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# A signal-on electrochemical DNA biosensor based on potential-assisted Cu(I)-catalyzed azide-alkyne cycloaddition mediated labeling of hairpin-like oligonucleotide with electroactive probe

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ABSTRACT

A novel electrochemical biosensor was developed for the signal-on detection of sequence-specific DNA by exploiting potential-assisted Cu(I)-catalyzed azide-alkyne cycloaddition (φCuAAC) as an efficient approach for the labeling of hairpin-like oligonucleotide (hairpin) with electroactive probe. The hairpins, dually labeled with thiol and azide at either terminal, were firstly self-assembled on gold electrode and served as the capture probes for the specific recognition of target DNA. Upon hybridization with target DNA, the surface-confined hairpins were unfolded, liberating the azide-containing terminals away from electrode surface. Subsequently, the unfolded hairpins were conveniently and efficiently labeled with ethynylferrocene (EFC) via the φCuAAC. The quantitatively labeled EFC was finally measured via differential pulse voltammetry (DPV) for the signal-on electrochemical detection of sequence-specific DNA. The biosensor presented a good linear response over the range from 1 pM to 1 nM with a detection limit of 0.62 pM. Results also revealed that it was highly specific and held a good detection capability in serum samples. Furthermore, the ability to chemoselectively label hairpin-like oligonucleotide with signal reporter by electrical addressing, together with the simplicity and efficiency of the φCuAAC, makes it compatible with microfluidic devices and microelectrode arrays to achieve the miniaturized and multiplexed detections.



## 1. Introduction

The ability to fast and sensitively detect sequence-specific DNA of electrochemical DNA biosensors has enabled them to find widespread applications in medical diagnostics [1], forensic investigations [2], agricultural and food sciences [3], and environmental monitoring [4]. Compared with conventional optical methods, electrochemical DNA biosensors are fast, portable, highly sensitive and selective, cost-effective, and amenable to miniaturization [5,6]. Recently, hairpin-like oligonucleotides (hairpins) have been extensively used in electrochemical DNA biosensors due to their inherent simplicity and specificity [7–10]. Pioneering work was reported by Fan et al., in which a ferrocene-labeled hairpin was adopted for the electrochemical interrogation of DNA hybridization [8]. It emblematized a significant progress in the electrochemical DNA detection with the superiorities of fast response, easy to use, low cost, high reusability and direct use in complicated samples. Generally, hairpin-based electrochemical DNA biosensors can be divided into two types: signal-off and signal-on. Since the former is susceptible to false positives and the obtained sensitivities are insufficient for the PCR-free applications, the latter is more preferred to avoid these possible disadvantages. To date, most of the reported signal-on methods required the utilization of enzymes and/or nanoparticles for the purpose of signal amplification [9,11]. However, natural enzymes usually have poor long-term stability in vitro, and their activities are easily affected by the protein conformation, leading to susceptibility to external conditions (e.g. temperature, pH, ionic strength, etc.) [12]. Besides, additional signal amplification methods need expensive biochemicals and the protocols are complicated, tedious and time-consuming, which greatly restrict the potential clinical applications, where simplicity and economy are preferred. Therefore, further improvements are still in great demand to simplify the protocols while maintain its sensitivity.

The Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has found broad applications in the fields ranging from surface functionalization to bioconjugation [13–17], benefiting most from its high yield, excellent chemoselectivity and orthogonality to diverse

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functional groups, and mild reaction conditions [13–19]. In a standard CuAAC reaction, the active Cu(I) catalyst is often generated from the Cu(II)/ascorbate system, allowing for its convenience and effectiveness at generating and maintaining the catalytically active Cu(I) oxidation state [13,14]. Unfortunately, it is biologically detrimental, for the oxidative cleavage of biomolecules (e.g. DNA, RNA, proteins, etc.) by the highly reactive hydroxyl radicals of Fenton reaction [20]. As a result, alternative methods based on electrochemistry have been developed to obtain the active Cu (I) catalyst without employing ascorbate. For example, selective functionalization of independently addressable electrodes with electroactive probe via electrochemistry-based CuAAC has been achieved [15], in which the active Cu(I) was directly obtained from electrochemical activation of Cu(II) under a negative potential. However, to the best of our knowledge, there has been no report on incorporating it with hairpin-like oligonucleotide for the signal-on electrochemical detection of sequence-specific DNA.

