-stranded stem of the target unbound form, thus the azido moiety can be localized close to the surface of the electrode, and the loop part can specially recognize tDNA and hybridize with it [38,39]. After hybridizing with tDNA, the hairpins were unfolded and experienced a big conformational change, making the azido moieties more available to conjugated with alkynyl-containing polymer, which was formed by 1,4-diacetylenebenzene (DEB) and 4,4′-Diazido-2,2′-stilbenedisulfonic acid disodium salt tetrahydrate (DSDA) via CuAAC. The DNA probes were tested by EIS to monitor the hybridization, and compared with other DNA detection methods, this technology has a great improvement in detection sensitivity. Meanwhile, the results of the detection capability in serum samples indicate that the biosensor has good detection selectivity and reliability in serum samples. Owing to the simplicity, efficiency, and low cost, this method is considered to possess great potential in bioanalytical applications to sensitively detect of HTLV-II DNA.

2. Experimental section

2.1. Materials and reagents

1,4-diacetylenebenzene (DEB), 6-mercapto-1-hexanol (MCH) and 4,4′-Diazido −2,2′-stilbenedisulfonic acid disodium salt tetrahydrate (DSDA) were purchased from aladdin reagent Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS, Defined) was purchased from Shanghai YiJi Industrial Co., Ltd (Shanghai, China). Copper(II) sulfate was purchased from Tianjin RuijinTe Chemical Reagent Manufacturing Co., Ltd. (Tian Jin, China), L-ascorbic acid (AA) was purchased from Tianjin Hengxing Chemical Regent (Tianjin, China). Tris-EDTA buffer (TE, 10 mM Tris-HCl, 1 mM EDTA, pH = 8) was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). All the nucleotide sequences used in the experiment were purchased from Sangon Biotech Co., Ltd (Shanghai, China), their sequences are listed in Table 1. In addition, the other chemical reagents used in the experiment were analytical purity and not further purified. Ultrapure water (≥18.25 MΩ•cm) was used throughout all experiments.

2.2. Apparatus

The Electrochemical impedance spectroscopy (EIS) and Cyclic voltammetry (CV) tests were performed at room temperature using a three-electrode system, which comprising a saturated calomel electrode, a platinum wire electrode and a gold electrode (diameter = 2 mm), and they were used as reference electrode, counter electrode and work electrode, respectively. The electrochemical experiment was performed on Autolab potentiostat/galvanostat PGSTAT204 (Metrohm, Netherlands). The UV spectra were measured by using a UV-2600 spectrophotometer (Tianmei, Shanghai,China). The static water contact angle was measured by droplet shape analyzer-DSA100 (KRÜSS, Germany). The morphologies of modified electrode surface were characterized with field-emission scanning electron microscopy (FE-SEM, SU8010, Hitachi, Japan).

Table 1
DNA sequences used in experiments.

Type	Sequences of DNA
Hairpin capture DNA(Hairpins)	5′-SH-$(CH_2)_6$-CCACGCTCCCCGACCCAATTTCCACCTTGGCGTGG-N_3-3′
HTLV-II DNA(tDNA)	5′-CGAAGGTGGAAATTGGGTCGGGGAG-3′
Single base mismatched ssDNA(SBM)	5′-CGAAGATGGAAATTGGGTCGGGGAG-3′
Three bases mismatched ssDNA(TBM)	5′-CGCAGGTGGAATTTGGGTAGGGGAG-3′
Control ssDNA (Control)	5′-GAGGGCCTGCAGGATCATTGGCTTT-3′

2.3. Preparation of modified electrode

To obtain a smooth surface, the Au electrode was first polished with alumina (0.3 μm) before use. And then the electrode was washed with ultrapure water and followed by ultrasonic for 2 min in anhydrous ethanol and ultrapure water respectively. Subsequently, the electrode was sonicated in a newly prepared Piranha solution (98% H_2SO_4 and 30% H_2O_2, 3:1 in v/v) for 10 min. At last, the gold electrode was treated by cyclic voltammetry in H_2SO_4 solution (0.5 M) with the scanning potential of −0.2 V-1.5 V and the scan rate of 0.1 V/s until a stable cyclic voltammetry curve was obtained [20,40].