Herein, inspired by these pioneering studies mentioned above, a novel hairpin-based electrochemical biosensor for the signal-on detection of sequence-specific DNA was developed for the first time by exploiting potential-assisted CuAAC (φCuAAC) as an efficient approach to label the azide-terminated hairpin-like oligonucleotide with alkynyl-containing electroactive probe. The hairpins, dually labeled with thiol and azide at either terminal, were firstly self-assembled on gold electrode and served as the capture probes for the specific recognition of target DNA. Upon hybridization, the surface-confined hairpins were unfolded, liberating the azide-containing terminals away from electrode surface. Subsequently, the unfolded hairpins were labeled with ethynylferrocene (EFC) via the φCuAAC, where the active Cu(I) was directly obtained from in situ electrochemical activation of inert Cu(II) complexes with cyclic voltammetry (CV). The quantitatively labeled EFC was finally measured via differential pulse voltammetry (DPV) for the signal-on electrochemical detection of sequence-specific DNA. Compared with those time-consuming techniques adopted to introduce into electroactive probes, such as carbodiimides-mediated bioconjugation [21,22], labeling of electroactive probe via the φCuAAC could be completed conveniently with high efficiency within 30 min, and this process was also highly tolerant to reaction conditions. Moreover, the triazole linkage of cycloaddition is quite thermally and hydrolytically stable, as well as highly tolerant to redox reactions [23]. More importantly, unlike most of the coupling chemistries, the azido and alkynyl are chemoselective to each other, thus the possible interference due to the conjugation of EFC with any other functional groups, present in the system, could be eliminated. That is to say, it is highly tolerant to false positives. Without complicating the design of hairpin-like oligonucleotide, it realized the signal-on electrochemical detection of sequence-specific DNA. More importantly, the ability to chemoselectively label hairpin-like oligonucleotide with signal reporter by electrical addressing, together with the simplicity and efficiency of the φCuAAC, makes it compatible with microfluidic devices and microelectrode arrays to achieve the miniaturized and multiplexed detections.

## 2. Material and methods

### 2.1. Materials and reagents

The hairpin-like oligonucleotide and all other oligonucleotides used in this work were synthesized and purified by Sangon Biotech Co., Ltd. (Shanghai, China), and their sequences are listed below (mismatch(es) underlied):

Hairpin-like oligonucleotide (hairpin): 5′-SH-$(CH_2)_6$-CCA CGC TTG TGG GTC AAC CCC CGT GG-$N_3$-3′;

Target complementary oligonucleotide (target DNA): 5′-GGG GTT GAC CCA CAA G-3′;

Single-base mismatched oligonucleotide (SBM): 5′-GGG GTC GAC CCA CAA G-3′;

Three bases mismatched oligonucleotide (TBM): 5′-GGG GTC GTC GCA CAA G-3′;

Control oligonucleotide (Control): 5′-TTC AGC TCT ATC AAT C-3′.

Bathophenanthroline disulfonic acid disodium salt hydrate (BPDS, see Fig. S1 for its structure), ethynylferrocene (EFC), potassium hexafluorophosphate ($KPF_6$), and lithium perchlorate trihydrate ($LiClO_4$) were all purchased from J&K Scientific Ltd. (Shanghai, China). Copper(II) sulfate pentahydrate ($CuSO_4$) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS, Defined) was purchased from Shanghai YiJi Industrial Co., Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO), magnesium chloride ($MgCl_2$) and all other chemicals were of analytical reagent grade and used as received. Ultrapure water ($\geq$ 18.25 MΩ) was obtained from a Millipore Milli-Q water purification system and was used in all experiments.

Tris-EDTA buffer (TE, 10 mM Tris–HCl, 1 mM EDTA, pH = 8) was prepared and used as the stocking buffer and washing solution of oligonucleotides. TE buffer containing 0.1 mM $MgCl_2$ was used as the buffer for the immobilization of hairpin, since $Mg^{2+}$ ions have a powerful stabilizing influence on the double-stranded stems of hairpins [7]. Various different concentrations of oligonucleotides used in this work were prepared by serial dilution with TE buffer. Electrochemical characterization of the labeled EFC on the electrode with CV at different scan rates and differential pulse voltammetric measurements were all conducted in 1 M $LiClO_4$, allowing for its ability in stabilizing the ferrocenium, which is the oxidized form of ferrocene [8]. 1 mM Cu(II)/BPDS complexes (1:2 in molarity) was prepared by successively adding BPDS and $CuSO_4$ into DMSO/$H_2O$ (1:1 in volume) [15,24]. The labeling solution was freshly prepared by adding 0.5 mL DMSO, 1 mL 1 mM Cu(II)/BPDS complexes, and 1 mL 1 mM EFC (dissolved in DMSO/$H_2O$, 1:1 in volume) into 7.5 mL 0.1 M $KPF_6$, successively, thus the final concentrations of Cu(II), EFC and $KPF_6$ were 0.1 mM, 0.1 mM and 0.075 M, respectively.

### 2.2. Apparatus

All electrochemical experiments were carried out at room temperature with a conventional three-electrode system, consisting of a modified gold electrode (2 mm in diameter) as working electrode, a saturated calomel electrode (SCE) as reference electrode and a platinum wire as auxiliary electrode. Unless stated otherwise, all potentials mentioned in this work were referred to SCE. Cyclic voltammetry (CV), linear sweep voltammetry (LSV) and differential pulse voltammetry (DPV) were performed on a CHI 760D electrochemical workstation (Shanghai, China). Electrochemical impedance spectroscopy (EIS) measurements were carried out using an Autolab/PGSTAT302N (Eco Chemie, Netherlands). The EIS measurements were conducted within the frequency range of 0.1 Hz to 100 KHz in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M $KNO_3$ at open circuit potential of 0.18 V; the amplitude of the applied sine wave potential was 5 mV.

### 2.3. Preparation of the electrochemical DNA biosensor and electrochemical measurements

The gold electrode was carefully polished with 0.3 and 0.05 μm alumina slurries, followed by ultrasonic cleaning in ethanol and ultrapure water. After that, it was chemically treated in a fresh Piranha solution (98% $H_2SO_4$ and 30% $H_2O_2$, 3:1 in volume, *Caution!* it is dangerous and must be handled with great care) for

10 min. Subsequently, it was electrochemically cleaned in 0.5 M $H_2SO_4$ by CV within the potential of −0.2 V and 1.5 V until a reproducible cyclic voltammogram was achieved. After that, the electrode was rinsed thoroughly with ethanol and ultrapure water, and dried with nitrogen prior to use.

Immobilization of the hairpin-like oligonucleotides was achieved by pipetting the solution (0.5 μM in TE buffer that contained 0.1 mM $MgCl_2$, 5 μL) onto the gold electrode surface and kept at 37 °C for 1.5 h. Then, the electrode was moderately washed with TE solution. The possible nonspecific binding sites on the electrode were blocked with 2 mM MCH (dissolved in 70% ethanol), followed by rinsing with 70% ethanol and ultrapure water. DNA hybridization was fulfilled by incubating the obtained electrode in 10 μL of TE buffer that contained different concentrations of oligonucleotides at 37 °C for 1.5 h, followed by washing with TE solution to remove the unbound oligonucleotides.

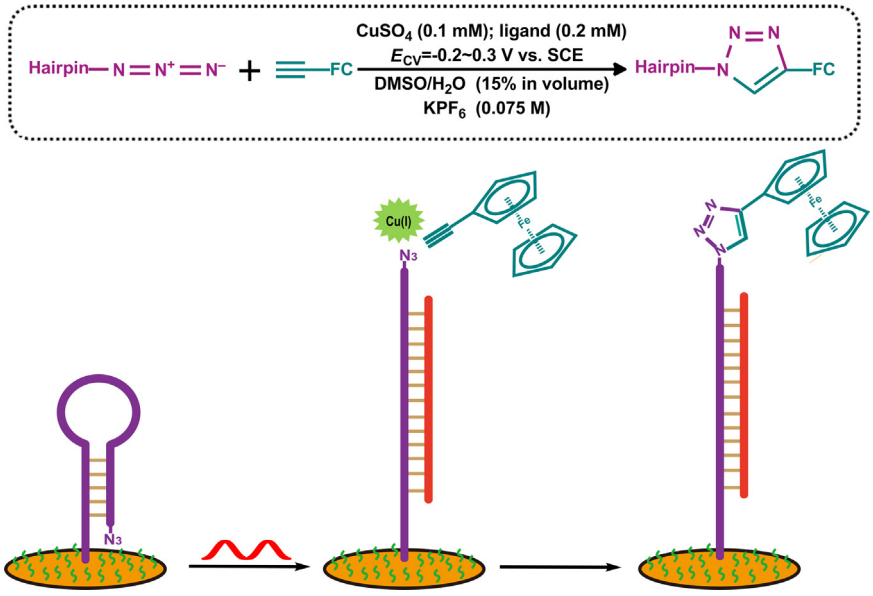
The labeling of the unfolded hairpins with EFC via the φCuAAC is described as follows. The modified electrode was firstly immersed in the labeling solution. Then, CV (see Fig. S3 for details) was applied to in situ electrochemically generate the catalytically active Cu(I)/BPDS complexes, and this process was repeated every 2 min during the 30 min labeling. Here, the standing time (2 min) following after every scan was intentionally reserved for the CuAAC. After the labeling was completed, the electrode was treated with LSV (see Fig. S4), and washed with ethanol, DMSO and ultrapure water to remove any non-covalently bound Cu(0) and EFC.