The prepared process of the modified electrode and the process of HTLV-II DNA detection via this strategy are illustrated in Scheme 1. Before modifying, the freshly prepared Au electrode was washed with ultrapure water and was dried with nitrogen, and then it was incubated in TE solution (5 μM, containing 1 μM hairpin DNA) for 90 min to immobilize the capture hairpin probes on the electrode surface. After removing the nonspecific binding hairpins with TE buffer, the modified electrode was immersed in MCH (2.0 mM, dissolved in 70% ethanol) for 30 min to block unbound sites. Then TE buffer and 70% ethanol were used to rinse the electrode, and 5 μL of certain concentration of tDNA was dropped on its surface and keep 90 min at 37 °C. After that, the tDNA can be identified and captured by the hairpins on the electrode surface. At the same time, the mixture solution consisting of DSDA (50 μL, 100 μM, dissolved in DMSO), DEB (50 μL, 100 μM, dissolved in DMSO), AA (100 μL, 10 mM, dissolved in 70% alcohol), $CuSO_4$ (25 μL, 20 mM, dissolved in 70% alcohol), and DMSO (4.775 ml) was stirred at 37 °C for 2 h. When this click reaction was finished, a linear alkynyl-containing polymer, which called P(DEB-DSDA), can be obtained. Subsequently, a new prepare mixed solution of 400 μL containing P(DEB-DSDA), AA, CuSO4, wherein AA concentration was 0.2 mM and CuSO4 concentration was 0.1 mM. After fully mixing, the modified electrode was immersed in this mixed solution and incubated at 37 °C for 2 h. Finally, the P(DEB-DSDA) obtained electrode based on click chemistry, was washed with anhydrous ethanol and ultrapure water respectively, and dried with nitrogen.