The electrode was finally immersed in 1 M $LiClO_4$, and then DPV was conducted under the potential from 0 V to 0.7 V (with a potential scan rate of 4 mV $s^{-1}$, a pulse amplitude of 50 mV, a pulse time of 0.05 s, and a quiet time of 120 s) to record the oxidation current of the labeled EFC.

## 3. Results and discussion

### 3.1. Principle of the electrochemical DNA biosensor

The scheme of preparation of the electrochemical DNA biosensor is illustrated in Scheme 1. Briefly, the hairpins, dually labeled with thiol and azide at either terminal, were firstly immobilized on a gold electrode via the facile gold-thiol self-assembly and served as the capture probes for the specific recognition of target DNA. The hairpin is designed such that it has five complementary bases at either terminal of its sequence, they are responsible for keeping the folded stem-loop form of the hairpin and this is important both for the low background interference and for the improved specificity toward genotyping of single-nucleotide polymorphisms (SNPs) when it is compared with that of linear oligonucleotide probes [7]; the rest part of its sequence is a completely complementary region for the specific recognition of the predetermined target DNA [7,25]. Initially, the hairpins were kept in a folded stem-loop form due to the formation of thermostable double-stranded stems, holding the azide-containing terminals in the proximity of the electrode surface. Thus, it was difficult for EFC to conjugate with azidos due to the remarkable steric hindrance of the densely packed self-assembled monolayer (SAM). In addition, EFC is highly hydrophobic and this is adverse to it to pass through the hydrophilic SAM. Upon hybridization with target DNA, the surface-confined hairpins were unfolded and spontaneously underwent a large conformational change, thus liberating the azide-containing terminals away from the electrode surface. As a result, the hairpins became free to be labeled with EFC via the φCuAAC. The active Cu(I) catalyst required to accelerate the cycloaddition was generated from in situ electrochemical activation of the inert Cu(II)/BPDS complexes via CV. By modulating the potential with CV, Cu(II) can be readily switched into Cu(I), leading to an adequate amount of Cu(I) catalyst was generated and maintained at the electrode surface. The water-soluble BPDS was utilized as the stabilizing ligand of Cu(I) [24,26–28] not only to protect it from being oxidized or disproportionated, but also to enhance its catalytic activity [24,26]. Finally, the quantitatively labeled EFC was exploited as the electroactive probe to monitor the DNA hybridization. As the unfolded hairpins were stoichiometrically labeled with EFC, electrochemical measurement of the latter via DPV enabled a reliable quantification of sequence-specific DNA. In this work, the electroactive probes were introduced into the system after DNA hybridization, thus it allowed a signal-on electrochemical detection. Of note, to suppress the nonspecific adsorption of oligonucleotides on the electrode due to the weak electrostatic interaction between gold and nitrogen-containing bases, MCH was employed to block the possible nonspecific binding sites and to help hairpins to stand up at the electrode surface in a highly hybridizable orientation [29].




**Scheme 1.** Schematic illustration of the hairpin-based electrochemical DNA biosensor for the signal-on detection of sequence-specific DNA.

### 3.2. Electrochemical characterizations

Initial experiments focused on in situ electrochemically generating and maintaining the active Cu(I) catalyst at the electrode surface. The modified electrode was immersed in the labeling solution, and then CV was carried out to investigate the redox potentials of the electroactive species in the labeling solution at the modified electrode surface. As shown in Fig. S2, a weak oxidation current within the potential of 0.1–0.3 V was observed, and it could be ascribed to the kinetically very slow conversion of Cu (I) into Cu(II) [15]; meanwhile, a gradual decrease in current from −0.1 V to −0.4 V was detected, indicating the reduction of Cu(II) to Cu(I) in the presence of the BPDS ligand [15,16]. The redox couple at the apparent formal potential ($E^{\circ\prime}$) of 0.343 V (vs. SCE), as estimated from $E_{1/2}=(E_{red}+E_{ox})/2$, is consistent with the ferrocene/ferrocenium redox process [15]. Additionally, the absence of a well-defined Cu(II/I) redox couple is presumably due to the complicated monolayer which impedes the interfacial electron transfer [16]. Therefore, in the subsequent experiments, CV was conducted within the potential of −0.2 V to 0.3 V to in situ electrochemically activate the inert Cu(II)/BPDS complexes (see Fig. S3). As the applied low/final potential (−0.2 V vs. SCE) was slightly negative of the Cu(II)/Cu(I) standard potential [15], Cu(II) could be effectively switched into Cu(I), leading to an adequate amount of Cu(I) catalyst generated and maintained at the electrode surface. In addition, the deactivated copper species resulting from the oxidation or disproportionation could also be readily reactivated due to the cycling of the exerted potential on the modified electrode. This process is quite biocompatible, since no oxidative cleavage of biomolecules by the highly reactive hydroxyl radicals will occur due to the absence of ascorbate. The possible damage from the highly active Cu(I) can also be eliminated, because the ligand is capable of sequestering it away from biomolecules.

The labeling of the unfolded hairpins with EFC correlates with the enrichment of the electroactive probes at the electrode surface. To investigate the feasibility of the φCuAAC, CV was conducted within the potential of −0.3 V to 0.45 V to real-time monitor the labeling process. As shown in Fig. 1A, a clear oxidation peak current increased gradually at the potential around 0.38 V (vs. SCE). The potential value fell into the typical redox potential range of ferrocene, and the oxidation current was a result of the electrochemical oxidation of ferrocene into ferrocenium [8,30,31]. The increase of ferrocene signal indicated that EFC had been successfully labeled to the unfolded hairpins mediated by the φCuAAC. In addition, the kinetically slow oxidation current at the potential around 0.25 V observed in Fig. 1A provided further evidence of the presence of active Cu(I) catalyst. The labeled EFC on the electrode was further characterized with CV in 1 M $LiClO_4$ at different scan rates (Fig. 1B). The peak current varied linearly along with scan rate from 0.01 to 1 V $s^{-1}$, revealing that EFC had been covalently attached to the electrode surface [32,33]. The obtained results strongly guarantee that EFC can be successfully labeled to the unfolded hairpins mediated by the φCuAAC. In other words, the labeling of the unfolded hairpins with EFC via the φCuAAC is quite feasible.

To verify the reliability of the biosensor in the electrochemical detection of DNA, control experiments were also performed. They were carried out in an identical manner but with one of the required reagents was absent. DPV was conducted in 1 M $LiClO_4$ to detect the labeling amounts of EFC on different modified