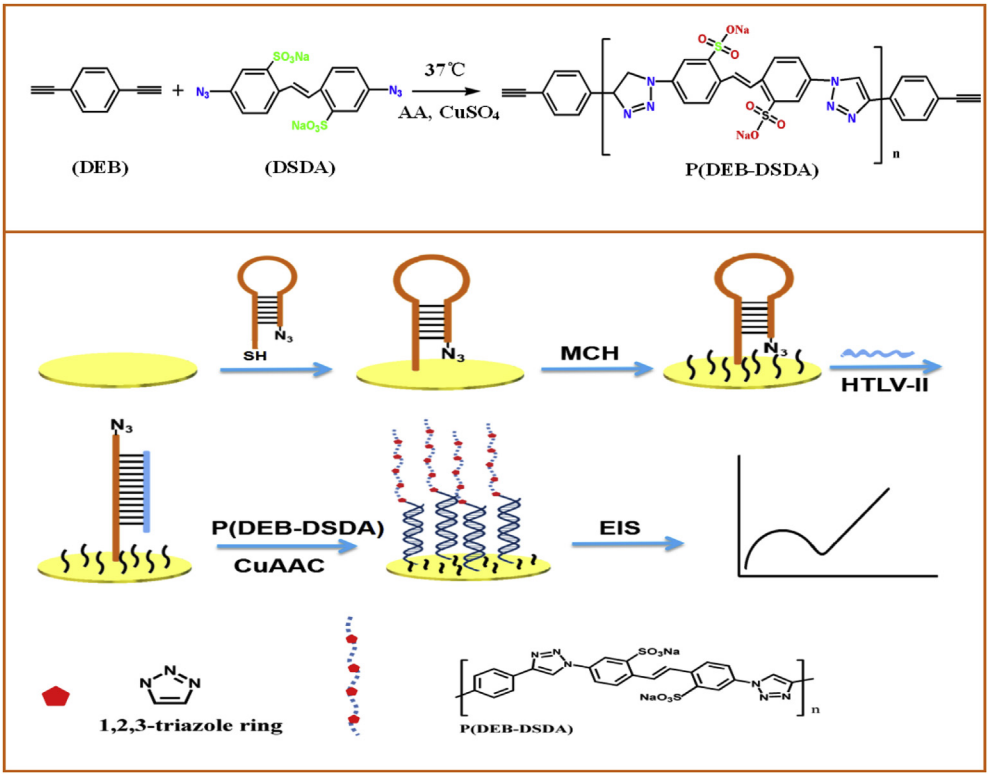
2.4. Electrochemical measurements

The performance analysis of the new manufactured sensors were carried out with CV and EIS in $[Fe(CN)_6]^{3-/4-}$ solution (5.0 mM, containing 0.1 M KNO_3). CV measurement was conducted in the potential range of −0.2 V- 0.6 V. The EIS measurement was conducted in the frequency range of 0.1 Hz−100 kHz with an open circuit potential of 0.18 V and an alternate voltage of 5 mV.

3. Results and discussion

3.1. Surface characterization

The water contact angle (WCA) can effectively characterize the change of chemical groups on the surface of materials. Fig. 1 shows the change process of the WCA of the surface of the modified electrode with different steps. It can be observed from Fig. 1A that the WCA of bare gold electrode is 68°. The WCA decreased to 61.55° when hairpin DNA is introduced into gold electrode, which because DNA contains a lot of hydrophilic groups (Fig. 1B). The WCA increases to 62.7° slightly when MCH is self-assembled on a gold electrode, this may be because that MCH consists of hydrophobic carbon chains and hydrophilic hydroxyl groups, so the WCA is not changed obviously when both of them act synchronously (Fig. 1C). After hairpin DNA and tDNA hybridization, the WCA decreased to 57.85° because of the increase in hydrophilic groups(Fig. 1D). Finally, the WCA increased to 70.4° dramatically after incubation of



Scheme 1. Schematic illustration of the prepared process of the modified electrode and the process of HTLV-II DNA detection via click chemistry-mediated of electroactive probes.

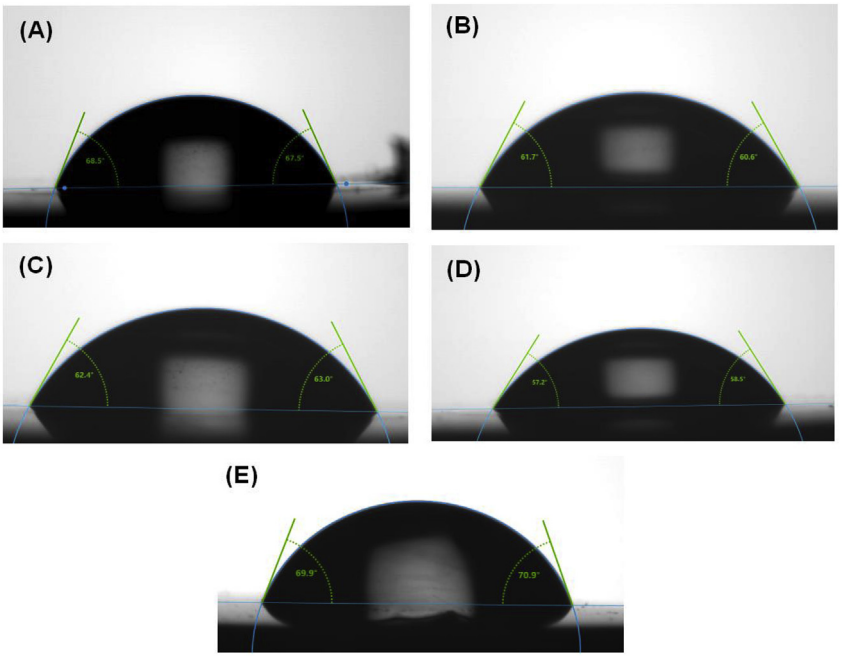


Fig. 1. (A) Water droplet on the bare gold electrode, (B) hairpins, (C) hairpins/MCH, (D) hairpins/MCH/tDNA, and (E) hairpins/MCH/tDNA/P(DEB-DSDA) modified gold electrode. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