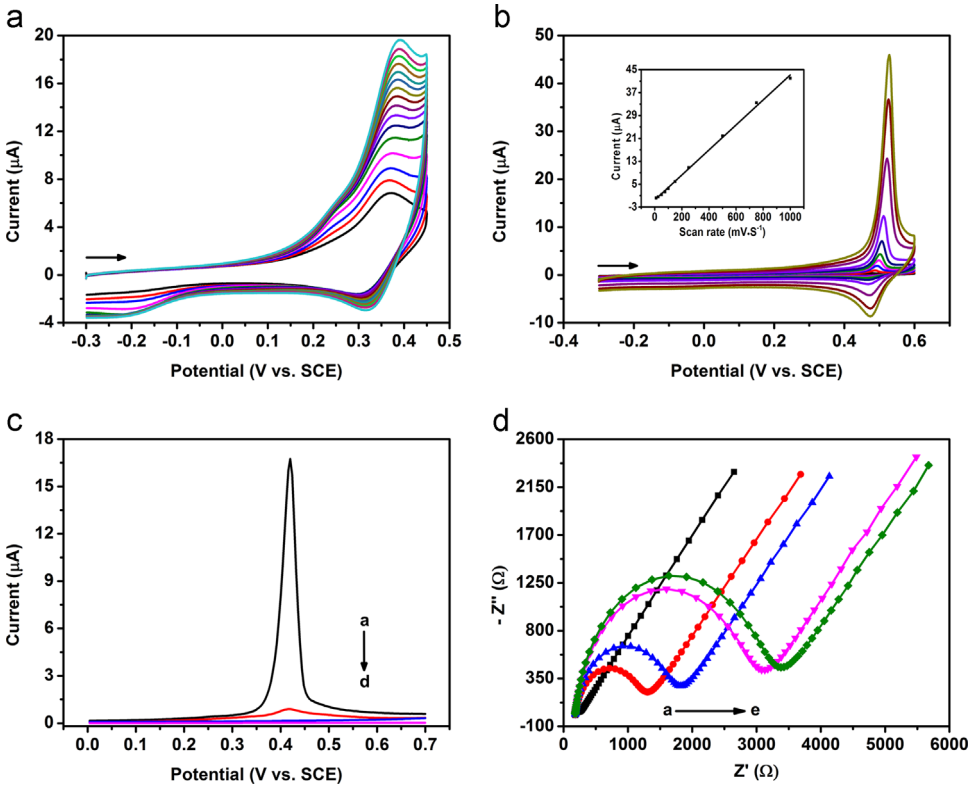



**Fig. 1.** (A) Cyclic voltammograms of the modified electrode in the labeling solution for the real-time monitoring of the labeling process (from inside to outside). Arrow indicates start of the scan. Scans taken every 2 min in the labeling solution during the 30 min labeling of the hairpin with EFC. (B) Cyclic voltammograms of the labeled EFC on the electrode surface at the scan rates of 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.25, 0.5, 0.75 and 1 V $s^{-1}$ (from inside to outside). Inset: The linear relationship between anodic peak current and scan rate. Arrow indicates start of the scan. The electrolyte is 1 M $LiClO_4$. (C) Differential pulse voltammograms of the different modified electrodes in the presence of all reagents (a), and in the absence of target DNA (b), $CuSO_4$ (c), and EFC (d). The electrolyte is 1 M $LiClO_4$. (D) The Nyquist plots of EIS of the bare gold electrode (a), and hairpins (b), hairpins/MCH (c), hairpins/MCH/target DNA (d) and hairpins/MCH/target DNA/EFC (e) modified gold electrode with $[Fe(CN)_6]^{3-/4-}$ as the redox probe. The concentration of target DNA used for all the experiments was 0.5 nM.

electrodes (Fig. 1C). If no EFC was added into the labeling solution, except the faint background charging current, almost no current peak was observed (Fig. 1C,d). Likewise, no observable signal enhancement was detected provided that $CuSO_4$ was absent from the labeling solution (Fig. 1C,c). This was mainly because that the primitive cycloaddition of azides with alkynes; namely without the catalysis of Cu(I), to give triazoles is typically less efficiency [14,15]. And the result also confirmed that the possible interference due to the conjugation of EFC with any other functional groups available in the system could be eliminated; in other words, the biosensor is highly tolerant to false positives. In addition, only a very low oxidation current would appear on condition that target DNA was not added (Fig. 1C,b). This was because that the hairpins were kept in the folded form in the target-unbound form, and therefore it was difficult for EFC to conjugate with azidos due to the remarkable steric hindrance of the densely packed SAM. In addition, EFC is highly hydrophobic and this is adverse to it to pass through the hydrophilic SAM. In contrast, a strong ferrocene oxidation peak would come out at the potential of 0.42 V (vs. SCE) when all the reagents were added (Fig. 1C,a). The obtained results evidenced that the electrochemical signal of ferrocene appeared only when the hairpins had been unfolded by the target DNA, and the φCuAAC was effectively enough in labeling of the hairpins with EFC. Thus, it can be concluded that the biosensor is quite reliable in the signal-on electrochemical detection of DNA.

In addition, we have also used EIS to monitor the preparation process of the electrochemical DNA biosensor, since it is highly sensitive to the microscopic interfacial changes occurring at the electrode surface [34]. In EIS, the diameter of the semicircle, measured at high frequency, corresponds to the electron-transfer resistance ($R_{et}$); it represents the electron transfer kinetics of the redox probe at the electrode surface [35]. Fig. 1D shows the Nyquist plots of impedance spectra of the same electrode at different stages of the preparation process. The bare gold electrode exhibited a smallest $R_{et}$ due to the fast electron-transfer process of the pretreated electrode (Fig. 1D,a). After immobilization of hairpins onto the electrode surface, the $R_{et}$ increased significantly and this is because the densely-packed negatively-charged hairpin layer is adverse to the interfacial electron transfer (Fig. 1D,b). Subsequently, the possible nonspecific binding sites on the electrode were blocked with MCH, and the $R_{et}$ increased accordingly (Fig. 1D,c). This can be interpreted as that more densely packed monolayer allows a slower electron transfer [36]. Furthermore, DNA hybridization resulted in a substantial increase in $R_{et}$ owing to the introduction of negatively charged target DNA (Fig. 1D,d). Finally, increase in $R_{et}$ was detected after the labeling was finished (Fig. 1D,e), indicating that EFC had been successfully labeled to the unfolded hairpins via the φCuAAC. The results highlighted that the modification process proceeded successfully.