modified electrode within the P(DEB-DSDA) polymer solution under the catalysis of Cu(I)(Fig. 1E), proved that the P(DEB-DSDA) polymer has strong hydrophobic.

The AFM images of modified electrode as shown in Fig. 2 were carried out in the gold plated pad silicon wafer. The modified electrode based on hairpins/MCH/tDNA presents a maximum height of 12.2 nm(Fig. 2A). Subsequently, significant changes in morphology or height were observed after the hairpins/MCH/tDNA modified electrode conjugate with alkynyl-containing polymer(-maximum height of 29.0 nm) in Fig. 2B.

The SEM images of different steps of the modified electrode were shown in Fig. 3, the film changes to higher density and thicker from Fig. 3A - C, especially the hairpins/MCH/tDNA/P(DEB-DSDA) (Fig. 3C) modified electrode. This phenomenon proved that we have succeeded in modifying DNA and the P(DEB-DSDA) polymer onto the electrode. Moreover, the hairpins/MCH/P(DEB-DSDA) (Fig. 3D) modified electrode did not change much of the electrode morphology compared with hairpins/MCH (Fig. 3A), suggesting that the P(DEB-DSDA) polymer cannot be bonded to hairpins in the absence of tDNA.

3.2. Electrochemical characterization of the modified electrode

The EIS, which can provide a wealth of information about the subtle changes in the electrode surface, was used to characterize the modified electrodes. Fig. 4A is the Nyquist plots of the impedance spectrum at different stages of the preparation process. The electron transfer resistance (R_{ct}) of the electrode can be expressed by the diameter of the semicircle, and the linear part represents the solution diffusion resistance (W) [20]. Compared with the modified electrodes, the bare gold electrode has the smallest electron transfer resistance and the highest electron transfer ability (curve a). The increase in R_{ct} of the hairpin DNA-modified electrode may be attribute to the fact that the electron transfer was impeded by the film on the electrode formed by the hairpin DNA, while the negatively charged hairpin DNA also inhibited the redox reaction of the redox probe $[Fe(CN)_6]^{3-/4-}$ (curve b). After modifying by MCH, a denser film was formed on the electrode surface, and the electron transfer ability was further impeded, thus the R_{ct} increased (curve c). Upon hybridization, the R_{ct} value continued to increase (curve d), indicating the successful pairing between tDNA and hairpin DNA.

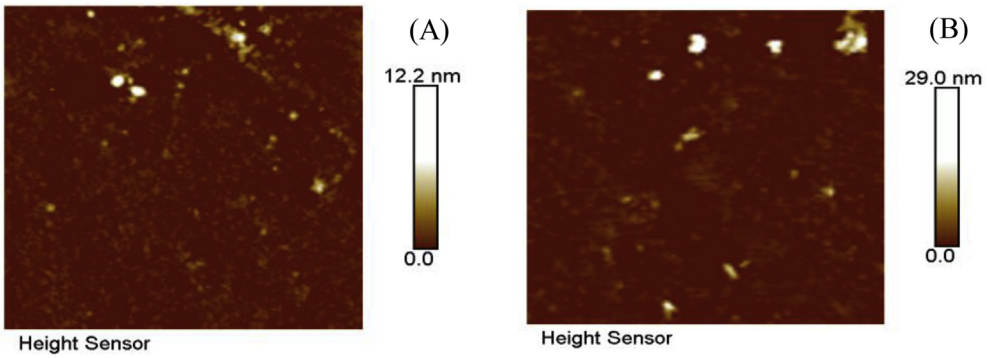


Fig. 2. AFM images for the electrode modified with the hairpins/MCH/tDNA(A), hairpins/MCH/tDNA/P(DEB-DSDA)(B). Scan area of 2μm×2 μm.

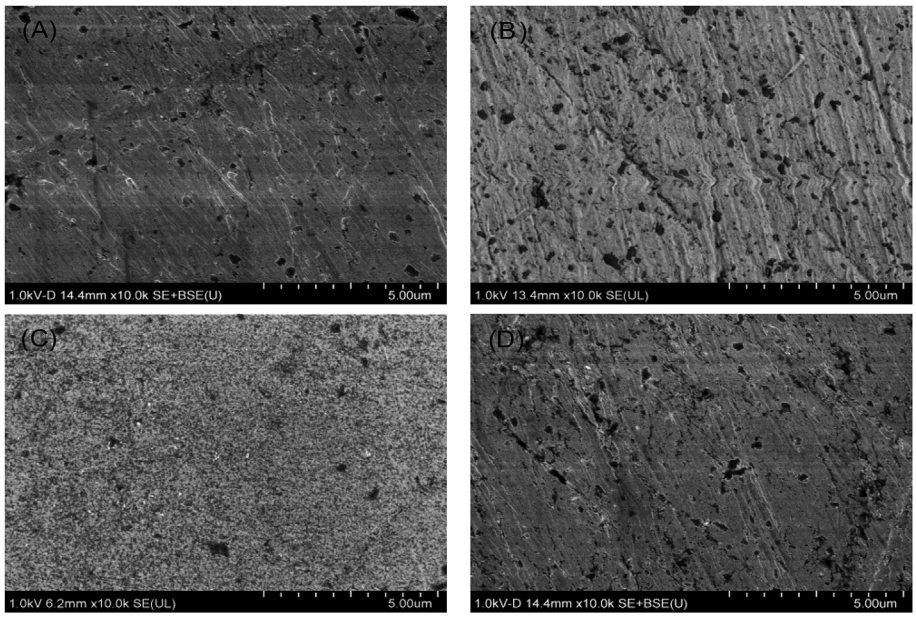


Fig. 3. (A) SEM images of the hairpins/MCH, (B) hairpins/MCH/tDNA, (C) hairpins/MCH/tDNA/P(DEB-DSDA), (D) hairpins/MCH/P(DEB-DSDA) modified electrode.

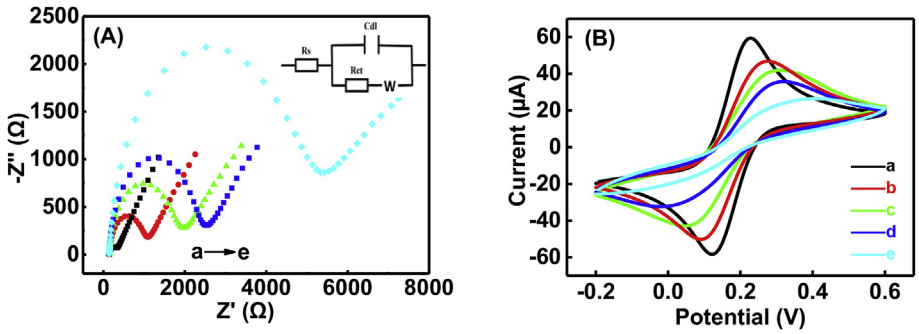


Fig. 4. (A) EIS and (B) CVs curve of the bare gold electrode (a) and the electrodes modified by hairpins (b), hairpins/MCH (c), hairpins/MCH/tDNA (d), and hairpins/MCH/tDNA/P(DEB-DSDA)(e). The reaction time of the tDNA (1 nM) and the polymer solution (80 μL) was 60 min. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

After click reaction, the obtained polymer and the azide group on the hairpin DNA interacted through covalent bonds, the thickness of the film increased greatly, resulting the R_{ct} value increases significantly (curve e) [40].