### 3.3. Analytical performance of the electrochemical DNA biosensor

The analytical performance of the biosensor was evaluated. As shown in Fig. 2, the DPV curves showed a well-defined oxidation peak at the potential of 0.42 V (vs. SCE), and the peak current increased linearly along with the increase of target DNA concentration over a range from 1 pM to 1 nM. The calibration plot showed a good linear relationship between the peak current and logarithm of target concentration, with a detection limit as low as 0.62 pM ($S/N=3$). The linear regression equation was $I$ (μA)$=2.32+5.10\lg[C_{DNA}/pM]$ ($R^2=0.9996$), with an acceptable relative standard deviation (RSD) of 4.83% ($n=6$). Since the sample volume for the electrochemical detection of target DNA was 10 μL, the minimum amount of target DNA could be accurately detected was roughly 6.2 amol. The detection limit is better than many other hairpin-based DNA biosensors (as summarized in Table 1) [8,11,31,37,38]. The improved sensitivity can be ascribed to the relatively low background signal of the biosensor, since it is a signal-on method and thus is tolerant to false positives. The labeling of hairpins with EFC occurred only when the azide-containing terminals had been liberated away from the electrode surface as a result of DNA hybridization, because there were no other possible binding sites available for the attachment of EFC due to the excellent chemoselectivity and orthogonality of AAC. In addition, the protocol of the biosensor for the quantitative analysis of target DNA has also been largely simplified and becomes more robust, affordable than that of the signal amplification methods, because it requires no complicated scheme or expensive biochemicals. Importantly, the ability to chemoselectively label hairpin-like oligonucleotide with signal reporter by electrical addressing together with the simplicity and efficiency of the φCuAAC makes it an ideal method for the multiplexed detection of biomolecules within microfluidic devices and on microelectrode arrays. Therefore, the analytical performance of the biosensor is quite acceptable.

### 3.4. Selectivity, real applicability, and stability of the electrochemical DNA biosensor

To evaluate the selectivity of the biosensor, the electrochemical responses of three kinds of non-complementary oligonucleotides, including SBM, TBM, and Control, were compared with that of the target DNA. As shown in Fig. 3A, it can found that the peak current from SBM was only 26.8% of the value obtained from target DNA,

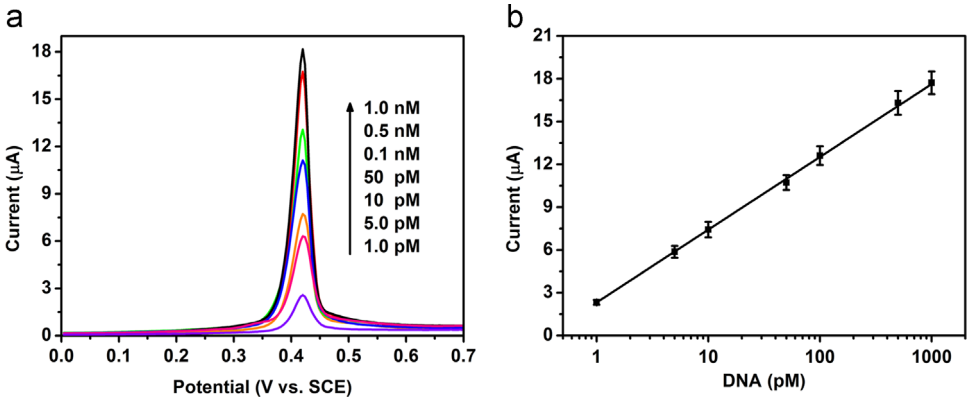



**Fig. 2.** (A) Differential pulse voltammograms of the electrochemical response of the biosensor toward different concentrations of target DNA in the range from 1 pM to 1 nM. (B) The calibration plot between peak current and logarithm of target DNA concentration. Error bars show the standard deviations of measurements taken from independent experiments ($n=6$).

**Table 1**
Comparison of the analytical performance of the biosensor with other hairpin-based methods.

| Electroactive probe | Technique | Linear range (M) | Detection limit (pM) | Signal | Ref |
|---|---|---|---|---|---|
| Methylene blue | DPV | $2.3\times10^{-12}$–$2.3\times10^{-9}$ | 1.7 | On | [11] |
| Ferrocene | DPV | $5.0\times10^{-12}$–$5.0\times10^{-9}$ | 3.5 | Off | [37] |
| Methylene blue | SWV* | $5.0\times10^{-11}$–$1.0\times10^{-6}$ | 25.1 | Off | [31] |
| Ferrocene | DPV | $3.5\times10^{-10}$–$2.5\times10^{-9}$ | 27.5 | Off | [38] |
| Ferrocene | CV | $1.0\times10^{-9}$–$1.0\times10^{-5}$ | 1000 | Off | [8] |
| Ferrocene | DPV | $1.0\times10^{-12}$–$1.0\times10^{-9}$ | 0.62 | On | This work |

* SWV: square wave voltammetry.