Moreover, the modified electrode at each step was also characterized by CV technique. As can be seen from Fig. 4B, the bare gold electrode has a large peak current (curve a), this may be mainly caused by its smooth surface and the fast electron-transfer process. The peak current decreased obviously when hairpins was immobilized on the electrode surface (curve b). The nonspecific sites on the surface of the electrode were blocked after self-assembly of MCH on the modified electrode, resulting a further decreased of the peak current (curve c). As seen from curve d, a smaller peak current was observed when the hybridization was accomplished. While the current was dramatically decreased (curve e) after the polymer was immobilized on the hairpin DNA. In conclusion, all the results demonstrate that the electrode was modified successfully.

3.3. Optimization

The analytical performance of this strategy is highly depended on the signal amplification process, therefore, the incubation time between P(DEB-DSDA) and hybridization DNA, as well as the volume of P(DEB-DSDA) are the main experimental parameters for our optimization. Firstly, the incubation time from 30 to 160 min was investigated to monitor its effect on resistances for the aptasensor. As shown in Fig. 5A, the resistances of modified electrode increased drastically with the increase of reaction time, and achieved equilibrium after 120 min, after that there was no significant changes in resistances value, which might be due to the fact that the reaction between the free polymer and the azide group on the electrode complete with the prolong of the reaction time. Thus, 120 min was selected as the optimal time for the subsequent studies. Meanwhile, because of the significant effects on the analytical performance of biosensor, the dosage of polymers was undoubtedly the other important parameter to optimize. Therefore, the effect of the volume of polymers on the electrochemical performance was investigated by adding different volume of polymer with certain concentration. As shown in Fig. 5B, the R_{ct} of value increased gradually with the volume of polymer increased from 40 μL to 80 μL, and then no significant changes were observed with the increase of the volume of the polymer. Therefore, 80 μL was picked as the optimal volume for further experiments.

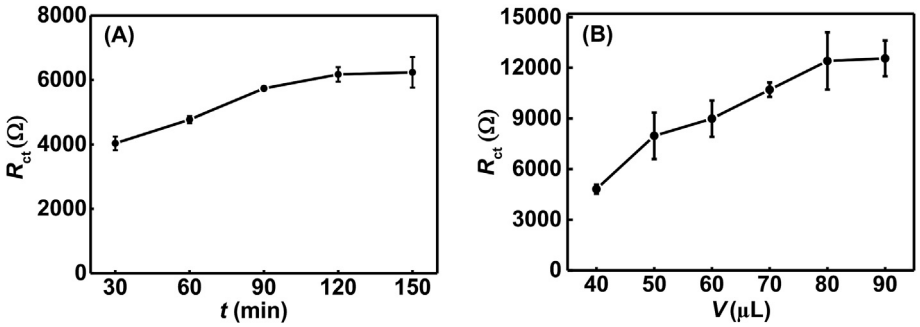


Fig. 5. (A) The optimizations of incubation time between P(DEB-DSDA) and hybridization DNA (B) the volume of P(DEB-DSDA). (1 nM tDNA, error bars = SD, n = 3).

3.4. Analytical performance

A range of different concentrations of HTLV-II was used to evaluate the analytical performance of the prepared biosensor. As the concentration of HTLV-II increased in the range of 0–1 nM, the R_{ct} value increased significantly (Fig. 6A), which indicated that various amounts of polymers were deposited on the hairpin DNA successfully. From Fig. 6B we can see that, there is a good liner relationship between R_{ct} value and the logarithms of tDNA concentrations in the range of 1 pM-1 nM, and the detection limit was 0.171 pM (S/N = 3). The linear regression equation can be expressed as $R_{ct}(\Omega) = 2758\lg(C_{HTLV\text{-}II}/pM) + 2689$ ($R^2 = 0.986$). Three measurements were performed in parallel, and an average value was reported. Some analysis parameters of developed DNA sensors in recent years are listed in Table 2. Compared with these developed DNA sensors, this sensor has no complicated fabrication procedures and has a lower detection limit, in absence of enzymes and nanomaterials.