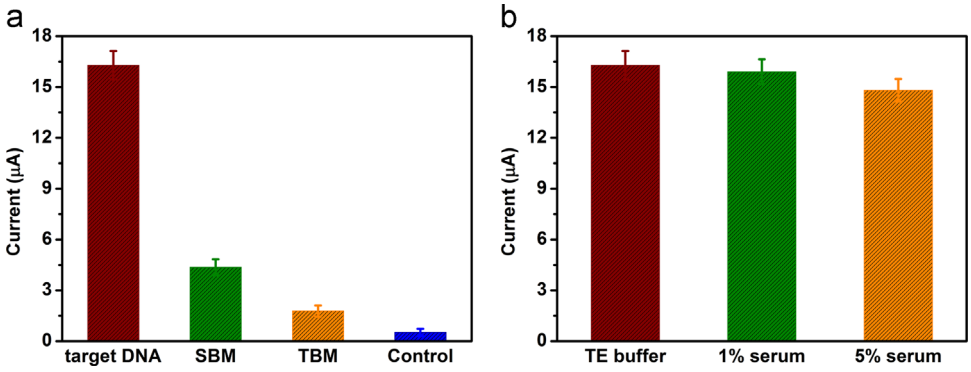



**Fig. 3.** (A) Selectivity of the biosensor toward four different oligonucleotides. (B) Detection capability of the biosensor in complicated serum samples. Error bars show the standard deviations of measurements taken from independent experiments ($n=6$). The concentrations of target DNA, SBM, TBM, and Control used in the experiments were all 0.5 nM.

suggesting that the biosensor has high specificity toward even a single base mismatch in the full sequence. The electrochemical response from TBM was nearly 89.0% lower off than that of the target DNA, while the current intensity from Control was not significantly different from the background signal. The notable differences in electrochemical response can be attributed to the hybridization efficiency of different oligonucleotides with the immobilized hairpins, since the presence of the stem makes it thermodynamically unfavorable for the hybridization of mismatched oligonucleotide with the loop portion of the folded hairpins [39]. Such results highlighted that the biosensor has high ability to effectively discriminate complementary and mismatched oligonucleotide fragments, indicating that it is highly specific and shows great potential toward genotyping of SNPs.

In addition, the real applicability of the electrochemical DNA biosensor in complicated biological fluids has also been evaluated. The samples were prepared by dissolving target DNA into FBS, and the interference effect of the complicated biological fluids on its analytical performance was tested. The electrochemical response from FBS samples were compared with that from TE buffer. As shown in Fig. 3B, the current intensity from 1% and 5% serum samples were about 97.6% and 91.0% of the value from TE buffer, respectively. Such results revealed that the biosensor has acceptable analytical reliability and application potential in clinical applications due to its good detection capability in complicated in serum samples. Furthermore, the simplicity and efficiency of the labeling strategy also make it suitable for clinical applications.

In addition, the stability of the electrochemical DNA biosensor was further challenged by long-term storage assay. After the modified electrode was stored at 4 °C for a month, the biosensor retained 95.8% of the initial response for 0.5 nM target DNA. The result revealed that the biosensor possessed satisfactory stability and this is because that the triazole linkage of cycloaddition is thermally and hydrolytically stable, as well as highly tolerant to redox reactions [23].

## 4. Conclusions

In summary, a novel hairpin-based electrochemical biosensor for the signal-on detection of sequence-specific DNA was developed by exploiting the φCuAAC as an efficient approach to label the hairpin-like oligonucleotide with electroactive probe. Assisted by the potential-mediated in situ activation of Cu(II), the active Cu (I) catalyst could be generated in a biologically friendly manner under mild conditions. The labeling reaction proceeded rapidly and selectively, it allowed the labeling of electroactive probe completed conveniently with high efficiency within the time scale of minutes. The biosensor is highly specific and holds a good detection capability in complicated serum samples. Its analytical performance is quite acceptable and the protocol for the quantitative analysis of sequence-specific DNA has also been largely simplified and becomes more robust and affordable. Without complicating the design of hairpin-like oligonucleotide, it offers a simple and convenient method to the signal-on electrochemical detection of sequence-specific DNA. Importantly, the ability to chemoselectively label hairpin-like oligonucleotide with signal reporter by electrical addressing together with the simplicity and efficiency of the φCuAAC makes it an ideal method for the multiplexed detection of biomolecules within microfluidic devices and/or on multielectrode arrays.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.10.039.

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