3.5. Specificity and stability of this strategy

The specificity of this strategy in detection of HTLV-II viruses was evaluated by comparing the electrochemical responses of tDNA with three other types of non-complementary ssDNA, including SBM, TBM, and control. The R_{ct} value of SBM, TBM and control DNA were about 42.1%, 37.3%, and 31.7% of that from tDNA, respectively (Fig. 7). These results were mainly due to the hybridization efficiency between the loops of the immobilized hairpins and different types of ssDNA, taking into account the ssDNA wouldn't particularly and thoroughly bind to the loop portions of the hairpins and unfold them, while the tDNA could. In addition, compared with the hairpins/MCH/Au modified electrode, the impedance signal produced by three other types of non-complementary ssDNA was not significantly increased compared with the hairpins/MCH/Au modified electrode. These results demonstrated that the novel

Table 2
Comparison of analytical performance between this sensor and other types of DNA sensors.

method	linear range	LOD	ref.
DPV	4.4×10^{-11} to 2.0×10^{-9} M	1.55×10^{-12} M	[41]
EIS	1×10^{-8} to 2×10^{-6} M	1×10^{-8} M	[42]
DPV	1×10^{-12} to 1×10^{-9} M	2.96×10^{-13} M	[40]
EIS	1×10^{-11} to 2×10^{-8} M	7×10^{-12} M	[43]
EIS	1×10^{-12} to 1×10^{-6} M	1.5×10^{-13} M	[44]
EIS	1×10^{-12} to 1×10^{-9} M	1.71×10^{-13} M	This work

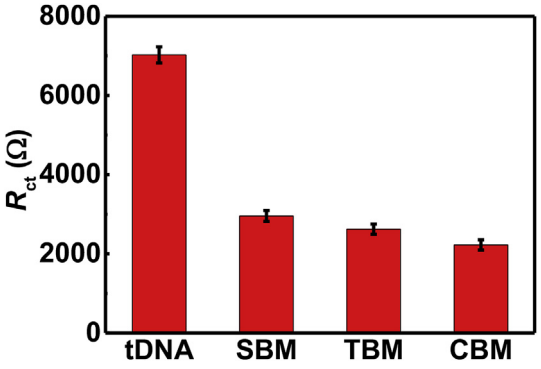


Fig. 7. Impedance responses of this strategy toward tDNA, SBM, TBM and control with a concentration of 50 pM. (error bars = SD, n = 3).

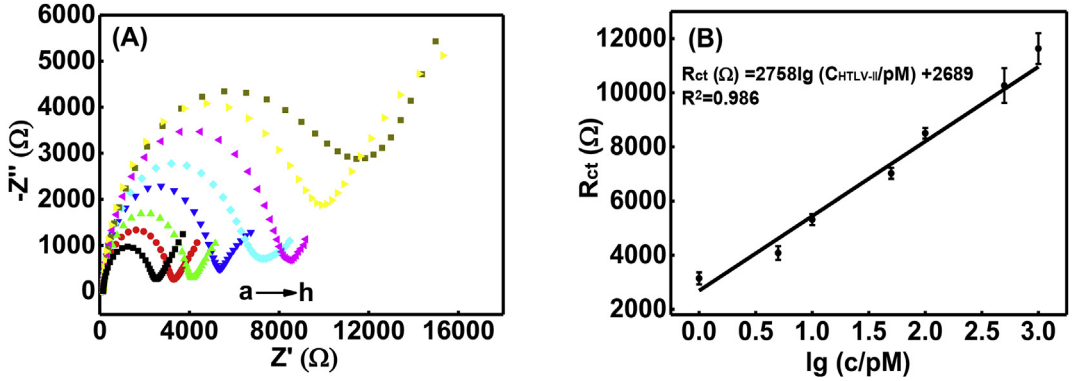


Fig. 6. (A) EISs of this strategy toward different tDNA concentrations 0 (a), 1 pM (b), 5 pM (c), 10 pM (d), 50 pM (e), 100 pM (f), 500 pM (g), 1000 pM (h). (B)The calibration plot between Rct and the logarithm of tDNA concentration from 0 to 1 nM. (error bars = SD, n = 3).

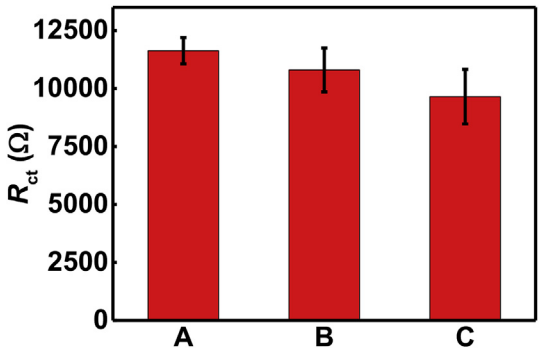


Fig. 8. Impedance responses of this method toward 1 nM tDNA in TE buffer (A), 1%. serum sample (B), and 5% serum sample (C). (error bars = SD, n = 3).

strategy possesses excellent specificity in discriminating mismatched and complementary oligonucleotide fragments and potential application value in detecting of HTLV virus.

Furthermore, stability is one of the factors affecting the practicality of biosensor. To evaluate the stability of this strategy, the obtained electrode was preserved at 4 °C for a long-term storage experiment. After two weeks, the EIS signal was approximately 92.2% of that from the initial signal. The decrease of the impedance might be due to the decreased biological activity of hairpin DNA. These results demonstrated that the obtained electrode has a good stability.

3.6. Detection capability in serum samples

Finally, the reliability and potential application of this novel method in real serum were evaluated by adding the tDNA to the fetal bovine serum (FBS) and observing the interference effect of FBS on its analytical performance. In this experiment, the tDNA was dissolved in FBS and then the product was diluted to 1% and 5%, respectively. Comparing the R_{ct} of tDNA in FBS samples with that obtained from tDNA in TE buffer, and the results were shown in Fig. 8. The R_{ct} of 1% FBS sample (B) was approximately 91.1% of that from TE buffer (A), and the R_{ct} of 5% FBS sample (C) was approximately 88.7% of that from TE buffer (A). These results indicated that this strategy possesses excellent anti-interference ability and good detection capability in real samples, which make it has reliable analytical ability and great application potential in clinical diagnosis.

4. Conclusion

In conclusion, a novel, sample, and label-free amplification electrochemical impedance strategy for quantitative detection of HTLV-II via click chemistry -mediated of hairpin DNA probes with alkynyl-containing polymers was developed. The hairpins were used as the immobilized capture to specific recognize tDNA, resulting the specificity of this strategy improved significantly. What's more, the independence signal can be amplified multiply via efficient click-chemistry reaction and there is no any enzymes or nanomaterials needed. In addition, the strategy exhibited a low detection limit of 0.171 pM with a good liner in the range of 1pM -1 nM under optimal conditions and it exhibited good selectivity and reliability for biosening of tDNA in serum samples. The strategy exhibits superior analytical performance, such as simplicity, high sensitivity, reliability and low cost, and it can be expanded to detect more kinds of DNA through replacing the sequence of hairpins, implicating that it exhibits great potential for clinical diagnosis and DNA detection.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Compliance with ethical standards

The author(s) declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.01.027.

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